ANIMAL REPRODUCTION

Official journal of the Brazilian College of Animal Reproduction

v.16, n.3 **July/September** **2019**

Contents

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Comandatuba Island, Bahia, Brazil, August 15th to 18th, 2019, and 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Conferences papers

Abstracts

Conference abstracts

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Comandatuba Island, Bahia, Brazil, August 15th to 18th, 2019, and 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

From the SBTE President

Dear colleagues,

I am really happy to welcome all of you to the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE-2019), at the Transamerica Resort, Comandatuba Island, – Bahia, Brazil. It is also a pleasure to present the meeting proceedings, considering that this is our fifth joint publication with the Association of Embryo Technology in Europe (AETE). I am strongly optimistic about this ongoing partnership and we are grateful to AETE for their efforts in contributing to the pronounced quality of these proceedings.

My kind acknowledgments to the Scientific Committee: Vilceu Bordignon, Luiz Pfeifer and Bernardo Gasperin, who organized a wonderful program with an outstanding team of speakers, enabling the discussion of top issues regarding embryo production and fertility. I would also like to thank our speakers and our workshop coordinators, for their kindness in sharing their time and knowledge, thus greatly contributing to the remarkable quality of the meeting.

We also greatly thank our sponsors: CNPq, CAPES and partner companies, particularly the partner companies of the SBTE condominium, for their great contribution in making this meeting possible.

Thank you all SBTE members, for supporting our society in so many ways through the decades.

Finally, my highest sincere and special thanks to all SBTE directors, whose nice job has made this meeting possible.

Best regards,

Marcelo Marcondes Seneda
SBTE President (2018-2019)

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Comandatuba Island, Bahia, Brazil, August 15th to 18th, 2019, and 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

From the AETE President

Dear Friends, dear Colleagues,

It is with great pleasure that I invite you to our 35th Annual Scientific Meeting of the Association of Embryo Technologies in Europe. This year we go to Murcia, Spain where we will be welcomed by a fantastic Local Organizing Committee, chaired by Dr Rakel Romar. They prepared a wonderful venue for us with a great social program, starting with a warm Welcome Reception on Thursday evening 12th of September and ending on Saturday 14th with a real beach party. Of course, between these two events a lot of scientific content is programmed as well. We call it "scientific" program, while in real life, we do our utmost best to bring an ideal mix of fundamental science with very applied topics, ready to be used by practitioners and other stakeholders from the field.

The board of the AETE now contains three scientific committee members, focusing on the scientific programs of the next meetings. If you have suggestions, please do not hesitate to advise us. For this year's meeting we are pleased to announce 4 very interesting keynote speakers. Dr Peter Hansen (Department of Animal Sciences, University of Florida) will expand on the importance of heat stress in cattle breeding and how a changing climate may affect our embryo technology business. Dr Hansen promised us to focus on solutions and valuable advice. His lecture will be generously supported by our main sponsor Vétoquinol. This is of course very well appreciated by the Society. Next, following our yearly tradition, the SBTE will be represented by Dr Felipe Perecin (Faculty of Animal Sciences and Food Engineering, Veterinary Medicine Department, University of Sao Paulo, Brazil), who will speak about cellular interactions between the oocyte and somatic cells. Also Dr Perecin promised us to provide sufficient hands-on advise translated from the great fundamental knowledge he and his team generated over the years. The intense collaboration between AETE and SBTE is very well appreciated by the board and all members. As you know, this year we jointly publish already for the fifth time our abstract proceedings and invited lectures in Animal Reproduction. At the Affiliated Society meeting at IETS, this collaboration and joint publication efforts between AETE and SBTE were mentioned and really appreciated.

Dr Olli Peltoniemi (Faculty of Veterinary Medicine, University of Helsinki, Finland) will, as a third invited speaker, expand on recent advances in reproductive management and biotechnology in the pig industry. Our fourth speaker, Dr Sylvie Chastant (National Veterinary School of Toulouse, France), will speak about the importance of inflammation and disease on the quality of the oocyte and the embryo and on reproductive performances.

Next to the invited speakers, we will have more than 15 short oral presentations. Furthermore, I can already tell you that more than 30 abstracts were submitted in the Student Competition section. Only a shortlist of the best 5 abstracts will be selected to be presented during the meeting in our prestigious Student Competition session. In total, this year, we have about 110 abstracts submitted.

As every year we will have two interactive workshops. The first workshop will focus on the ethical aspects of our embryo technology sector. The importance of public opinion is growing and we should, as a society, prepare ourselves and formulate a strong opinion. This workshop will be chaired by Dr Roger Sturmey (Hull-York Medical School, Hull, UK). The second workshop will be moderated by Dr Zvi Roth (The Hebrew University of Jerusalem, Israel) and focuses on the practical aspects of heat stress in our embryo technology business. How can we anticipate and avoid the negative effects of climate change.

The highlight of our meeting in Murcia is of course the AETE Pioneer Award. It is a great honor for all members of the AETE board, and for me personally, to present Prof Dr Poul Hyttel (Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark) as the 2019 AETE Medalist winner. Since 1990, he has been the Professor of Anatomy at the Department of Anatomy and Physiology at the Royal Veterinary and Agricultural University; now Department of Veterinary and Animal Sciences (DVAS), Faculty of Health and Medical Sciences at University of Copenhagen. Poul Hyttel's current research activities are directed towards pluripotent animal and human stem cells and in vitro embryo production. A fully equipped stem cell laboratory approved for genetically modified cells has been established at DVAS. The major contemporary projects focus on (1) establishment of human patient-specific neural in vitro cell models ("microbrains") for modeling of neurodegenerative disorders by the use of induced pluripotent stem cells (iPSC); (2) establishment of animal iPSCs for the development of the domestic animals as a translational model for iPSC-based cell therapy; and (3) in vitro production of bovine embryos, where the technologies for oocyte maturation, fertilization and embryo culture in vitro are optimized and genomics is applied for precise selection of high quality embryos. He has authored more than 280 refereed international articles and has been the principal supervisor of more than 40 PhD-students and postdocs. In 1997-1998 Prof. Hyttel was President of the International Embryo Transfer Society, in 2011 he was awarded Doctor Honoris Causa at University of Antwerp, Belgium, in 2015 he was knighted 1st Class Order for his services to Denmark, and in 2018 he was awarded Doctor Honoris Causa at Estonian University of Life Sciences, Tartu, Estonia.

And finally, we cannot underestimate the great importance of our Sponsors in our Society. We are very grateful to (20 May 2019):

Main: [Vetoquinol]; General: [Ceva Santé Animal]; Exhibitors: [imv TECHNOLOGIES] [EGG TECH] [ivf BIOSCIENCE] [IVF tech] [Minitüb] [PETS] [CONTROLTECNICA Instruments] [SPARMED APS] [ART Lab Solutions] [SCR Europe srl] [WTA] [REPBIOTECH Marie-Skłodowska Curie ITN]; Supporters: [IVI Murcia]

Stay with us for the latest updates on the program at www.aete.eu and I hope to welcome you all in Murcia.

Jo Leroy
President of the AETE

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Comandatuba Island, Bahia, Brazil, August 15th to 18th, 2019, and 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

From the Chairs of the SBTE Scientific Committee

Dear Friends and Colleagues,

We are delighted to welcome you to the 33rd Annual Meeting of the Brazilian Society of Embryo Technology (SBTE), which will be held from August 15 to 18, 2019, in the Comandatuba Island, Bahia, Brazil.

This special issue of Animal Reproduction contains the full papers from invited speakers of both the SBTE and the Association of Embryo Technology in Europe (AETE) annual meetings. We express our gratitude to the editorial board and staff of the Animal Reproduction Journal for their support and collaboration in organizing and publishing the joint proceedings of the annual meetings of the SBTE and AETE.

For SBTE 2019, we have prepared a special meeting with two complementary programs: on August 16, a technology-oriented program will cover topics of major interest to professionals working in the field, and on August 17, a science-oriented program will cover fundamental aspects on reproductive biology, oocyte/embryo development and animal fertility. We thank all the speakers for their commitment and efforts in preparing the review articles, their kind willingness to comply with our deadlines, and for their oral presentations during the SBTE meeting.

The SBTE 2019 program will start on the afternoon of August 15, with four workshops that were planned and developed to serve the diverse interests of all SBTE members. Participants will have the opportunity to attend the workshop of their interest where renowned professionals and scientists will be sharing their experiences and research findings. We are grateful to the workshop presenters and coordinators for their enormous contributions, especially their dedication to the planning and developing of each topic.

The SBTE program will continue during the opening ceremony on August 15 when Dr. Pascale Chavatte-Palmer, President of the International Embryo Transfer Society (IETS), will present an update on worldwide embryo production.

We would like to express our gratitude to the coordinators of the abstract sessions, to the reviewers of abstracts and manuscripts, and to colleagues who evaluated the abstracts for the competitions and awards. This year, more than 170 abstracts were received and carefully evaluated. Thank you all for your time and effort dedicated to the review process.

We would like to highlight the tremendous work, dedication and contributions of all members of the SBTE Board in making this meeting possible.

Finally, we thank all the SBTE members and participants for their attendance at the SBTE 2019 annual meeting.

Bernardo Garziera Gasperin
Luiz Francisco Machado Pfeifer
Vilceu Bordignon
Co-Chairs of the SBTE Scientific Committee (2018-2019)

SBTE Board Members (2018-2019)

President: **Marcelo Marcondes Seneda** (Universidade Estadual de Londrina, PR)
Vice president: **Flávio Meirelles** (FZEA/USP - Pirassununga, SP)
1st Secretary: **Luciana Rafagnin Marinho** (Universidade Estadual de Londrina, PR)
2nd Secretary: **Fabiana Bressan** (FZEA/USP - Pirassununga, SP)
1st Treasurer: **Márcio de Oliveira Marques** (Geraembryo - Cornélio Procópio, PR)
2nd Treasurer: **José Nélio Sales** (UFLA - Lavras, MG)
Communications Director: **Marcos Roberto Chiaratti** (UFSCar - São Carlos, SP)
Communications Director: **Fabiana Melo Sterza** (UEMS - Aquidauana, MS)
Business Director: **Manoel Sá Filho** (Alta Genetics - Uberaba, MG)
Veterinary Practitioners Representative: **Gustavo Gomes dos Santo**s (Sheep Embryo - Reprodução Animal - Londrina, PR)
Scientific Committee Co-Chair: **Bernardo Garziera Gasperin** (UFPel - Pelotas, RS)
Scientific Committee Co-Chair: **Luiz Francisco Pfeifer** (Embrapa Rondônia - Porto Velho, RO)
Scientific Committee Co-Chair: **Vilceu Bordignon** (McGill University - Canada)

AETE Board Members (2018-2019)

President: **Jo Leroy**, Belgium
Vice President: **Marja Mikkola**, Finland
Treasurer: **Jan Detterer**, Germany
Secretary: **Teresa Mogas**, Spain
Annual statistics: **Hélène Quinton**, France
Newsletter: **Roger Sturmey**, United Kingdom
AETE website: **Hilde Aardema**, The Netherlands
Scientific Committee: **Jane Morrell**, Sweden
French foundation and of ET Industry: **Daniel Le Bourhis**, France
Student Affairs: **Lotte Stroebech**, Denmark

SBTE Proceedings General Editors

Bernardo Garziera Gasperin
Luiz Francisco Machado Pfeifer
Vilceu Bordignon

AETE Proceedings General Editors

Jo Leroy
Teresa Mogas

SBTE Workshop Chairs

Amauri Alfieri
Felipe Perecin
José Nélio Sales
Mateus Sudano
Vilceu Bordignon

AETE Workshop Chairs

Roger Sturmey
Zvi Roth

SBTE Manuscript Reviewers

Arnaldo Diniz Vieira
Augusto Schneider
Evelyn Andrade
Fábio Gallas Leivas
Fabíola Freitas de Paula Lopes
Flavia Lombardi Lopes
Guilherme Pugliesi
Gustavo Zamberlam
Hernan Baldassarre
José Buratini Jr
José Nélio Sales
Juliano Coelho da Silveira
Lígia Margareth Cantarelli Pegoraro
Lindsay Unno Gimenes
Luiz Francisco Machado Pfeifer
Luiz Gustavo Bruno Siqueira
Marcella Pecora Milazzoto
Marcos Henrique Barreta
Maria Angelica Miglino
Mario Binelli
Rogério Ferreira
Vilceu Bordignon
Werner Giehl Glanzner

AETE Manuscript Reviewers

Hilde Aardema
Marijke Beltman
Jessie De Bie
Patrick Lonergan
Manel López Béjar
Dominique Maes
Roberto Sartori
Martin Sheldon
Ann Van Soom
Marc Yeste

SBTE Abstract Session Coordinators

Augusto Schneider
Fernando Mesquita
Guilherme Pugliesi
Karina Gutierrez
Ligia Margareth Cantarelli Pegoraro
Lindsay Unno Gimenes
Luiz Gustavo Bruno Siqueira
Monique Tomazele Rovani
Rafael Gianella Mondadori
Werner Giehl Glanzner

AETE Abstract Session Coordinators

Hilde Aardema
Daniel Le Bourhis
Marja Mikkola
Teresa Mogas
Jane Morrell
Hélène Quinton
Lotte Stroebech
Roger Sturmey

SBTE Abstract reviewers

Adnan Darin
Alejo Menchaca
Alexandre Henryli de Souza
Alfredo Quites Antoniazzi
Amanda Prudêncio Lemes
André Luís Rios Rodrigues
Andrei Guedes Fidelis
Angela Maria Gonella-Diaza
Anthony Cesar Souza Castilho
Arnaldo Diniz Vieira
Bernardo Garziera Gasperin
Bruno Freitas
Carine Dahl Corcini
Carlos Antônio Carvalho Fernandes
Carlos Eduardo Leonardi
Carlos Jose Hoff de Souza
Carolina Bespalhok Jacometo
Carolina Capobiango Romano Quintão
Cassio Cassal Brauner
Clara Slade Oliveira
Claudia Maria Bertan Membrive
Daniela dos Santos Brum
Danila Barreiro Campos
Diego Velasco Acosta
Eduardo Kenji Nunes Arashiro
Eliza Rossi Komninou
Emiliana de Oliveria Santana Batista
Everton Lopes
Fabio Morato Monteiro
Fabio Vasconcellos Comim
Fabrício Mozzaquatro
Felipe Zandonadi Brandão
Fernando Caetano de Oliveira
Francielli Weber Santos Cibin
Guilherme de Paula Nogueira
Guilherme Pugliesi
Gustavo Guerino Macedo
Gustavo Zamberlam
Henderson Ayres
Joao Alvarado Rincon
João Henrique Moreira Viana
Jorgea Pradiee
José Felipe Spricigo
Juliana Germano Ferst
Juliano Coelho da Silveira
Juliano Rodrigues Sangalli
Kalyne Bertolin
Karina Gutierrez
Kleber Menegon Lemes
Ligia Margareth Cantarelli Pegoraro
Lindsay Unno Gimenes
Lucas Hax
Luciana Simões Rafagnin Marinho
Luiz Francisco Machado Pfeifer
Luiz Gustavo Bruno Siqueira
Marcelo Tigre Moura
Marcos Henrique Barreta
Margot Alves Nunes Dode
Maria Gabriela Tavares Rheingantz
Mariana Marques Groke

Mariana Priotto de Macedo
Mariana Sponchiado
Mateus Jose Sudano
Milena Lopes Oliviera
Miller Pereira Palhão
Moana Rodrigues França
Nadja Gomes Alves
Naiara Zoccal Saraiva
Naomi Dicks
Natália Avila Castro
Nelcio Antonio Tonizza de Carvalho
Paulo Roberto Antunes da Rosa
Pedro Augusto Silva Silveira
Pedro Leopoldo Monteiro Jr
Rafael Bisinotto
Rafael Ulguim
Rafael Vilar Sampaio
Ricarda Maria Dos Santos
Roberta Machado Ferreira
Rodolfo Daniel Mingoti
Rodrigo Camponogara Bohrer
Sabine Wohlres Viana
Sandra Mara Fiala Rechsteiner
Suellen Gonzalez
Thales Ricardo Rigo Barreiros
Thiago Martins
Thomaz Lucia Jr
Tiago Henrique Camara de Bem
Vera Fernanda Hossepian de Lima
Vitor Braga Rissi
Werner Giehl Glanzner

AETE Abstract Reviewers

Rafi Ahmad
Essraa Mohsen Al-Essawe
Ziyad Makram Al-Kass
Carmen Almiñana Brines
Luis Anel-Lopez
Christine Aurich
Renée Båge
Nathalie Beaujean
Frank Becker
Ann-Sofi Bergqvist
Heinrich Johann Bollwein
Aurélie Bonnet
Marleen Broekhuijse
Jean-François Bruyas
Diego Bucci
Jose Buratini
Andreina Cesari
Gilles Charpigny
Sylvie Chastant
Fabienne Constant
Rozenn Dalbies-Tran
Alline de Paula Reis
Sourabh Deori
Karolien Desmet
Jesus Dorado
Véronique Duranthon
Sebastien Elis
Francis Fieni
Bart M. Gadella
Bénédicte Grimard
Hakan Guerler
Yongzhi Guo
Benoit Guyonnet
William Vincent Holt
Miel Hostens
Isabelle Hue
Anders Johannisson
Alice Jouneau
Iris Kaimio
Rebecca L Krisher
Arumugam Kumaresan
Denise Laskowski
Bart Leemans
Henry John Leese
Jo Leroy
Heli Lindeberg
Felipe Martínez Pastor
Paul Joseph McKeegan
Marja Mikkola
Teresa Mogas
Frøydis Deinboll Myromslien
Thanapol Nongbua
Theodoros Ntallaris
Fabienne Nuttinck
Jaana M H Peippo
Nicole Picard-hagen
Ralf Poehland
Karine Reynaud
Christophe Richard
Bernard Roelen
Rakel Romar
Marie Saint-Dizier
Pascal Salvetti
Olivier Sandra
Alexei Santiani
Eli Sellem

Sponsors SBTE

Se é Bayer, é bom

AETE

Main Sponsor

Thanks to all our sponsors!

vetoquinol
ACHIEVE MORE TOGETHER

General Sponsor

ceva

Exhibitors

EGG TECH

minitube

ivf BIOSCIENCE

imv TECHNOLOGIES

CONTROLTECNICA

sparMED
GOosafe in IVF Laboratories

PETS
Professional Embryo Transfer Supply, Inc.

ART LAB SOLUTIONS
SOLUTIONS FOR CATTLE BREEDING

IVFtech®

Allflex®
Livestock Intelligence

WTA

OC Biomedical
one cause... one health

Rep-Biotech

This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 675526.

Supporter

IVI

DOI: 10.21451/1984-3143-AR2019-0050

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Challenges to increase the AI and ET markets in Brazil

Pietro Sampaio Baruselli*, Bruna Lima Chechin Catussi, Laís Ângelo de Abreu, Flavia Morag Elliff, Laísa Garcia da Silva, Emiliana de Oliveira Santana Batista

Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil.

Abstract

Artificial insemination (AI) and embryo transfer (ET) are the most widely used biotechnologies in the world with the goal of increasing genetic gain and improving reproductive efficiency of beef and dairy herds. The protocols for ovulation synchronization for timed AI (TAI) or ET (TET) are tools that allow artificial insemination or transfer of a high number of embryos in a pre-established moment and without the necessity of estrous detection. Currently, 86% of inseminations in Brazil are performed using TAI (13.6 million TAI out of a total of 15.4 million doses of semen marketed in 2018). With the use of TAI, it was possible to verify that the percentage of artificially inseminated females in Brazil went from 5.8% in 2002 to 13.1% in 2018. The ET market also presented considerable growth in the last 20 years. There was an increase of approximately 8 fold in the number of produced embryos, escalating from 50,000 in 1999 to 375,000 in 2017. In this period, there was a significant increase on the *in vitro* embryo production, which represented 92.1% of embryos produced in Brazil in 2017. Also, in this period, there was an increase on the embryo production of dairy breeds and reduction on the embryo production of zebu breeds in comparison to data from the early 2000's. TET increases significantly the number of recipients suitable to receive an embryo. After synchronization, 75 to 85% of recipients present a suitable CL for ET without estrous detection. Currently, many synchronization and resynchronization protocols for TAI/TET have been studied to attend different managements, breeds and animal categories, with predictable and satisfactory results. With the intensification of the use of these biotechnologies, it is possible to obtain elevated reproductive efficiency with increase on the genetic gain, which determines greater productivity and economic return for dairy and beef farms. However, the challenge to keep the market growing in the next decade could depend on some factors, such as: increase of the extension services for producers and of the extension training for specialists, improvement of the technological advances to develop more efficient and cost-effective products and practical protocols, increase the integration between universities, research institutes, veterinarians and industries and also, asses market demand for production of animal protein with higher quality, efficiency and environmental and economic sustainability.

Keywords: Artificial insemination, embryo transfer, synchronization, reproductive efficiency, economic return.

Introduction

The accelerated growth of the world population is generating a significant increase in the demand for food, causing concern for the production of animal proteins to meet the growing number of people on the planet (FAO, 2017). In this context, Brazil is relevant because it is the fifth largest country in territorial extension and has the largest commercial cattle herd in the world (221.81 million heads, IBGE, 2018).

In 2017, the number of bovine slaughters in Brazil reached 39.2 million, with an estimated production of 9.71 million tons of carcass equivalent, representing 14.4% of world meat production (ABIEC, 2018). Despite this positive scenario, Brazilian beef cattle production still has low production efficiency, and ranks second in the world classification of meat production, led by the United States, which produce 17.9% of the world meat production (ABIEC, 2018).

The national production of fluid milk was 33.5 billion liters, out of a total of 17 million milked cows, corresponding to the yield of 1,943 liters of milk per cow per year (IBGE, 2017). In this context, each animal contributed with only 5.4 liters of milk produced per day, evidencing the low efficiency of this activity in Brazil (IBGE, 2017). These numbers rank Brazil as the fourth largest milk producer (falling behind the United States, India and China), despite having the world's largest cattle herd. Also, Brazil is not self-sufficient in the production of cattle milk, and in this scenario, importation is necessary to supply the domestic market (IBGE, 2017).

Thus, it is essential to develop and improve technologies that collaborate with increasing productivity on farm, optimizing the breeding systems and the profitability of the herds. Among the developed technologies, reproduction biotechnologies are noticeable.

Artificial insemination (AI) is the most widely used reproductive biotechnology in the world and its application brings great benefits to the herds when compared to the use of natural service (Lima *et al*., 2010; Lamb and Mercadante, 2016; Baruselli *et al*. al., 2018a). The technique allows the use of the semen of genetically superior bulls, accelerating the genetic gain and resulting in more productive calves, which generate greater economic return to the meat and milk producer (Baruselli et al., 2017b). In addition, AI prevents the transmission of venereal diseases (Vishwanath, 2003) and allows better control of the herd, increasing the uniformity of calves when compared to natural service

*Corresponding author: barusell@usp.br
orcid.org/0000-0002-6773-4450
Received: May 3, 2019
Accepted: June 9, 2019

(Rodgers *et al*., 2015; Baruselli *et al*., 2017a).

In order to facilitate the use of AI in rural properties, timed artificial insemination (TAI) was developed (Pursley *et al*., 1995). This reproductive biotechnology eliminates the necessity of estrous detection and allows anestrous cows to be inseminated, increasing the reproductive efficiency of cows and heifers (Rhodes *et al*., 2003). Furthermore, the use of TAI anticipates and concentrates conception at the beginning of the breeding season, increasing the reproductive and productive efficiency of farms (Baruselli *et al*., 2002; Baruselli et al., 2004; Bo et al., 2007; Sá Filho *et al*., 2013; Sartori *et al*., 2016).

Embryo transfer (ET) allows the dissemination of high value genetic material from both males and females, increasing the genetic gain of animal breeding programs when compared to AI. Furthermore, ET associated with the advent of genomic technology and endocrine markers (AMH) allows the use calves as oocyte donors to produce embryos from young cattle, which is an important strategy to accelerate genetic gain by decreasing generation intervals (Batista et al., 2016). The ET has presented considerable growth in the last decades, mainly due to the scientific and technological development of innovative processes of embryo production. Currently, *in vitro* embryo production (IVEP) represents 92.1% of the embryos produced in Brazil, and 66% of the embryos produced in the world (Viana *et al*., 2018; IETS, 2018). In addition to the increase on the IVEP, there was the development of synchronization techniques for fixed-time embryo transfer (TET), which increases the number of recipients suitable for receiving an embryo and eliminates the necessity for estrous detection, allowing the establishment of this biotechnology in beef and dairy farms (Baruselli *et al*., 2000; Bo *et al*., 2002; Baruselli *et al*., 2010).

The evolution of artificial insemination

The Brazilian market for artificial insemination traded approximately 7.0 million doses of semen in 2002. In 2018, this market reached 15.4 million semen doses marketed (ASBIA INDEX, 2019), with a 220% growth in that period. Still, compared to the previous year (2017 to 2018), the semen market grew 13.7%. For the calculation of the number of semen doses marketed in Brazil, data from the ASBIA INDEX were considered (representing 90% of the Brazilian semen market), with adjustments to 100% of the market (Baruselli *et al*., 2019a). These data clearly demonstrate that artificial insemination has gained ground in Brazil over the years.

The increase in the AI market in Brazil occurred simultaneously with the introduction of TAI technology. In 2002, according to data obtained by the Animal Reproduction Department of FMVZ/USP (Baruselli *et al*., 2012), the number of protocols marketed was 100,000, which shows that only 1% of the inseminations in Brazil were performed by TAI that year. In 2018, the number of TAI reached 13.3 million procedures, indicating that 86% of the inseminations were performed using TAI in Brazil (Baruselli 2019a; Fig. 1). The TAI market growth in 2018 also showed a significant increase of 16.1% when compared to 2017 (11.4 million TAI). It is possible to verify that the TAI has grown 130 fold in the last 16 years, bringing great advances and benefits to the entire meat and milk production chain.

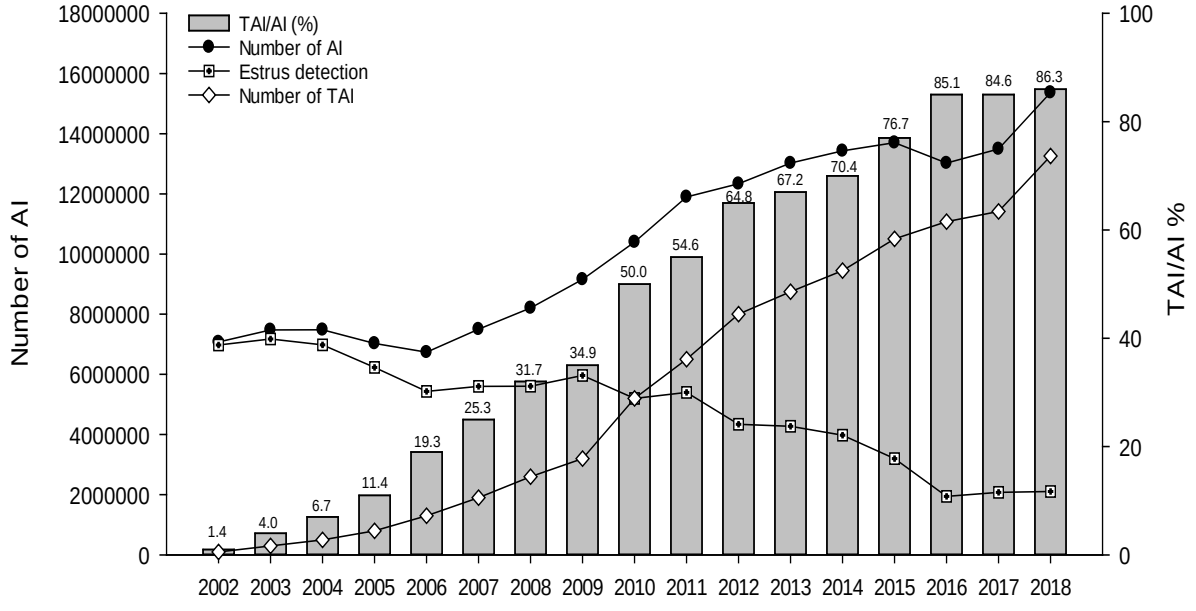


Figure 1. Evolution of timed artificial insemination (TAI) or artificial insemination with estrous detection in cattle in Brazil (adapted from Baruselli, 2019a).

Based on the number of breeding cows and heifers in Brazil (ANUALPEC, 2018) and the number of commercialized doses of semen (ASBIA, 2019), it was possible to estimate the evolution of the AI market in Brazil from 2002 to 2018 (Fig. 2). In 2002, which coincides with the beginning of data collection (presented in Baruselli *et al*., 2012), only 5.8% of the bovine females of the Brazilian herd were inseminated, taking into account the use of 1.6 doses of semen per breeding female. However, in 2018 this figure reached 13.1% of the total number of females in reproduction of the Brazilian herds (Fig. 2), demonstrating a significant advance in the use of this technology. This increase was mainly due to the use of timed protocols, which are highly reproductive efficient and facilitate the dissemination of artificial insemination. In 2018, it is estimated that approximately 9.5 million females were artificially inseminated in Brazil, contributing to increase the genetic, productive and economic gains of livestock.

Furthermore, statistical data collected in Brazil are similar to those of neighboring countries, according to the information reported by Argentina and Uruguay (2016/2017 breeding season). Uruguay has approximately 3 million breeding females and 300,000 TAI, demonstrating that approximately 10% of breeding females in reproduction are inseminated. In total, more than 15 million breeding females were inseminated using TAI in Brazil, Argentina and Uruguay in the year 2017 (Mapletoft *et al*., 2018). This information indicates the consolidation of the technology in the market, resulting in economic gains and positive perspectives for livestock.

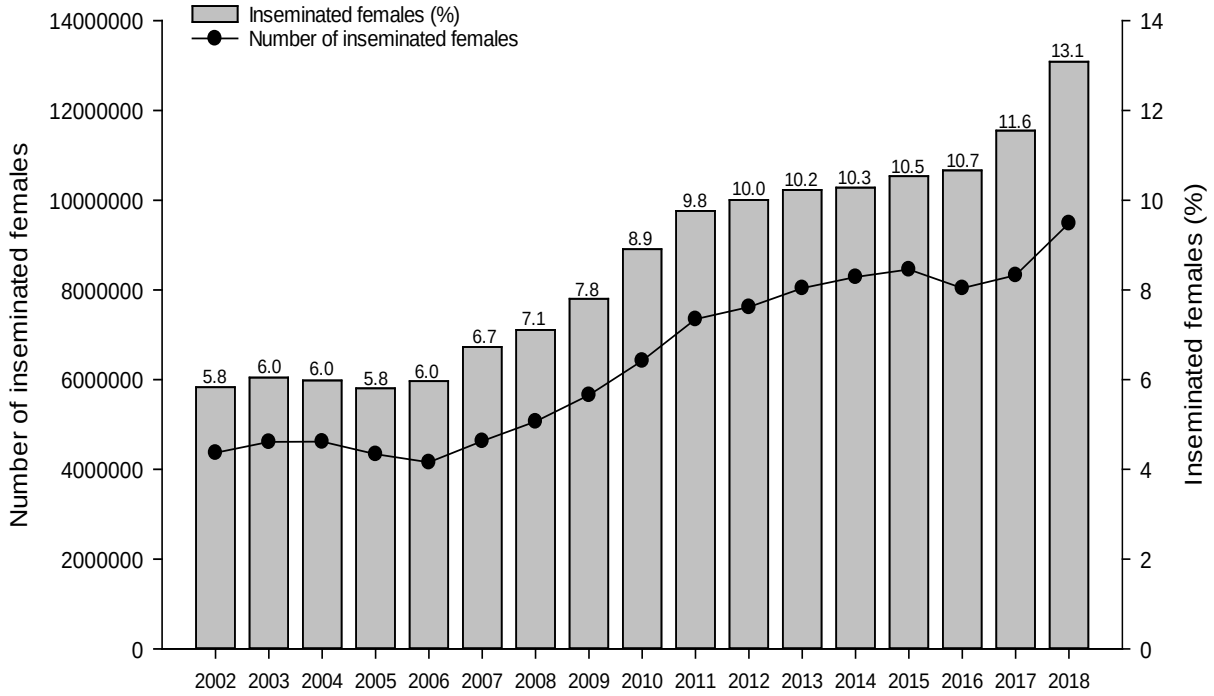


Figure 2. Evolution of the number and percentage of females inseminated in Brazil based on the number of beef and dairy breeding cows and heifers (ANUALPEC, 2018) and the number of marketed doses of semen (ASBIA, 2019). An average of 1.6 inseminations per breeding female was considered.

According to the number of bovine females in reproduction (cows and heifers) in Brazil (ANUALPEC, 2012 and 2018), the percentage of beef (Fig. 3) and dairy (Fig. 4) artificially inseminated females from 2002 to 2018 were calculated. These data were confronted with the number of semen doses commercialized for beef and for dairy herds disclosed by the Brazilian Association of Artificial Insemination (ASBIA, 2019). For the analysis, 1.4 doses of semen per artificially inseminated beef female and 2.4 doses of semen per artificially inseminated dairy females were considered. The data show that 13.6% of the beef females and 10.8% of the dairy females are artificially inseminated in Brazil. Unexpectedly, there was a drop in the percentage of inseminated dairy cows in the last 5 years. On the other hand, the percentage of artificially inseminated beef females has increased almost two times in the last 10 years.

According to preliminary data from 2017 (IBGE), 76.2% of livestock establishments in Brazil had less than 50 head. Since there are many small producers, the low incidence of technology in small farms is proportionately higher, creating a budget constraint for the producer to invest in technology. For dairy cattle, 85.9% of all properties have less than 50 head (IBGE, 2017), which can explain the reduction on the use of AI in the last 5 years (Fig 4). The number of establishments with 50 head or less is also high for beef farms in Brazil (68.1%), however, the number of large properties (441,133 farms, IBGE 2017) is almost 3-fold the number of large dairy properties (164,568), which is indicative of a higher

incidence of technology for beef farms. Furthermore, according to the profile of lactating cows in Brazil, 54% of the total is composed of genetics without milk production specialization (Nogueira, 2019). Additionally, in the last 4-years, an increase in the cost of the food for dairy cows was observed and therefore, there was a reduction on the economic gain of the dairy farms (CNA, 2019), discouraging the use of technology in the milk production sector. These facts corroborate with the evolution of the AI market, where it is possible to observe that beef cattle have a growing number of inseminated animals, and dairy cattle, in the last four years (2014 to 2018), have had a decrease on the number of inseminated females.

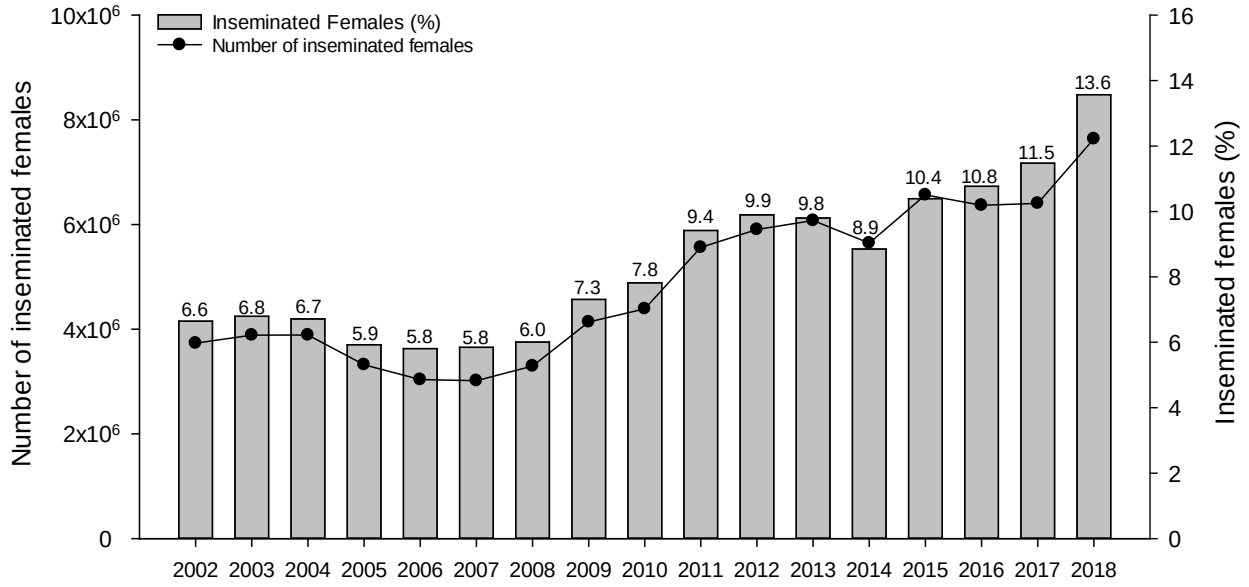


Figure 3. Evolution of the number and percentage of beef females inseminated in Brazil based on the number of beef breeding cows and heifers (ANUALPEC, 2018) and the number of commercialized semen doses (ASBIA, 2019). An average of 1.4 inseminations were considered per breeding female.

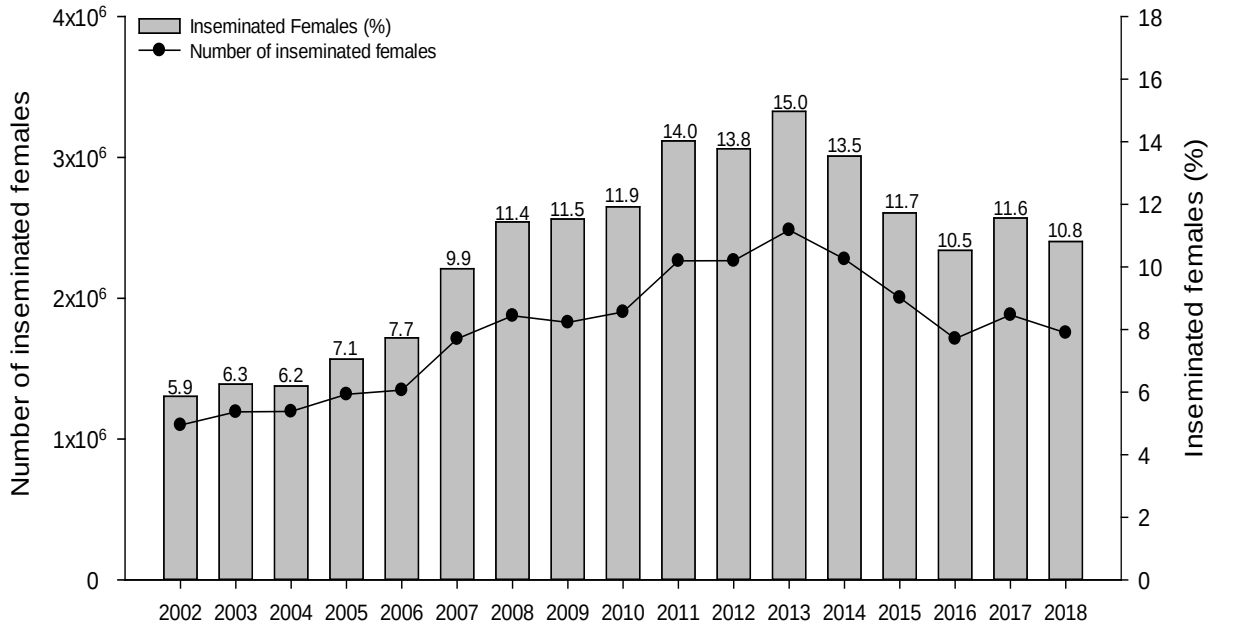


Figure 4. Evolution of the number and percentage of dairy females inseminated in Brazil, based on the number of dairy breeding females (ANUALPEC, 2018) and the number of commercialized doses (ASBIA, 2019). An average of 2.4 inseminations were considered per breeding female.

The economic impact of TAI in Brazil

In the year of 2018, it is estimated that TAI generated approximately R$ 3.5 billion (approximately 1 billion dollars) for the Brazilian beef and dairy production chain. It is calculated that this activity counts with 3,788 veterinarians specialized in animal reproduction acting on the farms (considering 3,500 TAI per professional, Baruselli, 2019b). Based on these data, it is estimated that TAI moved R$ 796 million for its execution in Brazil. The veterinary service corresponds to 33% of the value for execution of TAI (R$ 265.2 million), considering the cost of R$ 20 per synchronized animal. The companies selling semen and pharmaceuticals represent 66% (R$ 796 million) of the total value, considering 13.3 million TAI performed in the year at an average price of R$ 20 for synchronization drugs and R$ 20 for semen dose (Baruselli, 2019b).

In addition to these direct economic impacts, there are benefits in increasing productivity that must be taken into consideration. In beef herds, there is an increase in the quantity and quality of calves produced with the introduction of TAI technology. Considering that TAI is used in 10.2 million beef females, an increase of 8% is estimated in calf production when compared to natural service (Sá Filho *et al*., 2013, Baruselli *et al*., 2018a), with additional production of 816 thousand calves per year, adding R$ 979 million in the meat production chain (TAI calf price = R$ 1,200.00). Also, because of the high genetic merit and anticipation of the calving provided by the use of TAI comparing to natural service, these studies showed an additional gain of 20 kg in the weaning weight, which represents an extra gain of R$ 490 million (Kg of the calf = R$ 6.00). Also, from weaning to slaughter, calves from TAI show an additional gain of 15 kg of carcass, totaling R$ 600 million (approximately 4 million animals slaughtered with R$ 150.00 per 15 kg of carcass). Thus, TAI generates an impact of additional R$ 2.1 billion per year on the beef production chain when compared to natural service (Baruselli, 2019b).

In dairy herds, TAI also has great economic impact. Studies have shown that there is a reduction of approximately 1 month in the inter calving interval (ICI) of animals receiving TAI when compared to animals submitted to traditional systems of estrous detection or natural mating (Nebel, 2003; Caraviello *et al*., 2006; Teixeira, 2010; Baruselli *et al*., 2017a). With this reduction of the ICI, there is a 10% increase in the annual milk yield of the farm. Considering that 3.1 million dairy cows are submitted to TAI in Brazil, with an estimated production of 3,000 liters per lactation (estimative for farms that use AI), an increase of 917 million liters of milk per year is estimated, with additional revenues of 1.3 billion additional income per year. In addition, the use of genetically superior bulls through AI adds 350 liters of milk per lactation, corresponding to 131 million liters per year (R$ 1.39 per liter, average of 2018 CEPEA/USP, published in 2019) and a turnover of R$ 166 million per year. Thus, it was estimated that the impact of the TAI on dairy farming generates an additional R$ 1.5 billion per year when compared to traditional breeding systems with estrous detection or natural mating.

From these data it is possible to calculate the return on investment of this biotechnology. Each R$ 1.00 invested on the TAI technology, there is a return of R$ 4.50 for the beef and dairy production chain in Brazil. (Baruselli, 2019b). These figures clearly demonstrate that the investment in TAI technology generates significant gains for Brazilian livestock.

Evolution of synchronization and resynchronization protocols for TAI

The first TAI protocols for cattle appeared in the mid - 1990s with the development of the Ovsynch protocol (GnRH - 7 days/PGF - 48 hours/GnRH - 16 hours before AI; Pursley *et al*., 1995). Currently, TAI programs underwent various modifications to facilitate management and improve pregnancy rates. In Brazil, the protocol based on estradiol (E2) and progesterone (P4) is the most used for TAI (Baruselli *et al*., 2002; Sá Filho *et al*., 2009; Baruselli *et al*., 2012). Numerous protocols have been developed for different breeds, animal categories and for the producer to adapt the best reproductive program to the farm production system.

Regarding the available TAI programs, many different protocols can be used with similar efficiency. The time of permanence of the P4 device may vary from 5 to 9 days (Bó *et al*., 2007; Baruselli *et al*., 2012, Baruselli *et al*., 2017a; Bo *et al*., 2018). Regarding the number of managements required to perform the synchronization for TAI, there are studies that have developed systems with 3 or 4 animal handlings. In general, the goal of the additional management is to administer prostaglandin (PGF), anticipating luteolysis, and reducing serum P4 concentrations at the end of the protocol in cycling animals. The additional treatment with PGF in cyclic heifers and cows 2 or 3 days before the device removal (four animal handlings) increases the dominant follicle growth and the ovulation and pregnancy rates (Mantovani *et al*., 2005, 2010; Sá Filho *et al*., 2009; Meneghetti *et al*., 2009). However, some studies have demonstrated that it is possible to perform PGF treatment on day zero (D0) of the protocol (3 animal handlings), causing luteolysis in animals with presence of a responsive corpus luteum (CL) at the beginning of synchronization, reducing blood P4 during the protocol, increasing the follicular growth, ovulation and conception and facilitating the management of TAI (Carvalho *et al*., 2008). However, recent studies have shown association between decrease in circulating progesterone concentration during the synchronization protocol and increase of dry matter intake (Batista *et al*., 2015). Therefore, the positive effect of the PGF treatment to induce early luteolysis during the TAI protocol could be reduced in animals submitted to high dry matter intake.

The EB has been successfully used for inducing ovulations at the end of the synchronization protocol (Hanlon *et al*., 1997; Cavalieri *et al*., 2002). Estradiol cypionate (EC) is another ester of E2 with low

water solubility. Despite differences in pharmacodynamics, both esters of estradiol (EB and EC) administered either at P4 device removal (EC) or 24 h later (EB) were effective in inducing synchronized LH surge and ovulations and similar P/AI in suckled *Bos indicus* beef cows submitted to TAI (EB = 57.5%; 277/482 vs. EC = 61.8%; 291/471; Sales *et al*., 2012). Furthermore, in lactating Holstein cows the EC administered at P4 device removal (3 animal handlings) or GnRH 48 h later (4 animal handlings) presented similar LH surge (time of the LH peak averaged 43.6 h after P4 device removal) and P/AI (TAI performed 58h after P4 device removal for both treatments, EC = 30.0%; 117/390 vs. GnRH = 31.4%; 123/392; Souza *et al*., 2009). The use of EC as the ovulatory stimulus given at the time of P4 device removal in the TAI protocol reduces the handling, without reducing fertility.

Furthermore, to achieve better genetic and production gains, reproductive strategies should focus on improving service rates and reducing the interval between inseminations, without compromising the viability of the previously established gestation (Sá Filho *et al*., 2014). Based on this concept, ovulation resynchronization protocols were developed for females that did not become pregnant. In these reproductive programs, the non-pregnant females from the previous TAI were identified as soon as possible and inseminated again, therefore increasing the proportion of pregnant cows per AI (Baruselli *et al*., 2017b). This procedure promotes the anticipation of conception in the breeding season; concentrating parturitions at the beginning of the calving season and increasing reproductive efficiency at the subsequent breeding season (Sá Filho *et al*., 2013; Bo and Baruselli, 2014; Bo *et al*., 2016).

Conventional resynchronization is initiated at the time of pregnancy diagnosis (28 to 32 days after TAI; Marques *et al*., 2012; Stevenson *et al*., 2003). With this method, it is possible to perform three inseminations with an 80-days interval. Subsequently, early resynchronization was developed, which is initiated in all females (independently of the pregnancy diagnosis) 22 days after the TAI. On day 30 all females are submitted to pregnancy diagnosis and only the non-pregnant females carry on the TAI protocol, receiving the next artificial insemination on day 32 (Sá Filho *et al*., 2014). With this method, it is possible to perform three inseminations with a 64-days interval. Recently the super-early resynchronization protocol was developed, in which it is possible to perform three inseminations in 48 days. This resynchronization starts in all females 14 days after TAI. On the 22nd, all the non-pregnant females were diagnosed using Doppler ultrasonography (Vieira *et al*., 2014), analyzing the presence and vascular flow of the CL (Pugliesi *et al*., 2017; Siqueira *et al*., 2013).

According to Vieira *et al*. (2014), the application of 1.5 mg of estradiol benzoate (EB) on days 13 to 14 after previous TAI induced luteolysis and reduced conception rate of the first insemination differently from resynchronized females at 22 days post-TAI, which do not present luteolysis after the treatment with estradiol benzoate at the beginning of the resynchronization protocol (Sá Filho *et al*., 2014). Thus, super-early resynchronization is based on the use of injectable P4 at the time of insertion of the P4 device (14 after TAI) to induce the emergence of a new wave of follicular growth (Cavalieri, 2018) without impairing the gestation established in the previous AI (Rezende *et al*., 2016; Guerreiro *et al*., 2018; Gonçales-Junior *et al*., 2018). However, recently Motta *et al*. (2018) reported that 16- to 18-month-old Nellore heifers submitted to super-early resynchronization with 1 mg of EB at day 14 showed early luteolysis, but there was no reduction in the conception rate of the first TAI. This information is indicative of conflicting scientific findings and justify further studies to evaluate the effect of treatment with estradiol at the onset of the super-early resynchronization protocol (Day 14).

Embryo Transfer Market

The *in vivo* embryo production (*in vivo* derived – IVD) in Brazil had its onset in 1977 (Rubin, 2005), reaching a production of 24,085 embryos in 1997 (IETS, 1998). In the next 7 years, the IVD embryo production increased more 4 fold, with a production of 102,100 embryos in 2004 (IETS 2005). As of this moment, the *in vitro* production (IVP) of embryos, which was implemented in Brazil in 1993 (Rubin, 2005), becomes noticeable, approaching the IVD embryo production (IVD: 102,100 vs. IVP: 80,833). In 2004, 30% of transferred embryos in the world were derived from the IVP of embryos (IVD: 789,000 vs. IVP: 239,813).

In 2007, 46,694 IVD embryos and around 200,000 IVP embryos were transferred in Brazil, clearly showing the exponential growth of the *in vitro* embryo production market (IETS, 2008). At that time, over 90% of these embryos (both IVD and IVP) were produced in beef herds. In 2008, Brazil accounted for 86% of the world production of IVP embryos (IETS, 2009). These data demonstrate that Brazil was a world leader in the use of this technology.

While the IVD embryo production maintained constancy between 2007 (46,694) and 2013 (50,455), the *in vitro* embryo production continued enhancing, reaching its peak production in 2013 with 366,517 IVP embryos (IETS, 2014).

In addition, according to recent data published by the IETS in 2018 (Viana, 2018), there has been an increase of 48.9% in the number of IVP embryos in the world in comparison to the previous year. On the other hand, the number of IVD embryos production had a reduction of 21.7%. Nowadays, the number of IVD embryos produced in the world (495,054) is two times lower than IVP embryos (992,289; Viana, 2018). This increase on the IVP embryos in 2017 occurred mainly in North America (82.7%) and in Europe (164.7%). For the first time since 1999, the number of IVP embryos in North America overcame the number of IVP embryos in South America.

In South America, Brazil and Argentina stand out as the largest bovine herds and largest embryos producers (Viana *et al*, 2018). In 2017, Brazil was

responsible for the production of 345,528 IVP embryos, which represents 76.2% of all IVP embryos in South America.

In 2017 in Brazil, the IVP embryos was higher for dairy (180.475 embryos) than for beef (165,053 embryos) herds, and in Argentina (2nd in the ranking for IVP embryos in South America), the number of IVP embryos for beef herds was higher than for dairy herds (31,034 vs. 1,710).

In South America the IVD embryo production is of 49,230 embryos, with an average of 5.5 embryos per flushing. Brazil is responsible for approximately 60% of the IVD embryos produced in South America. The second in the ranking is Argentina, comprising 36% of this production. In Brazil, there is a noticeable difference in the proportion of IVD embryos in dairy and beef herds in the last 3 years (Tab. 1). In beef herds, the IVD embryos represents nowadays only 24% of the Brazilian production, the remaining (76%) belongs to the dairy herds. In Argentina, this proportion is reversed, where the beef herds comprise 92% of the IVD embryos market.

Table 1. Proportion of IVD and IVP embryos for beef and dairy herds in Brazil from 2015 to 2017.

	In vivo			*In vitro*		
	Beef	Dairy	Total embryos	Beef	Dairy	Total embryos
2015	73%	27%	22,355	43%	57%	353,539
2016	48%	52%	31,683	46%	54%	346,817
2017	24%	76%	29,533	48%	52%	345,528

Evolution of the synchronization and resynchronization protocols for TET

The technique of synchronization of ovulation for timed embryo transfer (TET) significantly increases the amount of recipients suitable for receiving an embryo, making it feasible and easy to use this technology for beef and dairy herds (Baruselli *et al*., 2000; Bo *et al*., 2002; Nasser *et al*., 2004). After synchronization, 75 to 85% of the recipients present a CL that is suitable for ET without the need for estrous detection (Baruselli *et al*., 2010). TET can be used in cyclic or anestrous recipients, optimizing the reproductive efficiency in beef and dairy herds (Rodrigues *et al*., 2010).

In addition to optimizing the dissemination of high value genetic material, the ET improves the reproductive efficiency of repeat-breeder cows and reduces fertility impact caused by heat stress (Baruselli *et al*., 2010). The TET can be used in repeat-breeder cows to increase conception rates mainly during warm periods of the year (Freitas *et al*., 2010, Rodrigues *et al*., 2010; Ferreira *et al*., 2011). In studies conducted in Brazilian tropical conditions, it was observed that the oocyte quality is affected by heat stress during the summer (Torres-Junior *et al*., 2008), which interferes with initial embryo development, especially in repeat-breeder cows (Ferreira *et al*., 2011; Ferreira *et al*., 2016). Studies have indicated that it is possible to obtain a larger numbers of pregnant females in dairy herds with the use of ET during the summer and in repeat-breeder cows.

In beef herds, studies evaluated the reproductive efficiency of synchronization and resynchronization techniques used simultaneously for TAI and TET. In one study (Martins et al., 2014), 634 lactating Nellore cows were submitted to four treatments: two consecutive TAI (2TAI; n = 160), TAI followed by TET (TAI/TET; n = 160); TET followed by TAI (TET/TAI; n = 158) and two consecutive TET (2TET; n = 156). The embryos were produced *in vitro* and transferred fresh. The pregnancy rate after the first service was higher for managements with 2TAI (59.4%, 95/160) and TAI + TET (59.4%, 95/160) compared to TET + TAI (31.7%, 50/158) and 2TET (32.7%, 51/156, Figure 5). Furthermore, the accumulated pregnancy rate (1st + 2nd service) was lower for the animals that received 2 TET ($P < 0.0001$; Fig. 5) when compared to animals that received 2 TAI and TAI+TET. However, no differences were observed between 2 TAI and TAI+TET reproductive programs. These data are indicative of high reproductive efficiency for programs associating TET with TAI.

In order to reduce the negative effects of pregnancy rate using TET, another study was carried out with two resynchronizations (early resynchronization protocol starting 22 days after AI and with diagnosis of gestation at day 30) in Nellore lactating cows (n = 360; unpublished data). The animals received a TET, followed by 2 TAI in a 64-day breeding season. The pregnancy rate for TET was 44.2%, followed by 55.7% for the first TAI (second service) and 47.1% for the second TAI (third service), comprising 86.9% pregnancy rate at the end of 64 days breeding season. These data are indicative that it is possible to use TET on the first service, followed by resynchronizations with TAI, with satisfactory results in the herds breeding season.

Aiming to improve the efficiency of reproductive programs, research groups have studied the use of Doppler ultrasonography (to evaluate the vascularization of the CL) for TET. Several studies have observed the effect of the CL blood flow on embryo recipients pregnancy (Pinaffi, 2015; Pugliesi *et al*., 2016), which allows the use of Doppler ultrasonography to rule out recipients presenting CL without vascularization, directing desirable embryos to more fertile cows (Pugliesi *et al*., 2017).

The possibility of initiating resynchronization after the early pregnancy diagnosis using Doppler was

also evaluated. A total of 165 embryo recipients were diagnosed at 21 days, aiming to improve reproductive management of TET programs (Guimarães *et al*., 2015). The accuracy and sensitivity of the early diagnosis at 21 days was of 88.3% and 100%, respectively. This early diagnosis with Doppler enabled 80% of the non-pregnant recipients to be diagnosed at 21 days of gestation and thus resynchronized early for a new TET program.

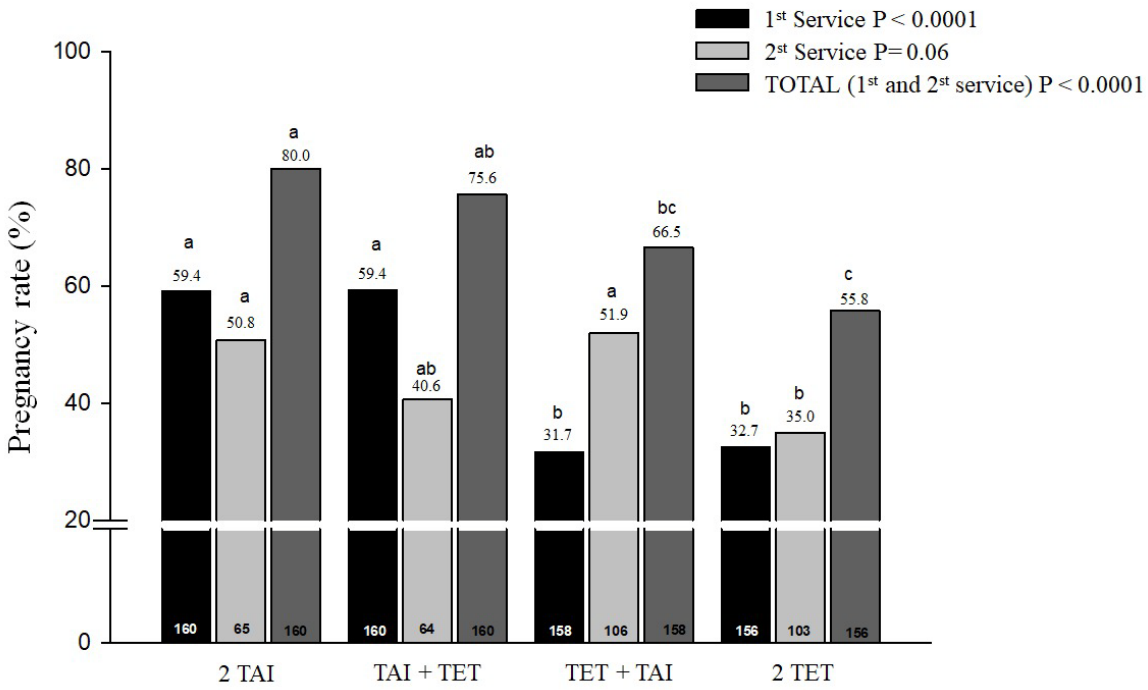


Figure 5. Pregnancy rate of the first and second services and cumulative pregnancy rate (first + second service) in Nelore (*Bos indicus*) lactating cows submited to four reproductive managements: 2TAI (n = 160), TAI followed by TET (TAI/TET; n = 160), TET followed by TAI (TET/TAI; n = 158) and two TET (n = 156). Adapted from Martins *et al*., (2014).

Challenges to increase the use of reproductive biotechnologies in Brazil

The markets for AI and ET in Brazil have grown considerably in the last 20 years. The technological development of the reproductive processes has reached high efficiency with favorable cost-benefit when applied in the farms for meat and milk production. However, the challenge to keep the market growing in the next decade could depend on some factors, such as:

- Extension services for producers: transfer the scientific and technical knowledge related to increased farm productivity and economic benefits of the application of reproductive biotechnologies on farms, providing a clear message to the users (many farmers do not know the advantages of using reproductive biotechnologies involved with breeding programs).

- **Extension training for specialist:** the educational programs for training new specialists to apply assisted reproductive technologies on farms to support the continuous growth of these technologies (to reach the world average of inseminated cattle in Brazil, 4000 veterinarians working on the farms are necessary).

- **Technological development:** continue to develop more efficient and cost-effective products and practical protocols to apply on the farm to enhance productivity and profitability. Conduct more research to increase the efficiency of AI and ET programs taking into account the interaction between nutrition and reproduction, genetics and reproduction, health and reproduction and environment and reproduction.

- **Increase the integration:** increase the cooperation between universities, research institutes, veterinarians, industries for cattle AI and ET, allied animal production industries, farm associations and producers with the intent of more effectively transferring the use of reproductive technologies to the field.

- Market demand: in the world there is a strong demand for production of animal protein with higher efficiency and environmental and economic sustainability. The use of reproductive biotechnologies increases productivity per unit of land and significantly contributes to improve the efficiency of livestock. Therefore with the intensification of the use of reproductive biotechnologies it is possible to enhance production with reduced environmental impact. This scenario contributes to change the production system and for farmers to seek new alternatives, in which reproductive biotechnologies are included.

Conclusions

The use of reproductive biotechnologies (AI and ET) to multiply superior individuals results in significant genetic advancement of the herds and

increased productivity. Despite all the scientific and technological advances that have occurred in recent years, these biotechnologies are still used in small extent in rural properties. Only 13% of Brazilian cows and heifers are artificially inseminated, compared to 20-22% in the world (Thibier and Wagner, 2002; Vishwanath *et al*., 2003). The Brazilian AI market needs to grow 5% per year in the next 10 years to reach the world average of inseminated bovine females. Although Brazil is the largest producer of embryos in the world, the intensity of use of ET is low (number of embryos transferred/total of the herd), ranking the country in the 11th position in the world (Viana *et al*., 2017). Therefore, the Brazilian livestock needs to intensify the use of reproductive biotechnologies for the genetic and productive improvement of the herd. To change this scenario, efficient reproductive programs that aim to increase the use of artificial insemination and embryo transfer on farms have been developed. These programs are currently established and present a positive economic return (Baruselli *et al*., 2018b), generating higher meat and milk production per area and more value for the beef and dairy production chain.

Author contributions

PSB: Conceptualization, Resources, Supervision, Visualization, Writing – review & editing; Bruna Lima Chechin Catussi (BLCC): Formal analysis, Visualization, Writing – original draft; LAA:Visualization, Writing – original draft; FME: Visualization, Writing – original draft, Writing – review & editing; LGS: Visualization, Writing – original draft; EOSB: Formal analysis, Visualization, Writing – review & editing.

Conflict of interest

There is no conflict of interest. Authors are required to include a statement disclosing any potential conflicts of interest.

Acknowledgments and Funding disclosure statement

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001, Fapesp and CNPq .

References

Anuário da Pecuária Brasileira (ANUALPEC). 2012. Rebanho bovino brasileiro. Sumário. São Paulo: FNP.

Anuário da Pecuária Brasileira (ANUALPEC). 2018. Rebanho bovino brasileiro. Sumário. São Paulo: FNP.

Associação Brasileira das Indústrias Exportadoras de Carnes (ABIEC). 2018. Sumário. Available on: http://abiec.siteoficial.ws/images/upload/sumario-pt-010217.pdf.

Associação Brasileira de Inseminação Artificial (ASBIA). 2019. Index ASBIA Mercado. Available on: https://www.lancerural.com.br/vendas-de-semen-bovino-crescem-no-1o-semestre-de-2018/presidente-da-asbia-sergio-saud-anuncia-aumento-nas-vendas-de-semen.

Baruselli PS, Marques MO, Madureira EH, Bó GA, Neto C, Pinto W, Grandinetti RR. 2000. Superestimulação ovariana de receptoras de embriões bovinos visando o aumento de corpo lúteo, concentração de P4 e taxa de prenhez. Arquivos da Faculdade de Veterinária - UFRGS, 28(1):218.

Baruselli PS, Marques MO, Carvalho NAT, Madureira EH, Campos Filho EP. 2002. Efeito de diferentes protocolos de inseminação artificial em tempo fixo na eficiência reprodutiva de vacas de corte lactantes. *Rev Bras Reprod Anim*, 26:218-221.

Baruselli PS, Reis EL, Marques MO, Nasser LF, Bó GA. 2004. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. *Anim Reprod Sci*, 82:479-486.

Baruselli PS, Ferreira RM, Sá Filho MF, Nasser LFT, Rodrigues CA, Bó GA. 2010. Bovine embryo transfer recipient synchronization and management in tropical environments. *Reprod Fertil Dev*, 22:67-74.

Baruselli PS, Sales JNS, Sala RV, Vieira LM, Sá Filho MF. 2012. History evolution and perspectives of timed artificial insemination programs in Brazil. *Anim Reprod*, 9:139-152.

Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Freitas BG. 2017a. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*, 14:558-571.

Baruselli PS, Marques MO, Borges A, Penteado L. 2017b. Impactos econômicos do uso de tecnologia reprodutiva na fazenda. In: Encontro dos Encontros da Scot Consultoria, 4, 2017. Anais... Ribeirão Preto: Suprema Gráfica e Editora. Available on: https://www.scotconsultoria.com.br/loja/livros/125/anais-do-encontro-dos-encontros-da-scot-consultoria-2017.

Baruselli PS, Ferreira RM, Sá Filho MF, Bó GA. 2018a. Review: Using artificial insemination v. natural service in beef herds. *Animal*, 12:45-52.

Baruselli PS, Souza AH, Sá Filho MF, Marques MO, Sales JNS. 2018b. Genetic market in cattle (Bull, AI, FTAI, MOET and IVP): financial payback based on reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*, 15:247-255.

Baruselli PS. 2019a. Avaliação do mercado de IATF no Brasil. Boletim Eletrônico do Departamento de Reprodução Animal/FMVZ/USP, 1ed., 2019. Available on: http://vra.fmvz.usp.br/boletim-eletronico-vra.

Baruselli PS. 2019b .IATF gera ganhos que superam R$ 3,5 bilhões nas cadeias de produção de carne e de leite. Boletim Eletrônico do Departamento de Reprodução Animal/FMVZ/USP, 2ed., 2019. Available on: http://vra.fmvz.usp.br/boletim-eletronico-vra.

Batista EOS, Macabelli CH, Chiaratti MR, Yasuoka MM, Sala RV, Ortolan MDDV, Jesus EF, Del Valle TA, Rennó FP, Baruselli PS. 2015. Impact of bovine genetic group (Bos indicus vs Bos taurus) and level of dry matter intake (high vs. low) on gene expression of liver enzymes related to progesterone metabolism. *Anim Reprod*, 12:642. (Abstract).

Batista EOS, Guerreiro BM, Freitas BG, Silva JCB, Vieira LM, Ferreira RM, Rezende RG, Basso AC, Lopes RNVR, Rennó FP, Souza AH, Baruselli PS.

2016. Plasma anti-Müllerian hormone as a predictive endocrine marker to select Bos taurus (Holstein) and Bos indicus (Nelore) calves for in vitro embryo production. *Domestic Animal Endocrinology*, 54:1–9.

Bó GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tríbulo R, Tríbulo H, Mapletoft RJ. 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology*, 57:53- 72.

Bó GA, Cutaia L, Peres LC, Pincinato D, Maraña D, Baruselli PS. 2007. Technologies for fixed-time artificial insemination and their influence on reproductive performance of Bos indicus cattle. *Soc Reprod Fertil Suppl*, 64:223-236.

Bó GA, Baruselli PS. 2014. Synchronization of ovulation and fixed-time artificial insemination in beef cattle. Animal, 8(s1):144–150.

Bó GA de la Mata JJ, Baruselli PS, Menchaca A. 2016. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology* 86(1):388-396.

Bó GA, Huguenine E, Mata JJ, Núñez-Olivera R, Baruselli PS, Menchaca A. 2018. Programs for fixed-time artificial insemination in South American beef cattle. *Anim Reprod*, 15(Suppl.1):952-962.

Caraviello DZ, Weigel KA, Fricke PM, Wiltbank MC, Florent MJ, Cook NB, Nordlund KV, Zwald NR, Rawson CL. 2006. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. *J Dairy Sci*, 89(1):4723-35.

Carvalho JBP, Carvalho NAT, Reis EL, Nichi M, Souza AH, Baruselli PS. 2008. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus x Bos taurus, and Bos taurus heifers. *Theriogenology*, 69:167-175.

Cavalieri J, Coleman C, Rodrigues H, Macmillan KL, Fitzpatrick LA. 2002. The effect of timing of administration of oestradiol benzoate on characteristics of oestrus, timing of ovulation and fertility in Bos indicus heifers synchronised with a progesterone releasing intravaginal insert. *Aust Vet J*, 80(4):217-23.

Cavalieri J. 2018. Effect of treatment of Bos indicus heifers with progesterone 0, 3 and 6 days after follicular aspiration on follicular dynamics and the timing of oestrus and ovulation. *Anim Reprod Sci*, 193:9-18.

Centro de Estudos Avançados em Economia Aplicada (CEPEA). Boletim do leite, 2019. Available on: https://www.cepea.esalq.usp.br/br/categoria/boletim-do-leite.aspx. Access on: 22/04/2019.

Confederação da Agricultura e Pecuária do Brasil (CNA). 2019. Available on: https://www.cnabrasil.org.br/assets/arquivos/boletinstecnicos/Comunicado-T%C3%A9cnico-08.ed-14-mar.pdf. Access: 24/06/2019.

Ferreira RM, Ayres H, Chiaratti MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, Vireque AA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS. 2011. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J Dairy Sci*, 94(5):2383-2392.

Ferreira RM, Chiaratti MR, Macabelli CH, Rodrigues CA, Ferraz ML, Watanabe YF, Smith LC, Meirelles FV, Baruselli PS. 2016. The infertility of repeat-breeder cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes in oocytes. *Biol Reprod*, 94(3):1-10.

Food and Agriculture Organization (FAO). 2017. Cenário da demanda por alimentos no Brasil. Rome: FAO, 2017. Available on: http://www.fao.org/brasil/noticias/detail-events/pt/c/901168/. Access: 03/02/2019.

Freitas BG, Rodrigues CA, Sales JNS, Teixeira AA, Ferreira RM, Ayres H, Ranieri AL, Baruselli PS. 2010. Embryonic loss (between 30 and 60 days) followed to artificial insemination or embryo transfer in high production Friesian dairy cattle. *Acta Sci Vet*, 38:781. (Abstract).

Gonçales-Junior WA, Colli MHA, Catussi BLC, Rezende RG, Andrea DL, Castro MW, Freitas BG, Guerreiro BM, Baruselli PS. 2018. Effect of treatment with injectable progesterone at the onset of super precocious TAI ressincronization protocol on TAI pregnancy rates of Nellore heifers. *Anim Reprod*, 15(3):337. (Abstract).

Guerreiro BM, Freitas BG, Colli MHA, Rezende RG, Penteado L, Gonçales-Junior WA, Baruselli PS. 2018. P4 serum concentration at the time of TAI in Nelore females treated with 50 vs. 100 mg of injectable P4 (short or long action) in the super precocious resynchronization protocol. *Anim Reprod*, 15(3):317. (Abstract).

Guimarães, CRB, Oliveira, ME, Rossi, JR, Fernandes, CAC, Viana, JHM, Palhao, MP. 2015. Corpus luteum blood flow evaluation on Day 21 to improve the management of embryo recipient herds. *Theriogenology*, 84:237-241.

Hanlon DW, Williamson NB, Wichtel JJ, Steffert IJ, Craigie AL, Pfeiffer DU. 1997. Ovulatory responses and plasma luteinizing hormone concentrations in dairy heifers after treatment with exogenous progesterone and estradiol benzoate. *Theriogenology*, 47:963-975.

Instituto Brasileiro de Geografia e Estatística (IBGE). 2017. Sistema de Recuperação Automática (SIDRA). Efetivo do rebanho brasileiro. Available on: https://sidra.ibge.gov.br/pesquisa/ppm/quadros/brasil/2017. Access on: 05/04/2019.

Instituto Brasileiro de Geografia e Estatística (IBGE). 2018. Sistema de Recuperação Automática (SIDRA). Efetivo do rebanho brasileiro. Available on: https://sidra.ibge.gov.br/tabela/3939#resultado. Access on: 02/04/2019.

International Embryo Technology Society (IETS). 1998. Data Retrieval Annual Report. Available on: https://www.iets.org/pdf/comm_data/December1998.pdf. Access: 15/04/2019.

International Embryo Technology Society (IETS). 2005. Data Retrieval Annual Report. Available on: https://www.iets.org/pdf/comm_data/December2005.pdf. Access: 15/04/2019.

International Embryo Technology Society (IETS). 2008. Data Retrieval Annual Report. Available on: https://www.iets.org/pdf/comm_data/December2008.pdf.

Access: 15/04/2019.

International Embryo Technology Society (IETS). 2009. Data Retrieval Annual Report. Available on: https://www.iets.org/pdf/comm_data/December2009.pdf. Access: 15/04/2019.

International Embryo Technology Society (IETS). 2014. Data Retrieval Annual Report. Available on: https://www.iets.org/pdf/comm_data/December2014.pdf. Access: 15/04/2019.

International Embryo Technology Society (IETS). 2018. Embryo Technology Newsletter, 36(4):46p. Available on: https://www.iets.org/pdf/Newsletter/Dec18_IETS_Newsletter.pdf.

Lamb GC, Mercadante VRG. 2016. Synchronization and artificial insemination strategies in beef cattle. *Vet Clin North Am Food Anim Pract*, 32:335-334.

Lima FS, Vries ADE, Risco CA, Santos JEP, Thatcher WW. 2010. Economic comparison of natural service and timed artificial insemination breeding programs in dairy cattle. *J Dairy Sci*, 93:4404-4413.

Mantovani AP, Reis EL, Gacek F, Bó GA, Binelli M, Baruselli PS. 2005. Prolonged use of a progesterone releasing intravaginal device (CIDR®) for induction of persistent follicles in bovine embryo recipients. *Anim Reprod*, 2:272-277.

Mantovani AP, Nichi M, Sá Filho MF, Ayres H, Vettorato LF, Bó GA. 2010. Follicular growth and plasma progesterone patterns in Bos indicus x Bos taurus heifers submitted to different PGF2α/progesterone-based synchronization protocols, *Anim Reprod*, 7:91-96.

Mapletoft RJ, Bó GA, Baruselli PS, Menchaca A, Sartori R. 2018. Evolution of knowledge on ovarian physiology and its contribution to the widespread application of reproductive biotechnologies in South American cattle. *Anim Reprod*, 15(Suppl.1):1003-1014.

Marques MO, Ribeiro Júnior M, Silva RCP, Sá Filho MF, Vieira LM, Baruselli PS. 2012. Ressincronização em bovinos de corte. In: Simpósio Internacional de Reprodução Animal Aplicada (SIRAA), 5, 2012, Londrina, PR, Brazil. Londrina: UEL, p.82-92.

Martins CM, Reis, PO, Vieira, JH, Soares, JG, Vieira, LM, Sá Filho, MF, Baruselli, PS. 2014. Effect of association of FTET and TAI in reproductive programs of Nelore females. *Anim Reprod*, 11(3):347.

Meneghetti M, Sá Filho OG, Peres RFG, Lamb GC, Vasconcelos JLM. 2009. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: basis for development of protocols. *Theriogenology*, 72:179-189.

Motta IG, Rocha CC, Bissinoto DZ, Arantes G, Junior A, Melo GD, Lafuente BS, Bastos MR, Kleber ML, Madureira EH, Pugliesi G. 2018. A novel and safe strategy for resynchronization using estradiol 14 days after timed-ai in beef heifers. *Anim Reprod*, 15(3):323. (Abstract).

Nasser LF, Reis EL, Oliveira MA, Bó GA, Baruselli PS. 2004. Comparison of four synchronization protocols for fixed-time bovine embryo transfer in Bos indicus x Bos taurus recipients. *Theriogenology*, 62(9):1577-1584.

Nebel RL. 2003. The key to a successful reproductive management program. *Adv Dairy Technol*, 15:1-16.

Nogueira, MP. 2019. Até quando vai o bom momento da pecuária leiteira? Available on: www.rallydapecuaria.com.br. Access: 03/06/2019.

Pinaffi FL, Santos ÉS, Silva MGD, Maturana Filho M, Madureira EH, Silva LA. 2015. Follicle and corpus luteum size and vascularity as predictors of fertility at the time of artificial insemination and embryo transfer in beef cattle. *Pesq Vet Bras*, 35:(5):470-476.

Pugliesi G, Silva JCB, Nishimura T, Miyai D, Silva LA, Binelli M. 2016. Use of Color-Doppler ultrasonography to improve selection of higher fertility beef recipiente cows for embryo transfer. *Anim Reprod*, 13(3):454. (Abstract).

Pugliesi G, Rezende RG, Da Silva JCB, Lopes E, Nishimura TK, Baruselli PS, Madureira EH, Binelli M. 2017. Uso da ultrassonografia Doppler em programas de IATF e TETF em bovinos. *Rev Bras Reprod Anim*, 41(1):140-150.

Pursley JR, Mee MO, Wiltbank MC. 1995. Synchronization of ovulation in dairy cows using PGF2α and GnRH. *Theriogenology*, 44:915-923.

Rezende RG, Freitas BG, Mingoti RD, Colli MHA, Carvalho JBP, Sá Filho MF, Motta JCL, Macedo GG, Baruselli PS. 2016. Follicular dynamics of nelore cows submitted to resynchronization 14 days after tai using injectable p4 for synchronization of follicular wave. *Anim Reprod*, v.13(3):233. (Abstract).

Rhodes FM, McDougall S, Burke CR, Verkerk GA and Macmillan KL. 2003. Treatment of cows with an extended postpartum anestrous interval. *J Dairy Sci*, 86:1876-1894.

Rodgers JC, Bird SL, Larson JE, DiLorenzo N, Dahlen CR, DiCostanzo A, Lam GC. 2015. An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. *J Anim Sci*, 10:1297-1308.

Rodrigues CA, Teixeira AA, Ferreira RM, Ayres H, Mancilha RF, Souza AH, Baruselli PS. 2010. Effect of fixed-time embryo transfer on reproductive efficiency in high-producing repeat-breeder Holstein cows. *Anim Reprod Sci*, 118:110-117.

Rubin MIB. 2005. Histórico dos 20 anos da Sociedade Brasileira de Tecnologia de Embriões (1985-2005). *Acta Scientiae Veterinariae*, 33 (Suppl.1):35-54.

Sá Filho MF, Penteado L, Reis EL, Reis TANPS, Galvão KN, Baruselli PS. 2013. Timed artificial insemination early in the breeding season improves the reproductive performance of suckled beef cows. *Theriogenology*, 79(4):625-632.

Sá Filho MF, Marques MO, Girotto R, Santos FA, Sala RV, Barbuio JP, Baruselli PS. 2014. Resynchronization with unknown pregnancy status using progestin-based timed artificial insemination protocol in beef cattle. *Theriogenology*, 81:284-290.

Sá Filho OG, Meneghetti M, Peres RFG, Lamb GC, Vasconcelos JLM. 2009. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows II: Strategies and factors affecting fertility. *Theriogenology*, 72:210-218.

Sales JNS, Carvalho JBP, Crepaldi GA, Cipriano RS, Jacomini JO, Maio JRG, Souza JC, Nogueira GP, Baruselli OS. 2012. Effects of two estradiol esters (benzoate and cypionate) on the induction of synchronized ovulations in Bos indicus cows submitted to a timed artificial insemination protocol. *Theriogenology*, 78:510-516.

Sartori R, Prata AB, Figueiredo ACS, Sanches BV, Pontes GCS, Viana JHM, Pontes JH, Vasconcelos JLM, Pereira MHC, Dode MAN, Monteiro Jr PLJ, Baruselli PS. 2016. Update and overview on assisted reproductive technologies (ARTs) in Brazil. *Anim Reprod*, 13(3):300-312.

Siqueira LG, Areas VS, Ghetti AM, Fonseca JF, Palhao MP, Fernandes CA, Viana JH. 2013. Color Doppler flow imaging for the early detection of nonpregnant cattle at 20 days after timed artificial insemination. *J Dairy Sci*, 96:6461-6472.

Souza AH, Viechnieski S, Lima FA, Silva FF, Araujo R, Bo GA, Wiltbank MC, Baruselli PS. 2009. Effects of equine chorionic gonadotropin and type of ovulatory stimulus in a timed-AI protocol on reproductive responses in dairy cows. *Theriogenology*, 72:10-21.

Stevenson JS, Cartmill JA, Hensley BA, El-Zarkouny SZ. 2003. Conception rates of dairy cows following early not-pregnant diagnosis by ultrasonography and subsequent treatments with shortened Ovsynch protocol. *Theriogenology*, 60:475-483.

Teixeira AA. 2010. Impacto da Inseminação Artificial em Tempo Fixo na eficiência reprodutiva de vacas de leite de alta produção. 71p. Dissertação para obtenção do título de mestre em Ciências Veterinárias. Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo.

Thibier M, Wagner HG. 2002. World Statistics for artificial insemination in cattle. Livest Prod Sci, 74(2):203-2012.

Torres-Júnior JDS, De FA Pires M, De Sá WF, Ferreira ADM, Viana JHM, Camargo LSA, Ramos AA, Folhadella IM, Polisseni J, de Freitas C, Clemente CAA, de Sá Filho MF, Paula-Lopes FF, Baruselli PS. 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in Bos indicus cattle. *Theriogenology*, 69(2):155-166.

Viana JHM, Figueiredo ACS, Siqueira LGB. 2017. Brazilian embryo industry in context: pitfalls, lessons, and expectations for the future. *Anim Reprod*, 14:476-481.

Viana JHM, Figueiredo ACS, Gonçalves, RLR, Siqueira LGB. 2018. A historical perspective of embryo-related technologies in South America. *Anim Reprod*, 15(Suppl.1):963-970.

Viana, J. 2018. Statistics of embryo production and transfer in domestic farm Animals. A publication of International Embryo Technology Society (IETS), *Embryo Technology Newsletter 2017*, 36(4):8-19.

Vieira LM, Sá Filho MF, Pugliesi G, Guerreiro BM, Cristaldo MA, Batista EOS, Freitas BG, Carvalho FJ, Guimaraes LHC, Baruselli PS. 2014. Resynchronization in dairy cows 13 days after TAI followed by pregnancy diagnosis based on corpus luteum vascularization by color doppler. *Anim Reprod*, 11:378. (Abstract).

Vishwanath R. 2003. Artificial insemination: the state of the art. *Theriogenology*, 59:571-584.

DOI: 10.21451/1984-3143-AR2019-0075

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Aspects of embryo-maternal communication in establishment of pregnancy in cattle

José M. Sánchez, Constantine A. Simintiras, Patrick Lonergan*

School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland.

Abstract

Establishment of pregnancy in mammals requires reciprocal molecular communication between the conceptus and endometrium that modifies the endometrial transcriptome and uterine luminal milieu to support pregnancy. Due to the small size of the early embryo and elongating conceptus relative to the volume of the uterine lumen, collection of endometrium adjacent to the developing conceptus is difficult following conventional uterine flushing methods in cattle. Use of endometrial explants in culture can overcome this challenge and reveal information about the dialogue between the developing embryo and the uterus. The aim of this short review is to summarize some of our recent findings in relation to embryo maternal interaction during bovine pregnancy establishment and to put them in the wider context of fertility in cattle.

Keywords: conceptus, fertility, bovine, uterus, progesterone.

Introduction

Embryo mortality is a major contributor to poor reproductive efficiency in dairy and beef cows. A significant proportion of embryonic loss in cattle, particularly lactating dairy cows, occurs during the first 2-3 weeks after conception, before maternal recognition of pregnancy, which occurs around Day 16. In a recent comprehensive review, Wiltbank *et al*. (2016) described four pivotal periods of pregnancy loss during the first trimester of gestation and discussed possible causes for pregnancy failure during these periods. Despite a relatively high fertilization rate (>85%), 20%-50% of high-producing lactating dairy cows experience pregnancy loss during the first week of gestation. From Days 8 to 27, concomitant with embryo elongation and maternal recognition of pregnancy, losses average approximately 30%. From Days 28 to 60, losses of approximately 12% occur while in the fourth period, during the third month of pregnancy, pregnancy losses are reduced (approximately 2%), but may be elevated in some cows, particularly in those carrying twins in the same uterine horn (Wiltbank *et al*., 2016).

Communication between the developing embryo and the mother is vital for the successful establishment and maintenance of pregnancy but the requirement for this dialogue is temporal in nature and in reality only becomes absolutely essential from around Day 15-16 onwards. Pregnancies are routinely established in commercial embryo transfer following the transfer of 6-7 day old embryos, often produced in vitro, to synchronized recipients. Indeed, pregnancy can be established following the transfer of elongated conceptuses up to at least Day 16 (Betteridge *et al*., 1980; Kimura and Matsuyama 2014) indicating that the uterus does not need to receive any signals from the embryo prior to that stage in order for pregnancy occur.

Up to the blastocyst stage (Day 7-8), the bovine embryo is relatively autonomous, as blastocysts can be produced in vitro in the absence of contact with the female reproductive tract and are capable of establishing pregnancy after transfer to a synchronous uterus. In contrast to primates and rodents, in which implantation occurs shortly after the blastocyst enters the uterus, in ungulates, such as ruminants and pigs, the early conceptus undergoes a phase of rapid growth and elongation before implantation, the latter occurring 2-3 weeks after fertilization. In cattle, conceptus elongation is initiated around Day 13 of gestation when the hatched bovine blastocyst transitions sequentially from a spherical- to ovoid-, then tubular- and finally filamentous-shaped structure that primarily involves proliferation of the conceptus trophectoderm cells. During this time, the elongating conceptus secretes interferon tau (IFNT), the maternal pregnancy recognition signal in ruminants (Bazer and Thatcher 2017). While there is a strong positive correlation between conceptus length and IFNT secretion (Rizos *et al*., 2012), surprisingly, threshold concentrations of IFNT required to overcome luteolysis are as yet not known (Forde and Lonergan 2017; Spencer *et al*., 2017).

Elongation is necessary to ensure sufficient concentrations of IFNT are secreted and to expand the conceptus surface area for maximal vascular exchange with maternal tissues after implantation. An inability of the conceptus to optimally elongate undoubtedly results in embryonic loss and is believed to significantly contribute to reproductive failure in cattle (Wiltbank *et al*., 2016; Moraes *et al*., 2018). In contrast to pre-hatching development, elongation is predominantly maternally-driven, dependent on substances in the uterine lumen fluid (ULF; or histotroph). As evidence for this, blastocysts do not elongate in vitro (Brandão *et al*., 2004) and the absence of uterine glands in vivo results in failure of blastocysts to elongate following embryo transfer (Gray *et al*., 2002).

Spatial and temporal changes of the endometrial transcriptome and histotroph composition are necessary to establish uterine receptivity to implantation and, in turn, are pivotal to the likelihood of successful pregnancy in cattle. Those modifications are

*Corresponding author: pat.lonergan@ucd.ie

orcid.org/0000-0001-5598-5044

Received: June 5, 2019
Accepted: June 27, 2019

primarily regulated by the steroid hormone progesterone (P4) derived from the corpus luteum (CL) which acts via the endometrium to promote conceptus growth and implantation, as well as conceptus-derived IFNT, which prevents development of the endometrial luteolytic mechanism (Forde and Lonergan 2012; Brooks *et al*., 2014). The role of P4 in uterine receptivity is unequivocal (Lonergan *et al*., 2016; Spencer *et al*., 2016). Low circulating P4 concentrations in the first week after ovulation, as frequently occurs in high-producing lactating dairy cows, are associated with under-developed conceptuses (Forde *et al*., 2012) with an altered transcriptomic signature (Barnwell *et al*., 2016) and a low likelihood of establishing pregnancy (Wiltbank *et al*., 2016). On the other hand, elevated concentrations of circulating P4 in the period immediately after conception have been associated with advanced conceptus elongation (Carter *et al*., 2008), increased IFNT production (Mann and Lamming 2001), and greater pregnancy rates in cattle and sheep (Ashworth *et al*., 1989; Stronge *et al*., 2005; McNeill *et al*., 2006). Despite the definitive association between P4 and conceptus elongation, significant natural variation in age-matched in vivo- (Betteridge *et al*., 1980) and in vitro-derived conceptuses occurs, even amongst conceptuses developing in the same uterus (Clemente *et al*., 2009; Sánchez *et al*., 2019a; b). This would suggest that part of the ability to elongate is intrinsic to the embryo and may be related to oocyte and/or blastocyst quality.

The aim of this short review is to summarize some of our recent findings in relation to embryo maternal interaction during bovine pregnancy establishment and to put them in the wider context of fertility in cattle. Several of the recent studies referred to below have used an endometrial explant co-culture system to elucidate this fine dialogue by examining changes in endometrial gene expression induced by blastocysts (Passaro *et al*., 2018, 2019) or by an elongating conceptus (Mathew *et al*., 2019; Sánchez *et al*., 2019b; Bagés-Arnal *et al*., 2019). Due to the maintenance of normal cellular and extracellular architecture in endometrial explants (Borges *et al*., 2012), some of the limitations of traditional cell culture can be overcome; for example, uterine explants allow the communication between resident populations of endometrial cells which cannot be achieved with current 2D and 3D cell culture technologies.

Role of progesterone in uterine receptivity and conceptus elongation

Progesterone from the CL induces both temporal and spatial changes in the endometrial transcriptome necessary to establish uterine receptivity, when implantation in the uterus is possible (Forde *et al*., 2009). These changes include down-regulation of the nuclear progesterone receptor (PGR) in the luminal and then glandular epithelium (Okumu *et al*., 2010), which allows expression of genes and secretion of their protein products, as well as active transport of other molecules, required for conceptus elongation.

The capacity of the uterus to stimulate conceptus elongation is primarily dependent on secretions from the luminal and glandular epithelium. However, the timing of conceptus elongation is clearly associated with concentrations of P4 in circulation, which acts via the uterus (Clemente *et al*., 2009) to alter the timing of PGR downregulation and thus onset of expression of key genes required for elongation in cattle (Forde *et al*., 2009) and sheep (Satterfield *et al*., 2006). Consequently, P4 has an indirect effect on the secretion of IFNT by the conceptus, given the strong positive correlation between conceptus length and IFNT production (Rizos *et al*., 2012). In order for P4 output from the CL to be maintained, sufficient quantities of IFNT must be produced by the conceptus by Day 16 to abrogate the luteolytic mechanism and maintain CL function and induce expression of both classical and nonclassical interferon-stimulated genes (ISG) in the different cellular compartments of the endometrium that are proposed to regulate conceptus elongation.

Temporal changes in uterine gene expression occur irrespective of whether the cow is pregnant or not (Forde *et al*., 2009) and it is only during maternal recognition of pregnancy, around Day 16, by which time the conceptus is secreting copious amounts of IFNT (Forde and Lonergan 2017) that major changes in gene expression between cyclic and pregnant endometrium become apparent (Forde *et al*., 2011; Bauersachs *et al*., 2012).

Blastocyst-induced changes in the endometrium

Pregnancy recognition in cattle is initiated around Day 15-16, both at the physiological and transcriptomic level. Nonetheless, the first week of development is critical as evidenced by the fact that, at least in high-producing dairy cows, about 50% of embryos are no longer viable by Day 6-7 (Sartori *et al*., 2010). Whether communication between the embryo and endometrium at this stage is really important remains to be demonstrated convincingly. There is unequivocal evidence that when development occurs in vivo, blastocyst quality is improved in terms of ultrastructure (Rizos *et al*., 2002a), gene expression profiles (Lonergan *et al*., 2003a, b; Gad *et al*., 2012), cryotolerance (Rizos *et al*., 2002b) and pregnancy rate after transfer (Hasler *et al*., 1995) compared to when blastocysts are produced in vitro. However, evidence of a reciprocal effect of a single embryo on the cells of the uterus is more difficult to detect. As mentioned earlier, the fact that blastocysts can be produced routinely in vitro in the absence of contact with the reproductive tract and subsequently establish a pregnancy after transfer to a recipient supports the notion that exposure of the reproductive tract to the early embryo, or vice-versa, is not required for pregnancy.

In vitro studies have demonstrated that preimplantation embryos secrete a variety of biochemical messengers, embryotropins, that act in an autocrine manner to promote embryonic development (reviewed by Wydooghe *et al*., 2015). For many of these factors, expression of corresponding receptors in

the uterus has been identified, the activation of which could lead to cellular and tissue responses in regions that are in close physical contact with the embryo. Others have reported that the early bovine embryo (from Day 5 to Day 9) induces an anti-inflammatory response in uterine epithelial cells and immune cells in vitro (Talukder *et al*., 2017). Therefore, if factors secreted by the pre-elongating embryo enhance changes in the transcriptome and in the proteome of the endometrium, those changes are most likely to be local in nature and may not be detectable using crude methods of sample collection. Use of an explant model allows the interrogation of cells that were in direct contact with the embryo(s) facilitating the detection of such local embryo-induced changes in the endometrium during the very early stages of pregnancy.

Recently, local embryo-induced alterations in the endometrial transcriptome from spatially-defined regions in response to the presence of a Day 7 bovine embryo were reported (Sponchiado *et al*., 2017). In that study, the presence of an embryo altered the abundance of 12 transcripts in the cranial part of the uterine horn ipsilateral to the CL, including classical ISG (*ISG15, MX1, MX2, OAS1Y*), genes involved in prostaglandin biosynthesis (*PTGES, HPGD, AKR1L4*), water channels (*AQP4*) and a solute transporter (*SLC1A4*); however, the extent of change was relatively minor in nature ranging from 1.35- to 2-fold). Based on this, we hypothesized that the blastocyst induces local changes in the endometrial transcriptome through the production of IFNT and potentially other diffusible factors. Using co-culture of endometrial explants in the absence or presence of blastocysts or medium conditioned by blastocysts, we demonstrated that bovine endometrium responds to the presence of 8-day old blastocysts by upregulating expression of classical ISG (Passaro *et al*., 2018). This effect was (i) specific to the blastocyst stage - earlier stages did not induce gene expression changes, (ii) dependent on the number of blastocysts present - a minimum of 5 blastocysts were required to detect such changes, and (iii) independent of direct contact - the effect was induced by embryos co-cultured on endometrial explants using a cell culture insert (preventing direct contact) as well as by blastocyst-conditioned medium (Passaro *et al*., 2018). While others have reported differential expression of a small number of other transcripts in the endometrium in vivo, induced by the presence of a single blastocyst (Sponchiado *et al*., 2017), or in cultured endometrial cells (Talukder *et al*., 2017; Gómez *et al*., 2018), we failed to detect in endometrial explants using qPCR (Passaro *et al*., 2018).

To extend these findings, Passaro *et al*. (2019) used RNA sequencing to investigate global changes in the transcriptome of endometrial explants induced by exposure to blastocysts. Exposure of bovine endometrium to blastocyst-stage embryos resulted in the upregulation of 40 transcripts in blastocyst-exposed endometrial explants compared to the control. Comparison of this list of differentially expressed genes (DEG) with the common list of genes altered in endometrial explants following culture with 100 ng/ml IFNT or a Day 15 conceptus (from Sánchez *et al*., 2019b; Fig. 1) indicated that all of the DEG induced in the endometrium by blastocyst-stage embryos are IFNT-stimulated, in contrast to Day 15 when a significant number of IFNT-independent genes are induced (Mathew *et al*., 2019; Sánchez *et al*., 2019b – see below).

These results support the concept that the early embryo is capable of communicating with the reproductive tract. The effect on the endometrial transcriptome is dependent on the stage of embryo development and appears to be due solely to IFNT. The functional significance, if any, of such induced changes remains to be fully elucidated given that it is possible to transfer embryos from Day 7 onwards to a uterus that has not previously been exposed to an embryo and achieve normal pregnancy rates.

Response of the endometrium to in vivo or in vitro derived conceptuses

It has been elegantly shown that the endometrium can act as a 'sensor', with its transcriptome reflective of the type and developmental competency of the conceptus present (Bauersachs *et al*., 2009; Mansouri-Attia *et al*., 2009). These studies compared the endometrial responses to bovine conceptuses produced by somatic cell nuclear transfer, in vitro fertilization or artificial insemination (AI) and suggested that placental failure in bovine clone pregnancies may originate from abnormal embryo-maternal communication during the peri-implantation period (Day 18-20).

As stated above, it is generally accepted that blastocysts produced in vitro are inferior in quality to in vivo-derived embryos. This difference is reflected in the fact that in commercial embryo transfer, the majority of in vitro-produced blastocysts are transferred fresh while the majority of in vivo-derived blastocysts are transferred frozen (Viana, 2018).

Mathew *et al*. (2019) compared the transcriptomic response of the endometrium following exposure to IFNT or a conceptus derived from the transfer of an in vivo-derived (superovulation and AI) or in vitro-produced (IVF) blastocyst in order to identify novel transcripts dependent and independent on IFNT, conceptus origin and conceptus sex. IVF- or AI-produced blastocysts were transferred into recipient heifers on Day 7 of the estrous cycle. On Day 15, IVF- or AI-derived conceptuses were obtained by uterine flushing and individually placed on endometrial explants in media for 6 h. Explants were also cultured with media alone as a control or media containing 100 ng/mL recombinant ovine IFNT. Incubation of endometrium with IFNT or IVF- or AI-derived conceptuses altered the expression of 491, 498 and 576 transcripts, respectively, compared to the control. Further, 369 DEG were common between explants exposed to IFNT or a conceptus. 240 DEG were uniquely altered by conceptuses (IVF- and AI-derived) but not IFNT. Of these transcripts, 46 were shared between the IVF and AI groups, while 61 and 133 were specific to IVF and AI conceptuses, respectively. Five

genes (*MLPH, PROM2, MYADM, VN1R4L, HTR1A*) were more abundant in endometrium exposed to female compared to male conceptuses while a single gene (*ARL4C*) was more abundant in response to male conceptuses than female conceptuses.

These data support the hypothesis that conceptus regulation of gene expression in the endometrium is complex and involves factors other than IFNT that may have a biological role in pregnancy establishment. The findings are consistent with the presence of unique proteins in ULF of pregnant heifers on Day 16 and produced by short-term in vitro cultured Day 16 conceptuses (Forde *et al*., 2015) and those of Bartol *et al*. (1985) who demonstrated that the fully elongated bovine conceptus produces a significant number of proteins when cultured in vitro. Further, Spencer *et al*. (2013) demonstrated that the bovine conceptus produces prostaglandins, which can modify the endometrium prior to pregnancy recognition.

Effect of conceptus length on the endometrial response

Significant variation in the length and morphology of age-matched conceptuses exists, even when multiple conceptuses are recovered from the same uterine environment (Clemente *et al*., 2009; O'Hara *et al*., 2014), despite the fact that embryos were produced in vitro under the same conditions until the blastocyst stage and were of similar morphological quality at the time of transfer on Day 7. Conceptus length on a given day in the period around pregnancy recognition is thought to be indicative of its quality and the likelihood of establishing and maintaining a pregnancy (Barnwell *et al*., 2016), although this has yet to be definitively established. While significant differences in the transcriptomes of long and short Day 15 conceptuses have been reported (Barnwell *et al*., 2016), the interaction between such divergent conceptuses and the endometrium had, until recently, not been described. We hypothesized that bovine endometrium exposed to long vs. short Day 15 conceptuses would exhibit a different transcriptome profile reflective of potential for successful pregnancy establishment. To test this hypothesis we used a combination of in vitro production of bovine blastocysts, multiple embryo transfer and conceptus-endometrial explant co-culture to investigate the response of the endometrium to age-matched conceptuses of different sizes collected from the same uterine environment (Sánchez *et al*., 2019b). The main findings were that: (i) Day 15 conceptuses vary significantly in length, even when derived from the same uterine environment; and (ii) the endometrium responds in an IFNT-dependent and independent manner to conceptuses of different sizes which likely reflects the ability to successfully establish pregnancy (Fig. 1). These data complement nicely the data on the conceptus transcriptome mentioned above describing differential patterns of mRNA expression between short (mean length of 4.2 ± 0.1mm) and long (24.7 ± 1.9 mm) bovine conceptuses recovered on Day 15 of gestation (Barnwell *et al*., 2016). In that study, a total of 348 genes were differentially expressed related to metabolism and biosynthesis. These genes and cellular pathways involved in enhanced conceptus elongation, as well as the endometrial response blueprint to short and long conceptuses (Sánchez *et al*., 2019b), may ultimately serve as markers of successful pregnancy.

Whether or not smaller conceptuses on a given day are actually abnormal or whether they are simply slower in development is unclear; however, it is likely that they are compromised compared to longer (normal) conceptuses. Definitive proof will come from the recovery and retransfer of long and short age-matched conceptuses to establish their ability to initiate and maintain pregnancy.

While the differences in conceptus length are due, at least in part, to intrinsic differences in the embryo/conceptus, likely related to oocyte quality, it would be wrong to completely discount a role for the uterus in contributing to variation in conceptus length and pregnancy establishment. Using a model of repeated embryo transfer originally described by McMillan *et al*. (1999), Geary *et al*. (2016) classified heifers based on pregnancy success following serial embryo transfer as high fertile (HF), subfertile (SF), or infertile (IF). Conceptus survival and growth to Day 14 was not compromised in SF and IF heifers. However, pregnancy rate on Day 28 was higher in HF (70.4%) than in heifers with low fertility (36.8%; SF and IF). In a follow-up study (Moraes *et al*., 2018), pregnancy rate on Day 17 was substantially higher in HF (71%) and SF (90%) than IF (20%) heifers. Furthermore, elongating conceptuses were about twofold longer in HF than SF heifers. Taken together, these data suggest that the uterus impacts conceptus survival and programs conceptus development, and effects of dysregulated conceptus-endometrial interactions elicit loss of the post-elongation conceptus in SF cattle during the implantation period of pregnancy.

In summary, bovine endometrium responds differently in terms of its gene expression signature to age-matched long and short conceptuses, in an IFNT-dependent and independent manner, which may be critical for embryo survival. In particular, short conceptuses failed to alter the expression of a large number of ISG that were altered by both IFNT and long conceptuses, suggesting that insufficient IFNT production is a major contributory factor to lower survival of such conceptuses. Furthermore, the alteration of >100 endometrial transcripts uniquely by long conceptuses suggests that other aspects of maternal-embryo communication at this critical time are IFNT-independent.

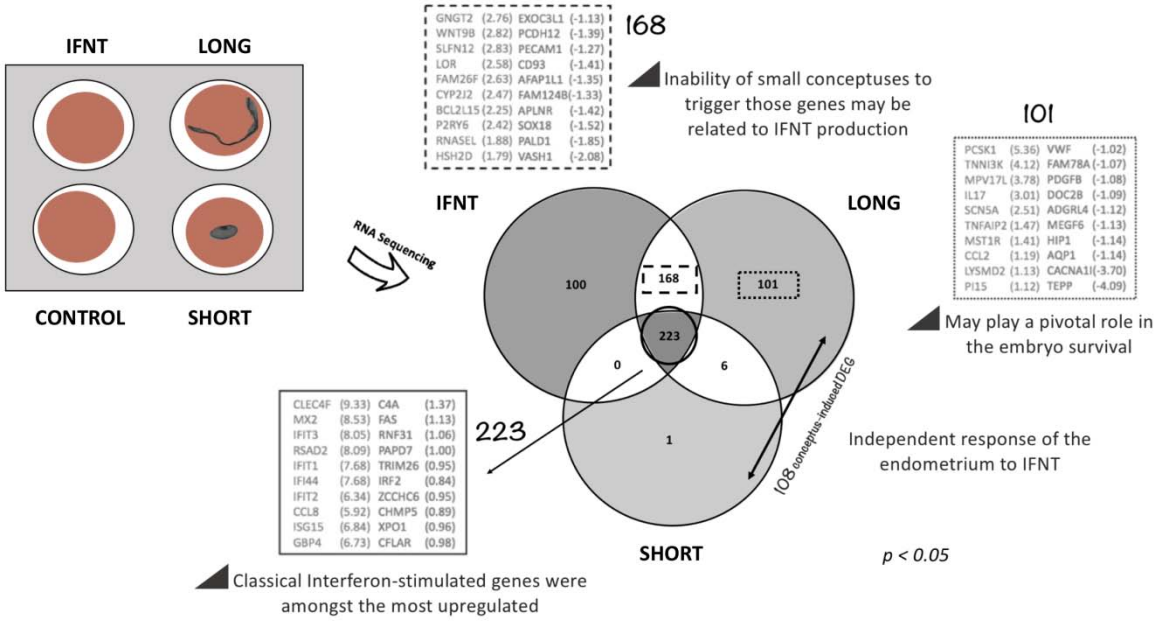


Figure 1. Use of an *ex-vivo* uterine endometrial explant-conceptus co-culture system to elucidate conceptus-induced effects on the endometrium both dependent and independent of interferon-tau (IFNT). Uterine explants taken from the same uterus were exposed to (i) medium alone (control), (ii) 100 ng/ml recombinant ovine IFNT, (iii) a long Day 15 conceptus, or (iv) a short Day 15 conceptus. Numbers of differentially expressed genes indicated for each group are relative to the Control. Modified from Sánchez *et al*. (2019b).

Differential response of endometrium ipsilateral and contralateral to the CL

Embryo transfer studies established that the incidence of embryo loss is higher following transfer to the uterine horn contralateral to the ovary containing the CL compared to transfer to the ipsilateral horn (Christie *et al*., 1979). Whether these differences are manifest in conceptus growth and elongation in the critical window preceding maternal recognition of pregnancy is unknown. Knowledge of differences in gene expression between the uterine horns during the estrous cycle could further enhance our understanding of uterine receptivity and the process of conceptus elongation, key events for the maternal recognition of pregnancy and, in turn, successful pregnancy establishment.

We hypothesized that differences in the endometrial transcriptome between the ipsilateral and contralateral horns throughout the cycle exist, and those differences would be correlated with differences in conceptus elongation after embryo transfer (Sánchez *et al*., 2019a). Endometrial samples from both horns were collected from synchronized heifers slaughtered on Day 5, 7, 13 or 16 post-estrus and subjected to RNA sequencing. Main findings were that: (i) day of the estrous cycle contributed to the greatest variation in the endometrial transcriptome; (ii) there were many more altered genes between the uterine horns ipsilateral and contralateral to the CL in the early (Day 5 and 7) as compared to late (Day 13 and 16) luteal phase; (iii) signalling pathways regulating pluripotency of stem cells were highly dysregulated when both uterine horns were compared, regardless of the day of luteal phase. In a separate experiment within the same study, ten Day 7 in vitro produced blastocysts were transferred into the uterine horn ipsilateral or contralateral to the CL or into both horns (i.e., bilateral) of synchronized recipient heifers. Reproductive tracts were recovered at slaughter on Day 14 and the number and dimensions of recovered conceptuses were recorded for each horn. Site of embryo transfer did not affect recovery rate (48.0%, 168/350) or length of conceptuses. Thus, although differences in gene expression exist between the endometrium of uterine horns ipsilateral and contralateral to the CL in cattle, these differences were not associated with a reduced ability of the uterus to support conceptus survival or development to Day 14 after embryo transfer on Day 7.

In a follow-on study, we asked whether the endometrium from the uterine horn ipsilateral or contralateral to the CL responds differently to an elongating conceptus. Bagés-Arnal *et al.* (2019) compared the local response of the ipsilateral and contralateral endometrium to a Day 14 conceptus. Although no differences in gene expression were detected between ipsilateral and contralateral endometrium, the response of the endometrium to a Day 14 conceptus was distinct in each uterine horn. Interestingly, more genes were differentially expressed in the contralateral than in the ipsilateral endometrium after exposure to a conceptus 239 vs. 61 DEG, respectively). Many of the biological processes enriched in the DEG between both horns in response to a conceptus were associated with immune response and response to stimuli. This observation is consistent with the study of Moraes *et al*. (2018), where relatively few differences were detected in the

endometrial transcriptome of non-pregnant high-fertile, subfertile and infertile heifers; however, the response of the endometrium from high-fertile and subfertile animals to pregnancy was remarkably different (3422 vs. 1095 DEG, respectively).

These data extend those of Sánchez *et al*. (2019a) describing temporal changes in the transcriptome of the endometrium ipsilateral and contralateral to the CL during a nonpregnant estrous cycle by describing differential response of the endometrium in both uterine horns to an elongating conceptus. The large difference in the number of DEG between the endometrium ipsilateral and contralateral to the CL in response to a Day 14 conceptus may be related to the differences in P4 concentrations during the first days after ovulation (Takahashi *et al*., 2016), since, as mentioned earlier, P4 is one the major regulators of the uterine receptivity through changes in the endometrium transcriptome.

Uterine lumen fluid compositon

The composition of ULF during the preimplantation period has been extensively studied in sheep (see review by Bazer *et al*., 2015 and references therein). Data in cattle are more limited although various studies have reported on aspects of ULF composition under various physiological states (Mullen *et al*., 2012; Faulkner *et al*., 2013; Forde *et al*., 2015).

We recently metabolically interrogated ULF flushes on Days 12-14 - the window of conceptus elongation-initiation - from cyclic heifers, either (i) supplemented with P4 on Day 3 post-estrous (high P4 cohort), or (ii) not (normal P4 cohort; physiological control). The former group is an established model of conceptus elongation rate acceleration (Carter *et al*., 2008; Clemente *et al*., 2009; O'Hara *et al*., 2014). Given that conceptus elongation coincides with a period of significant bovine pregnancy loss, our aim was to achieve a better understanding of the biochemical landscape surrounding the peri-elongation conceptus. Over 5000 metabolites were screened for by high-throughput untargeted ultra-high-performance liquid chromatography tandem mass spectroscopy, with 233 consistently identified, clustering within 8 super-pathways: amino acids, carbohydrates (Simintiras *et al*., 2019a), lipids (Simintiras *et al*., 2019b), cofactors, vitamins, nucleotides, peptides, energy substrates, and xenobiotics (Simintiras *et al*., 2019c). A global analysis of this dataset revealed three core 'strategies' likely utilised by the bovine endometrium to facilitate conceptus elongation, discussed below.

Firstly, indicative of the changing biochemical requirements of the conceptus around the initiation of elongation, a metabolic shift in the ULF of normal P4 heifers after Day 12 was observed (Simintiras *et al*., 2019d), to which fructose and mannitol/sorbitol were central. More specifically, only these two metabolites increased on Days 13 and 14 vs. 12 within the normal P4 group. Moreover, fructose and mannitol/sorbitol were elevated by 18.4 and 28.4-fold, respectively, in the ULF of high vs. normal P4 heifers on Day 12 (Simintiras *et al*., 2019a) - the greatest differences observed throughout the study - suggestive of a key role for these metabolites in sustaining, in addition to initiating, conceptus elongation.

Secondly, sub-pathway enrichment and representation analyses revealed that metabolic cascades of likely importance to conceptus elongation-initiation revolve around phospholipids, polyamines, and purines. Regarding the former, membrane biogenesis is intuitively essential to the ~30-fold increase in trophoblast length between Days 12-15 (Betteridge *et al*., 1980; Brooks *et al*., 2014). As 47% of identified lipids were intricately linked to membrane biogenesis, it seems reasonable to suspect that endometrial lipid secretions contribute to conceptus membrane fusion, and, thus, elongating conceptus membrane biogenesis is not entirely de novo (Simintiras *et al*., 2019b). The latter, polyamines and purines, are discussed below within the context of adenosine monophosphate signaling.

Thirdly, P4 supplementation amplified the total mean metabolite abundance on Day 14 ($P \leq 0.0001$); however, just 19 metabolites (8.2% of total) were elevated ($P \leq 0.05$) on Day 14 in high vs. normal P4 heifers, and are, therefore, largely responsible for raising the mean (Simintiras *et al*., 2019d). The biochemical properties of the these 'selectively' amplified 19 metabolites - including glucose (primary fuel source), trimethylamine-N-oxide (protein-stabilizing osmolyte), and phenol sulfate (relatively metabolically inert yet acidic molecule) - (i) support the notion that optimal conceptus elongation is contingent on biophysical and physicochemical, in addition to metabolic, cues, and (ii) contribute to the generation of our hypothesis pertinent to the molecular bases of conceptus elongation initiation, discussed below.

These findings combined, coupled with previous data on the enzymatic profile of bovine ULF (Muñoz *et al*., 2012; Forde *et al*., 2014), give rise to the hypothesis that conceptus elongation internally hinges on 5' adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma (PPARγ) activity, and is modulated by glucose, adenine, and adenosine mono- (AMP), di- (ADP), and tri-phosphate (AMP) influx (discussed in Simintiras *et al*., 2019d). Additional observations worth highlighting include: (i) that total ULF metabolite abundance (Fig. 2A) is not indicative of activity in terms of total day effects (Fig. 2B), P4 effects (Fig. 2C), or day by P4 interactions (Fig. 2D), and (ii) the identification of a plethora of microbiome-associated molecules in ULF, some of which were responsive to P4 (Simintiras *et al*., 2019c), highlights a need for further research into the influence of the uterine microbiome in uterine metabolism and maternal-embryo communication.

Conclusion

The period of early embryo development and pregnancy establishment is fascinating. This complex process encompasses ovulation, fertilization, blastocyst formation and growth into an elongated conceptus,

pregnancy recognition signalling, and development of the embryo and placenta. Despite the aforementioned advances in the field, there is still much to learn. The precise drivers of conceptus elongation remain unknown. While the process is dependent on the uterus - it does not occur in vitro - there is significant variation exhibited amongst conceptuses which is independent of the uterus and may point to variation in oocyte and early embryo quality. Furthermore, the role of the sire in determining embryo quality and in conceptus development is only beginning to be appreciated (Ortega *et al*., 2018).

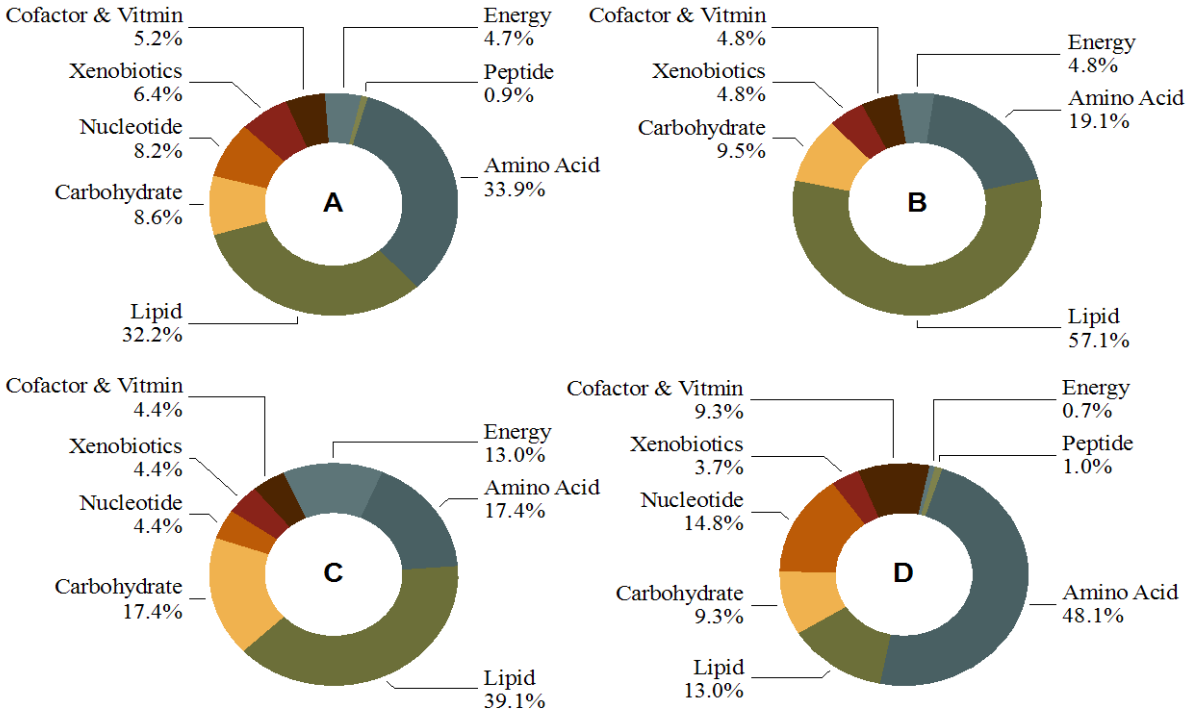


Figure 2. Breakdown of metabolites identified in uterine luminal fluid, by super-pathway, on Days 12-14 of cyclic heifers in terms of total: (A) abundance, (B) day effects, (C) progesterone (P4) effects, and (D) day by P4 interactions. Adapted from Simintiras *et al*. (2019d).

Author contributions

All authors (JMS, CAS, PL) contributed to the writing and revising of the manuscript.

Conflict of interest

Authors declare no conflicts of interest.

Acknowledgments and Funding disclosure statement

This work was supported by grants from Science Foundation Ireland (to PL; Grant number: 13/IA/1983), an Irish Research Council Government of Ireland Postdoctoral Fellowship (to CAS; grant number GIOPD/2017/942), and a University College Dublin Career Development Award (to CAS; Grant number: CDA54580).

References

Ashworth CJ, Sales DI, Wilmut I. 1989. Evidence of an association between the survival of embryos and the periovulatory plasma progesterone concentration in the ewe. *J Reprod Fertil*, 87:23-32.

Barnwell CV, Farin PW, Ashwell CM, Farmer WT, Galphin SP, Farin CE. 2016. Differences in mRNA populations of short and long bovine conceptuses on Day 15 of gestation. *Mol Reprod Dev*, 83:424-441.

Bartol FF, Roberts RM, Bazer FW, Lewis GS, Godkin JD, Thatcher WW. 1985. Characterization of proteins produced in vitro by periattachment bovine conceptuses. *Biol Reprod*, 32:681-693.

Bauersachs S, Ulbrich SE, Zakhartchenko V, Minten M, Reichenbach M, Reichenbach H-D, Blum H, Spencer TE, Wolf E. 2009. The endometrium responds differently to cloned versus fertilized embryos. *Proc Natl Acad Sci USA*, 106:5681-5686.

Bauersachs S, Ulbrich SE, Reichenbach HD, Reichenbach M, Büttner M, Meyer HH, Spencer TE, Minten M, Sax G, Winter G, Wolf E. 2012. Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium. *Biol Reprod*, 29:86(2):46. Doi: 10.1095/biolreprod.111.094771.

Bagés-Arnal S, Sánchez JM, Fernández-Fuertes B, McDonald M, Behura SK, Spencer TE, Fair T,

Lonergan P. 2019. Differential response of bovine endometrium ipsilateral and contralateral to the corpus luteum to a Day 14 conceptus. *Annual Meeting of the Society for the Study of Reproduction*, San Jose, CA, USA, July 2019.

Bazer FW, Wang X, Johnson GA, Wu G. 2015. Select nutrients and their effects on conceptus development in mammals. Anim Nutr, 1(3):85-95. Doi: 10.1016/j.aninu.2015.07.005.

Bazer FW, Thatcher WW. 2017. Chronicling the discovery of interferon tau. Reproduction, 154:F11-F20.

Brandão DO, Maddox-Hyttel P, Løvendahl P, Rumpf R, Stringfellow D, Callesen H. 2004. Post hatching development: a novel system for extended in vitro culture of bovine embryos. *Biol Reprod*, 71:2048-2055.

Betteridge K, Eaglesome M, Randall GCB, Mitchell D. 1980. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. *J Reprod Fertil*, 59:205-216.

Borges AM, Healey GD, Sheldon IM. 2012. Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. *Am J Reprod Immunol*, 67:526-539.

Brooks K, Burns G, Spencer TE. 2014. Conceptus elongation in ruminants: Roles of progesterone, prostaglandin, interferon tau and cortisol. *J Anim Sci Biotechnol*, 5:1-12.

Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, Evans ACO, Kenny DA, Roche JF, Lonergan P. 2008. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod Fertil Dev*, 20:368-375.

Christie WB, Newcomb R, Rowson LE. 1979. Embryo survival in heifers after transfer of an egg to the uterine horn contralateral to the corpus luteum and the effect of treatments with progesterone or hCG on pregnancy rates. *J Reprod Fertil*, 56:701-706.

Clemente M, de La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, Rizos D, Lonergan P. 2009. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction*, 138:507-517.

Faulkner S, Elia G, O' Boyle P, Dunn M, Morris D. 2013. Composition of the bovine uterine proteome is associated with stage of cycle and concentration of systemic progesterone. *Proteomics*, 13:3333-3353.

Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, Bazer FW, McBride R, Boland MP, O'Gaora P, Lonergan P, Roche JF. 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol Reprod*, 81:784-794.

Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Okumu LA, McGettigan PA, Mehta JP, McBride R, O'Gaora P, Roche JF, Lonergan P. 2011. Conceptus-Induced Changes in the Endometrial Transcriptome: How Soon Does the Cow Know She Is Pregnant? *Biol Reprod*, 85:144-156.

Forde N, Lonergan P. 2012. Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? *J Reprod Dev*, 58(2):189-95.

Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, Lonergan P. 2012. Effects of low progesterone on the endometrial transcriptome in cattle. *Biol Reprod*, 87:124.

Forde N, McGettigan PA, Mehta JP, O'Hara L, Mamo S, Bazer FW, Spencer TE, Lonergan P. 2014. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. *Reproduction*, 147:575-587.

Forde N, Bazer FW, Spencer TE, Lonergan P. 2015. 'Conceptualizing' the endometrium: identification of conceptus-derived proteins during early pregnancy in cattle. *Biol Reprod*, 92:156.

Forde N, Lonergan P. 2017. Interferon-tau and fertility in ruminants. *Reproduction*, 154:F33-F43.

Gad A, Hoelker M, Besenfelder U, Havlicek V, Cinar U, Rings F, Held E, Dufort I, Sirard MA, Schellander K, Tesfaye D. 2012. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biol Reprod*, 87:100.

Geary TW, Burns GW, Moraes JG, Moss JI, Denicol AC, Dobbs KB, Ortega MS, Hansen PJ, Wehrman ME, Neibergs H, O'Neil E, Behura S, Spencer TE. 2016. Identification of Beef Heifers with Superior Uterine Capacity for Pregnancy. *Biol Reprod*, 95(2):47. Doi: 10.1095/biolreprod.116.141390.

Gómez E, Sánchez-Calabuig MJ, Martin D, Carrocera S, Murillo A, Correia-Alvarez E, Herrero P, Canela N, Gutiérrez-Adán A, Ulbrich S, Muñoz M. 2018. In vitro cultured bovine endometrial cells recognize embryonic sex. *Theriogenology*, 108:176-184.

Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction*, 124:289-300.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*, 43:141-152.

Kimura K, Matsuyama S. 2014. Successful Nonsurgical Transfer of Bovine Elongating Conceptuses and Its Application to Sexing. *J Reprod Dev*, 60:210-215.

Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P, Boland MP. 2003a. Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. *Reproduction*, 126:337-346

Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, de la Fuente J, Boland MP. 2003b. Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. *Biol Reprod*, 69:1424-1431.

Lonergan P, Forde N, Spencer T. 2016. The role of progesterone in embryo development in cattle. *Reprod*

Fertil Dev, 28:66-74.

Mann GE, Lamming GE. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction*, 121:175-180.

Mansouri-Attia N, Sandra O, Aubert J, Degrelle S, Everts RE, Giraud-Delville C, Heyman Y, Galio L, Hue I, Yang X, Tian XC, Lewin HA, Renard JP. 2009. Endometrium as an early sensor of in vitro embryo manipulation technologies. *Proc Natl Acad Sci USA*, 106:5687-5692.

Mathew DJ, Sánchez JM, Passaro, Charpigny G, Behura S, Spencer TE, Lonergan P. 2019. Interferon tau (IFNT)-dependent and -independent effects of the bovine conceptus on the endometrium during early pregnancy. *Biol Reprod*, 100:365-380.

McMillan WH, Donnison MJ. 1999. Understanding maternal contributions to fertility in recipient cattle: development of herds with contrasting pregnancy rates. *Anim Reprod Sci*, 57:127-140

McNeill RE, Diskin MG, Sreenan JM, Morris DG. 2006. Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. *Theriogenology*, 65:1435-1441.

Moraes JGN, Behura SK, Geary TW, Hansen PJ, Neibergs HL, Spencer TE. 2018. Uterine influences on conceptus development in fertility-classified animals. *Proc Natl Acad Sci USA*, 115:E1749-E1758.

Mullen MP, Elia G, Hilliard M, Parr MH, Diskin MG, Evans AC, Crowe MA. 2012. Proteomic characterization of histotroph during the preimplantation phase of the estrous cycle in cattle. *J Proteome Res*, 11:3004-3018.

Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, Martín D, Carrocera S, Gómez E. 2012. Proteome of the early embryo-maternal dialogue in the cattle uterus. *J Proteome Res*, 11:751-766.

O'Hara L, Forde N, Kelly AK, Lonergan P. 2014. Effect of bovine blastocyst size at embryo transfer on day 7 on conceptus length on day 14: Can supplementary progesterone rescue small embryos? *Theriogenology*, 81:1123-1128.

Okumu LA, Forde N, Fahey AG, Fitzpatrick E, Roche JF, Crowe MA, Lonergan P. 2010. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. *Reproduction*, 140:143-53.

Ortega MS, Moraes JGN, Patterson DJ, Smith MF, Behura SK, Poock S, Spencer TE. 2018. Influences of sire conception rate on pregnancy establishment in dairy cattle. *Biol Reprod*, 99:1244-1254.

Passaro C, Tutt D, Mathew DJ, Sánchez JM, Browne JA, Boe-Hansen GB, Fair T, Lonergan P. 2018. Blastocyst-induced changes in the bovine endometrial transcriptome. *Reproduction*, 156:219-229.

Passaro C, Tutt D, Bages-Arnal S, Maicas C, Laguna-Barraza R, Gutierrez-Adan A, Browne JA, Rath D, Behura SK, Spencer TE, Fair T, Lonergan P. 2019. Global transcriptomic response of bovine endometrium to blastocyst stage embryos. *Reproduction*. In press.

Rizos D, Fair T, Papadopoulos S, Boland MP, Lonergan P. 2002a. Developmental, qualitative, and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. *Mol Reprod Dev*, 62:320-327.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. 2002b. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-248.

Rizos D, Scully S, Kelly AK, Ealy AD, Moros R, Duffy P, Al Naib A, Forde N, Lonergan P. 2012. Effects of human chorionic gonadotrophin administration on day 5 after oestrus on corpus luteum characteristics, circulating progesterone and conceptus elongation in cattle. *Reprod Fertil Dev*, 24:472-481.

Sánchez JM, Passaro C, Forde N, Browne JA, Behura SK, Fernández-Fuertes B, Mathew DJ, Kelly AK, Butler ST, Spencer TE, Lonergan P. 2019a. Do differences in the endometrial transcriptome between uterine horns ipsilateral and contralateral to the corpus luteum influence conceptus development to Day 14 in cattle? *Biol Reprod*, 100:86-100.

Sánchez JM, Mathew DJ, Behura SK, Passaro C, Charpigny G, Butler ST, Spencer TE, Lonergan P. 2019b. Bovine endometrium responds differentially to age-matched short and long conceptuses†. *Biol Reprod*, 101(1):26-39. Doi: 10.1093/biolre/ioz060.

Sartori R, Bastos MR, Wiltbank MC. 2010. Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reprod Fertil Dev*, 22:151-158.

Satterfield MC, Bazer FW, Spencer TE. 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol Reprod*, 75:289-96.

Simintiras CA, Sánchez JM, McDonald M, Martins T, Binelli M, Lonergan P. 2019a. Biochemical characterization of progesterone-induced alterations in bovine uterine fluid amino acid and carbohydrate composition during the conceptus elongation window. *Biol Reprod*, 100:672-685.

Simintiras CA, Sánchez JM, McDonald M, Lonergan P. 2019b. Progesterone alters the bovine uterine fluid lipidome during the period of elongation. *Reproduction*, 157:399-411.

Simintiras CA, Sánchez JM, McDonald M, Lonergan P. 2019c. The influence of progesterone on bovine uterine fluid energy, nucleotide, vitamin, cofactor, and xenobiotic composition during the conceptus elongation-initiation window. *Sci Rep.* In press.

Simintiras CA, Sánchez JM, McDonald M, Lonergan P. 2019d. The biochemistry surrounding conceptus elongation. *Biol Reprod.* In press.

Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ, Lonergan P. 2013. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. *Reproduction*, 146:377-387.

Spencer TE, Forde N, Lonergan P. 2016. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy*

Sci, 99:5941-5950.

Spencer TE, Forde N, Lonergan P. 2017. Insights into conceptus elongation and establishment of pregnancy in ruminants. *Reprod Fertil Dev*, 29:84-100.

Sponchiado M, Gomes NS, Fontes PK, Martins T, Del Collado M, Pastore A de A, Pugliesi G, Nogueira MFG and Binelli M, Lonergan P. 2017. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. *PloS One*, 12:e0175954.

Stronge AJH, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG. 2005. Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology*, 64:1212-1224.

Takahashi H, Haneda S, Kayano M, Matsui M. 2016. Differences in progesterone concentrations and mRNA expressions of progesterone receptors in bovine endometrial tissue between the uterine horns ipsilateral and contralateral to the corpus luteum. *J Vet Med Sci*, 78:613-618.

Talukder AK, Yousef MS, Rashid MB, Awai K, Acosta TJ, Shimizu T, Okuda K, Shimada M, Imakawa K, Miyamoto A. 2017. Bovine embryo induces an anti-inflammatory response in uterine epithelial cells and immune cells in vitro: possible involvement of interferon tau as an intermediator. *J Reprod Dev*, 63:425-434.

Viana J. 2018. 2017 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter* 36:8-25.

Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, Ochoa JC, Santos JEP, Sartori R. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology*, 86:239-253.

Wydooghe E, Vandaele L, Heras S, De Sutter P, Deforce D, Peelman L, De Schauwer C and Van Soom A. 2015. Autocrine embryotropins revisited: how do embryos communicate with each other in vitro when cultured in groups? *Biol Rev Camb Philos Soc*, 92:505-520.

DOI: 10.21451/1984-3143-AR2019-0073

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Sanitary program to reduce embryonic mortality associated with infectious diseases in cattle

Amauri Alcindo Alfieri[1,2,*], Raquel Arruda Leme[1,2], Alais Maria Dall Agnol[1,2], Alice Fernandes Alfieri[1,2]

[1]National Institute of Science and Technology of Dairy Production Chain (INCT-Leite), Universidade Estadual de Londrina, Paraná, Brazil.
[2]Laboratory of Animal Virology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

Abstract

Among reproductive disorders in dairy and beef cattle worldwide, embryonic mortalities stand out as one of the most frequent. Because of the multifactorial etiology, the clinical and laboratory diagnoses of embryonic mortality causes in cattle are quite complex. Often, infectious causes may account for up to 50% of bovine embryonic mortality rates after 30 days of conception. This review will address the main causes of early and late embryonic mortality, with emphasis on infectious causes and, particularly, those more frequent in the Brazilian cattle herds. In addition, we will discuss ways of controlling and prophylaxis including those related to reproductive and sanitary management, with emphasis on immunoprophylaxis of the three most frequent reproductive infectious diseases in Brazilian dairy and beef cattle herds.

Keywords: bovine, reproduction, IBR, BVD, leptospirosis, vaccination.

Introduction

In a recent past of Brazilian cattle breeding, and especially of beef cattle, the embryonic mortality rate was a reproductive parameter that was very little evaluated in the production systems. The lack of information regarding this parameter was general both to part of producers and technicians responsible for the reproduction of the herds. However, with the advent and more recurrent use of reproductive biotechniques, primarily the Fixed-Time Artificial Insemination (FTAI), this important parameter of reproduction in cattle has been evaluated more frequently. The establishment of more standardized FTAI protocols suitable for different geographic regions and different production conditions enabled to compare results that facilitate the identification and quantification of reproductive failures and, in particular, embryonic mortalities. In this review, we approach sanitary programs focused on reducing embryonic mortality associated with infectious diseases. Features of embryonic mortality, related infectious causes, epidemiological profile of infectious reproductive diseases, sanitary programs, vaccination, and biosecurity are addressed.

Embryonic mortality

In the bovine species, the embryo is defined as the product obtained up to approximately 42-45 days after conception, which refers to the cell differentiation period (Committee on Reproductive Nomenclature, 1972). Consequently, this is the period used to evaluate the embryonic mortality rate in beef and dairy cattle herds. In practice, the return to estrus in an interval longer than 17-25 days reflects the occurrence of embryonic mortality (Ayalon, 1978; Abdalla *et al*., 2017). It should be emphasized that in the analysis of the embryonic mortality rate, one must eliminate fertilization failures.

In cattle, embryonic mortality is a multifactorial event that may involve genetic and environmental factors. Genetic factors are intrinsic to the embryo, and the most frequent are those caused by genetic defects, individual genes and genetic interactions that can lead to chromosomal abnormalities. In cattle, genetic defects may account for up to 20% of embryonic and fetal mortalities (Vanroose *et al*., 2000; Diskin and Morris, 2008).

A range of causes can be included as environmental factors of embryonic mortalities in cattle. Among the several causes, the following stand out: age; climate; hormonal imbalance; uterine environment, among others that can cause physiological and endocrine disorders that can lead to the death of the embryo (Chebel *et al*., 2004; Inskeep and Dailey, 2005; Walsh *et al*., 2011; Abdalla *et al*., 2017; Sánchez *et al*., 2018). In addition, the nutrition factor is also important, especially considering the possibility of postpartum cows present a negative energy balance that may impact the follicular dynamics due to changes in the gene expression of dairy cow granulosa cells at 60 days post-partum, reducing the reproductive performance of this animal category. Therefore, the effects of the negative energy balance may be felt even after the resolution of the problem (Butler, 2003; Girard *et al*., 2015; Lonergan *et al*., 2016; Rani *et al*., 2018). Also in regards to environmental factors, we will highlight the infectious causes.

Embryonic mortality can also be classified into two types based on the time elapsed after conception. Those mortalities that occur before 15 days post-conception are termed early embryonic mortalities and mortalities of 16 to 42-45 days are termed late embryonic

*Corresponding author: alfieri@uel.br
orcid.org/0000-0002-7024-4487
Received: July 30, 2019
Accepted: May 29, 2019

mortalities (Inskeep and Dailey, 2005). Comparatively, early embryonic mortalities are more frequent than the late ones (Dunne *et al*., 2000; Inskeep and Dailey, 2005). In cases of early embryonic mortality there will be no change in the period of the estrous cycle. It means that early embryonic mortalities are accompanied by a return to estrus at regular intervals. Conversely, one of the main clinical features of late embryonic mortalities is the return to estrus at irregular intervals (Silke *et al*., 2002).

Infectious causes of embryonic mortality in cattle

In this category we can include nonspecific causes, represented by a series of bacteria that can cause ascending infections. That is, these bacteria can be present in the vaginal mucosa itself or else in the penis, in cases of reproduction by natural mating, or in the semen in cases of artificial insemination. Often, these opportunistic bacteria cause inflammation in the uterus, resulting in endometritis, which can render the uterine environment inhospitable to the embryo (Bielanski *et al*., 2000; Vanroose *et al*., 2000; Silva *et al*., 2016; Sheldon and Owens, 2017; Rani *et al*., 2018). The frequency of nonspecific infections in bovine females that result in embryonic mortalities in dairy herds is low (Vanroose *et al*., 2000; Sheldon and Owens, 2017). As cases are sporadic, in general, specific measures for the control and prophylaxis of these infections are rarely adopted.

However, the reproductive tract of the bovine female is susceptible to a series of infectious processes caused by pathogenic organisms specific to the reproductive sphere. Infections can be caused by different classes of microorganisms, such as bacteria, viruses, and protozoa (Tab. 1). Although it is possible, fungal infections are rarely a problem of great magnitude in the reproduction of cattle (Vanroose *et al*., 2000; Givens and Marley, 2008; Walsh *et al*., 2011).

Infections caused by specific microorganisms can be venereally transmitted, occurring in cases that the organisms are present in the genital tract of the female or male, and by the hematogenous route, which occurs with those organisms that reach the uterus through the bloodstream (Vanroose *et al*., 2000). The epidemic or endemic presentation of the diseases depends on the geographical region, epidemiological scenario, reproductive management, vaccination and health status of the herds. In epidemiological terms, specific infections of the reproductive tract may present in an epidemic form or, more frequently in Brazil, in an endemic manner. The epidemic form prevails in specific pathogen-free herds, that is, they have never been in contact with the microorganism, either by active infection or vaccination. Since these animals lack the active humoral or cellular response specific to the target microorganism, the tendency is the development of the epidemic form of the infection (Alfieri and Alfieri, 2017). In this form, several animals can simultaneously manifest specific clinical signs of disease. In general, they are symptomatic and therefore easier to identify. However, those animals that had the previous infection or those that were previously vaccinated have specific immunological memory, which means that the infection likely will occur in the endemic form. In the endemic presentation, the clinical problems, primarily the embryonic mortalities, may compromise a smaller number of animals for a longer period of time (Alfieri and Alfieri, 2017).

The most important endemic infectious diseases in Brazil are infectious bovine rhinotracheitis, IBR (caused by *Alphaherpesvirus 1* - BoHV-1), bovine viral diarrhea, BVD (caused by *bovine viral diarrhea virus* – BVDV), leptospirosis (cause by *Leptospira* spp.), vulvovaginitis (caused by *Mycoplasma bovigenitalium* and *Ureaplasma diversum*), campylobacteriosis – caused by *Campylobacter fetus*), trichomoniasis (caused by *Tritrichomonas foetus*), and neosporosis (caused by *Neospora caninum*) (Vanroose *et al*., 2000; Grooms, 2006; Givens and Marley, 2008; Kumar *et al*., 2011; Almería and López-Gatius, 2013; Gates *et al*., 2013; Sanhueza *et al*., 2013; Michi *et al*., 2016; Alfieri and Alfieri, 2017; Fischer *et al*., 2018; Rani *et al*., 2018). However, regardless of the form of presentation, epidemic or endemic, both cause considerable economic losses for both dairy and beef cattle (Alfieri and Alfieri, 2017).

Table 1. Frequency of embryonic death associated with different infectious agents in beef and dairy cattle herds.

Microorganism		Disease	Infection		Embryonic death
Class	Species		Transmission	Persistence	
Virus	BoHV-1	IBR	Hematogenous	Viral latency	+++
	BVDV	BVD	Hematogenous	Persistent infection	++
Bacteria	*Leptospira* spp.	Leptospirosis	Hematogenous	Renal carrier	+++
	Campilobacter sp.	Campylobacteriosis	Genital	Asymptomatic carrier bull	+
	M. bovigenitalium	Mycoplasmosis	Genital	Asymptomatic carrier cow	+
	U. diversum	Ureaplasmose	Genital	Asymptomatic carrier cow	+
	Histophilus somni	Histophilosis	Hematogenous		+
Protozoa	*Tritrichomonas foetus*	Trichomoniasis	Genital	Asymptomatic carrier bull	+
	Neospora caninum	Neosporosis	Vertical	Oocyst	+

BoHV-1: Bovine alfaherpesvirus 1; BVDV: Bovine viral diarrhea virus. +: sporadic; ++: frequent; +++: highly frequent. Source: Vanroose *et al*., 2000; Grooms, 2006; Givens and Marley, 2008; Kumar *et al*., 2011; Almería and López-Gatius, 2013; Gates *et al*., 2013; Sanhueza *et al*., 2013; Michi *et al*., 2016.

Animal category in a herd

IBR, BVD, and leptospirosis are the three main and most frequent reproductive infectious diseases in the Brazilian cattle herds (Alfieri and Alfieri, 2017; Fischer *et al*., 2018). Particularly in relation to beef cattle, the serological profile for these three infectious diseases in a Brazilian cattle herd differs considerably due to the animal category. In most herds the percentages of non-vaccinated and seropositive animals for BoHV-1, BVDV, and *Leptospira* spp. increase according to the age of the animals. Therefore, the average percentage of nulliparous (heifers) seropositive for these three microorganisms can be considered smaller than the average percentage of primiparous, which is smaller than the average percentage of multiparous cows (Médici *et al*., 2000; Alfieri and Alfieri, 2017). In other words, the percentage of animals susceptible to field prime-infection in a breeding season is higher in heifers than in primiparous and multiparous cows, meaning that the two categories composed by the youngest animals in the herd are the most vulnerable. Consequently, sanitary management for reproductive infectious problems in beef cattle should focus on both heifers and primiparous, particularly when evaluating a vaccination program.

Epidemiological profile of infectious reproductive diseases

An action of special importance, particularly directed to the control of reproductive diseases, is to obtain information aiming to define the epidemiological profile of the animals and, mainly, stratifying them according to the animal categories. Serological tests should be performed in a percentage of animals that enable defining the seroepidemiology of IBR, BVD, and leptospirosis, for example. Some tests, including the enzyme-linked immunosorbent assay (ELISA) for viruses are qualitative and enable determining the presence/absence of infection in the herd and/or categories of animals that compose the herd. Other diagnostic tests have additional advantages. This applies to the virus neutralization test, especially because of its simultaneous qualitative and quantitative feature, which means that based on the tritation it is possible to establish the magnitude of antibody titers present in the blood serum. High titers evidence recent infection or viral circulation in the herd (Dubovi, 2013; Lanyon *et al*., 2014; Alfieri and Alfieri, 2017).

Even as distinct epidemiological situations the presence of infectious risk factors can influence the occurrence of reproductive problems (Souza *et al*., 2017). The risk of non-infectious early fetal loss appears to increase under the conditions of intensive management systems (Forar *et al*., 1995; Hanzen *et al*., 1999). The non-infectious risk factors, such as nutritional disorders, management failures, and environmental conditions, isolated or in association with infectious causes, may play important role in changes of the main parameters used to evaluate the reproductive efficiency in cattle herds (García-Ispierto *et al*., 2006, 2007a,b). The technical level used in the reproductive activity can influence the presence, frequency, and intensity of health problems, and can generate negative results in a production unit. Therefore, all possible causes of reproductive failures, including infectious, non-infectious, current and previous herd health status, and local and regional epidemiological features should be carefully considered for the resolution/control of the problems.

Furthermore, although the considerable benefits, the indiscriminate use of the biotechnologies of reproduction can generate undesirable consequences, especially when used without a careful analysis of the risk factors inherent to the management. Some factors may compromise the health of the herd, such as the intensive use of parturition areas, increased animal population density at certain periods, abundance and agglomeration of young animals, which are more susceptible to infections (Pegoraro *et al*., 2018). In these situations, the risks of environmental contamination with the main reproductive pathogens, that can be eliminated at the time of birth, are higher than those in traditional breeding systems and facilitate the spread of infections that compromise the reproductive system of cattle. As well, the purchase and sale of genetic material (semen, oocytes, and embryos) should follow some safety principles (Carvalho *et al*., 2007). When the material is imported/exported, clinical isolation and observation of donors, and serological and microbiological tests should be performed to ensure the absence of relevant diseases. These also include epidemiological surveillance in areas where artificial insemination is practiced with imported semen. With regard to embryos, proper collection, manipulation, and transfer techniques can prevent many pathogens of concern (Rufino *et al*., 2006; Carvalho *et al*., 2007). These are some of the examples of non-infectious risks that might be associated with embryonic mortality and other reproductive failures.

Before decision making regarding the health problems causing embryonic mortalities in bovine females, some issues should be raised, such as i) what is the history of the disease in the region and/or in the herd?; ii) how does the microorganism enter the herd?; iii) how is the infection transmitted?; iv) how does the disease remain or how is it kept in the herd (carriers)?; v) are there vectors?; vi) is there an effective treatment?, vii) is there a vaccine to control and prophylaxis?; viii) if it exists, is the vaccine effective?; ix) when and why to vaccinate?; x) are there other controlling forms? Only with answers to these questions is it possible to design an efficient Sanitary Program to reduce the rate of embryonic mortality in beef or dairy bovine herds.

Sanitary program

In animal production “Sanitary Program” can be defined as a thematic unit constituted by a set of actions developed aiming to promote and maintain the animal health. Unfortunately, particularly for cattle, it is usual to summarize or confuse Sanitary Program with Vaccination Program. When available, vaccines are

undoubtedly one of the main actions to be implemented in a Sanitary Program. However, besides the importance of prophylaxis as good practices in vaccinations, there are other important issues, specially the control of disease risk factors. Vaccination prophylaxis strategies may loss efficiency due to the maintenance of the risk factors within the herds. Therefore, complementary actions that promote or preserve animal health are of fundamental importance to obtain quantitative and, mainly, qualitative increase in the beef and dairy production chains. This is directly associated with the financial efficiency of production chains.

One of the main tools that, when available and depending on the epidemiological profile of infections, should be included in a Sanitary Program is the vaccine. In the context of a Sanitary Program for the control of embryonic mortalities in bovine herds, some vaccines should be used not as an additional and emergency measure, but should form a "Vaccination Program".

Considering the herd animal density and intensive management of a dairy herd and aiming to reduce the embryonic mortality caused by viruses (IBR and BVD), a "Vaccination Program" should be applied to all the females of the herd, regardless of the animal category they belong. Although most of the vaccine manufacturers indicate annual revaccination, biannual revaccinations can be recommended (Alfieri and Alfieri, 2017). It depends on the monitoring results of some reproductive parameters, as well as the qualitative and quantitative (titration) epidemiological profile of the herd. Similarly, the manufacturers' revaccination recommendation for leptospirosis control is biannual. However, in our personal experience and on the basis of epidemiological profile and, mainly, on the antibody titers of the seropositive animals we recommend quarterly revaccinations. Also in the regards of leptospirosis, in very specific situations such as high-producing beef and dairy herds, the therapeutic use of antibiotics may also be prescribed in addition to vaccine prophylaxis in order to reduce the time to control the infection in the herd (Alfieri and Alfieri, 2017).

Commercial vaccines against most of the infections associated with bovine embryonic mortality are available in Brazil. As the epidemiological profile of these infections varies considerably, "Vaccination Programs" should consider the individual variations of each herd. It is important to consider the nutritional management with body score analysis when defining a Vaccination Program, since optimal nutrition is important to enhance immunity and mount an appropriate response to vaccination. Therefore, sufficient protein, energy, minerals, and vitamins are all required to develop and maintain a strong immune system (Berge and Vertenten, 2017). As well, the type of reproductive management should also be considered in the establishment of a Vaccination Program, since pre-breeding vaccination program may improve the health of cows by preventing BVD, trichomoniasis, campylobacteriosis, and leptospirosis (Daly, 2006; Vasconcelos *et al*., 2017), for example. Vaccinating cows against BoHV-1, BVDV, and *Leptospira* spp., particularly when both doses are administered before AI or FTAI improve cow reproductive performance (Pereira *et al*., 2013; Vasconcelos *et al*., 2017). Therefore, a pre-breeding vaccination program aims to increase the chances the cow will breed and ultimately deliver a calf; help the cow become pregnant early in the breeding season; and protect the calf from becoming persistently infected with BVD (Campbell, 2011).

However, in general, infections with BoHV-1, BVDV and *Leptospira* spp. are widely disseminated in Brazilian beef cattle (Junqueira and Alfieri, 2006). Thus, these reproductive infectious diseases are more frequently manifested as endemic and only sporadically as epidemic. As mentioned earlier, in most of the herds, the infection rates among females of different categories vary considerably. It is observed a decreasing percentage rate of seronegative animals and, consequently, more susceptible animals in the nulliparous, primiparous, and multiparous female categories (Junqueira and Alfieri, 2006).

Another important aspect inherent to the pathogenesis of these three bovine reproductive infectious diseases is the tendency to chronicity. Throughout evolution, the etiological agents responsible for these infections have developed strategies to remain in the herds (Alfieri and Alfieri, 2017). BoHV-1, by means of viral latency mechanism (Nandi *et al*., 2009), is able to maintain the viral genome in the nucleus of infected cells, as provirus. Under this viral condition, the infected animal may remain without clinical signs of infection for a long period (Nandi *et al*., 2009). Eventually, the viral latency is broken and the infected animal may present clinical manifestations accompanied by viral re-excretion, perpetuating the infection in the herds (Jones and Chowdhury, 2007; Biswas *et al*., 2013). Regarding BVDV, this viral agent is able to persistently infect (PI) calves, which eliminate high BVDV titers throughout their productive lives, contributing to perpetuate the infectious process in a herd (Hamers *et al*., 1998; Moennig and Becher, 2018). Finally, bovine leptospirosis is considered a chronic infection since most of the times it evolves to chronic kidney disease. The clinical features of the infection, such as the immunological exclusion and intermittent bacterial shedding, make the infection control an important challenge in the herd, especially the serovar Hardjo, which is the most adapted to the bovine species (Adler, 2015; Balamurugan *et al*., 2018).

Concerning the two viral infections (IBR/BVD) this epidemiological feature has a very important practical implication. Depending on the rate of previously infected and, consequently, seropositive animals, in the category of multiparous females the decision to vaccinate or not will depend on a cost-effective analysis of the vaccination. If the decision is vaccinating, a single vaccination dose in the animals of that category is enough, since it is likely that these animals have active immunological memory to these viral agents (Van Drunen Little-van den Hurk, 2006). Additionally, also considering the immunological aspects related to a previous infection, the period between vaccination and artificial insemination may be

shorter, and may even adapt to the management on day zero of the FTAI (Daly, 2006; Vasconcelos *et al*., 2017). However, in the categories of more susceptible females, such as nulliparous and primiparous, which have lower rates of seroconversion, the ideal is that before the mating season the animals receive two doses of vaccine with a minimum interval of 21 days between doses (Aono *et al*., 2013; Pereira *et al*., 2013). Also in this situation the second dose, for management reasons may coincide with the day of initiation of the FTAI protocol.

Vaccination *vs*. reproductive performance of cattle

Hundreds of scientific articles published in peer-reviewed and indexed journals that are available in relevant scientific databases, including Web of Science, PubMed, CAB Abstracts, and others analyzed different variables regarding the vaccination of beef and dairy cattle to control reproductive infections, such as IBR and BVD. These several articles were found by the authors of two meta-analysis based-studies aimed to evaluate the results of vaccination against BoHV-1 and BVDV on the reproductive performance of cattle herds (Newcomer *et al*., 2015; Newcomer *et al*., 2017). In these studies, variables such as the type of reproductive management, body score, herd size, vaccination and/or revaccination program, infection epidemiology in the herds (rate of seropositive animals and antibody titers), type of vaccine (monovalent, polyvalent, inactivated, attenuated), among other less studied aspects were analized. Although the results considerably vary, most studies conclude that vaccination increases the reproductive performance of herds (Pospísil *et al*., 1996; Grooms, 2004; Ficken *et al*., 2006; Zimmerman *et al*., 2007; Aono *et al*., 2013; Pereira *et al*., 2013; Ridpath, 2013; Newcomer *et al*., 2015; Newcomer *et al*., 2017).

Newcomer *et al*. (2015) evaluated the impact of BVDV vaccination on three outcomes in cows: risk of fetal infection, abortion risk, and pregnancy risk. Forty-six studies in 41 separate papers matched the inclusion criteria. The analysis revealed a decrease in abortions of nearly 45% and a nearly 85% decrease in fetal infection rate in cattle vaccinated for BVDV compared with unvaccinated cohorts. Additionally, pregnancy risk was increased by approximately 5% in field trials of BVDV vaccinations. This meta-analysis provided quantitative support for the benefit of vaccination in the prevention of BVDV-associated reproductive disease.

The meta-analysis study developed by Newcomer *et al*. (2017) comprised the analysis of 15 experiments in 10 manuscripts involving more than 7,500 animals. The aim of this meta-analysis was to determine the cumulative efficacy of BoHV-1 vaccination to prevent abortion in pregnant cattle. Risk ratio effect sizes were used in random effects, weighted meta-analyses to assess the impact of vaccination. A 60% decrease in abortion risk in vaccinated cattle was demonstrated. This meta-analysis provided quantitative support for the benefit of BoHV-1vaccination in the prevention of abortion.

Brazilian vaccination based-studies

In the central-western region of Brazil, Aono *et al*. (2013) evaluated the reproductive efficiency of 16 herds of beef cattle, of which 13 herds did not vaccinate and three herds were regularly vaccinated for IBR, BVD, and leptospirosis. All animals were submitted to the same FTAI protocol and the pregnancy rate was determined by transrectal ultrasonography at 30 and 120 days post-FTAI. The mean rate of pregnancy loss was significantly lower in the animals of the vaccinated herds when compared with the mean rate of embryonic loss observed in animals from unvaccinated herds. Concerning the category of cows, authors also observed a reduction in the embryonic mortality at 30 and 120 days post-FTAI in vaccinated and non-vaccinated primiparous cows.

Also in the Midwest Brazilian region, the effect of vaccination against IBR, BVD, and leptospirosis was evaluated from six commercial herds of beef cattle. From a total of 1,968 cows, 953 were vaccinated and 1,015 were not-vaccinated. The body score was similar for both groups. The reproductive management and diagnosis of gestation were performed as in the previous experiment. The pregnancy rate was significantly higher in the vaccinated group at both 30 and 120 days of gestation. In the group of primiparous cows there was a significant reduction in embryonic mortality. However, vaccination had no effect in the multiparous cow group (Aono *et al*., 2013).

Alternative vaccination schemes for IBR, BVD, and leptospirosis were also compared. In this experiment, the influence of the day of the first vaccination in relation to the initiation of the FTAI protocol on the pregnancy and pregnancy loss rates in primiparous Nelore cows was analyzed. Two groups of vaccinated animals were constituted. In the first group (pre-vaccinated) the first dose of vaccine was administered 30 days before the initiation of the FTAI protocol and the second dose coincided with the initiation of the protocol. The second group of cows received the first dose on the day of initiation of the FTAI protocol and the second dose 30 days after the end of the protocol. There was an effect of the vaccination scheme used on the pregnancy rate at 30 and at 120 days, being higher in the pre-vaccinated group. However, the vaccination protocol had no effect on the rate of pregnancy loss or embryo mortality (Aono *et al*., 2013).

In a series of four experiments, Pereira *et al*. (2013) also evaluated the effect of different vaccination schemes against IBR, BVD, and leptospirosis in dairy herds of Minas Gerais and Paraná states. The four studies involved the total of 3,640 Holstein or Gir x Holstein lactating cows. All the animals of each study received two doses of vaccines, which were administered in different periods based on the beginning of the FTAI protocol. The results showed that pregnant rates were higher in the groups that received the two doses of vaccine before the time of the FTAI in relation to the control group. As well, pregnancy rates were higher in comparison with the group that received the

first dose of the vaccine at the moment of FTAI. Therefore, it was concluded that when both doses of vaccines are administered prior to AI there is improve of the reproductive efficiency in dairy production systems.

Biosecurity

Currently in Brazil, the concept of biosecurity is easily associated with animal health involving the pork and poultry production chains, particularly due to the higher animal density in these both production systems. In Brazilian cattle breeding, the concepts of biosecurity are still very little used. However, we have recently observed in dairy cattle the beginning, albeit timid, of the application of some standards of biosecurity, particularly in herds with high genetics and production. Nevertheless, in the vast majority of Brazilian beef cattle, this important action for the control and prophylaxis of infectious diseases is still neglected.

Important actions must be implemented and regularly monitored in order to increase the biosecurity of the herds. Some are easier to be implemented, while others are more complex. Even at reduced percentages, some of these actions can change production costs, while in others there is practically no additional cost.

Biosecurity can be divided into two types, external and internal. External biosecurity is related to measures aimed at preventing the entry of pathogens into the cattle herd. Measures related to this type of biosecurity include quarantine before the introduction of newly acquired animals, and transit restriction of vehicle, person, and animals. Meanwhile, internal biosecurity is related to the decrease in the chance of transmission of pathogens present within the same cattle herd. Measures should be taken to clean and disinfect the installation, to provide adequate facilities according to the different age groups, to separate diseased animals (isolated facilities), to control rodent and other synanthropic animals, to perform correct disposal of dead animals avoiding the transmission of infectious agents, and to promoted the animal welfare (Sarrazin *et al*., 2014; Sahlström *et al*., 2014; Pegoraro *et al*., 2018).

Conclusion

Depending on the epidemiology of BoHV-1, BVDV and *Leptospira* spp. infection in a cattle herd, especially in certain categories of females of the herd, as well as the vaccination scheme used for the control and prophylaxis of these reproductive diseases the use of vaccines can contribute considerably to the increase the pregnancy rates and reduce embryonic mortality rates in both the Brazilian beef and dairy cattle herds.

Author contributions

AAA: provided the funding acquisition and conception of the article, made critical revisions related to important intellectual content of the manuscript and approved the final version of the article to be published; RAL and AMDA: designed and drafted the article; and AFA: participated on the conceptualization, supervision, review and editing of the manuscript.

Conflict of interest

The authors declared no potential conflicts of interest relative to the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank the following Brazilian Institutes for financial support: the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), the National Council of Technological and Scientific Development (CNPq), Financing of Studies and Projects (FINEP), and the Araucaria Foundation (FAP/PR). AA Alfieri and AF Alfieri are recipients of CNPq fellowships. RA Leme is a recipient of the CAPES fellowship. AM Dall Agnol Leme is a recipient of the FAP/PR fellowship.

Funding

This work was supported by the INCT-Leite under Grant (Number 465725/2014-7).

References

Abdalla H, Elghafghuf A, Elsohaby I, Nasr MAF. 2017. Maternal and non-maternal factors associated with late embryonic and early fetal losses in dairy cows. *Theriogenology*, 15(100):16-23.

Adler B. 2015. History of leptospirosis and leptospira. *Curr Top Microbiol Immunol*, 387:1-9.

Alfieri AA, Alfieri AF. 2017. Infectious diseases that impact the bovine reproduction [In Portuguese]. *Rev Bras Reprod Anim*, 41(1):133-9.

Almería S, López-Gatius F. 2013. Bovine neosporosis: clinical and practical aspects. *Res Vet Sci*, 95(2):303-309.

Aono FH, Cooke RF, Alfieri AA, Vasconcelos JL. 2013. Effects of vaccination against reproductive diseases on reproductive performance of beef cows submitted to fixed-timed AI in Brazilian cow-calf operations. *Theriogenology*, 79(2):242-248.

Ayalon N. 1978. A review of embryonic mortality in cattle. *J Reprod Fert*, 54: 483-493.

Balamurugan V, Alamuri A, Bharathkumar K, Patil SS, Govindaraj GN, Nagalingam M, Krishnamoorthy P, Rahman H, Shome BR. 2018. Prevalence of Leptospira serogroup-specific antibodies in cattle associated with reproductive problems in endemic states of India. *Trop Animal Health Prod*, 50(5):1131-1138.

Berge AC, Vertenten G. 2017. Whitepaper: The importance of preventive health and vaccination programmes in ruminant production. Available from https://www.timetovaccinate.com/. Accessed in: July 7th. 2019.

Bielanski A, Devenish J, Phipps-Todd B. 2000. Effect of *Mycoplasma bovis* and *Mycoplasma bovigenitalium*

in semen on fertilization and association with in vitro produced morula and blastocyst stage embryos. Theriogenology, 53(6):1213-1223.

Biswas S, Bandyopadhyay S, Dimri U, Patra PH. 2013. Bovine herpesvirus-1 (BHV-1) - a re-emerging concern in livestock: a revisit to its biology, epidemiology, diagnosis, and prophylaxis. *Vet J*, 33(2):68-81.

Butler WR. **2003**. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. *Livest Prod Sci*, 83(2-3): 211-218.

Campbell J. 2011. Pre-breeding vaccinations. Available from https://www.drovers.com/article/pre-breeding-vaccinations. Accessed in July 8th. 2019.

Carvalho LFR, Melo CB, Drummond VO. 2007. Procedures for the exportation and importation of genetic animal material to Brazil. [In Portuguese]. *Rev Bras Reprod Anim*, 31(3):415-422.

Chebel RC, Santos JEP, Reynolds JP, Cerri RL, Juchem SO, Overton M. 2004. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. *Anim Reprod Sci*, 84(3-4): 239-255.

Committee on Reproductive Nomenclature Recommendations for standardizing bovine reproductive terms. 1972. *Cornell University College of Veterinary Medicine*. 62:216-237.

Daly R. 2006. Timing of reproductive vaccinations in beef cattle herds. In: Proceedings of Applied Reproductive Strategies in Beef Cattle, 2006, Rapid City, South Dakota, USA. p.190-195.

Diskin MG, Morris DG. 2008. Embryonic and early foetal losses in cattle and other ruminants. *Reprod Domest Anim*, 43(2):260-267.

Dubovi EJ. 2013. Laboratory diagnosis of bovine viral diarrhea virus. *Biologicals*, 41(1):8-13.

Dunne LD, Diskin MG, Sreenan JM. 2000. Embryo and foetal loss in beef heifers between day 14 of gestation and full term. *Anim Reprod Sci*, 58(1-2):39-44.

Ficken MD, Ellsworth MA, Tucker CM. 2006. Evaluation of the efficacy of a modified-live combination vaccine against abortion caused by virulent bovine herpesvirus type 1 in a one-year duration-of-immunity study. *Vet Ther*, 7(3):275-282.

Fischer G, Rodrigues R, Pappen FG, Zanela MB, Ribiero MER, Almeida LL, Souza GN, Weissheimer CF, Pegoraro LMC, Pradieé J. 2018. Principais doenças da bovinocultura leiteira. [In Portuguese]. In Pegoraro, LMC (Ed.). Biosecurity in dairy cattle. Pelotas, BR: Embrapa. pp. 13-27.

Forar AL, Gay JM, Hancock DD. 1995. The frequency of endemic fetal loss in dairy cattle: a review. *Theriogenology*, 43(6):989-1000.

García-Ispierto I, López-Gatius F, Santolaria P, Yániz JL, Nogareda C, López-Béjar M, De Rensis F. **2006**. Relationship between heat stress during the peri-implantation period and early fetal loss in dairy cattle. *Theriogenology*, 65(4):799-807.

García-Ispierto I, López-Gatius, F, Bech-Sabat G, Santolaria P, Yániz JL, Nogareda C, De Rensis F, López-Béjar M. 2007a. Climate factors affecting conception rate of high producing dairy cows in northeastern Spain. *Theriogenology*, 67(8):1379-1385.

García-Ispierto I, López-Gatius F, Santolaria P, Yániz JL, Nogareda C, López-Béjar M. 2007b. Factors affecting the fertility of high producing dairy herds in northeastern Spain. *Theriogenology*, 67(3):632-638.

Gates MC, Humphry RW, Gunn GJ. 2013. Associations between bovine viral diarrhoea virus (BVDV) seropositivity and performance indicators in beef suckler and dairy herds. *Vet J*, 198(3):631-637.

Givens MD, Marley MS. 2008. Infectious causes of embryonic and fetal mortality. *Theriogenology*, 70(3):270-285.

Girardi A, Dufort I, Sirard MA. 2015. The effect of energy balance on the transcriptome of bovinegranulosa cells at 60 days postpartum. *Theriogenology*, 84:1350-1361.

Grooms DL. 2004. Reproductive consequences of infection with bovine viral diarrhea virus. *Vet Clin North Am Food Anim Pract*, 20(1):5-19.

Grooms DL. 2006. Reproductive losses caused by bovine viral diarrhea virus and leptospirosis. *Theriogenology*, 66(3):624-628.

Hamers C, Lecomte C, Kulcsar G, Lambot M, Pastoret PP. 1998. Persistently infected cattle stabilise bovine viral diarrhea virus leading to herd specific strains. *Vet Microbiol*, 61(3):177-82.

Hanzen C, Drion PV, Loutrie O, Depierreux C, Christians E. 1999. Embryonic mortality. Part 1. Clinical aspects and etiological factors on the bovine species. *Ann Med Vet*, 143(2):91-118.

Inskeep EK, Dailey RA. 2005. Embryonic death in cattle. *Vet Clin North Am Food Anim Pract*, 21(2):437-641.

Jones C, Chowdhury S. 2007. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Ver*, 8(2):187-205.

Junqueira JRC, Alfieri AA. 2006. Reproductive failures in beef cattle breeding herds with emphasis for infectious causes [In Portuguese]. *Semin Cienc Agr*, 27(2):289-298.

Kumar A, Verma AK, Rahal A. 2011. Mycoplasma bovis, a multi disease producing pathogen: na overview. *Asian J Anim Vet Adv*, 6(6):537-546.

Lanyon SR, Hill FI, Reichel MP, Brownlie J. 2014. Bovine viral diarrhoea: pathogenesis and diagnosis. *Vet J*, 199(2):201-209.

Lonergan P, Fair T, Forde N, Rizos D. 2016. Embryo development in dairy cattle. *Theriogenology*, 86(1):270-277.

Médici KC, Alfieri AA, Alfieri AF. 2000. Prevalence of neutralizing antibodies against bovine herpesvirus type 1, due natural infection, in herds with reproductive problems [In Portuguese]. *Cienc Rural*, 30(2):347-350.

Michi AN, Favetto PH, Kastelic J, Cobo ER. 2016. A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. *Theriogenology*, 85(5):781-791.

Moennig V, Becher P. 2018. Control of Bovine Viral

Diarrhea. *Pathogens*, 7(1). pii: E29.

Nandi S, Kumar M, Manohar M, Chauhan RS. 2009. Bovine herpes virus infections in cattle. *Anim Health Res Ver*,10(1):85-98

Newcomer BW, Walz PH, Givens MD, Wilson AE. 2015. Efficacy of bovine viral diarrhea virus vaccination to prevent reproductive disease: a meta-analysis. *Theriogenology*, 83(3):360-365.

Newcomer BW, Grady Cofield L, Walz PH, Givens MD. 2017. Prevention of abortion in cattle following vaccination against bovine herpesvirus 1: A meta-analysis. *Prev Vet Med*, 138:1-8.

Pegoraro LMC, Nunes e Souza G, Pradieé J, Weissheimer CF, Dereti RM, Viegas DP, Saalfed MH, Zanela MB, Almeida LL. 2018. Biosecurity measures - internal and external. [In Portuguese]. In Pegoraro, LMC (Ed.). Biosecurity in dairy cattle. Pelotas, BR: Embrapa. pp.28-41.

Pereira MH, Cooke RF, Alfieri AA, Vasconcelos JL. 2013. Effects of vaccination against reproductive diseases on reproductive performance of lactating dairy cows submitted to AI. *Anim Reprod Sci*, 37(3-4):156-162.

Pospísil Z, Krejcí J, Machatková M, Zendulková D, Lány P, Cíhal P. 1996. The efficacy of an inactivated IBR vaccine in the prevention of intra-uterine infection and its use in a disease-control programme. *Zentralbl Veterinarmed B*, 43(1):15-21.

Rani P, Dutt R, Singh G, Chandolia RK. 2018. Embryonic mortality in cattle – A review. *Int J Curr Microbiol App Sci*, 7(7):1501-1516.

Ridpath JF. 2013. Immunology of BVDV vaccines. *Biologicals*, 41(1):14-9.

Rufino FA, Seneda MM, Alfieri AA. 2006. Bovine herpesvirus 1 and bovine viral diarrhea virus impact in the embryo transfer [In Portuguese]. *Arch Vet Sci*, 11(1):78-84.

Sahlström L, Virtanen T, Kyyrö J, Lyytikäinen T. 2014. Biosecurity on Finnish cattle, pig and sheep farms - results from a questionnaire. *Prev Vet Med*, 117(1):59-67.

Sánchez JM, Mathew DJ, Passaro C, Fair T, Lonergan P. 2018. Embryonic maternal interaction in cattle and its relationship with fertility. *Reprod Domest Anim*, 53(2):20-27.

Sanhueza JM, Heuer C, West D. 2013. Contribution of Leptospira, Neospora caninum and bovine viral diarrhea virus to fetal loss of beef cattle in New Zealand. *Prev Vet Med*, 112(1-2):90-98.

Sarrazin S, Cay AB, Laureyns J, Dewulf J. 2014. A survey on biosecurity and management practices in selected Belgian cattle farms. *Prev Vet Med*, 117(1):129-139.

Sheldon IM and Owens. 2017. Postpartum uterine infection and endometritis in dairy cattle. In Proceedings of the 33rd Annual Scientific Meeting of the European Embryo Transfer Association (AETE), 2017, Bath, United Kingdom. *Anim* Reprod, 14(3):622-629.

Silke V, Diskin MG, Kenny DA, Boland MP, Dillon P, Mee JF, Sreenan JM. 2002. Extent, pattern and factors associated with lateembryonic loss in dairy cows. *Anim Reprod Sci*, 71(1-2):1-12.

Silva JR, Ferreira LF, Oliveira PV, Nunes IV, Pereira ÍS, Timenetsky J, Marques LM, Figueiredo TB, Silva RA. 2016. Intra-uterine experimental infection by *Ureaplasma diversum* induces TNF-α mediated womb inflammation in mice. *An Acad Bras Cienc*, 88(1):643-652.

Souza, GN; Pegoraro, LMC; Weissheimer, CF; Fischer, G; Dellagostin, O; Bialves, TS; Gindri, P; Lucas, RM; Muller, L; Cavalcanti, F; Weiller, OH. 2017. Situação epidemiológica e fatores de risco para problemas reprodutivos em bovinos leiteiros localizados em diferentes mesorregiões do Estado do Rio Grande do Sul 2016/2017. Embrapa Gado de Leite. Boletim de Pesquisa e Desenvolvimento, 36. Juiz de Fora: Embrapa Gado de Leite, 2017. 22p.

Van Drunen Little-van den Hurk S. 2006. Rationale and perspectives on the success of vaccination against bovine herpesvirus-1. *Vet Microbiol*, 113(3-4):275-82.

Vanroose G, de Kruif A, Van Soom A. 2000. Embryonic mortality and embryo-pathogen interactions. *Anim Reprod Sci*, 60-61:131-43.

Vasconcelos, JLM, Carvalho R, Peres RFG, Rodrigues ADP, Junior IC, Meneghetti M, Aono FH, Costa WM, Lopes CN, Cooke RF, Pohler KG. 2017. Reproductive programs for beef cattle: incorporating management and reproductive techniques for better fertility. In Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Cabo de Santo Agostinho, PE, Brazil, 2017, Anim Reprod, 14(3):547-557.

Walsh SW, Williams EJ, Evans AC. 2011. A review of the causes of poor fertility in high milk producing dairy cows. *Anim Reprod Sci*, 123(3-4):127-138.

Zimmerman AD, Buterbaugh RE, Herbert JM, Hass JM, Frank NE, Luempert Iii LG, Chase CC. 2007. Efficacy of bovine herpesvirus-1 inactivated vaccine against abortion and stillbirth in pregnant heifers. *J Am Vet Med Assoc*, 231(9):1386-9.

DOI: 10.21451/1984-3143-AR2019-0058

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Intensive use of IVF by large-scale dairy programs

Bruno Valente Sanches[1], Amanda Fonseca Zangirolamo[2,3], Marcelo Marcondes Seneda[2,3,*]

[1]Vytelle IVF, LLC, Hermiston, OR, USA.
[2]Universidade Estadual de Londrina, Laboratório de Reprodução Animal, DCV-CCA-UEL, Londrina, Parana, Brazil.
[3]National Institute of Science and Technology for Dairy Production Chain (INCT–LEITE), Universidade Estadual de Londrina, Rodovia Celso Garcia Cid-Campus Universitário, Parana, Brazil.

Abstract

The number of embryos produced by *in vitro* fertilization (IVF) has grown exponentially in recent years. Recently, for the first time, the number of embryos produced and transferred *in vitro* was significantly higher than the number developed *in vivo* worldwide. In this context, a particular boost occurred with ovum pick-up (OPU) and *in vitro* embryos produced in North America, and this technology is becoming more prominent for commercial dairy farms. However, despite many advances in recent decades, laboratories and companies are looking for methods and alternatives that can be used in collaboration with the existing process to improve it. Among the strategies used to improve the dairy industry are the use of genomic analysis for the selection of animals with desired traits or as an evaluation tool of oocyte and embryo quality, the optimization of the collection and use of gametes from prepubertal females and males, the effective use of sexed semen, and improvements in culture media and methods of embryo cryopreservation. Thus, this review aims to discuss the highlights of the commercial use of IVF and some strategies to increase the application of this technique in large-scale dairy programs.

Keywords: IVF, bovine, dairy, commercial use, genomic analysis.

Introduction

The dairy industry plays an essential role in the global socioeconomic scenario. Although growth in global milk production has been limited in recent years, it is projected to increase by 22% in 2027 compared to 2015-2017 (OECD and FAO, 2018). The dairy industry is the leader among the food animal sector in the successful application of advanced technologies (Wiggans *et al*., 2017). Therefore, practices and alternatives that improve the production of dairy cattle are increasingly required.

The increase in the productive efficiency and quality of animal products from livestock has been possible due to the use of reproductive biotechniques (Hansen *et al*., 2014). In this context, *in vitro* fertilization (IVF) is a useful tool when performing the selection and breeding of genetically superior animals (Hansen *et al*., 2014), which is becoming more prominent in commercial dairies (Sirard, 2018).

According to the Embryo Transfer Newsletter (Viana, 2017), for the first time, the number of bovine embryos produced and transferred *in vitro* was significantly higher than those *in vivo* produced worldwide (Figs. 1-2; Viana, 2017).

Furthermore, for the first time since 1999, North America has reported more *in vitro* produced (IVP) embryos than South America, the region that led the use of IVF in the past decade (Fig. 3; Viana, 2018). Notably, the United States (US) had the highest number of IVP embryos within North America, at approximately 95.5% (Viana, 2018). The further development of the embryo industry in North America seems to resemble what happened in South America, in which the contribution of *in vivo* embryos has been linear, and the use of IVP embryos has resulted in a substantial increase in numbers (Viana *et al*., 2018).

However, despite advances in IVF, the embryo production rate from the total cumulus oocyte complexes (COC), the embryo production rate remains at 30 to 40% (Lonergan *et al*., 2016). Thus, laboratories and companies have been looking for alternatives that collaborate to improve the existing process and to optimize methods to use IVF in large-scale dairy programs.

The genomic testing of cattle is now significantly affecting IVF programs. Genomic selection has revolutionized dairy farming, shortening the breeding interval, increasing selection accuracy, and reducing the previous costs of progeny testing (Wiggans *et al*., 2017). The commercial interest in performing genomic analysis and collecting gametes from prepubertal animals that have desired traits is increasing (Moore and Hasler, 2017). The small ultrasound OPU probes currently available allow IVP embryos from younger females to be grown (Sirard, 2018). Additionally, genomic analysis has been used to evaluate the quality and viability of oocytes, and even the embryos, before transfer procedures (Moore and Hasler, 2017).

An embryo culture media have been developed to mimic what happens in the maternal organism. Several studies have been performed to investigate the addition of different products and molecules in the culture medium, such as cytokines, growth factors, and antioxidants, and many advances have been obtained.

Sexing technology is another practice used to improve IVF results.; The use of sexed semen enables the birth of offspring of a predetermined sex, as well as increase the efficiency of producing donors with the right genetic background. Furthermore, IVF is the most

*Corresponding author: marcelo.seneda@uel.br
orcid.org/0000-0002-5097-5119
Received: May 10, 2019
Accepted: July 26, 2019

common application of sexed semen, which has superior efficacy compared with its use in other areas of biotechnology (Morotti *et al*., 2014).

Due to the increasing number of IVP embryos, cryopreservation methods provide a good alternative for the storage of surplus products. However, some limitations exist, which may hamper the use of cryopreservation on a large scale. In this context, among the different protocols, the process of thawing and the direct transfer of embryos together make the cryopreservation protocol more efficient for commercial use by facilitating logistics in the field (Sanches *et al*., 2016).

Thus, this review aims to discuss some strategies to increase the useful application of IVF in large-scale dairy programs, as well as the trends, challenges, and highlights of the commercial use of the IVF program.

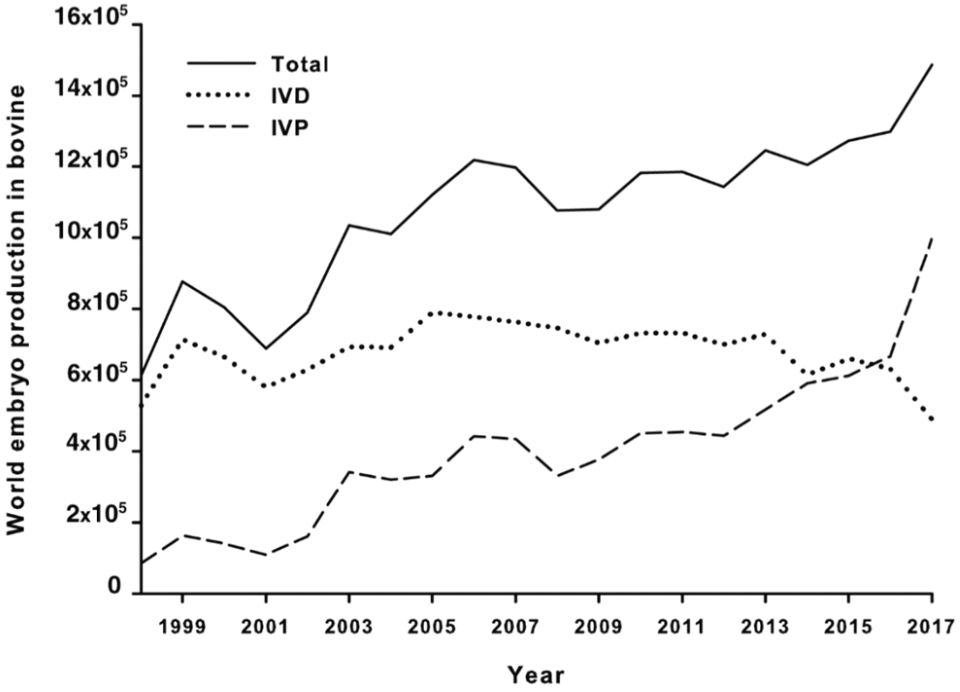


Figure 1. The number of bovine embryos produced (*in vivo* - IVD, *in vitro* - IVP, and total) recorded in the period 1998 - 2017 (Data sourced from Viana, 2017; Viana, 2018).

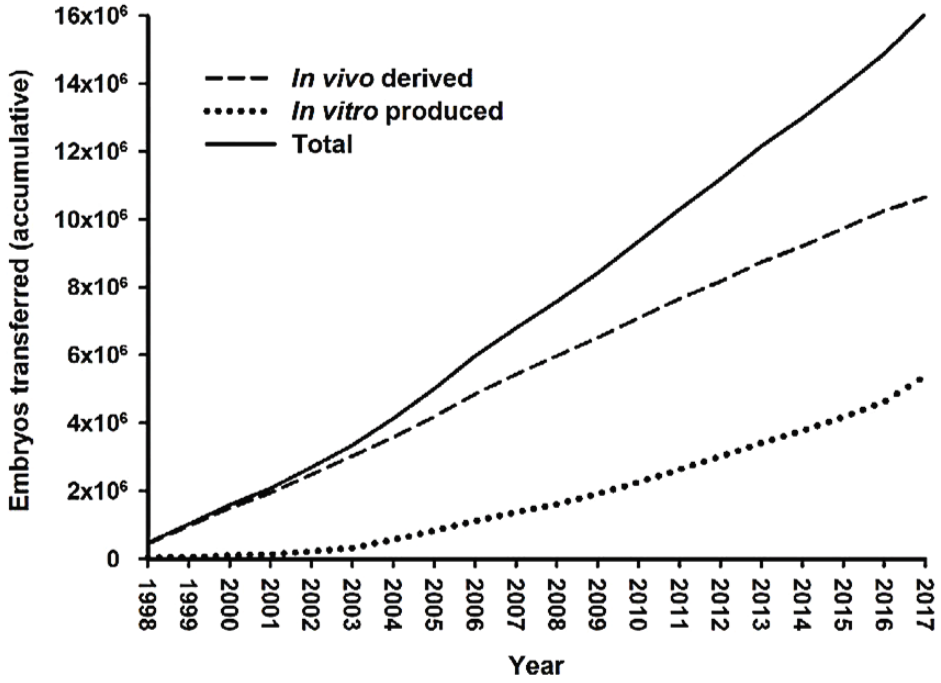


Figure 2. The accumulated number of bovine embryos transferred in the period 1998 - 2017, based on *in vivo* or *in vitro* production methods (Data sourced from Viana, 2017; Viana, 2018).

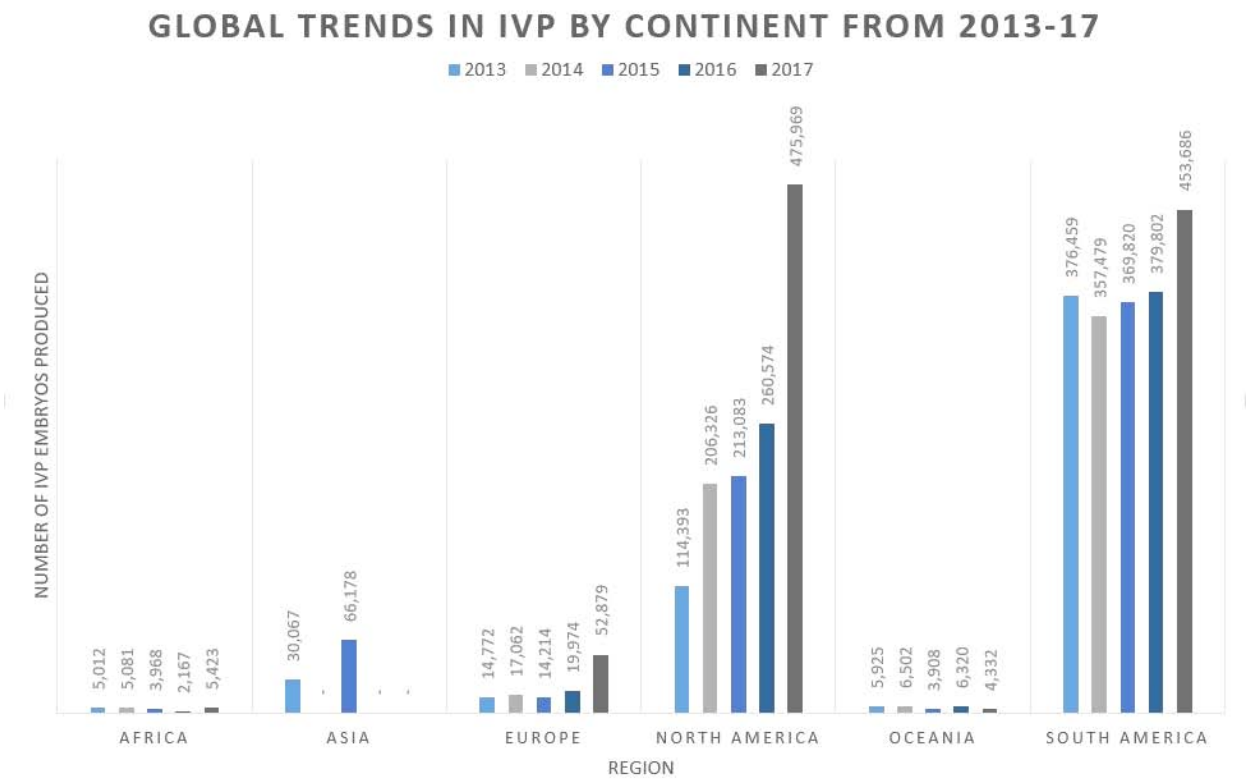


Figure 3. The accumulated number of IVP bovine embryos in the period 2013 - 2017 by continent (Data sourced from Viana, 2017; Viana, 2018).

Genomic analysis: from animal selection to oocyte and embryo evaluation

Currently, genomic analysis is driving the development of several IVF laboratories in North America and other places in the world (Sirard, 2018). The best effect of genomic selection to date has been to double the rate of genetic progress for traits of economic interest. Genetic improvement occurs through the increased accuracy of genetic merit for young animals (Wiggans *et al*., 2017).

With genomic analysis performed soon after birth, the genetic value of the bull is determined early, and as soon as semen is produced, such high genetic merit sperm, can be used for IVF. Moreover, it has increased the commercial demand for producing embryos from young heifers and calves (Sirard, 2018). Currently, the collection of oocytes from donors before puberty is possible with relatively high success (Landry *et al*., 2016). Additionally, genomic selection is helpful for choosing better embryo recipients according to the genes involved with gestation maintenance.

Genomic evaluations for Holsteins, Jerseys, and Brown Swiss became official in 2009 at the USDA, and since that time, more than 1 million animal genotypes have received genetic evaluations (Council on Dairy Cattle Breeding, 2016). Due to the popularity of genotyping chips, microsatellites have been replaced by SNPs, and the accessibility to chips of lower cost has made whole herd genotyping common in the US (Wiggans *et al*., 2017). The scenario of genotyped animals included in US genomic evaluations for dairy cattle is shown in Fig. 4.

Additionally, dairy cattle can be selected for any combination of traits, but total genetic progress will be fastest using an index because many traits affect profitability. In this context, the lifetime net merit (NM$) index, elaborated by the U.S. Department of Agriculture (USDA), ranks dairy animals based on their combined genetic merit for economically important traits (VanRaden, 2018). The NM$ index includes economically important traits related to health, yield, longevity and calving ease, and because it is calculated using Holstein values, it is, therefore, widely used for this breed (VanRaden, 2018). The weighting and composition of the 14 traits that make up the net merit in the year 2018 are shown in Fig. 5.

However, the relative importance of traits differed slightly between the production systems. Organic dairy producers, for example, tend to prefer health traits as the pillar of selection, even though the increase in the genetic gain in disease resistance is achieved at the expense of milk production, since they cannot use any medicine or chemical in animals (Fall *et al*., 2008). Moreover, the CM$ provides longevity and somatic cell score data for producers whose milk is made into cheese or other dairy products (VanRaden, 2017). Although the Jersey produces less milk than the Holstein, it produces milk with more fat, milk protein, and a higher energy content (Aikman, *et al*., 2007). Therefore, for Jersey cows, the CM$ would possibly be more interesting.

Another application of genomic analysis is the use of micro-array or RNAseq technologies on embryos

submitted to different culture conditions, with the intention of comparing *in vivo* control embryos and, thus, improving culture methods (Sirard, 2018). Furthermore, embryo culture medium can provide a source of material for noninvasive embryonic genetic testing (without biopsies, for example). However, whether the DNA found represents the genetic state of the embryo remains unknown. Thus, this potentially noninvasive approach must be validated by additional experiments (Liu *et al.*, 2017; Smith *et al.*, 2019), and once confirmed, it can lead to other methods to evaluate the quality of embryos.

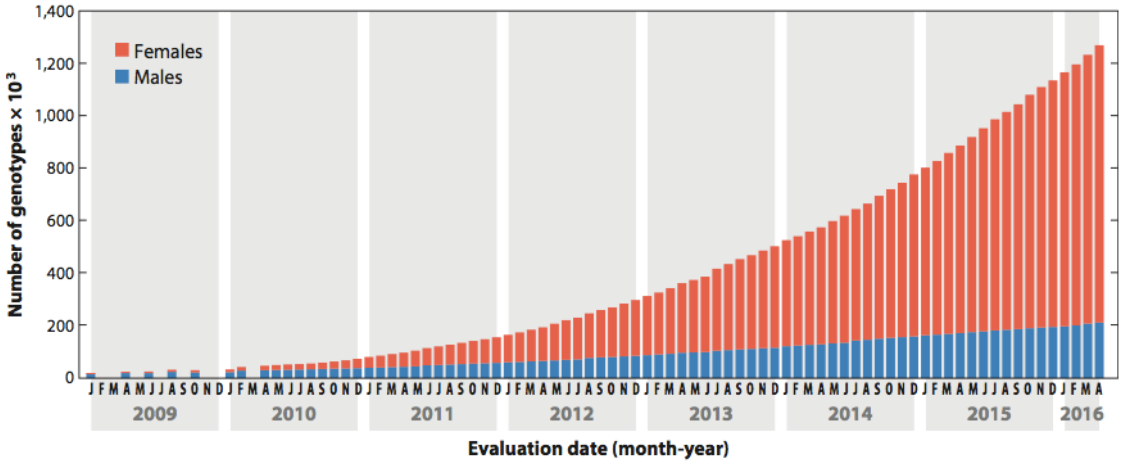


Figure 4. The number of genotyped animals included in US genomic evaluations for dairy cattle since January 2009 (Data sourced from the Council on Dairy Cattle Breeding, 2016; Wiggans *et al.*, 2017).

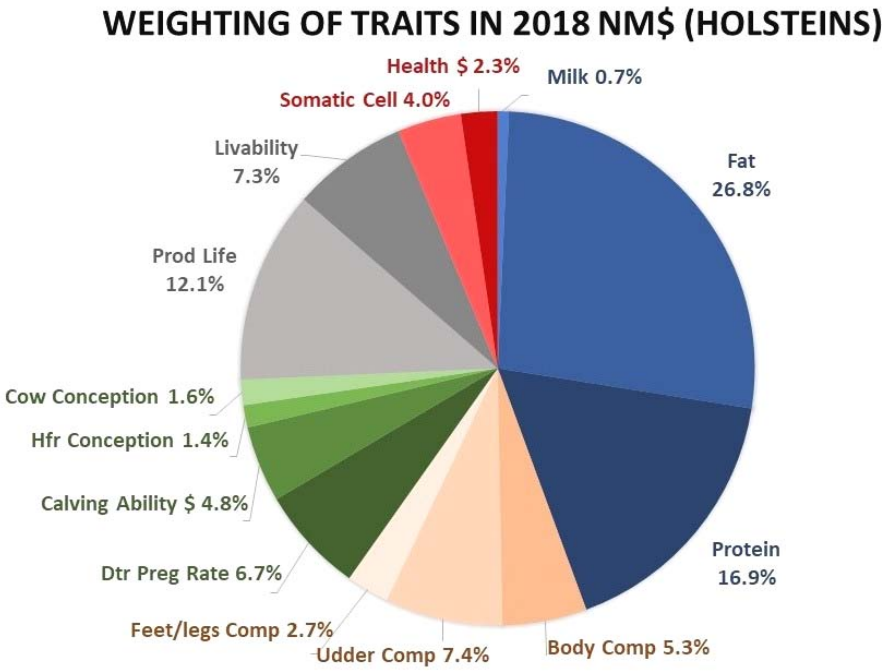


Figure 5. Composition and weighting of the 14 traits in 2018 Net Merit. (Available on: https://hoards.com/article-23717-net-merit-$-index-updated-to-include-health-traits.html. Accessed on April 10, 2019).

Strategies to improve oocyte competence before OPU

The quality of the oocyte is the central factor interfering with the blastocyst yield, as well as the potential explanation for the limited success rates of IVF (Lonergan and Fair, 2016). The oocyte competence, and consequently the development to the blastocyst stage, is positively associated with the size of the antral follicle (Lonergan *et al.*, 1994) and whether the blastocyst was produced *in vivo* or *in vitro* (Rizos *et al.*, 2002).

One strategy for manipulating follicular growth and affecting developmental competence is Coasting, which is a period between hormonal stimulation and ovary collection (Nivet *et al.*, 2012). In adult females, this approach allowed a high rate of blastocyst development after IVF, suggesting an increase in oocyte quality (Blondin *et al.*, 2002). Animals that received six

injections of FSH, followed by a 48-h coasting period and an injection of LH 6 h before ovum pick-up (OPU), presented an 80% rate of blastocyst stage occurrence (Blondin *et al*., 2002). More recently, the same group showed that the ideal period was 54 ± 7 hours, where a well-defined period of competence to recover oocytes of the highest quality is of paramount importance (Nivet *et al*., 2012).

During OPU oocytes are recovered on random days of the estrous cycle, i.e., follicles that are at different stages of development (Wit *et al*., 2000). In these conditions, more than 85% of the aspirated ovarian follicles present some degree of atresia due to the apoptosis process (Hendriksen *et al*., 2000). Recently, follicular wave synchronization before OPU was observed to provide an increase in embryo production rates and post-transfer conception for the recipients (Cavalieri *et al*., 2018).

Collection and quality of oocytes from prepubertal heifers and calves

Recently, there has been an increase in the commercial interest in producing bovine embryos from prepubertal heifers and calves. The interest in breeding the best animals at younger ages is to accelerate the genetic advancement rate of genetic gain (Baldassarre *et al*., 2018). With the emergence of genomic technologies in recent years, the prediction of better phenotype production has been possible after birth of the animal (Ponsart *et al*., 2013).

In the early 1990s, the development of transvaginal oocyte recovery procedures in cattle improve the IVF method (Pieterse *et al*., 1991). Initially, due to animal size issues, the collection of oocytes by OPU was very difficult or not possible, and laparoscopic ovum pick-up (LOPU) rapidly became the method of choice for small animals such as calves and pre-pubertal heifers (Cognie *et al*., 2004). Currently, small ultrasound OPU probes are available and allow IVP embryos from younger females to be grown (Moore and Hasler, 2017).

Several studies have shown that bovine calf oocytes are significantly less capable of developing into embryos compared with oocytes from adult cows (Baldassarre and Bordignon, 2018). Prepubertal females have immature and nonfunctional hypothalamus-pituitary-ovarian axes and, therefore, are unable to achieve full follicular development and ovulation (Sanchez and Smitz, 2012). Thus, different research groups are directing efforts seeking to improve quality and increase oocyte competence in young heifers.

Studies described the recovery of a high number of oocytes from females 2-6 months of age who were stimulated with gonadotropins to increase the proportion (and size) of large follicles (Baldassarre and Bordignon, 2018). In some cases, the number of oocytes was higher than what was recovered from adult cows.

B. taurus and *B. indicus* aging from 2 to 4 months did not exhibit an improvement in IVF results when stimulated with 140 mg of FSH (Batista *et al*., 2016). However, recently, more prolonged FSH stimulation (three days) was shown to increase the development competence of Holstein calf oocytes, which was associated with a higher proportion of follicles larger than 5 mm (Currin *et al*., 2017).

A previous study showed that Holstein calves aging from 5 to 7 months had more oocytes than cycling heifers aging from 16 to 18 months. Although the blastocyst rate was higher in the cycling heifers than in the calves, the number of embryos (6–8) was not different (Landry *et al*., 2016). Further studies are necessary to investigate the beneficial effects of exogenous gonadotropins to prepubertal heifers and calves.

Other possible future efforts of research include the development of *in vitro* maturation (IVM) protocols with strategies for delaying nuclear and improving cytoplasmic maturation. Additionally, another research target includes the supplementation of IVM medium with substances or molecules that improve oocyte development for step embryo transfer (Baldassarre and Bordignon, 2018). We also believe that the epigenetic changes or even the nutrition of the mother, which can interfere in the quality of the oocytes of the daughters, will be an area for future research.

Thus, with the advent of genomic analysis, the extraordinary growth of IVF technologies in recent years and high interest by dairy producers and the use of elite females that are as young as possible (from 2 months of age) for embryo production has the potential to help IVF become a viable practice very soon.

Advances in embryo culture media

For *in vitro* embryo production (IVEP), specific media are used for maturation, fertilization, and *in vitro* culture to mimic what occurs physiologically in the organism. In cattle, approximately 90% of immature oocytes, recovered from follicles at unknown stages of the estrous cycle (ovaries from slaughterhouse), undergo nuclear maturation *in vitro* and approximately 80% undergo fertilization (Lonergan and Fair, 2016).

The media used may be a determinant factor in the production and quality of blastocysts and embryo cryotolerance (Sanches *et al*., 2013). Changes in the culture conditions such as the addition of lipolytic chemical substances and the adjustment of fetal calf serum in the medium have been proposed to increase the embryo cryotolerance (Sanches *et al*., 2017). In this context, several studies show forskolin and phenazine ethosulfate (PES), as substances which reduces lipid accumulation (Sudano *et al*., 2011; Paschoal *et al*., 2017).

Although modest improvements have occurred in the development and composition of IVM media (addition of different products, cytokines, growth factors, antioxidants, and other substances), the blastocyst rate rarely exceeds 40–50% (Lonergan and Fair, 2016). Thus, the yield of oocytes developing to the blastocyst stage remains very similar to that in the years 1990 to 2000, in which it reached a plateau at 30-40% (Sirard, 2018).

Another strategy to improve embryo culture media is to try to keep what occurs physiologically in the follicular environment of the oocyte, in which the arrest of meiosis is maintained. Several meiotic inhibitors can delay the resumption of *in vitro* meiosis. Thereby, the continuous accumulation of mRNA and proteins within the oocyte allows a better cytoplasmic maturation (Bilodeau-Goessels, 2012).

However, despite the many protocols and tested methods *in vitro*, attention has turned toward the source of oocytes as the cause of the limited success rates of IVF (Sirard, 2018).

Use of sexed semen and its advantages

In the dairy industry, the production of overweight calves from undesirable sex (i.e. male) is a particularly important issue (Holden and Butler, 2018). The use of sexed semen in association with reproductive biotechnologies represents a significant advance in the global livestock industry. In this context, the predetermination of the sex of the animal optimizes production and profitability in dairy herds (Morotti *et al*., 2014).

Among the reproductive biotechnologies, the most common application of sexed semen is IVF due to good blastocyst rates can be achieved (Matoba *et al*., 2014). Moreover, IVF to require far less sperm per oocyte to make acceptable fertilization rates compared with AI (Holden and Butler, 2018). In the US, more than 90% of 4.5 to 5 million straws of sexed semen were from milk dairy bull sires in 2016 (Moore and Hasler, 2017).

However, studies show that the blastocyst rates are lower than those obtained with conventional semen (Seidel Jr. 2014). On the other hand, Cottle *et al*. (2018) identified a significant profit advantage for using sexed semen in the context of a high-output, dairy system of spring births in Ireland. The authors concluded that the use of sexed semen is more appropriate for those farms that already have an excellent fertility performance. Thus, the lowest rates associated with sexed semen can be less acceptable for farms with sub-optimal dairy herds fertility (Cottle *et al*., 2018).

In any case, genetic targeting of the dairy herd to achieve desired sex animals justifies the expansion of the use of sexed semen in the dairy sector.

Advances in cryopreservation with the use of DT

Despite the IVF advantages, cryopreservation represents a challenge for commercial laboratories. The low cryotolerance of IVP embryos is a limiting factor to the use of the cryopreservation process in an IVF program (Sudano *et al*., 2011). In addition, after the cryopreservation is well established, we believe the number of field technicians trained to do the transfer process will not be sufficient. Thus, efforts should be made to overcome all limitations involving the use of IVP embryo cryopreservation on a large scale and globally.

Among cryopreservation techniques, vitrification is more often used worldwide due to the speed and low cost (Dode, 2013). However, its method requires a high concentration of cryoprotectants and a trained professional to evaluate embryo quality prior to the transfer (Vajta and Kuwayama, 2006).

In contrast, the direct transfer (DT), a method used since the 1990s to simplify the post-thawing rehydration step of *in vivo* embryos, has been proving to be a useful alternative for commercial use in IVP embryos.

In a study with Girolando donors (1/2 Gir and 1/2 Holstein), the conception rates obtained were 51.35 ± 1.87% (133/259) for the fresh embryos, 35.89 ± 3.87% (84/234) for the vitrified embryos, and 40.19 ± 4.65% (125/311) for the embryos submitted to DT (Sanches *et al*., 2016). Possibly, IVP embryos with sexed semen could be directly transferred with similar conception rates to those submitted to vitrification.

The low concentration of cryoprotectants is the main advantage of this technique because of the reduced toxicity to the embryos (Voelkel and Hu, 1992). Furthermore, the DT eliminates the evaluation before transfer and, therefore, is more practical than vitrification (Sanches *et al*., 2017). Finally, due to the promising results, DT has been implemented in large-scale operations, mainly in the US and Brazil.

Final comments

The genomic selection of young animals, associated with sexed semen and frozen IVP-blastocysts and following direct transfer protocols, is driving a new era of IVF in the dairy sector (Sirard, 2018). However, since many of these processes are sensitive to operators or even the environment, the challenge of making IVF fully business-grade remains.

In the US, some large dairies have already left behind the commercially available genomic tests and have begun to implement their own genomic assessments and methods for identifying the best animals to be reproduced. Therefore, this behavior of the industry indicates that companies in the business of IVF also need to invest in innovation to develop a more personalized product because, ultimately, they must go beyond the goal of delivering a quality embryo and/or ensuring a high pregnancy rate.

Author contributions

AFZ: Conceptualization, Writing, Data curation; BVS: Conceptualization, Editing; MMS: Supervision.

Conflict of interest

Authors declare there is no conflict of interest.

Acknowledgments and Funding disclosure statement

MMS: supported by CNPq 403862/2016-7 and CNPq 308460/2018-9. National Institute of Science and Technology for Dairy Production Chain.

References

Aikman PC, Reynolds CK, Beever DE. 2007. Diet digestibility, rate of passage, and eating and rumination behavior of jersey and holstein cows. *J Dairy Sci*, 91:1103-1114.

Baldassarre H, Bordignon V. 2018. Laparoscopic ovum pick-up for *in vitro* embryo production from dairy bovine and buffalo calves. *Anim Reprod*, 15:191-196.

Baldassarre H, Currin L, Michalovic L, Bellefleur A, Gutierrez K, Mondadori RG, Glanzner WG, Schuermann Y, Bohrer RC, Dicks N, Lopez R, Grand FX, Vigneault C, Blondin P, Gourdon J, Bordignon V. 2018. The interval of gonadotropin administration for *in vitro* embryo production from oocytes collected from Holstein calves between 2 and 6 months of age by repeated laparoscopy. *Theriogenology*, 116:64-70.

Batista EOS, Guerreiro BM, Freitas BG, Silva JCB, Vieira LM, Ferreira RM, Rezende RG, Basso AC, Lopes RNVR, Rennó FP, Souza AH, Baruselli PS. 2016. Plasma anti-Müllerian hormone as a predictive endocrine marker to select *Bos taurus* (Holstein) and *Bos indicus* (Nelore) calves for *in vitro* embryo production. *Domest Anim Endocrinol*, 54:1-9.

Bilodeau-Goeseels S. 2012. Bovine oocyte meiotic inhibition before *in vitro* maturation and its value to *in vitro* embryo production: Does it improve developmental competence? *Reprod Domest Anim*, 47:687-93.

Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard MA. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod*, 66:38-43.

Cavalieri FLB, Morotti F, Seneda MM, Colombo AHB, Andreazzi MA, Emanuelli IP, Rigolon LP. 2018. Improvement of bovine *in vitro* embryo production by ovarian follicular wave synchronization prior to ovum pick-up. *Theriogenology*, 57:70-117.

Cognie Y, Poulin N, Locatelli Y, Mermillod P. 2004. State-of-the-art production, conservation and transfer of in-vitro-produced embryos in small ruminants. *Reprod Fertil Dev*, 16:437-445.

Cottle DJ, Wallace M, Lonergan P, Fahey AG. 2018. Bioeconomics of sexed semen utilization in a high-producing Holstein-Friesian dairy herd. *J Dairy Sci*, 101: 4498-4512.

Council on Dairy Cattle Breeding. 2016. Genotypes Included in Evaluations by Breed, Chip Density, Presence of Phenotypes (Old vs. Young), and Evaluation Year-Month (Cumulative). Bowie, MD: Council Dairy Cattle Breed. https://www.cdcb.us/Genotype/cur_density.html.

Currin L, Michalovic L, Bellefleur AM, Gutierrez K, Glanzner W, Schuermann Y, **Bohrer RC, Dicks N, da Rosa PR, De Cesaro MP, Lopez R, Grand FX, Vigneault C, Blondin P, Gourdon J, Baldassarre H, Bordignon V**. 2017. The effect of age and length of gonadotropin stimulation on the *in vitro* embryo development of Holstein calf oocytes. *Theriogenology*, 104:87-93.

Dode MAN, Leme LO, Spricigo JFW. 2013. Criopreservação de embriões bovinos produzidos *in vitro*. *Rev Bras Reprod Anim*, 37:145-150.

Fall N, Forslund K, Emanuelson U. 2008. Reproductive performance, general health, and longevity of dairy cows at a Swedish research farm with both organic and conventional production. *Livest Sci*, 118:11-19.

Hansen P. 2014. Current and Future Assisted Reproductive Technologies for Mammalian Farm Animals. *Adv Exp Med Biol, Advances in Experimental Medicine and Biology*, 752:1-22.

Hendriksen PJ, Vos PL, STeenweg WN, Bevers MM, DIeleman SJ. 2000. Bovine follicular development and its effect on the *in vitro* competence of oocytes. *Theriogenology*, 53:11-20.

Holden SA, Butler ST. 2018. Review: Applications and benefits of sexed semen in dairy and beef herds. *Animal*, 12(s1):s97-s103

Landry DA, Bellefleur AM, Labrecque R, Grand FX, Vi- gneault C, Blondin P, Sirard MA. 2016. Effect of cow age on the *in vitro* developmental competence of oocytes obtained after FSH stimulation and coasting treatments. *Theriogenology*, 86:1240-1246.

Liu W, Liu J, Du H, Ling J, Sun X, Chen D. **2017**. Non-invasive preimplantation aneuploidy screening and diagnosis of beta thalassemiaIVSII654 mutation using spent embryo culture medium. *Ann Med*, 49:319-328.

Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. *Mol Reprod Dev*, 37:48-53.

Lonergan P, Fair T. 2016. Maturation of Oocytes *in vitro*. *Annu Rev Anim Biosci*, 4:10.1-10.14.

Lonergan P, Fair T, Forde N, Rizos D. 2016. Embryo development in dairy cattle. *Theriogenology*, 86:270-277.

Matoba S, Yoshioka H, Matsuda H, Sugimura S, Aikawa Y, Ohtake M, Hashiyada Y, Seta T, Nakagawa K, Lonergan P. 2014. Optimizing production of *in vivo*-matured oocytes from superstimulated Holstein cows for *in vitro* production of embryos using X-sorted sperm. *J Dairy Sci*, 97:743-753.

Moore SG, Hasler JF. 2017. A 100-Year Review: Reproductive technologies in dairy science. *J Dairy Sci*, 100:10314-10331.

Morotti F, Sanches B, Pontes J, Basso A, Siqueira E, Lisboa L, Seneda M. 2014. Pregnancy rate and birth rate of calves from a large-scale IVF program using reverse-sorted semen in *Bos indicus*, *Bos indicus-taurus*, and *Bos taurus* cattle. *Theriogenology*, 81:696-701.

Nivet A-L, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard M.A. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction*, 143:165-171.

Organisation for Economic Co-operation and Development (OECD), Food and Agriculture Organization (FAO). 2018. Agricultural Outlook 2018-2027: Dairy and dairy products. 2018:29pp.

Paschoal DM, Sudano MJ, Schwarz KRL, Maziero RRD, Guastali MD, Crocomo LF, Magalhães LCO,

Martins Jr, A, Leal ALV, Landim-Alvarenga FC. 2017. Cell apoptosis and lipid content of *in vitro*-produced, vitrified bovine embryos treated with forskolin. *Theriogenology*, 87:108-114.

Pieterse MC, Vos PL, Kruip TA, Wurth YA, van Beneden TH, **Willemse AH, Taverne MA**. 1991. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology*, 35:857-862.

Ponsart C, Le BD, Knijn H, Fritz S, Guyader-Joly C, Otter T, Lacaze S, Charreaux F, Schibler L, Dupassieux D, Mullaart E. 2013. Reproductive technologies and genomic selection in dairy cattle. *Reprod Fertil Dev*, 26:12-21.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-48.

Sanches BV, Marinho LSR, Filho BDO, Pontes JHF, Basso AC, Meirinhos MLG, Silva-Santos KC, Ferreira CR, Seneda MM. 2013. Cryosurvival and pregnancy rates after exposure of IVF-derived Bos indicus embryos to 17 forskolin before vitrification. *Theriogenology*, 80:372-377.

Sanches BV, Lunardelli PA, Tannura JH, Cardoso BL, Pereira MHC, Gaitkoski G, Basso AC, Arnold DR, Seneda MM. 2016. A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, 85:1147-1151.

Sanches BV, Zangirolamo AF, Silva NC, Morotti F, Seneda MM. 2017. Cryopreservation of *in vitro*-produced embryos: challenges for commercial implementation. *Anim Reprod*, 14:521-527.

Sanchez F, Smitz J. 2012. Molecular control of oogenesis. *Biochim Biophys Acta*, 1822:1896-1912.

Seidel Jr, GE. 2014. Update on sexed semen technology in cattle. *Animal*, 8:160-164.

Smith HL, Stevens A, Minogue B, Sneddon S, Shaw L, Wood L , Adeniyi T, Xiao H, Lio P, Kimber SJ, Brison DR. 2019. Systems based analysis of human embryos and gene networks involved in cell lineage allocation. *BMC Genomics*, 20:171.

Sirard M. 2018. 40 years of bovine IVF in the new genomic selection context. *Reproduction*, 156:R1-R7.

Sudano MJ, Paschoal DM, Rascado TD, Magalhaes LCO, Crocomo LF, Lima-Neto JF, Landim-Alvarenga FD. 2011. Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology*, 75:1211-1220.

Vajta G, Kuwayama M. 2006. Improving cryopreservation systems. *Theriogenology*, 65**:**236-244.

VanRaden PM. 2017. Net merit as a measure of lifetime profit: 2017 revision. *AIP Research Report*, NM$6:2-17.

VanRaden PM. 2018. Net merit as a measure of lifetime profit: 2018 revision. *AIP Research Report*, 7:5-18.

Viana JHM. 2017. Statistics of embryo collection and transfer in domestic farm animals. Embryo Transfer. *Newsletter*, 36:08-25.

Viana JHM, Figueiredo ACV, Gonçalves RLR, Siqueira LGB. 2018. A historical perspective of embryo-related technologies in South America. *Anim Reprod*, 15:963- 970.

Voelkel SA, Hu YX. 1992. Direct transfer of frozen-thawed bovine embryos. *Theriogenology*, 37:23-37.

Wiggans GR, Cole JB, Hubbard SM, Sonstegard TS. 2017. Genomic Selection in Dairy Cattle: The USDA Experience. Ann *Rev Anim Biosci*, 5:13.1-13.19.

Wit AAC, Wurth YA, Kruip AM. 2000. Effect of ovarian and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J Anim Sci*, 78:1277-1283.

DOI: 10.21451/1984-3143-AR2019-0041

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Pre-TAI protocol strategies to increase reproductive efficiency in beef and dairy cows

José Nélio de Sousa Sales[1,*], Luiz Manoel Souza Simões[1], Raphael Evangelista Orlandi[1], Eduardo Alves Lima[1], Ana Paula Castro Santos[1], Miguel Pizzolante Bottino[1], Luiz Augusto Capellari Leite da Silva[1], José Camisão de Souza[2], Marcelo Maronna Dias[3], João Paulo Martinelli Massoneto[4], Luiz Antônio Scandiuzzi Jr.[4], Bruno Gonzalez Freitas[5], Bruna Martins Guerreiro[5], Michele Ricieri Bastos[5]

[1]Department of Veterinary Medicine, UFLA, Lavras, MG, Brazil.
[2]Department of Animal Science, UFLA, Lavras, MG, Brazil.
[3]Genex, CRI Genética Brasil, São Paulo, SP, Brazil.
[4]Agua Preta Farm, Cocalinho, MT, Brazil.
[5]Ouro Fino Saúde Animal, Ribeirão Preto, SP, Brazil.

Abstract

Ovulation synchronization protocols are well established in beef and dairy cows. However, the protocol response rate is around 70-90%. In beef cows, factors such as inadequate nutrition and calf presence negatively impact the response of progesterone (P4)/estradiol-based ovulation synchronization protocols by interfering with GnRH release and consequently reducing LH pulsatility and final follicular development. In dairy cows, protocols based on GnRH and prostaglandin (Ovsynch) are the most widely used in the world. However, the efficiency of Ovsynch is dependent on the presence of a large follicle at the time of administration of the first GnRH. In these ovulation synchronization protocols, pre-synchronization protocols (Prostaglandins, Double Ovsynch and P4synch) are usually attempted in an effort to increase responses. Thus, the objective of this review was to discuss pre-ovulation synchronization strategies (administration of injectable P4 or energetic/protein supplementation or pre-synchronization with intra-vaginal progesterone devices) aiming to increase the LH pulsatility in beef cows or induce the formation of a GnRH-responsive follicle at the beginning of the Ovsynch protocol in dairy cows.

Keywords: Conception rate; timed artificial insemination, LH, P4, pre-synchronization.

Introduction

Currently, the TAI protocols in beef and dairy cattle are well established, in which pregnancy rates between 30 and 65% are observed (Baruselli *et al*., 2012; Sales *et al*., 2015; Wiltbank *et al*., 2015; Sales *et al*., 2016; Baruselli *et al*., 2017). However, the response to the TAI protocol (ovulation of largest follicle by inducer) based on estrogen and P4 is approximately 80% in *Bos indicus* lactating beef cows (Sales *et al*., 2012) and approximately 85% in dairy cows in GnRH-based protocols and PGF2α (Souza *et al*., 2008; Silva *et al*., 2018).

In *Bos indicus* lactating beef cows, a long period of postpartum anestrous is observed characterized by normal initial follicular growth sustained by the release of FSH, reduction of the final growth of the dominant follicle and, consequently, absence of ovulation (Baruselli *et al*., 2004). These changes in the final follicular growth are due to the reduction of LH pulsatility after follicle deviation due to the calf presence and the reduced availability of forage (Jolly *et al*., 1995; Yavas and Walton, 2000). In cows in which the nutritional requirement is not met because of low feed availability, deficient GnRH secretion and consequently, LH release are observed (Jolly *et al*., 1995; Montiel and Ahuja, 2005). The reduction of GnRH secretion occurs due to the negative feedback in the hypothalamus promoted by the increase in the concentrations of neuropeptide Y, NEFA and beta-hydroxybutyrate produced by the mobilization of body fat (Hess *et al*., 2005). In addition to the nutritional effects, the calf presence blocks the secretion of GnRH by the hypothalamus through the action of released endogenous opioids (Malven *et al*., 1986; Williams *et al*., 1996). Under this condition, part of the cows do not respond to the TAI protocol due to a drastic reduction in LH pulsatility observed mainly in primiparous cows (Sales *et al*., 2016) and in undernourished cows with low body condition score (Grimard *et al*., 1995; Diskin *et al*., 2003).In *Bos indicus* lactating cows it is necessary to stimulate the hypothalamus to produce GnRH to increase LH pulsatility which would allow for the final growth of the dominant follicle and ovulation. The positive effects of ovulation synchronization protocols in anestrous cows are mainly due to the stimulation of exogenous P4 on the pulsatility of GnRH and LH (Rhodes *et al*., 2002), allowing the ovulation of a pre-ovulatory follicle in the recent postpartum period (Baruselli *et al*., 2017). During the early postpartum period, progesterone reduces the expression of estradiol receptors in the hypothalamus by interfering with the hormone receptor-negative feedback in LH secretion (Ireland and Roche, 1982; Day, 2004). However, in underfed cows with body condition score <2.5 or primiparous, the final growth of the dominant follicle is lesser, resulting in small follicles at the time of TAI (Sales *et al*., 2016). Thus, in females that do not respond to the TAI protocol, the period of exposure to P4 during the ovulation synchronization protocol may not be sufficient to increase the LH pulsatility needed for ovulation to occur. Therefore, supplementation with P4 prior to the protocol is an alternative to improve the

*Corresponding author: znlogan@yahoo.com.br
orcid.org/0000-0002-8217-755X
Received: April 15, 2019
Accepted: July 25, 2019

reproductive efficiency of beef cows submitted to TAI protocols (Simões *et al*., 2018). In addition to the effects of P4 in *Bos indicus* beef cows, energy/protein supplementation may increase the reproductive efficiency of beef cows submitted to the TAI protocol (Peres *et al*., 2016; Orlandi *et al*., 2018).

In dairy cows, the protocols based on GnRH and PGF2α are predominant due to the ban on the use of esters of estradiol in some countries. The standard protocol used based on GnRH and PGF2α is Ovsynch (Pursley *et al*., 1995). In spite of attending to the ovulation synchronization assumptions, there is low synchronization efficiency (64%) in this protocol when administered on a random day of the estrous cycle (Vasconcelos *et al*., 1999). The best results to start the Ovsynch protocol is between the 5th and 12th day of the estrous cycle, as at this period is common to have a dominant follicle responsive to the GnRH treatment (Vasconcelos *et al*., 1999). Thus, pre-synchronization protocols are used to increase the proportion of cows with a responsive dominant follicle at the first GnRH of the ovsynch protocol (Moreira *et al*., 2001). Among the pre-synchronization protocols, Double-Ovsynch has presented a better synchronization result, with ovulation rates at the first GnRH of around 82% and pregnancy rates of 49.7% (Souza *et al*., 2008). However, some limitations (long protocol of 28 days and many animal handling) prevent extensive use of this protocol. In addition, to stimulating LH pulsatility, P4 (single intravaginal P4 devices) may be an alternative to induce the formation of large follicles that responds to the first GnRH of the Ovsynch protocol (Silva *et al*., 2018). Cows with P4 devices develop follicular persistence due to absence of pre-ovulatory peak of LH and maintenance of sub luteal concentrations of progesterone (Cerri *et al*., 2009). Persistent follicles are capable of ovulating after long periods (15 days) of progestogen blocking (Chebel *et al*., 2006). Thus, the persistent follicle can be used as a pre-synchronization method for the Ovsynch protocol due to the constant follicular development and ovulatory capacity. Thus, the objective of this review was to propose strategies that increase the response to ovulation synchronization protocols in beef and dairy cows using P4 or protein/energy supplementation pre-protocol of TAI, aiming to increase LH pulsatility or induce a GnRH-responsive follicle at the beginning of the ovulation synchronization protocol.

Strategies to increase LH pulsatility prior to TAI protocols

Postpartum anestrous in cows is caused in part by a reduction in LH pulsatility after follicular divergence (Yavas and Walton, 2000). This gonadotropin depletion is caused by the strong negative feedbacks from progesterone and estrogens in late pregnancy. The period of anovulatory anestrus varies between cows and milk production level. In dairy cows, the interval between calving and first ovulation ranges from 19 to 22 days (Darwash *et al*., 2010). However, in dairy cows on grazing systems, this interval may increase up to 43 days (McDougall *et al*., 1995). In *Bos indicus* beef cows raised in a continuous grazing system, longer postpartum anestrous periods are observed (>100 days ; Baruselli *et al*., 2004). Under this management system, between 5 and 15% of the cows are cycling at the beginning of the breeding season (Sales *et al*., 2011; Baruselli *et al*., 2017). In this regards, strategies for stimulation of GnRH-induced LH secretion during early postpartum to reduce anestrous period were attempted, such as P4 (Simões *et al*., 2018) and energetic/protein (Peres *et al*., 2016; Orlandi *et al*., 2018) supplementation.

Progesterone

Progesterone increases LH pulsatility by reducing the expression of estrogen receptors in the hypothalamus, decreasing negative feedback for GnRH production and release (Anderson and Day, 1998; Day, 2004). Thus, treatment with P4 in anestrous cows increased follicular fluid estradiol concentration due to increased LH pulsatility and its LH receptors on granulosa and theca cells in pre-ovulatory follicles (Inskeep *et al*., 1988; Rhodes *et al*., 2002). Some studies have shown that the use of P4 stimulates cyclicity in lactating dairy cows (Fike *et al*., 1997; Lucy *et al*., 2001). Recently, our research group conducted studies to evaluate the effect of injectable P4 (P4i) on the reproductive efficiency of lactating *Bos indicus* cows submitted to TAI. In the first study (Simões *et al*., 2018) the effect of previous exposure to injectable progesterone (P4i) in TAI protocols on follicular growth and pregnancy rate of *Bos indicus* lactating cows was evaluated. In this study, 420 lactating anestrous Nelore cows were used. Cows were divided into one of three experimental groups (Control, P4, and P4GnRH), 10 days before (D-10) the beginning of the P4 and estrogen-based ovulation synchronization protocol (Sales *et al*., 2015). In the control group, cows were only submitted to the protocol based on P4 and estrogen. In the P4i group, cows received 150 mg of P4i (Sincrogest Injectable®, Ouro Fino, Brazil) intramuscularly on D-10 and were submitted to the same ovulation synchronization protocol as in the Control group. In the P4iGnRH group, cows received the same treatments of the P4 group associated with the administration of 10μg of buserelin (Sincroforte®, Ouro Fino, Brazil) on D0. In this study, the P4i treatment increased the follicular diameter at the beginning of the TAI protocol and on the day of removal of the P4 device. In addition, cows receiving pre-protocol P4 were 1.68 times more likely to become pregnant after TAI than the control group (Tab. 1). In *Bos taurus* beef cows (Simões, unpublished data), receiving P4i treatment previous to TAI protocol increased P/AI [Control 45.6% (118/259) and P4i 54.8% (142/259); P = 0.03]. In another study (Santos *et al*., 2018) using 988 lactating Nelore cows in adequate body condition score (~3.0), a P4 treatment preceding the ovulation synchronization protocol did not improve pregnancy rate [Control 64.7% (322/498) and P4i 62.9% (308/490); P = 0.55] and cyclicity 30 days after TAI [Control 39.8% (70/176) and P4i 39.6% (72/182) P = 0.78]. Thus, probably in cows

with adequate body condition LH pulsatility in postpartum should allow growth and ovulation of a preovulatory follicle. This difference in fertility after P4 treatment is probably due to the body condition of the animals in the different studies. In the study by Simões *et al*. (2018), the cows were nutritionally impaired which resulted in low body condition scores. Nutritionally deficient cows have lower postpartum LH pulsatility due to the formation of metabolites (NEFA, Beta-hydroxybutyrate and acetate), endorphins and peptides (mainly neuropeptide Y) known to produce negative feedback blocking hypothalamic GnRH (Hess, 2005). Thus, treatment with P4 prior to ovulation synchronization protocols may have increased LH secretion (Anderson and Day, 1998; Day, 2004), which resulted in higher pregnancy rates.

Based on the benefits reported here, we hypothesize that the prior use of P4 could replace eCG in TAI protocols (Simões, unpublished data). Research emphasizes the importance of treatment with eCG to increase both ovulation and pregnancy rates in TAI protocols (Baruselli *et al*., 2004; Sales *et al*., 2011; Sales *et al*., 2016). As shown previously, eCG has positive effects on recently calved anestrous cows (postpartum period less than 2 months) in animals with compromised body condition (Sales *et al*., 2011) and in cows with dominant follicle growth impairment due to high levels of progesterone at the end of the ovulation synchronization treatment (Baruselli *et al*., 2004). Despite the great benefits of eCG, the use of this gonadotrophin is banned in some countries and a resistance front has emerged because of the way it is extracted from mares. In addition, eCG has no activity pattern and its cost is extremely high. In the Simões study (unpublished data), 600 lactating Nelore multiparous cows were used and distributed in four experimental groups. In the control group (n = 150), cows were submitted to an ovulation synchronization protocol based on P4 and estrogen (D0 - 2mg estradiol benzoate (EB) + P4 device; D8 - withdrawal P4 device + 1mg estradiol cypionate (EC) + 500ug Cloprostenol; D10 - TAI). In the eCG group, cows were submitted to the same ovulation synchronization protocol of the Control group associated with the administration of 300 IU of eCG in D8. In the P4i group, the cows were submitted to the same TAI protocol of the control group associated with the administration of 150mg of injectable P4 (Sincrogest injectable®) 10 days before the initiation of the ovulation synchronization protocol. In the P4ieCG group, cows underwent the same TAI protocol from the Control group associated with the administration of 150mg of injectable P4 and 300UI of eCG in D8. The association of eCG with P4i prior to the protocol increased follicular diameter at day 10 of the TAI protocol. However, the use of P4i without the administration of eCG resulted in a lower pregnancy rate. However, there was an additive gain in pregnancy rate with the association of eCG and P4i prior to the protocol, similar to that previously observed in *Bos indicus* cows (Simões *et al*., 2018). Thus, in *Bos indicus* cows, P4 treatment prior to the TAI protocol is not a viable alternative to replace eCG.

Table 1. Effects of exposure to injectable progesterone previous to TAI protocol on follicular growth, CL diameter and ovulation rate of suckled Nelore cows.

	Control	P4i	P4iGnRH	P
Diameter (mm)				
LF on Day 0 (mm)	10.9 ± 0.2^{b}	12.7 ± 0.3^{a}	12.6 ± 0.4^{a}	0.001
LF on Day 8 (mm)	9.7 ± 0.2^{b}	10.4 ± 0.2^{a}	9.9 ± 0.2^{ab}	0.05
LF on Day 10 (mm)	12.6 ± 0.3	13.0 ± 0.3	12.6 ± 0.3	0.21
CL on Day 24 (mm)	19.7 ± 0.4^{ab}	20.1 ± 0.4^{a}	18.5 ± 0.4^{b}	0.001
Follicular growth rate (mm/day)	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	0.34
Ovulation rate (%)	78.2 (104/133)	80.3 (110/137)	75.2 (106/141)	0.62
CL presence on Day 8 (%)	0.0 $(0/136)^{b}$	0.0 $(0/140)^{b}$	26.4 $(38/144)^{a}$	0.001
P/AI	34.9 (78/223)^{b}	45.9 (105/229)^{a}	40.6 (93/229)^{ab}	0.01

Abbreviations: LF - largest follicle; CL - Corpus Luteum; P/AI - pregnancy per timed-AI. Control group - cows were only submitted to the conventional protocol based on P4 and estrogen P4i group - cows received 150mg of progesterone injectable intramuscularly 10 days before initiation of the ovulation synchronization protocol (D-10). P4iGnRH group - cows received the same treatments of the P4 group associated with the administration of 10µg of buserelin on D0. Different letters (a≠b) in the same line differ ($P < 0.05$; Simões *et al*., 2018).

Energetic and protein supplementation

Under feed restriction cows mobilize body energy reserves, resulting in increases in the concentration of neuropeptide Y (McShane *et al*., 1993) and NEFA from mobilization of body energy reserves (DiCostanzo *et al*., 1999) which, in turn, block the secretion of GnRH and, consequently, the release of LH (Schillo, 1992). In addition, cows in negative energetic balance have high concentrations of β-hydroxybutyrate and low glucose concentrations that reduce GnRH secretion by the hypothalamus (Mulliniks *et al*., 2013). Therefore, adequate nutrition during the pre-partum period and the amount of dry matter available in postpartum are key elements for the return to cyclicity in dairy and beef cows (Crowe *et al*., 2014). Studies have shown that cows with adequate body condition pre and postpartum have greater fertility after calving (Sa Filho *et al*., 2009; Ayres *et al*., 2014) and that energy and/or protein supplementation increases the conception

rate (Pescara *et al*., 2010). In a recent study by our research group (Orlandi *et al*., 2018), the effect of energy and protein supplementation on follicular growth and pregnancy rate of *Bos indicus* lactating cows was evaluated. In this study, 342 *Bos indicus* (Nelore) cows in anestrus were distributed in Control (non-supplemented cows) and Supplement (cows received 2.5 kg/day of an energy/protein supplement with 26.5% CP and 76.5% NDT for 26 days) groups. Supplementation was initiated 12 days prior to a standard P4 and estradiol based-TAI protocol and maintained for 14 days. After the first TAI, the non-pregnant cows were resynchronized and 10 days after the second TAI were exposed to Nelore clean-up bulls until the end of the breeding season, which lasted for 110 days. The diameter of the largest follicle at D0, D8, D10, CL diameter at D14 and ovulation rate were higher ($P < 0.05$) in the Supplement group. In addition, there were no differences ($P > 0.05$) between the treatments for P/AI at 1st and 2nd TAI or after the clean-up bull. However, the pregnancy rate at the end of the breeding season was greater in the Supplement group ($P = 0.02$; Fig. 1).

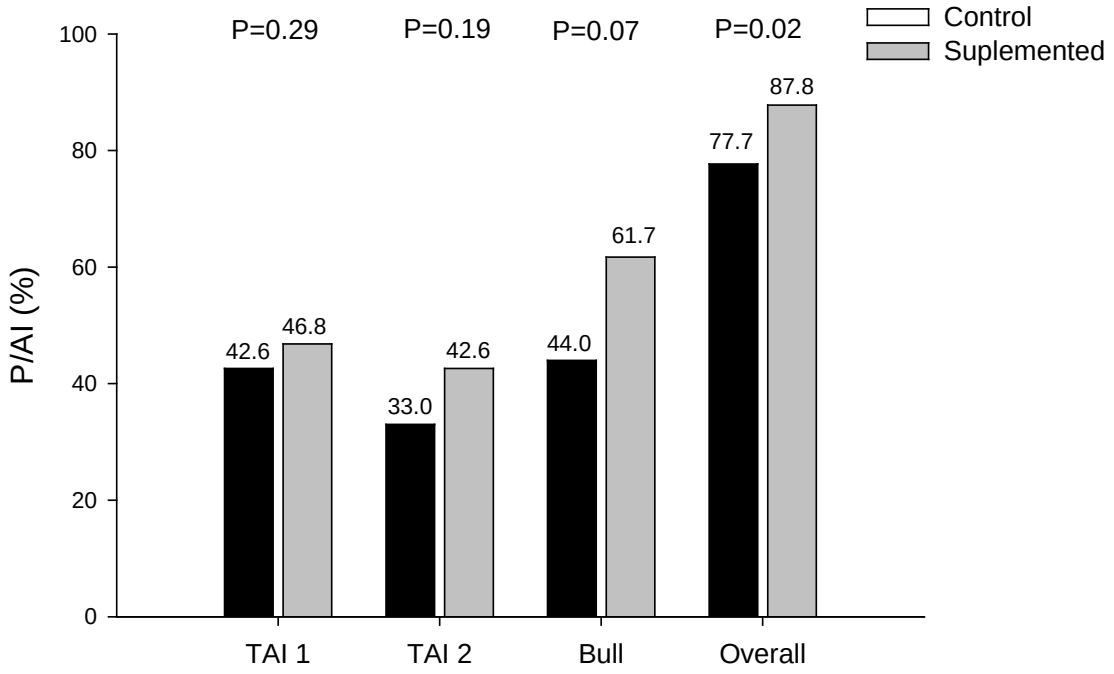


Figure 1. P/AI during a 110-day breeding season of lactating Nelore cows supplemented with energy and protein diet at the end of the dry period (Orlandi *et al*., 2018).

In another supplementation study in beef cows (Peres *et al*., 2016), the effect of corn-based supplementation was evaluated for 41 days. The supplementation started on the first day of insertion of the P4 device (D0) in the TAI protocol and remained until pregnancy check (D41). In this study two experiments were carried out to evaluate the hormonal profile and the fertility of Nelore females. In the study, the three-way TAI protocol with 11-day duration was used. In experiment 1,681 primiparous cows averaging 2.84 in BCS were used to evaluate the concentrations of IGF-1, leptin and GH and in experiment 2, 2395 Nelore females (648 heifers, 635 primiparous and 1112 multiparous) were submitted to the TAI protocol to evaluate fertility. In both experiments cows were distributed into two groups, Control (not supplemented) and Supplemented (1.0kg/cow/day of corn from D0 to D11 and 2.2kg/cow/day from D11 to D41). Both groups grazed on pastures with ad libitum access to water and mineral and TAI protocols started 35 days post calving. In experiment 1, the higher concentration of IGF-1 at TAI (138.4 *vs* 130.8ng/mL) and lower concentration on the day of the pregnancy diagnosis (135.5 *vs* 141.5ng/mL) were observed in the supplemented cows. In this study, cows with higher concentrations of IGF-1 at the time of TAI and leptin / GH at the beginning of the protocol had higher pregnancy rates, demonstrating that supplementation during the protocol may increase the pregnancy rate due to an increase in the concentration of IGF-1. In experiment 2, although corn-based supplementation did not interfere with the results of the first TAI (Control - 50.9% and Supplemented - 52.4%), there was a trend of higher pregnancy rate in the second TAI in supplemented cows (44.3% *vs*. 38.5%). In addition, primiparous cows had higher pregnancy rate at the end of the breeding season (77.8% vs. 65.7%), showing cumulative/late effects similar to those observed in Orlandi *et al*. (2018). However, in multiparous, the final pregnancy rate was lower in the supplemented group (87.0% vs. 92.0%). These results make it difficult to understand the effects of energy supplementation on beef cows during and after the TAI protocol. Thus, the positive effects of nutrition may occur due to changes in hormone concentrations (insulin and IGF-I) and metabolites (glucose, cholesterol and beta-hydroxybutyrate) related to reproductive efficiency

(Beam and Butler, 1998; Ospina *et al*., 2010; Mulliniks *et al*., 2013; Samadi *et al*., 2013). Thus, short duration(<30 days) energy and protein supplementation 12 days before TAI sufficient to animal maintenance increased fertility lactating beef cows in the post postpartum period, being an interesting alternative.

Strategies to increase response to the first GnRH of the Ovsynch protocol

Ovsynch is the standard GnRH/PGF2α-based protocol (Pursley *et al*., 1995) where a first GnRH is given on a random day of the estrous cycle (D0). Seven days later, PGF2α (D7) is given and 48 hours later, a second GnRH. Cows are inseminated 16 hours after the second GnRH. Although it meets the three premises of ovulation synchronization, this protocol presents low efficiency (64%) if administered in animals on a random day of the estrous cycle (Vasconcelos *et al*., 1999). In this study, ovulation rate was higher in cows that received the first GnRH of the Ovsynch protocol between days 5 - 9 and 17 - 21 days of the estrous cycle. In addition, there was a higher ovulation rate to the second GnRH of the Ovsynch protocol when the animals responded to the first GnRH (Vasconcelos *et al*., 1999). However, cows that did not respond to the first GnRH had a longer period of dominance of the ovulatory follicle (follicular persistence), compromising oocyte quality and early embryo development (Cerri *et al*., 2009). Such changes in follicular dynamics resulted in a lower pregnancy rate (Chebel *et al*., 2006). Thus, pre-synchronization protocols have been used to increase the response to the first GnRH of the Ovsynch protocol, (Moreira *et al*., 2001; Souza *et al*., 2008).

The first pre-synchronization protocol used two PGF2α with a 14-day interval, followed by Ovsynch 12 days after the second PGF2α (termed Presynch-Ovsynch; Moreira *et al*., 2001). Pre-synchronization in this study increased the conception rate (37% *vs* 49%) in heifers by 12 percentage points and other authors observed an increase of 18 percentage points in cyclic lactating cows (25% *vs* 43%; El-Zarkouny *et al*., 2001). In another study, using a similar protocol (twelve day intervals between PGF2α injections) conception rates at 42 days of gestation was 49.6% for the cows in the Presynch group and 37.3% for cows in the Ovsynch group (Navanukraw *et al*., 2004). Thus, such favorable results are attributed to a larger number of animals in the optimal phase of the estrous cycle receiving the Ovsynch protocol. However, only cyclic cows can benefit from the program with two PGF2α since the response depends on the presence of responsive corpus luteum (Chebel *et al*., 2006). Another limitation of the effectiveness of the Presynch-Ovsynch protocol would be the lack of precision in follicular synchronization and luteal stages, due to estrous variability and ovulation after PGF2α treatments (Ayres *et al*., 2013).

Among the pre-synchronization protocols, Double Ovsynch (Ovsynch protocol is performed as a pre-synchronization tool) has achieved better synchronization results (Souza *et al*., 2008). Double-Ovsynch comprises two Ovsynch protocols seven-days apart, with TAI after the last GnRH of the second protocol. Double-Ovsynch increases the ovarian response to hormone treatment and P4 concentrations during the Ovsynch of the TAI (Souza *et al*., 2008). In this study, 28% more cows with high progesterone (>3ng/mL) were observed at the time of PGF2α in the Double-Ovsynch group (78.1% *vs* 52.3%) when compared to the group treated with two PGF2α. In addition, there was a high ovulation rate at the first GnRH (82%) and a satisfactory pregnancy rate (49.7%). In another study (Herlihy *et al*., 2012), Double-Ovsynch reduced the proportions of primiparous and multiparous cows with low circulating P4 concentrations compared to Presynch-Ovsynch treated cows (3.3 *vs* 19.7% in primiparous and 8.8 *vs* 31.9% in multiparous). Cows with low concentrations of P4 at the time of PGF2α administration are more likely to have premature luteolysis, with consequent peak LH and ovulation prior to administration of the second Ovsynch GnRH (Vasconcelos *et al*., 1999). In both studies, the Double-Ovsynch protocol increased Ovsynch fertility compared to Presynch-Ovsynch. The ovulatory response to the first Ovsynch GnRH increases the circulating concentrations of progesterone and allows the development of the dominant follicle less variable and closer to the ideal size at the time of the second GnRH (Bello *et al*., 2006; Giordano *et al*., 2012). Increased circulating concentrations of P4 during follicular development may decrease LH pulsatility, possibly increase dominant follicle competence, oocyte and uterine environment qualities (Mihm *et al*., 1994; Revah and Butler, 1996). Other studies also related ovulation to the first Ovsynch GnRH and the presence of CL at the time of PGF2α with higher pregnancy rates at 30 and 60 days post artificial insemination (Vasconcelos *et al*., 1999; Chebel *et al*., 2006). However, such a protocol is too long (28 days) and difficult to implement on farm. Thus, there is still the need for the development of more practical and shorter pre-synchronization protocols.

Recently our research group developed a pre-synchronization protocol using a P4 sustained-release vaginal device (Silva *et al*., 2018) to induce a persistent dominant follicle to increase the response to the first GnRH of the Ovsynch protocol. In the experiment, 440 dairy cows (345 Holstein-Zebu crossbreds and 95 Holsteins) were randomly assigned to Double Ovsynch (Double-Ov; Souza *et al*., 2008) and P4synch. The P4synch protocol consisted of insertion of an intravaginal P4 device 10 days prior to the initiation of the Ovsynch protocol (D-10) and withdrawing the device on the day of PGF2α administration of the Ovsynch (D7) protocol. All cows were inseminated 16 hours after the second dose of GnRH from the Ovsynch protocol. No differences were observed between the groups for the pre-synchronization rate variables [presence of follicles with more than 12mm in the D0; P = 0.66), follicular diameter at the 1st GnRH (P = 0.28), ovulation rate at 1st GnRH (P = 0.50), synchronization rate (P = 0.84), follicular diameter at the 2nd GnRH (P = 0.48), ovulation rate in the 2nd GnRH (P = 0.48) and the diameter of the CL in D24 (P = 0.19)]. However, the

presence of CL on D0 was higher (P = 0.03) in the Double Ovsynch group (Tab. 2). In addition, there was no difference in pregnancy rates at 30 (P = 0.85), at 60 days of gestation (P = 0.41) and in gestational losses at 30 and 60 days of gestation (P = 0.13; Fig. 2). There was no difference in the percentage of cows with P4 <1ng/mL at D0 [Double-Ov 13.6% (3/22) and P4synch 5.0% (1/20); P = 0.37], for percentage of cows with P4 >1ng/mL in D7 (Double-Ov 77.3% and P4synch 95.0%; P = 0.14) and for P4 concentration in D24 (Double-Ov 4.7 ± 0.6 and P4synch 5.9 ± 0.9 ng/mL, P = 0.84). The P4synch protocol has the same reproductive efficiency as the Double Ovsynch protocol in lactating dairy cows. In another study by our research group (Lima, unpublished data), we compared the P4synch protocol with the protocol based on estrogen and P4 (Ferreira *et al*., 2013). In this study, similar results were reported between the P4synch and the P4-estradiol-based protocols (Tab. 3). Thus, P4synch may be an efficient alternative for ovulation synchronization in dairy cows.

Table 2. Effect of presynchronization (Double Ovsynch and P4synch) on the follicular dynamics of lactating crossbred dairy cows submitted to the Ovsynch protocol.

	Double-Ov	P4synch	P
Rates (%)			
Presynchronization	94.2 (49/52)	92.0 (46/50)	0.66
CL on Day 0	57.7 (30/52)	36.0 (18/50)	0.03
Ovulation to 1st GnRH	86.3 (44/51)	81.2 (39/48)	0.50
Follicular persistence	5.9 (03/52)	14.3 (07/49)	0.20
Synchronization of Day 9	84.6 (44/52)	86.0 (43/50)	0.84
Ovulation to 2nd GnRH	90.9 (40/44)	86.0 (37/43)	0.48
Diameters (mm)			
LF on Day 0	17.2 ± 07	18.6 ± 0.8	0.28
LF on Day 9	17.6 ± 0.5	17.9 ± 0.4	0.48
CL on Day 24	27.9 ± 0.7	29.4 ± 0.8	0.19

Abbreviations: LF, largest follicle; CL, Corpus Luteum. a) Presynchronization: presence of follicle >12mm on D0. b) Follicular persistence: presence of follicle >12mm on D0, absence of CL on D7 and follicle >20mm on D9. c) Synchronization: presence of a follicle >12 mm. The P4synch protocol consisted of insertion of an intravaginal P4 device 10 days prior to the initiation of the Ovsynch protocol (D-10) and withdrawing the device on the day of PGF2α administration of the Ovsynch (D7) protocol (Silva *et al*., 2018).

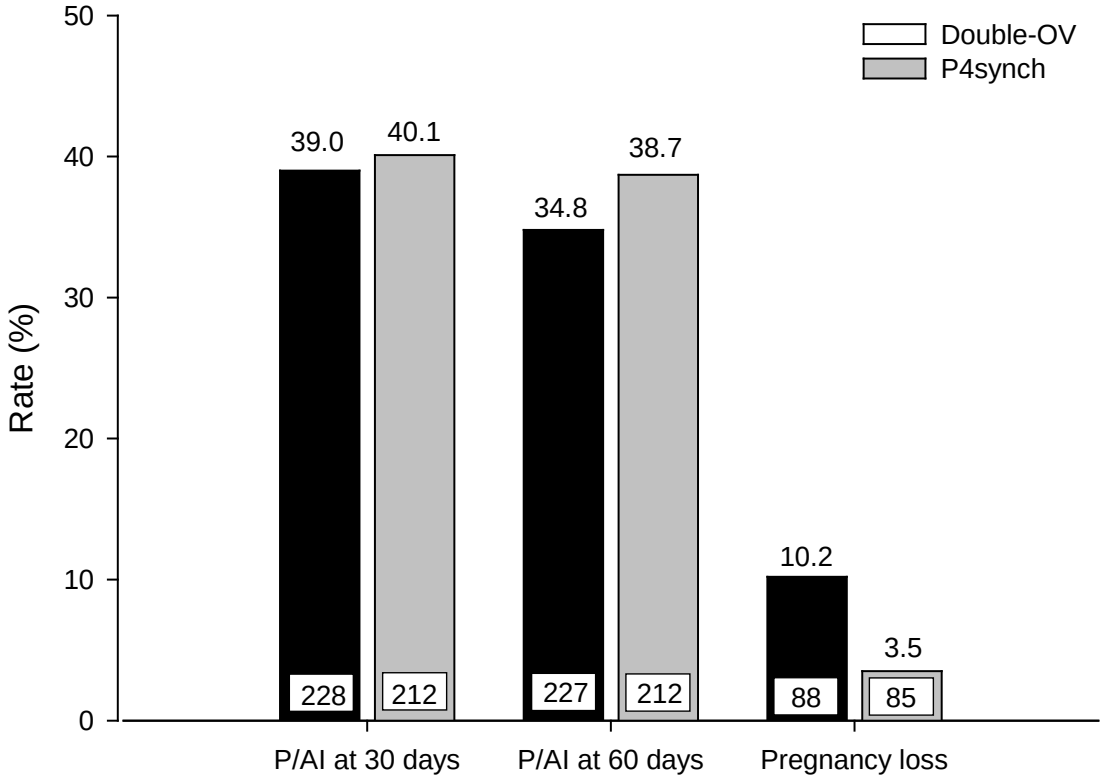


Figure 2. Effect of the presynchronization protocol (Double-Ov and P4synch) on the P/AI at 30 and 60 days and pregnancy loss (P > 0.05) in lactating crossbred dairy cows (Silva *et al*., 2018).

Table 3. Effect of protocols (P4E2 and P4synch) on the follicular dynamics and fertility of lactating dairy cows.

	P4E2	P4synch	P
Rates (%)			
Presynchronization	73.9 (34/46)	97.8 (45/46)	0.01
CL on Day 0	80.4 (37/46)	37.0 (17/46)	0.001
Ovulation to 1st GnRH	65.2 (30/46)	65.2 (30/46)	0.99
Follicular persistence	8.7 (4/46)	15.2 (7/46)	0.34
Synchronization on induction	76.1 (35/46)	80.4 (37/46)	0.61
Diameters (mm)			
LF on Day 0	15.0 ± 0.8	21.0 ± 0.8	0.001
LF on induction	13.9 ± 0.7	17.6 ± 0.6	0.001
LF on TAI	15.2 ± 0.7	17.2 ± 0.8	0.05
P/AI	37.4 (67/179)	42.4 (72/170)	0.35

Abbreviations: LF, largest follicle; CL, Corpus Luteum. a) Presynchronization: presence of follicle >12mm on D0. b) Follicular persistence: presence of follicle >12mm on D0, absence of CL on D7 and follicle >20mm on D9. c) Synchronization on induction: presence of a follicle >12 mm. The P4synch protocol consisted of insertion of an intravaginal P4 device 10 days prior to the initiation of the Ovsynch protocol (D-10) and withdrawing the device on the day of PGF2α administration of the Ovsynch (D7) protocol (Lima *et al*., unpublished).

Conclusion

The TAI protocols in beef and dairy cows are well established, but the response to protocols ranges from 70 to 90%. Currently, there are strategies to increase protocol response in cows with LH release impairment. The P4i strategy brought significant increase in fertility in Bos indicus and Bos taurus beef cows. Another strategy that has improved TAI results in Bos indicus beef cows is energy/protein supplementation before and during the protocol. In addition, the use of intravaginal P4 device is an efficient alternative of pre-synchronization to the Ovsynch protocol in dairy cows.

Despite these results, more studies are necessary to confirm these findings, especially in energy/protein supplementation.

Author contributions

JNSS: Conceptualization, Funding acquisition, Supervision, Project administration, Writing – original draft, Writing – review & editing, Formal analysis, Methodology, Investigation; LMSS, REO, EAL, APCS, MPB, LACLS, , BGF, BMG, MRB: Investigation; JPMM, LASJ, MMD: Investigation, Resources; JCS: Conceptualization, Writing – original draft, Methodology.

Conflict of interest

The authors claim no conflict of interest.

Acknowledgments and Funding disclosure statement

The authors are grateful to Agua Preta Farm and other farms of South and Southeast of Brazil for allowing the use of their animals and facilities for this study. This research was supported by Ouro Fino Agronegocio (pharmacological products), CNPq (Processo: 456398/2014-7 and scholarship), FAPEMIG (scholarship) and CAPES (scholarship).

References

Anderson LH, Day ML. 1998. Current Concepts on the Control of Puberty in Cattle. *J Anim Sci*, 76:1-15.

Ayres H, Ferreira RM, Cunha AP, Araujo RR, Wiltbank MC. 2013. Double-Ovsynch in high-producing dairy cows: effects on progesterone concentrations and ovulation to GnRH treatments. *Theriogenology*, 79:159-164.

Ayres H, Ferreira RM, Torres-Junior JR, Demetrio CG, Sa Filho MF, Gimenes LU, Penteado L, D'Occhio MJ, Baruselli PS. 2014. Inferences of body energy reserves on conception rate of suckled Zebu beef cows subjected to timed artificial insemination followed by natural mating. *Theriogenology*, 82:529-536.

Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. 2004. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. *Anim Reprod Sci*, 82-83:479-486.

Baruselli P, Sales JN, Sala R, Viera LM, Sá Filho M. 2012. History, evolution and perspectives of timed artificial insemination programs in Brazil. *Anim Reprod*, 9:139-152.

Baruselli P, Ferreira R, Colli M, Elliff F, Sá Filho M, Vieira L, Freitas B. 2017. Timed artificial insemination: Current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*, 14:558-571.

Beam SW, Butler WR. 1998. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. *J Dairy Sci*, 81:121-131.

Bello NM, Steibel JP, Pursley JR. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of ovsynch in lactating dairy cows. *J Dairy Sci*, 89:3413-3424.

Cerri RL, Rutigliano HM, Bruno RG, Santos JE. 2009. Progesterone concentration, follicular development and induction of cyclicity in dairy cows receiving intravaginal progesterone inserts. *Anim Reprod Sci*, 110:56-70.

Chebel RC, Santos JE, Cerri RL, Rutigliano HM, Bruno RG. 2006. Reproduction in dairy cows

following progesterone insert presynchronization and resynchronization protocols. *J Dairy Sci*, 89:4205-4219.

Crowe MA, Diskin MG, Williams EJ. 2014. Parturition to resumption of ovarian cyclicity: comparative aspects of beef and dairy cows. *Animal*, 1:40-53.

Darwash AO, Lamming GE, Wooliams JA. 2010. The phenotypic association between the interval to post-partum ovulation and traditional measures of fertility in dairy cattle. *Anim Sci*, 65:9-16.

Day ML. 2004. Hormonal induction of estrous cycles in anestrous Bos taurus beef cows. *Anim Reprod Sci*, 82-83:487-494.

DiCostanzo A, Williams JE, Keisler DH. 1999. Effects of short- or long-term infusions of acetate or propionate on luteinizing hormone, insulin, and metabolite concentrations in beef heifers. *J Anim Sci*, 77:3050-3056.

Diskin MG, Mackey DR, Roche JF, Sreenan JM. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. *Anim Reprod Sci*, 78:345-370.

El-Zarkouny SZ, Cartmill JA, Richardson AM, Medina-Britos MA. 2001. Presynchronization of estrous cycles in dairy cows before ovsynch + CIDR and resynchronization of repeat estrus using the CIDR. *Kansas Agricultural Experiment Station Research Reports*, 0:52-54.

Ferreira RM, Ayres H, Sales JN, Souza AH, Rodrigues CA, Baruselli PS. 2013. Effect of different doses of equine chorionic gonadotropin on follicular and luteal dynamics and P/AI of high-producing Holstein cows. *Anim Reprod Sci*, 140:26-33.

Fike KE, Day ML, Inskeep EK, Kinder JE, Lewis PE, Short RE, Hafs HD. 1997. Estrus and luteal function in suckled beef cows that were anestrous when treated with an intravaginal device containing progesterone with or without a subsequent injection of estradiol benzoate. *J Anim Sci*, 75:2009-2015.

Giordano JO, Wiltbank MC, Guenther JN, Pawlisch R, Bas S, Cunha AP, Fricke PM. 2012. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. *J Dairy Sci*, 95:639-653.

Grimard B, Humblot P, Ponter AA, Mialot JP, Sauvant D, Thibier M. 1995. Influence of postpartum energy restriction on energy status, plasma LH and oestradiol secretion and follicular development in suckled beef cows. *J Reprod Fertil*, 104:173-179.

Herlihy MM, Giordano JO, Souza AH, Ayres H, Ferreira RM, Keskin A, Nascimento AB, Guenther JN, Gaska JM, Kacuba SJ, Crowe MA, Butler ST, Wiltbank MC. 2012. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. *J Dairy Sci*, 95:7003-7014.

Hess B, Lake SL, Scholljegerdes EJ, Weston TR, Nayigihugu V, Molle JDC, Moss GE. 2005. Nutritional controls of beef cow reproduction. *J Anim Sci*, 83:90-106.

Inskeep EK, Braden TD, Lewis PE, Garcia-Winder M, Niswender GD. 1988. Receptors for luteinizing hormone and follicle-stimulating hormone in largest follicles of postpartum beef cows. *Biol Reprod*, 38:587-591.

Ireland JJ, Roche JF. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: changes in serum hormones, steroids in follicular fluid, and gonadotropin receptors. *Endocrinology*, 111:2077-2086.

Jolly PD, McDougall S, Fitzpatrick LA, Macmillan KL, Entwistle KW. 1995. Physiological effects of undernutrition on postpartum anoestrus in cows. *J Reprod Fertil Suppl*, 49:477-492.

Lucy MC, Billings HJ, Butler WR, Ehnis LR, Fields MJ, Kesler DJ, Kinder JE, Mattos RC, Short RE, Thatcher WW, Wettemann RP, Yelich JV, Hafs HD. 2001. Efficacy of an intravaginal progesterone insert and an injection of PGF2α for synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, peripubertal beef heifers, and dairy heifers. *J Anim Sci*, 79:982-995.

Malven PV, Parfet JR, Gregg DW, Allrich RD, Moss GE. 1986. Relationships among concentrations of four opioid neuropeptides and luteinizing hormone-releasing hormone in neural tissues of beef cows following early weaning. *J Anim Sci*, 62:723-733.

McDougall S, Burke CR, MacMillan KL, Williamson NB. 1995. Patterns of follicular development during periods of anovulation in pasture-fed dairy cows after calving. *Res Vet Sci*, 58:212-216.

McShane TM, Petersen SL, McCrone S, Keisler DH. 1993. Influence of food restriction on neuropeptide-Y, proopiomelanocortin, and luteinizing hormone-releasing hormone gene expression in sheep hypothalami. *Biol Reprod*, 49:831-839.

Mihm M, Baguisi A, Boland MP, Roche JF. 1994. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. *J Reprod Fertil*, 102:123-130.

Montiel F, Ahuja C. 2005. Body condition and suckling as factors influencing the duration of postpartum anestrus in cattle: a review. *Anim Reprod Sci*, 85:1-26.

Moreira F, Orlandi C, Risco CA, Mattos R, Lopes F, Thatcher WW. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *J Dairy Sci*, 84:1646-1659.

Mulliniks JT, Kemp ME, Endecott RL, Cox SH, Roberts AJ, Waterman RC, Geary TW, Scholljegerdes EJ, Petersen MK. 2013. Does beta-hydroxybutyrate concentration influence conception date in young postpartum range beef cows? *J Anim Sci*, 91:2902-2909.

Navanukraw C, Redmer DA, Reynolds LP, Kirsch JD, Grazul-Bilska AT, Fricke PM. 2004. A modified presynchronization protocol improves fertility to timed artificial insemination in lactating dairy cows. *J Dairy Sci*, 87:1551-1557.

Orlandi RE, Simões LMS, Bottino MP, Santos APC, Lima EA, Massoneto JPM, Scandiuzzi LA, Jr., Pádua PHBM, Sales JNS. 2018. Energy and protein

supplementation on follicular growth and pregnancy rate of Bos indicus lactating cows submitted to FTAI in a breeding season of 110 days. *Anim Reprod*, 15:329. (Abstract).

Ospina PA, Nydam DV, Stokol T, Overton TR. 2010. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. *J Dairy Sci*, 93:1596-1603.

Peres RFG, Carvalho R, Graff H, Furlan Junior JH, Rodrigues ADP, Franco GA, Keisler DH, Smith MF, Pohler KG, Vasconcelos JLM. 2016. Corn supplementation during 41 days after the begginning of TAI protocol and its effects on metabolic hormones and reproductive performance of Nelore females. *Anim Reprod*, 13:382. (Abstract).

Pescara JB, Sá Filho OG, Losi TC, Cooke RF, Vasconcelos JLM. 2010. Serum progesterone concentration and conception rate of beef cows supplemented with ground corn after a fixed-time artificial insemination protocol. *Arq Bras Med Vet e Zootec*, 62:130-135.

Pursley JR, Mee MO, Wiltbank MC. 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. *Theriogenology*, 44:915-923.

Revah I, Butler WR. 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. *J Reprod Fertil*, 106:39-47.

Rhodes FM, Burke CR, Clark BA, Day ML, Macmillan KL. 2002. Effect of treatment with progesterone and oestradiol benzoate on ovarian follicular turnover in postpartum anoestrous cows and cows which have resumed oestrous cycles. *Anim Reprod Sci*, 69:139-150.

Sa Filho OG, Meneghetti M, Peres RF, Lamb GC, Vasconcelos JL. 2009. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows II: strategies and factors affecting fertility. *Theriogenology*, 72:210-218.

Sales JNS, Crepaldi GA, Girotto RW, Souza AH, Baruselli PS. 2011. Fixed-time AI protocols replacing eCG with a single dose of FSH were less effective in stimulating follicular growth, ovulation, and fertility in suckled-anestrus Nelore beef cows. *Anim Reprod Sci*, 124:12-18.

Sales JNS, Carvalho JB, Crepaldi GA, Cipriano RS, Jacomini JO, Maio JR, Souza JC, Nogueira GP, Baruselli PS. 2012. Effects of two estradiol esters (benzoate and cypionate) on the induction of synchronized ovulations in Bos indicus cows submitted to a timed artificial insemination protocol. *Theriogenology*, 78:510-516.

Sales JNS, Carvalho JB, Crepaldi GA, Soares JG, Girotto RW, Maio JR, Souza JC, Baruselli PS. 2015. Effect of circulating progesterone concentration during synchronization for fixed-time artificial insemination on ovulation and fertility in Bos indicus (Nelore) beef cows. *Theriogenology*, 83:1093-1100.

Sales JNS, Bottino MP, Silva LA, Girotto RW, Massoneto JP, Souza JC, Baruselli PS. 2016. Effects of eCG are more pronounced in primiparous than multiparous Bos indicus cows submitted to a timed artificial insemination protocol. *Theriogenology*, 86:2290-2295.

Samadi F, Phillips NJ, Blache D, Martin GB, D'Occhio MJ. 2013. Interrelationships of nutrition, metabolic hormones and resumption of ovulation in multiparous suckled beef cows on subtropical pastures. *Anim Reprod Sci*, 137:137-144.

Santos APC, Orlandi RE, Bottino MP, Simões LMS, Lima EA, Silva AG, Guerreiro BM, Bastos MR, Freitas BG, Bertoncini FLS, Santos JA, Sales JNS. 2018. Effect of pre-exposition to injectable long acting progesterone on pregnancy rates of suckled beef cows submitted to the estrous synchronization protocol. *Anim Reprod*, 15:328. (Abstract).

Schillo KK. 1992. Effects of dietary energy on control of luteinizing hormone secretion in cattle and sheep. *J Anim Sci*, 70:1271-1282.

Silva L, Simoes LMS, Bottino MP, Santos APC, Santos G, Martinez IYH, Souza JC, Baruselli PS, Sales JNS. 2018. Presynchronization by induction of a largest follicle using a progesterone device in GnRH-based-ovulation synchronization protocol in crossbred dairy cows. *Theriogenology*, 119:233-237.

Simões LMS, Orlandi RE, Massoneto JPM, Scandiuzzi LA, Jr, Freitas BG, Bastos MR, Souza JC, Sales JNS. 2018. Exposure to progesterone previous to the protocol of ovulation synchronization increases the follicular diameter and the fertility of suckled Bos indicus cows. *Theriogenology*, 116:28-33.

Souza AH, Ayres H, Ferreira RM, Wiltbank MC. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. *Theriogenology*, 70:208-215.

Vasconcelos JL, Silcox RW, Rosa GJ, Pursley JR, Wiltbank MC. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. *Theriogenology*, 52:1067-1078.

Williams GL, Gazal OS, Vega GAG, Stanko RL. 1996. Mechanisms regulating suckling-mediated anovulation in the cow. *Anim Reprod Sci*, 42:289-297.

Wiltbank MC, Baez GM, Cochrane F, Barletta RV, Trayford CR, Joseph RT. 2015. Effect of a second treatment with prostaglandin F2alpha during the Ovsynch protocol on luteolysis and pregnancy in dairy cows. *J Dairy Sci*, 98:8644-8654.

Yavas Y, Walton JS. 2000. Postpartum acyclicity in suckled beef cows: a review. *Theriogenology*, 54:25-55.

DOI: 10.21451/1984-3143-AR2019-0042

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Strategies to increment *in vivo* and *in vitro* embryo production and transfer in cattle

Gabriel A. Bó[1,2,3]*, Andrés Cedeño[1,3,4], Reuben J. Mapletoft[5]

[1]Instituto de Reproducción Animal Córdoba (IRAC), Zona Rural General Paz, (5145) Córdoba, Argentina.
[2]Instituto de Ciencias Básicas, Medicina Veterinaria, Universidad Nacional de Villa María, Villa del Rosario, Córdoba, Argentina.
[3]Doctorado en Ciencias Mención Agroalimentos, Universidad Nacional de Villa Maria, Villa del Rosario, Córdoba, Argentina.
[4]Laboratorio de Biotecnologías de la Reproducción Animal, Medicina Veterinaria, Escuela Superior Politécnica Agropecuaria de Manabí (ESPAM), Calceta, Manabí, Ecuador.
[5] Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada.

Abstract

Knowledge of follicular wave dynamics obtained through the use of real-time ultrasonography and the development of the means by which follicular wave dynamics can be controlled have provided practical approaches for the *in vivo* and *in vitro* production and transfer of embryos in cattle. The elective control of follicular wave emergence and ovulation has had a great impact on the application of on-farm embryo transfer, especially when large groups of donors need to be superstimulated at the same time. Although estradiol and progestins have been used for many years, practitioners in countries where estradiol cannot be used have turned to alternative treatments, such as mechanical follicle ablation or the administration of GnRH for the synchronization of follicle wave emergence. *In vitro* embryo production also benefits from the synchronization of follicle wave emergence prior to Cumulus Oocyte Complexes (COCs) recovery. As *Bos indicus* cattle have high antral follicle population, large numbers of oocytes can be obtained by ovum pick-up (OPU) without superstimulation. However, synchronization of follicular wave emergence and superstimulation is necessary to obtain high numbers of COCs by OPU and blastocysts following *in vitro* fertilization in *Bos taurus* donors. Finally, embryos can now be transferred in commercial beef or dairy herds using efficacious synchronization and re-synchronization protocols that are easily implemented by farm personnel. These technologies can also be used to resolve reproductive problems such as the reduced fertility observed during summer heat stress and/or in repeat-breeder cows in commercial dairy herds.

Keywords: superstimulation, bovine embryos, fixed-time embryo transfer.

Introduction

The objective of ovarian superstimulatory treatments in cattle is to stimulate the growth of the maximum number of antral follicles that produce competent oocytes (Bó and Mapletoft, 2014). The usual regimen for *in vivo* embryo production has been twice daily intramuscular (i.m) treatments with FSH for 4 or 5 days (Bó and Mapletoft, 2014). However, previous experiments have indicated that follicle maturation and ovulation rate can be improved in at least some donors if FSH treatments are administered over 6 or 7 days (Bó *et al*., 2008; García Guerra *et al*., 2012). For *in vitro* embryo production (IVP), the requirement of superstimulation with gonadotropins prior to ovum pick-up (OPU) is still under discussion, and the approach may differ depending on whether the donors are of *Bos taurus* or *Bos indicus* breedings. Regardless of the method of embryo production, these technologies can be implemented in commercial beef and dairy operations for genetic improvement and even to increase fertility during the summer heat stress and/or in repeat-breeder dairy cows. The objective of this manuscript is to briefly summarize the existing protocols for superstimulating donors for *in vivo* and *in vitro* embryo production and to propose alternatives by which embryo transfer technologies can be implemented more widely in commercial herds.

Superstimulation and *in vivo* embryo production

Two very important factors influencing variability in superstimulatory response are the intrinsic number of antral follicles in donors, and the stage of follicular development at the beginning of FSH treatments. Response can be predicted by antral follicle count done with ultrasonography (Singh *et al*., 2004; Ireland *et al*., 2011), or measurement of circulating concentrations of anti-Müllerian hormone [AMH; *Bos taurus* (Rico *et al*., 2012; Monniaux *et al*., 2013), *Bos indicus* (Batista *et al*., 2014)]. High antral follicle counts have resulted in more ovulations and a greater number of transferable embryos following superstimulation with FSH than low antral follicle counts (Ireland *et al*., 2007). Similarly, the top quartile of circulating AMH values was associated with a greater superovulatory response than the lowest quartile (Souza *et al*., 2014). Therefore, selection of donors based on antral follicle counts or AMH concentrations may be important for predictable and economical embryo production.

Synchronization of follicle wave emergence for superstimulation

Transvaginal ultrasound-guided follicle ablation followed by FSH treatments 1 or 2 days later is

*Corresponding author: gabrielbo62@gmail.com
orcid.org/0000-0002-5853-3438
Received: April 17, 2019
Accepted: June 17, 2019

very efficacious in the synchronization of follicle wave emergence (Bergfelt *et al*., 1997; Baracaldo *et al*., 2000; Lima *et al*., 2007), but requires specialized skills to apply in the field. However, if donors are housed in an embryo production facility, follicle aspiration by OPU can be used to obtain COCs for IVP and at the same time synchronize follicle wave emergence for the production of *in vivo*-derived (IVD) embryos in the same donor (Surjus *et al*., 2014). The number of embryos produced in that study was higher when the interval from OPU to superstimulation was 2 days rather than 1 day. However, there was a concern that the number of recruited 3 to 5 mm follicles 2 days after OPU was lower than those counted at the time of OPU itself, which was performed at random stages of the estrous cycle (15.2 ± 2.3 *vs* 33.7 ± 2.3; P < 0.05; Surjus *et al*., 2014). However, a more recent report (Cirit *et al*., 2019) suggested that this can be overcome using a longer superstimulation treatment (i.e., 6 days) rather than the traditional 4-day FSH protocol. In this study, donors received 400 IU of equine Chorionic Gonadotropin (eCG) 1 day after OPU followed by a 5-day FSH treatment initiated 1 day later (Cirit *et al*., 2019). However, a critical study with a representative number of animals and an adequate control group is needed to confirm this notion. The practical application of producing embryos *in vitro* and *in vivo* in succession in the same donor has important practical implications because it potentially increases the production of embryos in a short period of time.

The preferred approach for synchronization of follicular wave emergence in South America is the administration of 2 mg estradiol benzoate (EB) or 5 mg estradiol-17ß and 50 – 100 mg progesterone (P4) and insertion of P4-releasing device 4 days before initiating FSH treatments (Bó *et al*., 1996, 2002a; Bó and Mapletoft, 2014). This protocol has been extensively reviewed (Bó and Mapletoft, 2014) and will not be discussed further in this manuscript. However, estradiol is not available in many other countries around the world, requiring the use of alternatives such as follicle ablation or GnRH to synchronize follicle wave emergence prior to superstimulation (reviewed in Bó and Mapletoft, 2014)

Attempts to synchronize follicular wave emergence for superstimulation with GnRH were initially unsuccessful; however, subsequent field data were more promising. In these cases, GnRH was administered 1.5 to 3.0 days after the insertion of an intravaginal P4-device which may have increased the probability of an LH-responsive follicle at the time of treatment with GnRH. Indeed, Bó *et al.* (2010) reported the strategic use of $PGF_{2\alpha}$, a P4-device and GnRH to induce ovulation prior to initiating FSH treatments. Basically, a persistent follicle was induced by treatment with $PGF_{2\alpha}$ at the time of progestin device insertion; following administration of GnRH 7 days later, ovulation occurred in more than 95% of the animals. Superstimulation initiated 36 hours after GnRH (with the P4-device remaining in place) resulted in a superovulatory response that did not differ from controls superstimulated on Days 8 to 12 of the estrous cycle. More recently, Hinshaw *et al*. (2015) reported no difference in superovulatory response whether GnRH was administered 2 or 7 days after insertion of a P4-device.

Extended superstimulatory treatment protocols

An earlier study provided rationale for the hypothesis that superstimulatory treatment may recruit follicles into the wave and allow small follicles to attain medium and large diameters (Adams *et al*., 1994). Based on this notion, attempts have been made to increase the superovulatory response by adding eCG treatment prior to initiating FSH treatments. Pre-treatment with eCG 2 days before the conventional FSH treatment protocol resulted in a numerically greater number of transferable embryos (6.7 ± 1.2 *vs* 4.9 ± 0.9) in an unselected group of donors (Caccia *et al*., 2000), and a significantly greater number of transferable embryos in donors that were defined as poor responders (3.6 ± 0.6 *vs* 1.0 ± 0.2; Bó *et al*., 2008).

A more recent study evaluated the superovulatory response and embryo recovery in donors treated with either a 4-day or a 7-day FSH treatment protocol utilizing the same total dose of 400 mg FSH (Folltropin-V; Vetoquinol Inc., Canada) administered twice daily at a constant daily dosage (García Guerra *et al*., 2012). The mean number of ovulations detected by ultrasonography was greater in the 7-day treatment group (30.9 ± 3.9 *vs* 18.3 ± 2.9, P = 0.01), consistent with a numerically greater number of follicles ≥ 10 mm just prior to ovulation (27.5 ± 4.1 *vs* 19.5 ± 2.6; P = 0.11). Moreover, ovulations occurred more synchronously in the 7-day group (93% of ovulations occurred 12 to 36 hours post-LH as compared to 66% in the 4-day group) suggesting that the superstimulated follicles were more mature and capable of responding to an LH stimulus. Although the total number of ova/embryos, fertilized ova and transferable embryos did not differ statistically, all end-points favored the 7-day group. In addition, when data from two cows with fertilization failure were removed, the number of transferable embryos tended to be higher in the 7-day group (7.6 ± 1.7 *vs* 4.2 ± 1.5; P = 0.07).

In another study (Dias *et al*., 2013a), a 7-day superstimulation protocol was used to investigate the influence of P4 on follicle growth, ovulation and oocyte competence. Beef cows were superstimulated with 25 mg of FSH twice-daily for 4 or 7 days. Again, the superstimulatory response (number of large follicles just prior to insemination) was greater (P < 0.05) in the 7-day group, and the numbers of ovulations (15.4 *vs* 11.6) and embryos (6.7 *vs* 5.9) were numerically higher in the 7-day group.

The duration of treatment rather than the FSH dose appears to be responsible for the increase in the superstimulatory response. In the two studies cited above, the number of ovulatory-sized follicles just prior to ovulation was greater following 7 days of superstimulation than 4 days, whether the total dose of FSH was greater (Dias *et al*., 2013a) or the same (García Guerra *et al*., 2012). In addition, there was no

evidence that more follicles were recruited; the total numbers of follicles at the end of FSH treatment was the same as that at the beginning of FSH treatment, the only difference was the distribution of follicle sizes. Furthermore, in a recent study of follicles undergoing a 4-day superstimulation protocol, gene expression in granulosa cells was altered compared to a single, naturally occurring dominant follicle (Dias *et al*., 2013b, 2014). Expression of growth-related genes similar to the pre-LH stage of follicle growth (even though LH had been administered) and those involved in oxidative stress response were up-regulated in granulosa cells of follicles undergoing a 4-day FSH superstimulation protocol, compared to a preovulatory follicle of an unstimulated follicular wave. Genes related to a disturbance in angiogenesis were also up-regulated in superstimulated follicles. Since the mean growth rate of follicles between initiation of treatment and ovulation was more similar to naturally cycling cattle in the 7-day group than in the 4-day group, we speculate that gene expression during the 7-day superstimulation protocol may be more similar to the naturally occurring single preovulatory follicle.

Use of eCG to replace the last four FSH applications

In search of possible improvements to the superstimulatory treatment protocol Price *et al.* (1999) demonstrated that during the superstimulatory treatment, LH pulse frequency diminish shortly after the first FSH injection and are accentuated during the last injections and the preovulatory period. This occurs as a consequence of the high steroidogenic activity and an increase in the concentrations of estradiol in superstimulated cows and may affect superovulatory response and embryo quality (Price *et al*., 1999). Therefore, a treatment that provides LH support at the end of the superstimulation treatment may be beneficial, since LH has been shown to be essential for the final growth of the superstimulated follicles and for the completion of oocyte maturation (Oliveira *et al*., 2014).

Equine Chorionic Gonadotropin is a complex glycoprotein that has FSH and LH activity in non-equid species (Murphy and Martinuk, 1991). A remarkable feature of eCG that has been exploited in multiple experimental and commercial contexts is its ability to express FSH and LH activity in the cow (Murphy, 2012). In cattle, this gonadotropin has a prolonged action time, due to the proportion of sialic acid (10 to 15%) present in its molecule (Murphy and Martinuk, 1991).

In the early days of bovine embryo transfer, eCG was used to induce superovulation in donors (Bó and Mapletoft, 2014). However, its long half-life, which was a feature for induction of superovulation with a single administration, resulted in multiple unovulated follicles and poor embryo quality at the time of embryo collection (reviewed in Bó and Mapletoft, 2014; Murphy, 2012). More recently, the last two doses of FSH in a superstimulation protocol have been replaced by different dosages of eCG, with the intention of providing more LH support to the growing follicles (reviewed in Barros *et al*., 2012). Some studies have shown beneficial effects of the association of FSH and eCG (Cifuentes *et al*., 2009; Reano *et al*., 2009; Mattos *et al*., 2011), whereas others showed no effect (Sartori *et al*., 2009; Davis *et al*., 2012).

Although the administration of eCG near the end of the FSH treatment protocol did not always improve the superovulatory response, it was not detrimental and raised some interest in its use to simplify the superstimulation protocol and to decrease the cost of the treatment, since eCG is usually less expensive than the pituitary extracts containing FSH. Therefore, we designed a study to evaluate the superovulatory response and embryo production in beef donors using twice daily FSH injections over 4 days or an alternative protocol in which the last 4 FSH treatments were replaced by a single injection of eCG (Barajas *et al*., 2019). Twelve (Experiment 1) and 18 (Experiment 2) mature Bonsmara donor cows were superstimulated twice at a 46-day interval in a crossover design. Follicular wave emergence was synchronized by the administration of estradiol-17β at the time of insertion of a P4-device and superstimulation was initiated 4 days later. Donors in the control group received 8 injections of FSH i.m. (total dose: 300 mg) in a twice-daily decreasing dosage schedule over 4 days, whereas donors in the FSH+eCG group received only the first 4 injections of FSH (total dose: 220 mg) and 48 h after initiating treatment, 800 IU of eCG i.m. in a single administration. All donors received $PGF_{2\alpha}$ i.m. with the eCG administration and again 12 h later. The P4-devices were removed in the AM of the next day. All cows received GnRH 24 hours after the removal of the P4-device and were inseminated with frozen/thawed semen from two bulls 12 and 24 hours later. Ova/embryos were collected and evaluated according to the IETS standards 7 days after the administration of GnRH. In Experiment 2, donors were treated only with FSH+eCG. The total dosage of FSH was 200 mg and the dosage of eCG was either 800 or 600 IU. Results of both experiments are presented in Table 1. In Experiment 1, the FSH (control) group produced a higher ($P < 0.01$) number of fertilized ova, but there were no differences in the number of transferable embryos. In Experiment 2, no differences were found between the FSH+800 eCG or FSH+600 eCG groups in any of the parameters evaluated. In conclusion, the replacement of the last 4 injections of FSH by a single administration of either 600 IU or 800 IU of eCG decreased the number of FSH treatments required in a superstimulation protocol without adversely affecting the production of transferable embryos.

Table 1. Embryo production (means ± SEM) in Bonsmara donors treated with FSH or FSH+eCG[£].

	n	Total ova/embryos	Fertilized ova	Transferable embryos
Experiment 1				
FSH	12	11.7 ± 2.5	10.5 ± 2.3[a]	5.7 ± 1.4
FSH+800 IU eCG	12	9.6 ± 1.5	6.8 ± 1.0[b]	5.3 ± 1.0
Experiment 2				
FSH+800 IU eCG	18	6.7 ± 0.7	5.4 ± 0.8	3.6 ± 0.7
FSH+600 IU eCG	18	6.1 ± 1.1	4.3 ± 1.0	3.7 ± 0.8

Different letters (a,b) within a column indicate significant difference ($P < 0.05$). [£]Donors were treated with 8 intramuscular injections of FSH administered at 12 h intervals (FSH group) or the last 4 FSH treatments were replaced by a single intramuscular injection of eCG (FSH + eCG group).

Manipulation of follicular development for *in vitro* embryo production (IVP)

The IVP of embryos, together with the technique of OPU, are reproductive biotechnologies that have advanced greatly in the last 10 years. This technology is highly developed in Brazil, where 57% of IVP embryos that are transferred in the world are produced (Viana *et al*., 2018). As indicated earlier, *Bos indicus* cattle have a higher number of follicles recruited per wave as compared to *Bos taurus* breeds and this has resulted in the recovery of a higher number of oocytes with OPU (Pontes *et al*., 2009; Baruselli *et al*., 2012; Watanabe *et al*., 2017). Likewise, field data from our laboratory has shown that Brahman-influenced synthetic breeds produce significantly more viable oocytes and transferable blastocysts following OPU/IVF than *Bos taurus* breeds (Bernal *et al*., 2016).

Several studies were designed to evaluate the effects of synchronizing follicular wave emergence and superstimulation on the number and quality of the COCs recovered by OPU and submitted to IVP (Ongaratto *et al*., 2015; Baruselli *et al*., 2016). The most important conclusions of these studies were: 1) Synchronizing follicle wave emergence prior to OPU increased the number of COCs obtained and blastocysts produced in *Bos taurus*, but not *Bos indicus* breeds; 2) Treatment with estradiol and P4 or the removal of the dominant follicle (DFR) were equally efficacious in the synchronization of follicular wave emergence for OPU; 3) Superstimulatory treatment with FSH increased the number and quality of COCs obtained by OPU in *Bos taurus* breeds, but not in *Bos indicus* breeds. In an experiment conducted in Brazil with Holstein donors (Vieira *et al*., 2014), all cows received a P4-device and 2 mg of EB (Day 0). Cows in the control group received no additional treatments, while cows in the FSH-treated group received twice daily treatments on Days 4 and 5 (total dose of 200 mg). On Day 7, the P4-device device was removed and the OPU was performed (40 h after the last FSH treatment). There were no differences between groups ($P = 0.92$) in the number of follicles that were aspirated per OPU session (17.2 ± 1.3 *vs* 17.1 ± 1.1 in the control and FSH-treated cows, respectively); however, COCs from FSH-treated cows yielded a higher blastocyst rate (34.5%, 89/258 *vs* 19.8%, 55/278, $P < 0.001$) and more transferable embryos per OPU session than the control group (3.0 ± 0.5 *vs* 1.8 ± 0.4, $P = 0.02$). It was concluded that superstimulation of Holstein donors with FSH before OPU increased the efficiency of IVP by increasing COC and embryo quality. In addition, non-lactating donors had a higher percentage of *in vitro* blastocyst development and produced more embryos per OPU session than lactating cows. In a later study, similar results were obtained when the four doses of FSH were replaced by a single i.m. injection of 200 mg of FSH diluted in a 0.5% hyaluronan solution (MAP-5, Vetoquinol; Vieira *et al*., 2015).

Two other studies were performed in Angus donors. In the first study (Ongaratto *et al*., 2011), administration of FSH resulted in a higher number of COCs obtained by OPU. In the second study (Ongaratto *et al*., *2019*), multiparous, non-lactating Angus cows, were randomly allocated into two treatment groups and treated twice in a cross-over design. Follicular wave emergence was synchronized with estradiol 17-β and progesterone, plus a P4-device. Four days later (Day 4) donors received either 160 mg FSH diluted in 4 ml of MAP-5 by a single i.m. injection or no FSH (Control group). COCs were obtained by OPU 72 h later (Day 7). Results are summarized in Table 2. The number of viable COCs was significantly higher in the FSH-treated donors than in controls.

Although administration of FSH prior to OPU has been a common practice for increasing the numbers of follicles available for OPU, most studies have adopted the conventional twice daily treatments with FSH, with either positive results in the number of COCs collected and embryos produced (Ongaratto *et al*., 2011; Blondin *et al*., 2012; Vieira *et al*., 2014; 2015) or no effect on COC or embryo production in Holstein cows (Oliveira *et al*., 2016). Obviously, the possibility of giving a single FSH injection instead of four prior to OPU in genetically superior animals is critically important for the widespread application of this technology in commercial herds, where personnel are not as familiar with intensive treatment protocols as producers working with purebred cattle.

Table 2. Mean (±SEM) numbers of total and viable cumulus oocyte complexes (COCs) recovered and number of blastocysts produced following superstimulation in Angus donors[£].

Group	COCs		Blastocysts
	Total	Viable	Total
Single FSH (n = 9)	21.4 ± 2.4[a]	14.1 ± 1.6[a]	4.2 ± 0 .8
Control (n = 9)	15.9 ± 2.7[b]	10.6 ± 2.0[b]	2.7 ± 0.7
P Value	0.02	0.02	0.13

Different letters (a,b) within a column indicate significant difference (P < 0.05). [£]Donors were treated with 5 mg estradiol 17-β and 50 mg of progesterone i.m. plus a P4-device on Day 0, followed by either 160 mg Folltropin-V diluted in 4 ml of MAP-5 by a single i.m. injection (Single FSH group) or no FSH (Control group) on Day 4. OPU was performed in both groups on Day 7.

Strategies for the application of *in vivo* and *in vitro* embryo transfer in commercial operations

The commercial embryo transfer industry began in North America in the early 1970s, and the technology soon spread to South America (Bó and Mapletoft, 2014). Brazil and Argentina have consistently ranked in the top five countries outside North America and Europe in the production IVD embryos. Viana (2018) has reported recently that more than 992,289 IVP and 495,054 IVD bovine embryos were produced worldwide in 2017. North America accounted for more than 59% (292,755) of the IVD embryos, while South America only accounted for 10% (49,230). On the other hand, the distribution of IVP embryos were similar in North (475,696; 48%) and South (453,685; 46%) America. This is the first report in which North America produced more IVP than IVD embryos, whereas in South America the number of IVP embryos has been higher than the number of IVD embryos for more than 10 years.

The application of a successful program using IVD or IVP embryos not only relies on a robust IVP system, but also on the implementation of a successful embryo transfer program. Nutrition, management and efficiency in the synchronization of estrus and ovulation are among the factors that affect the use of these technologies (Mapletoft and Bó, 2016). To avoid limitations associated with estrus detection, treatments that synchronize the time of ovulation in recipients, which were developed originally for fixed-time AI (FTAI), have been utilized for fixed-time embryo transfer (FTET; Bó *et al*., 2002a, 2012a). These treatments are generally divided into those that are GnRH-based (Ambrose *et al*., 1999) and those that are estradiol-based (Bó *et al*., 2002) and are selected depending on the availability of the hormones in different countries. In either case, the recipient protocols include the insertion of a P4-device for 7 or 8 days (Hinshaw, 1999; Bó *et al*., 2002b).

Estradiol and P4- (estradiol/P4) based treatments are the most commonly used protocols to synchronize follicle wave emergence and ovulation of recipients in South America (Baruselli *et al*., 2010). The simplified protocol used most commonly consists of insertion of a P4-device and the administration of 2 mg EB on Day 0, and $PGF_{2\alpha}$ at the time of insertion and removal of the P4-device if it is impregnated with >1 g of P4 and only at P4-device removal when it contains <1 g of P4. The P4-device is usually removed on Day 7 or 8 and 300 or 400 IU of eCG are administered at that time (Bó *et al*., 2002a). Ovulation is induced by the administration of 0.5 or 1 mg of estradiol cypionate (ECP) at the time of P4-device removal and all recipients with a corpus luteum (CL) 9 days later receive an embryo (i.e., 7 days after the expected time of estrus; Baruselli *et al*.. 2010, 2011; Bó *et al*., 2012b). Overall, 75 to 85% of the recipients treated with this protocol receive an embryo; P4 concentrations are high at the time of embryo transfer and pregnancy per embryo transfer (P/ET) range from 40 to 60%, when both embryos and recipients are of high quality (reviewed in Bó *et al*., 2002a; Baruselli *et al*., 2010, 2011).

Recent studies have suggested that increasing the interval from P4-device removal to FTAI may improve pregnancy per AI (P/AI) in a GnRH-based protocol (named 5-day CoSynch+CIDR) (Bridges *et al*., 2008) or estradiol/P4-based treatments (named J-Synch protocol; Bó *et al*., 2016). In both protocols, a second GnRH is administered 72 hours after the removal of the P4-device (prolonged proestrus). The benefits associated with the prolonged proestrus were a prolonged exposure to estradiol prior to ovulation and an increased ability of the uterus to support embryo development (reviewed in Bó and Cedeño, 2018).

Using a modified 5-day CoSynch+CIDR protocol (no GnRH at P4-device insertion, a single injection of PGF2α at P4 removal on Day 5 and GnRH on Day 8), Sala *et al.* (2016) reported similar P/ET rates with IVP embryos as in recipients synchronized with two $PGF_{2\alpha}$ treatments 14 days apart and estrus detection. Furthermore, Menchaca *et al.* (2015, 2016) reported higher pregnancy rates in beef recipients receiving Holstein IVP embryos and synchronized with the J-Synch protocol as compared to the conventional estradiol/P4 protocol in which ECP was given at P4-device removal. Although embryos can be transferred at a fixed time, without estrus observation, optimal P/ET and calving rates were obtained when tail paint was used to detect estrus and only recipients with their tail paint rubbed-off (i.e., in estrus) received embryos 7 days later (reviewed in Bó and Cedeño, 2018). The recommended protocols for FTET in recipients are shown in Figure 1.

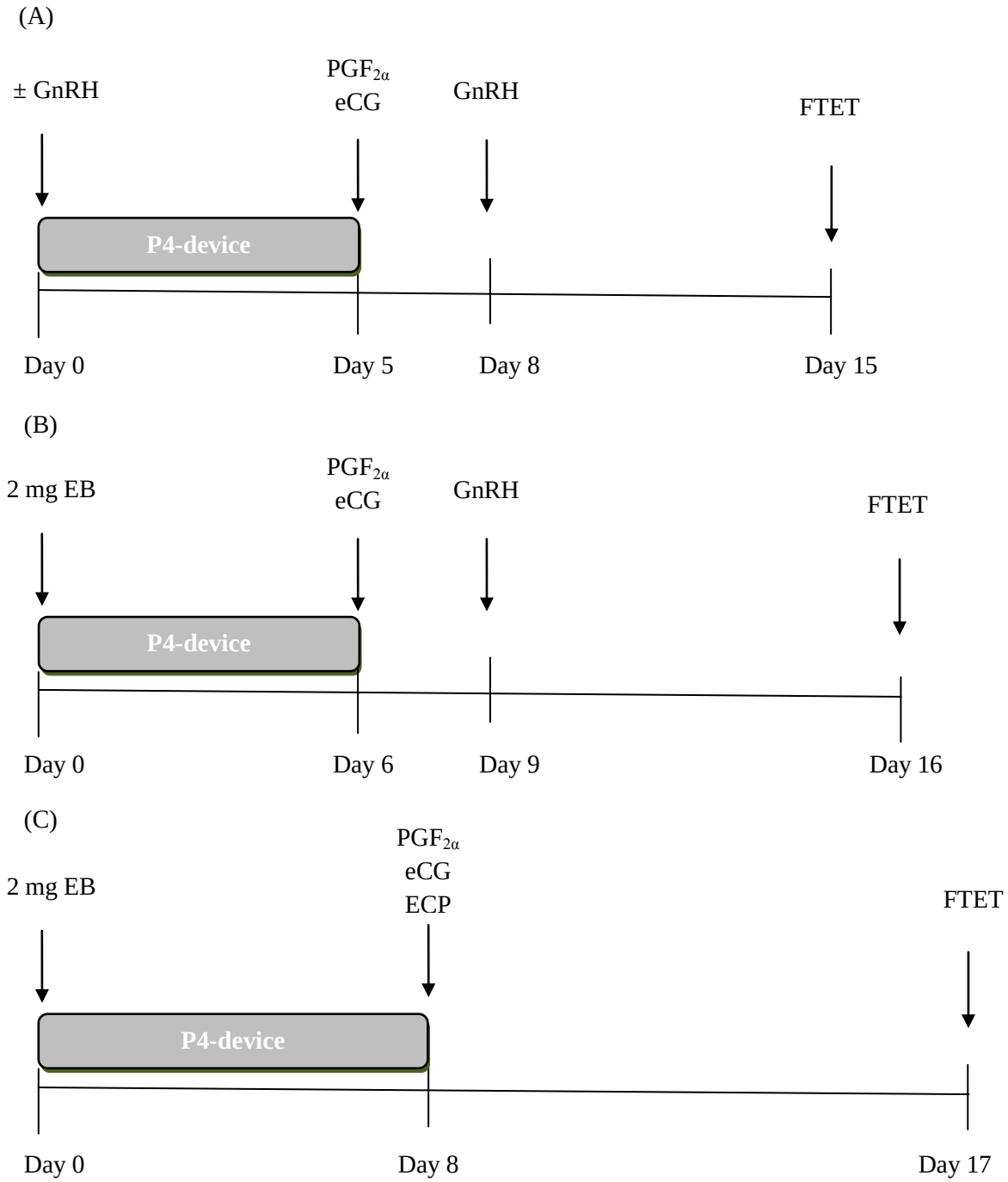


Figure 1. Recommended protocols for FTET in bovine recipients. A) Modified 5-day GnRH+P4 device protocol (P4 device on Day 0, PGF2α and eCG on Day 5 and GnRH on Day 8). B) J- Synch protocol (P4 device and EB on Day 0, PGF2α and eCG on Day 6 and GnRH on Day 9). C) Conventional ECP protocol (P4 device and EB on Day 0, PGF2α, eCG and ECP on Day 8) If estrus detection is implemented with tail patches or tail paint, recipients are observed 72 h after P4 device removal in protocols A and B and 54 h after P4 removal on protocol B.

Embryo Transfer in commercial beef herds

Although the numbers of embryos produced have increased over the years in several regions of the world (Viana, 2018), the main bottleneck for the widespread application of this technology has been the availability of recipients and the intensive management needed for embryo transfer in commercial herds. This problem has been changing as a consequence of the widespread application of successful FTAI in commercial herds. Therefore, in farms where personnel are already familiar with the application of synchronization and re-synchronization of ovulation for FTAI, the application of an embryo transfer program with the normal management of the cow herd seems feasible. Lactating cows and heifers can be synchronized at the beginning of the breeding season to receive IVD or IVP embryos and then resynchronized and inseminated or just simply exposed to clean-up bulls during the remainder of the breeding season.

A study was designed to examine pregnancy rates in *Bos indicus* cows that were synchronized for FTAI or FTET at the beginning of the breeding season (Martins *et al*., 2014). In this experiment, 634 lactating Nelore cows were randomly assigned to one of four treatment groups: 2 FTAI (n = 160), 2 FTET (n = 152), 1 FTAI followed by 1 FTET (FTAI/FTET; n = 160) and 1 FTET followed by 1 FTAI (FTET/FTAI; n = 158). All animals were treated with a P4-device for 8 days, EB on Day 0, and $PGF_{2\alpha}$, eCG and ECP on Day 8. Cows undergoing FTAI were inseminated 48 h after P4-device removal, whereas those receiving embryos were evaluated for the presence of CL 9 days after P4-device removal and those with a CL received IVP embryos. Cows in both groups were resynchronized using the same protocol that was used for the first service, 30 days after the first FTAI or 23 days after FTET. The post-partum period at the times of first service was 41.4 days for FTAI and 47.4 days for FTET and 82.8 days for FTAI and 89.8 days for FTET for the second service. Pregnancy diagnosis was performed by ultrasonography at 30 and 60 days of gestation. Pregnancy rates after the first service were higher ($P < 0.01$) in cows that were FTAI (59.4% and 59.4% for 2 FTAI and FTAI/FTET group respectively) than those that were FTET (31.7% and 32.7% for 2 FTET and FTET/FTAI groups, respectively). Similarly, pregnancy rates after the second service also differed ($P = 0.06$) among groups: 2 FTAI (50.8%), FTAI/FTET (40.6%) FTET/FTAI (51.9%) and 2 FTET (35.0%). Finally, the cumulative pregnancy rate (first + second service) was higher in the groups that receive 2 FTAI (80.0%) than those receiving 2 FTET (55.8%); pregnancy rates in the other groups that received the combined techniques were intermediate and did not differ (FTAI/FTET: 75.6% and FTET/FTAI: 66.5%, respectively). The conclusion of this study was that although the use of two consecutive FTET had a lower cumulative pregnancy rate than two consecutive FTAI, the association between FTAI and FTET programs can be considered as an alternate strategy to increase number of offspring from embryo transfer.

Other approaches have been implemented in the field. In one study (Bó, personal communication), an embryo transfer program was implemented in an extensive Hereford herd in Southern Argentina. In this herd, cows were synchronized with a conventional estradiol/P4-based synchronization protocol for FTET (n = 62) or were FTAI at the beginning of the breeding season (n = 300). In the FTET 49 cows (79.0%) had CL 9 days after P4-device removal and received IVD frozen/thawed embryos. The day after FTET all cows were exposed to clean up bulls for the remaining of the breeding season. Twenty cows (40.8%), were pregnant following FTET and the pregnancy rate to FTAI was 54% (162/300; $P < 0.05$). However, the overall pregnancy rate at the end of the breeding season was 91.0% and did not differ among groups.

These previous studies are examples of the many approaches that can be implemented using FTAI and FTET in commercial beef herds. Most of the protocols were designed for synchronization and resynchronization and have been published elsewhere (for recent reviews see Bó *et al*., 2016 and Baruselli *et al*., 2017). Two of the protocols for re-synchronization are called Resynch 22 and Resynch 14 (Baruselli *et al*., 2017). In the Resynch 22, cows receive 2 mg EB and heifers 1 mg EB at P4-device insertion on Day 22 after FTAI. Pregnancy diagnosis is performed at device removal (Day 30) and non-pregnant animals receive $PGF_{2\alpha}$ and ECP and are inseminated on Day 32. The Resynch 14 protocol involves the use of color Doppler ultrasonography for the detection of pregnancy based on the vascularization and size of the CL on Day 22 after the first AI. The resynchronization treatment starts 14 days after FTAI with the re-insertion of a used P4-device and the administration of 100 mg P4 IM (Rezende *et al*., 2016) at the same time. Cows are scanned with Doppler ultrasonography for pregnancy at P4-device removal (Day 22) and non-pregnant animals receive $PGF_{2\alpha}$ and ECP and are inseminated on Day 24. A proposed program using FTET and Resynch 22 for FTAI is illustrated in Figure 2.

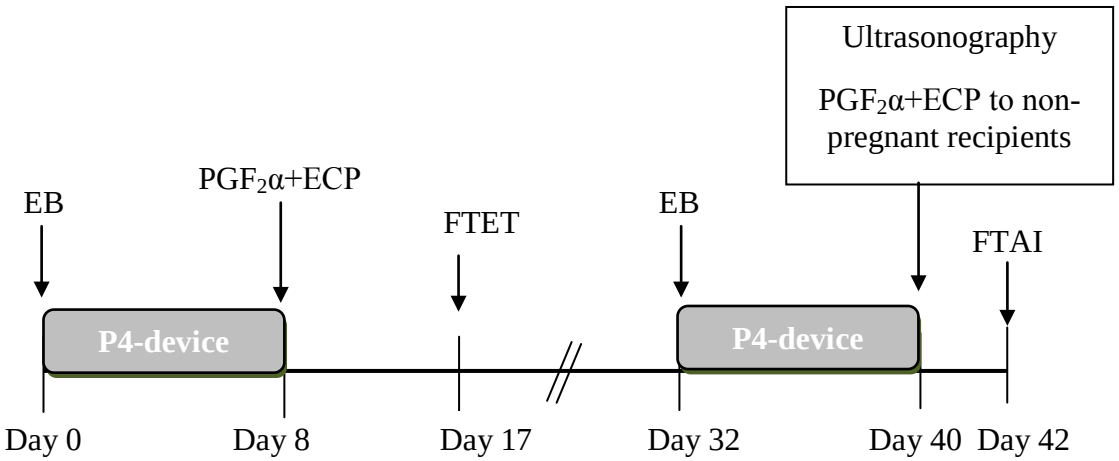


Figure 2. Simple proposal for FTET and resynchronization with FTAI in beef cattle. All cows treated on Day 0 (transferred or not on Day 17) are resynchronized on Day 32. All those recipients not pregnant by Day 30 of gestation (Day 40 of this protocol) are FTAI two days later.

Use of embryo transfer in dairy herds

Traditionally, embryo transfer has been implemented in dairy herds to reproduce animals of high genetic merit, but it has also been shown to improve the reproductive performance of high producing commercial herds. The main reason for the potential improvement is the higher fertility reported after embryo transfer in cows experiencing heat stress (Putney *et al*., 1989; Ambrose *et al*., 1999; Rodrigues *et al*., 2004, 2007a, 2007b; 2011; Baruselli *et al*., 2010) and those diagnosed as repeat-breeders (Rodrigues *et al*., 2007b; 2010; Dochi *et al*., 2008; Ferreira *et al*., 2011; Stewart *et al*., 2011). In a retrospective study that was performed using lactating Holstein cows, conception rates were higher across the year in cows receiving embryos as compared to those that were AI, but the differences were more pronounced in the warmer months of the year (November through April in the southern hemisphere) (Rodrigues *et al*., 2007a). In a subsequent study, embryonic loss between 30 and 60 days of pregnancy was also compared retrospectively in lactating Holstein cows subjected to AI or embryo transfer during summer and winter months (reviewed by Baruselli *et al*., 2011). Although pregnancy loss was higher for embryo transfer than for AI, cows receiving embryos had higher pregnancy rates after 60 days than those that were AI. Therefore, a useful management tool to maintain high pregnancy rates throughout the year would be to produce embryos during the cooler months and use them in embryo transfer during the periods of heat stress.

Another alternative that has tremendous application is to use embryos in repeat breeder cows. Repeat breeder cows are usually defined as cows that do not become pregnant over a period of time (usually after 3 or 4 unsuccessful breedings) that do not have any apparent abnormality that can be diagnosed by a veterinary examination. In a recent study, the transfer of embryos to repeat breeders resulted in increased pregnancy rates compared to AI, without differences in embryo/fetal losses between 30 and 60 days (Ferreira *et al*., 2010). In another retrospective study (Rodrigues *et al*., 2007b), conception rates in repeat breeder Holstein cows were greater after transfer of IVD embryos (41.7%; 1609/3858) than after AI (17.9%; 1019/5693), supporting the notion that the fertility problem in some repeat-breeders may be associated with oocyte quality and/or failure of early embryo development. Other reports have also shown significant improvements in pregnancy rates using IVD (Son *et al*., 2007; Dochi *et al*., 2008) or IVP embryos (Block *et al*., 2010) in repeat breeder cows. The strategy that will have the greatest impact on the fertility of the herd is to use FTET (without estrus detection; Rodrigues *et al*., 2010) and IVP or IVD embryos produced with sexed semen, to increase the number of female calves born in the herd. Certainly, IVP is an efficient method for using sexed semen (Wheeler *et al*., 2006; Pontes *et al*., 2010; Rasmussen *et al*., 2013; Pellegrino *et al*., 2016) and multiple embryos can be produced using a single straw of semen.

Concluding remarks

The use of protocols that control follicular development and ovulation have the advantage of allowing the widespread application of assisted reproductive technologies. The treatments used to synchronize follicle wave emergence for superovulation by many practitioners around the world have proven to be practical and easy to perform by field staff. Lengthened the superstimulation protocol is also an interesting alternative to produce embryos *in vivo*, especially in cows with reduced antral follicle populations, because the time necessary for all growing follicles to acquire the ability to ovulate is extended. With respect to the IVP of embryos, cattle with *Bos indicus* influence adapt very well to this technology because they have a high antral follicle population and, consequently, more COCs are obtained by OPU than in *Bos taurus* breeds. In *Bos taurus* donors, synchronization of follicular wave emergence and the use of FSH have resulted in a greater number of COCs per OPU and the IVP of a higher percentage of blastocysts. Nowadays *in vitro* and *in vivo* production of embryos can be combined with efficient synchronization and FTET programs that are easily implemented in the field and permit the inclusion of commercial beef herds in embryo transfer programs, combined with FTAI or simply with clean-up bulls. Although the embryo transfer technology has been used in dairy herds for many years, primarily for genetic improvement in a limited number of cows the technology can now be used to resolve reproductive problems such as the reduced fertility observed during the summer months and in repeat breeder cows.

Author contributions

GAB: Funding Acquisition, Writing – original draft, Supervision, Methology, Investigation, Writing – review & editing; AVC: Investigation, Formal Analysis; RJM: Data Curation, Writing – review & editing.

Conflict of interest

The authors have no conflicts of interest in relation to the data published in the present manuscript.

Acknowledgments and Funding disclosure statement

Research was supported by Fondo Nacional de Ciencia y Tecnología (FONCYT PICT 2017-4550) GAB; Instituto de Investigación Universidad Nacional de Villa María (UNVM, Programa Aplicación de Biotecnologías Reproductivas para Mejorar la Fertilidad en el Ganado Bovino de Carne y Leche) GAB; Instituto de Reproducción Animal de Córdoba (IRAC) GAB (Research funding), AVC (Scholarship), and Vetoquinol Canada GAB, RJM (Research funding, Folltropin-V); We also thank our colleagues at IRAC and UNVM for technical assistance. GAB, ACV, RJM.

References

Adams GP. 1994. Control of ovarian follicular wave dynamics in cattle Implications for synchronization and superstimulation. *Theriogenology*, 4:19-24.

Ambrose JD, Drost RL, Monson RL, Rutledge JJ, Leibfried-Rutledge ML, Thatcher MJ, Kassa T, Binelli M, Hansen PJ, Chenoweth PJ, Thatcher WW. 1999. Efficacy of timed embryo transfer with fresh and frozen *in vitro*-produced embryos to increase pregnancy rates in heat-stressed dairy cattle. *J Dairy Sci*, 82:2369-2376.

Baracaldo MI, Martínez M, Adams GP, Mapletoft RJ. 2000. Superovulatory response following transvaginal follicle ablation in cattle. *Theriogenology*, 53:1239-1250.

Barajas JL, Cedeño A, Andrada S, Ortega J., Oviedo JM., Tribulo A, Tribulo R, Tribulo H, Bó GA. 2019. Embryo production using follicle stimulating hormone (FSH) or FSH + equine chorionic gonadotropin in beef donors. *Reprod Fertil Dev*, 31:223. (Abstract).

Barros CM, Satrapa RA, Castilho ACS, Fontes PK, Razza EM, Ereno RL, Nogueira MFG. 2012. Effect of superstimulatory treatments on the expression of genes related to ovulatory capacity, oocyte competence and embryo development in cattle. *Reprod Fertil Dev*, 25:17-25.

Baruselli PS, Ferreira RM, Sá Filho MF, Nasser LFT, Rodrigues C, Bó GA. 2010. Bovine embryo transfer recipient synchronisation and management in tropical environments. *Reprod Fertil Dev*, 22:67-74.

Baruselli PS, Ferreira RM, Sales JNS, Gimenes LU, Sá Filho MF, Martins CM, Rodrigues CA, Bó GA. 2011. Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology*, 76:1583-1593.

Baruselli PS, Sá Filho MF, Ferreira RM, Sales JNS, Gimenes LU, Vieira LM, Mendanha MF, Bó GA. 2012. Manipulation of follicle development to ensure optimal oocyte quality and conception rates in cattle. *Reprod Dom Anim*, 47(Suppl. 4):134-141.

Baruselli PS, Batista EOS, Vieira LM, Ferreira RM, Guerreiro BG, Bayeux BM, Sales JNS, Souza A, Gimenes L. 2016. Factors that interfere with oocyte quality for *in vitro* production of cattle embryos: effects of different developmental & reproductive stages. *Anim Reprod*, 13:264-272.

Baruselli PS, Ferreira RM, Colli MHA, Elliff FM, Sá Filho MF, Vieira L, Gonzales de Freitas B. 2017. Timed artificial insemination: current challenges and recent advances in reproductive efficiency in beef and dairy herds in Brazil. *Anim Reprod*, 14:558-571.

Batista EOS, Macedo GG, Sala RV, Ortolan MDDV, Sa Filho MF, Del Valle TA, Jesus EF, Lopes RNVR, Renno FP, Baruselli PS. 2014. Plasma antimullerian hormone as a predictor of ovarian antral follicular population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. *Reprod Dom Anim*, 49:448-452.

Bergfelt DR, Bó GA, Mapletoft RJ, Adams GP. 1997. Superovulatory response following ablation-induced follicular wave emergence at random stages of the estrous cycle in cattle. *Anim Reprod Sci*, 49:1-12.

Bernal B, Revol J, Oviedo JM, Tribulo A, Tribulo H, Moreira G, Bó GA. 2016. Influence of breed and season on *in vitro* embryo production. *Reprod Fertil Dev* 28, 255-255. (Abstract).

Block J, Bonilla L, Hansen PJ. 2010. Efficacy of *in vitro* embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium. *J Dairy Sci*, 93:5234-5242.

Blondin P, Vigneault C, Nivet AL, Sirard MA. 2012. Improving oocyte quality in cows and heifers - What have we learned so far? *Anim Reprod*, 9:281-289.

Bó GA, Adams GP, Pierson RA, Mapletoft RJ. 1996. Effect of progestogen plus E-17ß treatment on superovulatory response in beef cattle. *Theriogenology*, 45:897-910.

Bó GA., Baruselli PS, Moreno D, Cutaia L, Caccia M, Tríbulo R, Tríbulo H, Mapletoft R.J. 2002a. The control of follicular wave development for self-pointed embryo transfer programs in cattle. *Theriogenology*, 57:53-72.

Bó GA, Cutaia L, Tríbulo R. 2002b. Tratamientos hormonales para inseminación artificial a tiempo fijo en bovinos para carne: algunas experiencias realizadas en Argentina. Segunda parte. *Taurus*, 15:17-32.

Bó GA, Carballo Guerrero D, Adams GP. 2008. Alternative approaches to setting up donor cows for superstimulation. *Theriogenology*, 69:81-7.

Bó GA, Carballo Guerrero D, Tríbulo A, Tríbulo H, Tríbulo R, Rogan D, Mapletoft R. 2010. New approaches to superovulation in the cow. *Reprod Fertil Dev*, 22:106-112.

Bó GA, Baruselli PS, Mapletoft RJ. 2012a. Increasing pregnancies following synchronization of bovine recipients. *Anim Reprod*, 9:312-317.

Bó GA, Peres LC, Cutaia LE, Pincinato D, Baruselli PS, Mapletoft RJ. 2012b. Treatments for the synchronization of bovine recipients for fixed-time embryo transfer and improvement of pregnancy rates. *Reprod Fertil Dev*, 24:272-277.

Bó GA, Mapletoft RJ. 2014. Historical perspectives and recent research on superovulation in cattle. *Theriogenology*, 81:38-48.

Bó GA, de la Mata JJ, Baruselli PS, Menchaca A. 2016. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. *Theriogenology*, 86:388-396.

Bó GA, Cedeño A. 2018. Expression of estrus as a relevant factor in fixed-time embryo transfer programs using estradiol/progesterone-based protocols in cattle. *Anim Reprod*, 15:224-230.

Bridges GA, Helser LA, Grum DE, Mussard ML, Gasser CL, Day ML. 2008. Decreasing the interval between GnRH and PGF2α from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. *Theriogenology*, 69:843-851.

Caccia M, Tríbulo R, Tríbulo H, Bó GA. 2000. Effect of eCG pretreatment on superovulatory response in CIDR-B treated beef cattle. *Theriogenology*, 53:495. (Abstract).

Cifuentes E, Quevedo L, Hoyos A, Carballo D, Piccardi M, Bó GA. 2009. Efecto de la aplicación de eCG en vacas donantes de embriones superovuladas con

Folltropin-V. VIII Simposio Internacional de Reproducción Bovina, Córdoba, Argentina, 26, 27 y 28 de Septiembre 2009, pp.7-9 (Abstract).

Cirit Ü, Özmen MF, Küçükaslan I, Köse M, Çınar EM. **Kutsal HG, Çınarc EM**. 2019. Effect of the interval from follicle aspiration to initiation of lengthened FSH treatment on follicular superstimulatory and superovulatory responses and embryo production in lactating Simmental cows. *Theriogenology*, 128:218-224.

Davis RL, Arteaga A, Hasler JF. 2012. Addition of equine chorionic gonadotropin to a traditional follicle stimulating hormone protocol for superovulation of *Bos taurus* beef cows. *Reprod Fertil Dev*, 24:224-225. (Abstract).

Dias FCF, Dadarwal D, Adams GP, Mrigank H, Mapletoft RJ, Singh J. 2013a. Length of the follicular growing phase and oocyte competence in beef heifers. *Theriogenology*, 79:1177-1183.

Dias FCF, Khan MIR, Sirard MA, Adams GP, Singh J. 2013b. Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction*, 146:181-191.

Dias FCF, Khan MIR, Adams GP, Sirard MA, Singh J. 2014. Granulosa cell function and oocyte competence: Super-follicles, super-moms and super-stimulation in cattle. *Anim Reprod Sci*, 149:80-89.

Dochi O, Takahashi K, Hirai T, Hayakawa H, Tanisawa M, Yamamoto Y, Koyama H. 2008. The use of embryo transfers to produce pregnancies in repeat-breeding dairy cattle. *Theriogenology***,** 69:124-128.

García Guerra A, Tribulo A, Yapura J, Singh J, Mapletoft RJ. 2012. Lengthening the superstimulatory treatment protocol increases ovarian response and number of transferable embryos in beef cows. *Theriogenology*, 78:353-60.

Hinshaw RH. 1999. Formulating ET contracts. In "Proceedings Annual Meeting Soc. for Theriogenology", Nashville, USA. Society for Theriogenology: USA. pp.399-404.

Hinshaw RH, Switzer ML, Mapletoft RJ, Bó GA. 2015. A comparison of 2 approaches for the use of GnRH to synchronize follicle wave emergence for superovulation. *Reprod Fertil Dev*, 27:263. (Aabstract).

Ireland JJ, Ward F, Jimenez-Krassel F, Ireland JL, Smith GW, Lonergan P, Evans ACO. 2007. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. *Hum Reprod*, 22:1687-1695.

Lima WM, Vieira AD, Thaller Neto A, Mezzalira A, Matos RC, Gregory RM. 2007. Improved superovulatory response in beef cattle following ovarian follicular ablation using a simplified transvaginal device**.** *Anim Reprod Sci*,100:364-370.

Menchaca A, Dutra S, Carrau JM, Sapriza F, Salazar J, de la Mata JJ, Bó GA. 2015. Improvement of pregnancy rate by using the 6-day J-Synch protocol in recipient cows transferred with *in vitro* produced embryos. *Anim Reprod*, 12, 651. (Abstract).

Menchaca A, Dutra S, Carrau JM, Sapriza F, Bó GA. 2016. Improvements of the 423 new J-Synch protocol used for fixed-time embryo transfer (FTET) in recipients transferred with *in vitro* produced embryos. In: International Congress on Animal Reproduction (ICAR 2016), 18 th, Tours, France, pp.506. (Abstract).

Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO. 2011. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reprod Fertil Dev*, 23:1-14.

Ferreira RM, Ayres H, Chiaratti MR, Rodrigues CA, Freitas BG, Meirelles F., Rodrigues CA, Baruselli PS. 2010. Heat stress and embryo production in high-producing dairy cows. *Acta Scientiae Veterinariae*, pp. s277-s315.

Ferreira RM, Ayres H, Chiaratti, MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, Vireque AA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS. 2011. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J Dairy Sci*, 94:2383-2392.

Martins CM, Reis PO, Vieira JH, Soares JG, Vieira LM, Sa Filho MF, Baruselli PS. 2014. Effect of association of FTET and TAI in reproductive programs of Nelore females. *Anim Reprod*, 11:347. (Abstract).

Mapletoft RJ, Bó GA. 2016. Bovine embryo transfer. In: International Veterinary Information Service (Ed.). IVIS Reviews in Veterinary Medicine. Ithaca: International Veterinary Information Service. (www.ivis.org; Document No. R0104.1106S).

Mattos MC, Bastos MR, Guardieiro MM, Carvalho JO, Franco MM, Mourão GB, Barros CM, 2011. Improvement of embryo production by the replacement of the last two doses of porcine follicle-stimulating hormone with equine chorionic gonadotropin in Sindhi donors. *Anim Reprod Sci*, 125:119-123.

Monniaux D, Drouilhet L, Rico C, Estienne A, Jarrier P, Touzé JL, Sapa J, Phocas F, Dupont J, Dalbies-Tran R, Fabre S. 2013. Regulation of anti-Mullerian hormone production in domestic animals. *Reprod Fertil Dev*, 25:1-16.

Murphy BD, Martinuk SD. 1991. Equine chorionic gonadotrophin. Endocrine Reviews 12:27-44.

Murphy BD. 2012. Equine chorionic gonadotropin: an enigmatic but essential tool. *Anim Reprod*, 9:223-230.

Oliveira ACS, Mattos MCC, Bastos MR, Trinca LA, Razza EM, Satrapa RA, Sartori R, Barros CM. 2014. Efficiency of superstimulatory protocol P-36 associated with the administration of eCH and LH in Nelore cows. *Theriogenology*, 82:715-719.

Oliveira LH, Sanches CP, Seddon AS, Veras MB, Lima FA, Monteiro PLJ Jr, Wiltbank MC, Sartori R. 2016. Short communication: Follicle superstimulation before ovum pick-up for *in vitro* embryo production in Holstein cows. *J Dairy Sci*, 99:9307-9312.

Ongaratto FL, Tribulo A, Ramos M, Rodríguez P, Bó GA. 2011. Oocyte recovery rates and *in vitro* blastocyst production in cattle treated with a single injection of folltropin-v diluted in a slow-release

formulation. *Repro Fert Dev*, 23 (1): 202-203

Ongaratto FL, Rodriguez VP, Tribulo A, Bó GA. 2015. Effect of follicle wave synchronization and gonadotropin treatments on the number and quality of cumulus-oocyte complex obtained by ultrasound-guided ovum pick-up in beef cattle. *Anim Reprod*, 12:876-883.

Ongaratto FL, Cedeño AV, Rodriguez VP, Tribulo A, Bó GA. 2019. Effect of FSH treatment on cumulus oocyte complex recovery by ovum pick up and in vitro embryo production in beef donor cows. *Anim Prod Sci*, (Submitted).

Pellegrino CA, Morotti F, Untura RM, Pontes JH, Pellegrino MF, Campolina JP, Seneda MM, Barbosa FA, Henry M. 2016. Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of *in vitro*-produced embryos in cattle. *Theriogenology*, 86:888-893.

Pontes JHF, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TRR, Oliveira JA, Hasler JF, Seneda MM. 2009. Comparison of embryo yield and pregnancy rate between *in vivo* and *in vitro* methods in the same Nelore (*Bos indicus*) donor cows. *Theriogenology*, 71:690-697.

Pontes JH, Silva KC, Basso AC, Rigo AG, Ferreira CR, Santos GM, Sanches BV, Porcionato JP, Vieira PH, Faifer FS, Sterza FA, Schenk JL, Seneda MM. 2010. Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and indicus-taurus dairy cows using sexed sperm. *Theriogenology*, 74:1349-1355.

Price CA, Carriére PD, Gosselin N, Kohram H, Guilbault LA. 1999. Effects of superovulation on endogenous LH secretion in cattle, and consequences for embryo production. *Theriogenology*, 51:37-46.

Putney DJ, Drost M, Thatcher WW. 1989. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology*, 31:765-78.

Rasmussen S, Block J, Seidel GE Jr, Brink Z, McSweeney K, Farin PW, Bonilla L, Hansen PJ. 2013. Pregnancy rates of lactating cows after transfer of *in vitro* produced embryos using X-sorted sperm. *Theriogenology*, 79:453-461.

Reano I, Carballo D, Tribulo A, Tribulo P, Balla E, Bó GA. 2009. Efecto de la adición de eCG a los tratamientos superovulatorios con Folltropin-V en la producción de embriones de donantes de embriones. In: VIII Simposio Internacional de Reproducción Animal-IRAC, 10th to 12th July de 2009, Córdoba. Argentina, pp.54. (Abstract).

Rezende RG, Freitas BG, Mingoti RD, Colli MHA, Carvalho JBP, Sá Filho MF, Motta JCL, Macedo GG, Baruselli PS. 2016. Follicular dynamics of Nelore cows submitted to resynchronization 14 days after TAI using injectable P4 for synchronization of follicular wave. *Anim Reprod*, 13:375. (Abstract).

Rico C, Drouilhet L, Salvetti P, Dalbie`s-Tran R, Jarrier P, Touze´ J-L, Pillet E, Ponsart C, Fabre S, Monniaux D. 2012. Determination of anti-Mullerian hormone concentrations in blood as a tool to select Holstein donor cows for embryo production: from the laboratory to the farm. *Reprod Fertil Dev*, 24:932-944.

Rodrigues CA, Ayres H, Nichi M, Bó GA, Baruselli PS. 2004. Artificial insemination and embryo transfer pregnancy rates in high production Holstein breeding under tropical conditions. *Anim Reprod*, 14:396. (Abstract).

Rodrigues CA, Ayres H, Ferreira RM, Teixeira AA, Mancilha RF, Oliveira MEF, Souza AH, Baruselli PS. 2007a. Conception rate after artificial insemination and embryo transfer in high producing holstein cows. *Acta Sci Vet*, 33:1254. (Abstract).

Rodrigues CA, Ayres H, Ferreira RM, Teixeira AA, Mancilha RF, Oliveira MEF, Souza AH, Baruselli PS. 2007b. Comparison of pregnancy rates after artificial insemination or embryo transfer in high producing repeat breeder Holstein cows. *Acta Sci Vet*, 35:1255. (Abstract).

Rodrigues CA, Teixeira AA, Ferreira RM, Ayres H, Mancilha RF, Souza AH, Baruselli P.S. 2010. Effect of fixed-time embryo transfer on reproductive efficiency in high-producing repeat-breeder Holstein cows. *Anim Reprod Sci*, 118:110-117.

Rodrigues CA, Ferreira RM, Vieira LM, Ranieri AL, Silva PRL, Baruselli PS. 2011. How FTAI and FTET impact reproductive efficiency of Brazilian dairy herds. *Acta Sci Vet*, 39:3-13.

Sartori R, Guardieiro MM, Barros CM, Bastos MR, Machado GM, Leme LO, Rumpf R. 2009. Lack of improvement on embryo production by the replacement of the last two doses of pFSH by eCG in superovulated Nelore heifers. *Reprod Fertil Dev*, 21:245. (Abstract).

Sala RV, Carrenho-Sala LC, Fosado M, Tosta LC, Tosta RD, Stoll M, Moreno JF, Monteiro BM, Baruselli PS, Garcia-Guera A, Wiltbank M. 2016. Comparison of methods for synchronizing recipients of *in vitro* produced embryos. *Reprod Fertil Dev*, 28:185-185. (Abstract).

Singh J, Domınguez M, Jaiswal R, Adams GP. 2004. A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology*, 62:227-243.

Son DS, Choe CY, Cho SR, Choi SH, Kim HJ, Hur TY, Jung YG, Kang HG, Kim IM. 2007. A CIDR-based timed embryo transfer protocol increases the pregnancy rate of lactating repeat breeder dairy cows. *J Reprod Dev*, 53:1313-18.

Souza AH, Carvalho PD, Rozner AE, Vieira LM, Hackbart KS, Bender RW, Dresch AR, Verstegen JP, Shaver RD, Wiltbank MC. 2014. Relationship between circulating anti-Müllerian hormone (AMH) and superovulatory response of high producing dairy cows. *J Dairy Sci*, 98:169-178.

Surjus RS, Prata AB, Borsato M, Mattos FC, Martins da Silveira MC, Mourão GB, Pires AV, Wiltbank MC, Sartori R. 2014. *In vivo* embryo production in cows superovulated 1 or 2 days after ovum pick-up. *Reprod Fertil Dev*, 26:527-532.

Viana J. 2018. 2017 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter*, 36:8-25.

Vieira LM, Rodrigues CA, Castro Netto A, Guerreiro BM, Silveira CRA, Moreira RJC, Sá Filho MA, Bó GA, Mapletoft RJ, Baruselli PS. 2014. Superstimulation prior to the ovum pick-up to improve

in vitro embryo production in lactating and non-lactating Holstein cows. *Theriogenology*, 82:318-324.

Vieira LM, Rodrigues CA, Castro Netto A, Guerreiro BM, Silveira CRA, Freitas BG, Bragança LGM, Marques KNG, Sá Filho MF, Bó GA, Mapletoft RJ, Baruselli PS. 2015. Efficacy of a single intramuscular injection of porcine FSH in hyaluronan prior to ovum pick-up in Holstein cattle. *Theriogenology*, 84:1-10.

Stewart BM, Block J, Morelli P, Navarette AE, Amstalden M, Bonilla L, Hansen PJ, Bilby TR. 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced *in vitro* with sex-sorted semen. *J Dairy Sci*, 94:3437-3445.

Watanabe Y, Souza H, Mingoti RD, Ferreira R, Batista EOS, Dayan A, Watanabe O, Meirelles FV, Nogueira MFG, Ferraz JBS, Baruselli PS. 2017. Number of oocytes retrieved per donor during OPU and its relationship with *in vitro* embryo production and field fertility following embryo transfer. *Anim Reprod*, 14(3):635-644.

Wheeler MB, Rutledge JJ, Fischer-Brown A, VanEtten T, Malusky S, Beebe DJ. 2006. Application of sexed semen technology to *in vitro* embryo production in cattle. *Theriogenology*, 65:219-227.

DOI: 10.21451/1984-3143-AR2019-0072

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Embryo competence and cryosurvival: Molecular and cellular features

Thamiris V. Marsico[1], Janine de Camargo[2], Roniele S. Valente[1], Mateus J. Sudano[1,2,*]

[1]Center for Natural and Human Sciences, Federal University of ABC, Santo André, SP, Brasil.
[2]School of Veterinary Medicine, Federal University of Pampa, Uruguaiana, RS, Brasil.

Abstract

Global cattle genetic market is experiencing a change of strategy, large genetic companies, traditionally recognized in the artificial insemination field, have also begun to operate in the embryo market. Consequently, the demand for *in vitro* produced (IVP) embryos has grown. However, the overall efficiency of the biotechnology process remains low. Additionally, the lack of homogeneity of post-cryopreservation survival results of IVP embryos still impairing a massive dissemination of this biotechnology in the field. A great challenge for *in vitro* production labs is to increase the amount of embryos produced with exceptional quality after each round of *in vitro* fertilization. Herein, we discuss the molecular and cellular features associated with the competence and cryosurvival of IVP embryos. First, morphofunctional, cellular and molecular competence of the embryos were addressed and a relationship between embryo developmental ability and quality were established with cryosurvival and pregnancy success. Additionally, determinant factors of embryo competence and cryosurvival were discussed including the following effects: genotype, oocyte quality and follicular microenvironment, *in vitro* production conditions, and lipids and other determining molecules. Finally, embryo cryopreservation aspects were addressed and an embryo-focused approach to improve cryosurvival was presented.

Keywords: *in vitro* production of embryos, bovine, embryo quality, cryopreservation, cryotolerance, pregnancy success.

Introduction

In the last few years, the importance of *in vitro* production of embryos (IVPE) has increased within the reproductive biotechnologies applied in dairy and beef cattle. As a consequence, for the first time in the last two decades, the number of *in vitro* produced (IVP) surpassed the number of *in vivo* derived (IVD) embryos globally (Fig. 1A).

However, despite the favorable scenario associated with the IVPE, it is important to highlight that even with great effort of the scientific community aimed to improve embryo development and competence in the last decades, the overall efficiency of the biotechnology process remains low (Lonergan *et al*., 2016; Sudano *et al*., 2019).

Additionally, embryos derived from *in vitro* production process generally present a reduced quality compared with the *in vivo* counterparts (Rizos *et al*., 2002; Sudano *et al*., 2012). Consequently, even with important advances in the last years, the cryopreservation of IVP embryos remains as one of the most challenges areas of the embryo technologies since the homogeneity of the achieved results is still not satisfactory. These results impact directly in the reduced number of IVP embryos cryopreserved and transferred to recipients, in relation to the total transferred, when compared with the IVD embryos in the world over the last two decades (Fig. 1B).

Therefore, the objective of this work was to address: *i*) morphofunctional, cellular and molecular competence of embryos; *ii)* determinant factors of embryo competence and cryosurvival, including the following effects: genotype, oocyte quality and follicular microenvironment, *in vitro* production conditions, and lipids and other determining molecules; and *iii*) embryo cryopreservation aspects and an embryo-focused approach to improve cryosurvival.

Embryo Competence

It is commonly accepted that *in vitro* produced (IVP) bovine embryos have lower developmental ability and quality than *in vivo*-derived (IVD) embryos (Hasler *et al*., 1995; Sudano *et al*., 2013a). The embryo developmental ability and quality are two crucial variables associated with cryosurvival, pregnancy establishment and maintenance that can be explained by morphofunctional, cellular and molecular competence of the embryos.

At the present review, the term embryo competence is used to describe the ability of the embryo to develop properly and with an exceptional quality that facilitates cryosurvival and/or pregnancy establishment.

Embryo morphofunctional competence

Since the establishment of the biotechniques used in assisted reproduction in mammals, a good relationship between morphological evaluation of the embryo quality and pregnancy success after transfer has been established (Lindner and Wright, 1983; Overström, 1996). In most mammalian species, the morphological evaluation of the embryo is the most used method to select embryos suitable for transfer, mainly in cattle and

*Corresponding author: mjsudano@gmail.com
orcid.org/0000-0002-7699-4449
Received: July 29, 2019
Accepted: May 28, 2019

humans (Lindner and Wright, 1983; Van Soom *et al*., 2001). Currently the embryo evaluation system recommended by the International Embryo Technology Society (IETS) is described by Bó and Mapletoft (2013), which classifies the embryos into four quality rankings: (1) excellent or good, (2) regular, (3) bad or (4) dead or degenerate. Drastic changes in embryo's morphology can directly affect its competence.

Assisted reproductive technologies such as *in vitro* production, nuclear transfer and gene transfer sheds light that imperceptible modifications in embryo structure can seriously compromise its quality. Two classical examples could be cited to illustrate this issue: the silicon/oil used to cover the microdrop culture affects the development of bovine embryos and their cryosurvival according to the product batch; however, embryos produced or not with this oil are morphologically similar. Another example is the parthenogenetic embryo, incapable of producing a successful pregnancy but impossible to be morphologic selected in the blastocyst stage from fertilized embryos (Van Soom *et al*., 2001).

Since embryos are mostly evaluated when it still confined in zona pellucida (ZP), this structure and the perivitelline space have some importance to discuss. The zona pellucida is a glycoprotein layer produced during oocyte growth phase surrounding the mammalian embryo between the zygote and the blastocyst stage. In average the thickness of ZP in mammals is 10 μm and its ultrastructure characteristics at oocytes (number and diameter of the pores) and embryos (thickness) can be associated with developmental competence (Santos *et al*., 2008; Báez *et al*., 2019) and hatching ability (Hoelker *et al*., 2006), respectively. It's well known that, the ZP thickness decrease further the embryo development (Van Soom *et al*., 2003) and the average diameter of the ZP is significantly smaller at hatching for IVD versus IVP blastocysts (Holm *et al*., 2002). Although no relationship between ZP thickness and embryo viability after transfer to recipients was established (Hoelker et a., 2006), the ZP remains crucial for embryo handling before transfer to recipients in the majority of reproductive technologies, and especially, during embryo cryopreservation. Another parameter to be evaluated is the perivitelline space (PS), because it has been recognized that IVD embryos have larger PS than IVP embryos, possibly by the swelling of the blastomeres in the latter. The reduction in size of the PS could be associated with lower embryo quality and related with problems during the morula compaction stage (Van Soom *et al*., 2003).

Specifically, in the embryo itself, Merton (2002) pointed out some important variables to be considered in the global morphological evaluation, which included: size and shape of blastomeres, presence of extruded or fragmented cells, compaction, color and stage of development which is reached at a certain time after fertilization.

In comparison to humans and mice, all ruminants have a larger accumulation of lipid droplets in the embryo, which cause a quite dark cytoplasm, even darker in pigs, cats and dogs (Van Soom *et al*., 2003). This opacity disturbs the possibility of quantification of number of pronuclei at the zygote stages and thus select against triploids, parthenotes or unfertilized embryos an excellent method for selecting human embryos with high implantation potential (Lundqvist *et al*., 2001). Besides that, a polarized distribution of the pronuclei during pronuclear alignment is related to chromosomally normal embryos in humans (Coskun *et al*., 2003).

Studies show that the presence of serum in the culture medium can induce accumulation of cytoplasmic lipid droplets in bovine embryos. These fluctuations of cytoplasmic lipid droplets make darker embryos and lesser cryotolerant. The Sudan Black staining method has been vastly used for identifying embryos with increased numbers of lipid droplets (Abe *et al*., 2002; Sudano *et al*., 2011). Serum also affects the duration from maximal compaction to blastulation, shortening 12h in comparison to IVD embryos. This short compaction period and early blastulation in embryos coincided with perturbed allocation of cells to the inner cell mass and trophectoderm and is caused by decreased expression of transcripts that are involved in the construction of tight junctions, all this together decreased cryosurvival of the subsequently formed blastocysts (Van Soom *et al*., 1997; Miller *et al*., 2003).

Timing of blastocyst formation is a good marker for embryo quality as well early cavitating embryos are superior in comparison with the latter cavitating embryos in regard to total cell number, allocation of inner cell mass and trophectoderm cells, and cryosurvival (Mahmoudzadeh *et al*., 1995; Van Soom *et al*., 1997). Although, reliable morphological predictors at the blastocyst stage for competence after embryo transfer are still lacking. Nevertheless, there is a consensus by many research groups and commercial companies that the greatest results of pregnancy rates are generally achieved after the transfer of bovine day 7 expanded blastocysts regardless of whether they were fresh or cryopreserved (Hasler 2000; Xu *et al*., 2006; Block *et al*., 2009; Sanches *et al*., 2013; Munoz *et al*., 2014; Mogas, 2019).

Embryos suffer considerable morphofunctional damage when they are cryopreserved. The challenge during the process of vitrification/freezing followed by warming/thawing is enormous, which includes osmotic, thermic and mechanical stress. The extent of the cryopreservation injuries depends on factors such as the membrane permeability, size and shape of the cells, and quality and sensitivity of the blastomeres (Vajta and Kuwayama, 2006). In order to obtain greater cryosurvival results, therefore, it is sound to cryopreserve following transfer expanded blastocysts of extraordinary superior quality. No signs of degeneration in the morphological evaluation of embryos would be recommended (Hasler 2000; Xu *et al*., 2006; Block *et al*., 2009; Sanches *et al*., 2013; Munoz *et al*., 2014; Mogas, 2019).

Embryo cellular competence

The initial development of embryos in mammals has unique physiological characteristics and

mechanisms of regulation. Modifications in the properties of cytoplasmic organelles, such as location, morphology and biochemical activity must occur to achieve high quality during development (Sirard *et al*., 2006; Mao *et al*., 2014).

Initially, mitochondria constitute the powerhouses of cells, responsible for energy to boost all cellular functions. This organelle has an important role in calcium homoeostasis, fatty acid oxidation and apoptosis (Lledo *et al*., 2018). The embryo only starts to replicate it at hatched blastocyst stage, so all stages before depends on the mitochondrias pre-existent in the oocyte (Spikings *et al*., 2006). Alterations in normal mitochondrial functions can lead to metabolic diseases affecting the preimplantation development (Van Blerkom, 2004; Schaefer *et al*., 2008). In the early cell division cycle, the embryo has a greater amount of mitochondrias in storage, which is diluted as the divisions progress. This is the reason for blastocysts have few copies per cell, considered the mechanism for high quality transmission by the bottle neck theory (Lee *et al*., 2012). Therefore, just only at the implantation period the embryo is capable of replicate mitochondrial DNA (mtDNA) (Thouas *et al*., 2005; St John *et al*., 2010).

The initial production of energy in the early stages of embryonic development occurs through aerobic and anaerobic respiration (Thouas *et al*., 2005; El Shourbagy *et al*., 2006; Spikings *et al*., 2007), and is critical in several species. Human (May-Panloup *et al*., 2005), murine (Liu *et al*., 2000; Thouas *et al*., 2004), bovine (Chiaratti *et al*., 2011), and porcine (Spikings *et al*., 2007) studies have shown that problems in mitochondrial function lead to reduced cleavage rates and aneuploidy occurrence. In addition, low production of ATP and decrease of mtDNA copies are related with poor quality embryos (Wai *et al*., 2010; Wakefield *et al*., 2011). Another important change is the remodelling of mitochondrial features, turning into a complex structure that includes development of cristae, denser matrix and elongated form. These transitions are crucial to change from glycolytic to aerobic metabolism (Schatten *et al*., 2014).

Response to exogenous stress is a crucial part of cellular physiology. The main mechanism to start the cellular stress response pathway is located in the endoplasmic reticulum (ER). This organelle is responsible for secreting various proteins involved in several biological processes and its quality control is responsible for detecting failures to maintain the functioning of the cells which include processes of cell division, homeostasis, and differentiation (Latham, 2015). Disturbance in the endoplasmic reticulum stress signalling, especially in the GRP78 / BiP protein, leads to problems in the pre-implantation period, failure in blastocyst hatching and defects in cell division and apoptosis of the inner cell mass (Luo *et al*., 2006).

Another cellular component is the structure of the cytoskeleton, essential in diverse cellular functions and embryonic development. The cytoskeleton is composed of microfilaments, intermediate filaments and microtubules. Damage to the cytoskeleton adversely affects cell viability and can cause disruption in development and survival, given the support for intracellular content to be organized (Mao *et al*., 2014). In Damiman *et al* (2013) study, the comparison between slow-freezing and non-cryopreserved embryos showed a decrease of tubulin, actin and nucleus structures in each stage compared in mouse, indicating that may the vitrification technique would cause less damage on cytoskeleton (Dasiman *et al*., 2013). The IVPE can also modify the cytoskeleton leading, in some cases, to aberrant actin organization that is responsible for decreased development in IVP embryos (Mao *et al*., 2014). It was described in the literature that the supplementation of melatonin, a cytoskeletal modulator, reverse the damage in actin organization related genes, such as Rho/Rac guanine nucleotide exchange factor 2 (*Arhgef2*), BCL2 apoptosis regulator (*Bcl2*), coronin 2B (*Coro2b*), filamin C (*Flnc*), and palladin cytoskeletal associated protein (*Palld*), providing a target to optimize existing *in vitro* production systems (Tan *et al*., 2015).

Membrane damage and DNA fragmentation are the most commonly injury caused by cryopreservation (Sudano *et al*., 2012). Both injuries are associated with the reduction in the total cell number after cryopreservation and also the disturbance in the proportion of inner cell mass (ICM) / trophectoderm (TE) cells ratio (Sudano *et al*., 2012; Gomez, 2009). The proper ICM/TE ratio and cells number is crucial for embryo development and pregnancy establishment (Van Soom *et al*., 1996; Leese *et al*., 1998). These cryoinjuries can severe reduce embryo survival and the ability for the pregnancy success.

Many ultrastructural damages are commonly related to embryo cryopreservation, including: plasma membrane disruption, abnormal mitochondrial cristae, matrix swelling of the endoplasmic reticulum, poorly developed desmosome, reduced number of microvilli, disintegrations of cell adhesion among TE cells, and increase incidence of cell death (Overstrom *et al*., 1993; Vajta *et al*., 1997; Fair *et al*., 2001).

Nevertheless, many of this cryoinjury can be avoided by a proper cryopreservation methodology and rigorous application of technique assuring correct time of exposure and temperature of the cryopreservation steps. Additionally, cryopreserve exceptional quality embryos tend to reduce cryoinjuries, and clear signs of regeneration and reorganization of embryonic structures are expected after warming/thawing and re-culture, including re-establishment of tight-junctions between TE cells and the return of normal mitochondrial morphology (Vajta *et al*., 1997).

Embryo molecular competence

Beyond morphological analyses, other techniques that reflect the functional and physiological state of the embryo have been proposed, such as the molecular analysis. Basically we can evaluate the embryos based into three groups of different stages of cellular functioning: (1) production of transcripts and proteins from specific genes of cellular products, (2) final products of cellular processes (metabolites) and (3)

gene expression regulatory transcripts, the so-called small non-coding RNAs (ncRNAs), such as: interference RNA (siRNA), micro RNAs (miRNA) and antisense RNA (Phillips, 2008; Rodgaard *et al*., 2015). The present review focused on mRNA transcripts evaluation and discussed briefly other molecular aspects.

Morphological embryonic development events as first cleavages, compaction and blastulation are accompanied by a loss of pluripotency and formation of lineages of trophectoderm and inner cell mass to further development of the three primary germ layers: endoderm, mesoderm and ectoderm (Stephenson *et al*., 2012; Schrode *et al*., 2013). During this period, not only embryo morphology undergoes modifications, changes in the transcripts levels for genes associated with early embryo development dynamic occur. Transcription factors required for maintenance of pluripotency, such as POU class 5 homeobox 1 (*POU5F1/Oct4*), SRY-box transcription factor 2 (*SOX2*) and Nanog homeobox (*NANOG*), expressed strictly in ICM cells, mediated by the action of the caudal type homeobox 2 (*CDX2*) gene product expressed on trophectoderm cells (Chambers *et al*., 2003; Ralston and Rossant, 2008) have been widely described.

During the embryo pre-implantation period, there is a massive degradation of the maternal RNA/proteins stored inside the oocyte and the gradual activation of the embryonic genome. Studies that inhibit RNA polymerase II have demonstrated that the time of the embryonic genome activation (EGA) is related to the rate of embryonic development. In bovine, EGA occurs between 8-16 cells and is associated with early differentiation, embryo implantation success, and fetal development. However, the regulation of the gene expression of bovine embryos remains an unresolved biological issue (Misirlioglu *et al*., 2006; Graf *et al*., 2014).

A correlation between EGA with increased abundances of mature forms of miR30a and miR-21, and the primary form of miR-130a from 1-cell to 8-cell zygotes have been described (Mondou *et al*., 2012). Tripurani *et al* (2013) pointed to another miRNA with increased expression in 2- to 8-cell embryos, miR-212, possibly a suppressor of the maternal factor in the germ line at the transition of transcripts and maternal proteins to those of the embryo, called the maternal-embryonic transition. Although specific mechanisms of EGA are poorly defined, some factors involved such as maternal cyclin A2 (*CCNA2*), retinoblastoma protein (*RB1*), catalytic subunit of the SWI/SNF-related chromatin remodelling complex (*BRG1*), and the SRY-box transcription factor 2 (*SOX2*) have recently been suggested in an EGA model in mice (Tripurani *et al*., 2013). Embryos that do not properly undergo genome activation fail in the further development.

It has already been described that biopsies derived from IVP blastocysts that resulted in calf delivery were enriched with transcripts necessary for implantation (cytochrome c oxidase subunit II, *COX2* and caudal type homeobox 2, *CDX2*), carbohydrate metabolism (arachidonate 15-lipoxygenase, *ALOX15*), growth factor (bone morphogenetic protein 15, *BMP15*), signal transduction by plasminogen activator urokinase (plasminogen activator urokinase, *PLAU*) and placenta (placenta–specific 8, *PLAC8*). Transcripts involved in protein phosphorylation (keratin 8, *KRT8*), plasma membrane (occludin, *OCLN*) and glucose metabolism (phosphoglycerate kinase 1, *PGK1* and aldo-keto reductase family 1 member B1, *AKR1B1*) were enriched in reabsorbed embryos. Embryos that did not resulted in pregnancy presented enriched transcripts involved with inflammatory cytokines (tumor necrosis factor, *TNF*), amino acid binding protein (eukaryotic translation elongation factor 1 alpha 1, *EEF1A1*), transcription factors (msh bomeobox 1, *MSX1*, and PTTG1 regulator of sister chromatid separation securing, *PTTG1*), glucose metabolism (phosphoglycerate kinase 1, *PGK1* and aldo-keto reductase family 1 member B1, *AKR1B1*), and implantation inhibitor (CD9 molecule, *CD9*) (El-Sayed *et al*., 2006).

Despite great improvement of the information availability of embryo competence, this knowledge was not enough to avoid pregnancy loss observed in all mammalian species, in which cause serious issues such as reproductive wastage in farm animals. It is generally expected pregnancy loss in cattle in 40% of the cases between day 8 and 18 (Diskin and Sreenan, 1980). This embryo mortality is caused by a series of events that includes intrinsic embryo's defects, unsatisfactory maternal environment and failure to synchronization between the cow and embryo.

Ghanem *et al*. (2011) identified 41 and 43 differently expressed genes between IVD blastocysts which resulted in no pregnancy *vs.* calf delivery and pregnancy loss *vs.* calf delivery, respectively. In general, genes related to placental development and maternal-embryo interaction (placenta–specific 8, *PLAC8*) were upregulated in embryos that had pregnancy to term. On the other hand, embryo's biopsies that did not end in successful pregnancy presented enriched transcripts related to mitochondrial transcripts (*Bos taurus* isolate FL405 mitochondrion, *Fl405*) and stress genes (heat shock protein family D (Hsp60) member 1, *HSPD1)*. However, both biopsies presented similar gene expression related to preimplantation development of embryo.

Another study also evaluating the molecular signatures of IVD embryo biopsies which resulted in no pregnancy and calf delivery identified 70 differently expressed genes: 32 transcripts levels were upregulated in the biopsies from calf delivery-derived embryos, such as sperm associated antigen 17 (*SPAG17/PF6*), ubiquitin conjugating enzyme E2 D3 (*UBE2D3P*), deafness autosomal recessive 31 (*DFNB31*), S-adenosylmethionine decarboxylase 1 (*AMD1*), dystrobrevin binding protein 1 (*DTNBP1*), and adp-ribosylation factor-like 8B (*ARL8B*); whereas 38 transcripts levels including RING1 and YY1 binding protein (*RYBP*), ring finger protein 34 (*RNF34*), karyopherin alpha 4 (*KPNA4*), and WD repeat domain 13 (*WDR13*) were increased in the biopsies of the no pregnancy-derived embryos indicating that the embryos are molecularly distinguishable (Salilew-Wondim *et al*.,

2010). The biopsy transcriptional profiles from cryopreserved blastocysts that resulted in calf delivery, no pregnancy, and pregnancy loss are still lacking.

Our group have recently evaluated the transcriptional profiles of IVP bovine embryos with high and low post-cryopreservation survival. Blastocysts with high cryosurvival were enriched in 27 genes associated with the following top five biological processes: a) predicted to be activated: organismal survival, cell death and survival, cellular growth and proliferation; and b) predicted to be inhibited: cellular movement and cell-to-cell signalling (data not published).

A favourable transcriptional profile for pregnancy establishment and maintenance, therefore, could be used as a marker of embryo competence. In addition, each gene and/or genes involved pathway should be studied in order to design strategies applied to the *in vitro* embryo production system to improve embryo quality, cryosurvival and embryo-derived pregnancy.

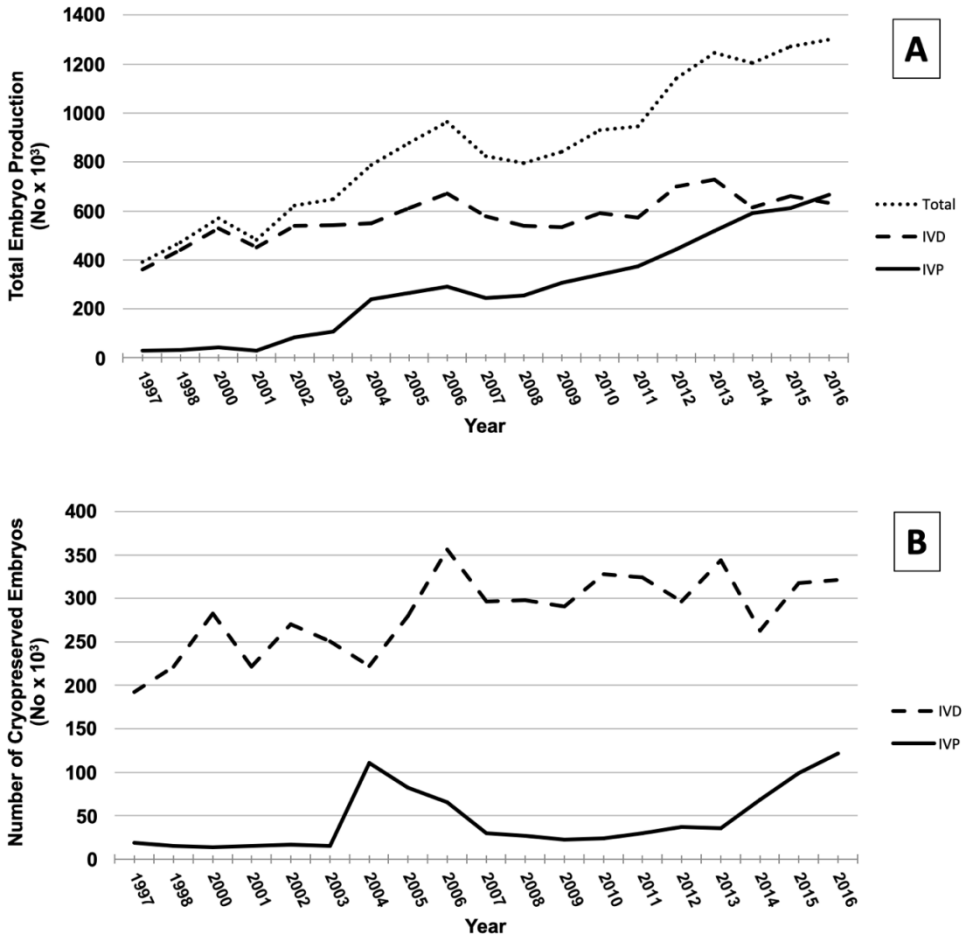


Figure 1. Total number, *in vivo* derived (IVD) and *in vitro* produced (IVP) bovine embryos in the world over the last 20 years (A). Number of cryopreserved IVD and IVP bovine embryos in the world in the last 20 years (B). Source: Data retrieval committee, International embryo technology society – IETS (https://www.iets.org/comm_data.asp?autotry=true&ULnotkn=true, accessed June 2019; and Perry, 2017).

Determinant effects of embryo competence and cryosurvival

Early embryo development period is complex, conserved, and well-orchestrated process involving dynamic molecular and structures changes. Embryos must undergo important events, such as fertilization, cleavage, epigenetic reprogramming, compaction, differentiation, and blastulation, for proper development and pregnancy establishment (Sudano *et al*., 2016a; Jiang *et al*., 2014).

Several factors can disrupt competence during this period and result in variations of the embryo phenotypes, including genotype and environmental effects. Because of this reason, a rigorous quality control should be used to perform all the steps involved in the IVPE in order to mimic as much as possible the *in vivo* environment conditions and reduce possible genotype effect. A putative model addressing cellular and molecular events required for acquisition of embryonic developmental competence and cryosurvival is presented at Figure 2.

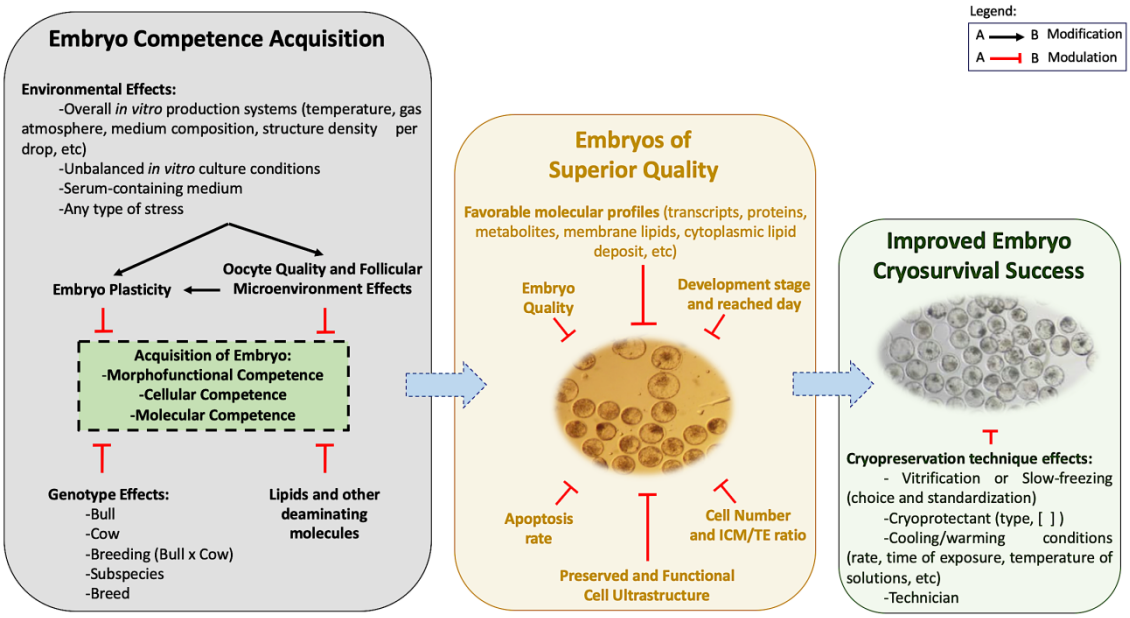


Figure 2. Cellular and molecular events required for embryonic developmental competence acquisition and its impact on cryosurvival. Interconnected steps (blue dashed arrows, from the left to the right) to *in vitro* produce an embryo of superior quality and improved embryo cryosurvival success. Overall environmental and genetic effects on embryo competence acquisition (Black Box). Determinant variables associated with the *in vitro* production of superior embryo quality (Gold Box, in detail fresh IVP blastocysts with great quality). Successful resumption of development after cryopreservation and cryopreservation technique effects (Green Box, in detail re-expanded IVP blastocysts after warming). All Box events (Black, Gold and Green) impact embryo cryosurvival.

Genotype effect

It is well documented in the literature the great variation in the developmental competence of IVP bovine embryos according to the sire (Brackett and Zuelke, 1993; Galli and Lazzari, 1996). Variables like cleavage and blastocyst production rates of a specific bull and/or semen batch (with unknow IVF results) are generally previously evaluated *in vitro* before the vastly use of this semen in the IVPE routine. Nevertheless, there are considerable variations of pregnancy rates observed after the embryo transfer according to the sire used for both fresh and cryopreserved embryos.

Our group recently evaluated the sire effect in the pregnancy rates following the transfer of fresh (N = 40,200) or cryopreserved (N = 9,858) IVP embryos. Pregnancy rates varying among 28.3 to 52.5 % (for fresh) and 7.7 to 61.6 % (for cryopreserved) were recorded after transfer IVP embryos derived from different bulls. We also have observed a variation according the cow, breeding, subspecies, and breed on pregnancy rates (Sudano *et al*., 2019). However, despite great amount of the variation was attributed to the genotype effect in this evaluation, it is also important to considerate the environmental effect in the determination of the reason of variation.

Variations in the membrane lipid profiles of phosphatidylcholine and sphingomyelin between *Bos taurus indicus* (Nellore) and *Bos taurus taurus* (Simmental) embryos and its impact on the embryo's cryosurvival have already been reported (Sudano *et al*., 2012; 2013a). After vitrification the evaluation of the percentage of structures hatching revealed results superior to *B. taurus taurus* (34.6%) compared to *B. taurus indicus* (20.2%) embryos (Sudano *et al*., 2013a). Arreseigor *et al*. (1998) also showed this difference between subspecies in the pregnancy rate. It seems that factors including the phospholipid cell membrane composition are associated with the determination of the physicochemical properties such as fluidity, permeability and thermal phase behavior, which are determinants for cryotolerance (Van Meer *et al*., 2008).

Oocyte quality and the follicular environment effects

The production of competent oocytes is dependent on complete nuclear and cytoplasmic maturation. The oocyte during its development undergoes rearrangements that make it capable of supporting fertilization and early embryonic development. During the nuclear maturation process, oocyte chromosomes go through diplotene of prophase I to metaphase II of meiosis. Among the main changes in cytoplasmic maturation are carbohydrate and lipid metabolism, mitochondrial function and location, reduction of oxygen radicals, epigenetic reprogramming, bidirectional communication between the cumulus-oocyte complexes (COC) and its secretion of growth factors (Brevini Gandolfi and Gandolfi, 2001). All this events are crucial for a proper oocyte competence acquisition.

In the commercial IVPE programs, the ultrasound-guided follicular aspiration (OPU) is routinely used for oocyte recovery, this biotechnology allows access a large number of female gametes in

mono-ovulatory species favoring the multiplication of animals with exponentially genetic merit (Nagai *et al*., 2015; Nagano, 2019). Investigations on the influence of the follicle microenvironment and size in the establishment of competent embryos have been conducted (Annes *et al*., 2018; Labrecque *et al*., 2016; Lonergan *et al*., 2016).

There is considerable effect of follicle conditions in the oocyte and embryo competence. Oocyte origin is the main factor affecting blastocyst yield (Rizos *et al*., 2002). Any type of stress conditions that oocytes face can potentially disrupts embryo development. Differences in the morphology (Nagai *et al*., 2015; Nagano, 2019), lipid composition, transcriptome, and embryo development (Labrecque *et al*., 2016; Annes *et al*., 2018) had already been described in the oocytes recovery from different follicle sizes. The bovine oocytes used for IVPE are recovered from follicles with 2 to 8 mm of diameter, before follicle divergence. Generally, oocytes recovered from large follicles (> 6mm) present a greater potential of reaching blastocysts compared to oocytes recovered from follicles of 2 to 6 mm (Rizos *et al*., 2002; Fair, 2003; Labrecque *et al*., 2016; Annes *et al*, 2018).

Despite embryo culture conditions is crucial for embryo quality and cryosurvival determination (Rizos *et al*., 2002), blastocysts that originate from oocytes matured *in vitro* result in lower rates of gestation compared to their *in vivo* counterparts (Peterson and Lee, 2003). Additionally, we recently identified a relationship between the cytoplasmic lipid content of oocytes and the lipid deposit of expanded blastocysts (Annes *et al*., 2018), i.e., oocytes derived from large follicles and containing greater amount of lipid droplets, originated day 7 expanded blastocysts, after IVPE, with increased lipid deposit. An experiment evaluating the cryosurvival of these blastocysts is still lacking, however, it is fair to speculate that this increased lipid deposit negatively impact embryo cryosurvival (Abe *et al*., 2002).

This results collaborate in the selection of follicles/oocytes of greater potential, since key events for embryonic development are dependent of oocyte status (Fair, 2010). A properly embryo developmental competence, therefore, could be increased in an oocyte focused manner.

In vitro production conditions effects

It is commonly accepted that IVP embryos have a reduced competence compared with the *in vivo* counterparts. *In vitro* production of embryos is a three-step procedure involving oocyte maturation, fertilization, and *in vitro* culture of embryos. The *in vitro* environment directly influences the embryonic phenotypes and results. It is very important, therefore, to mimic the conditions found *in vivo* in order to allow all events inherent to the early embryonic development occur (for review, see Sudano *et al*., 2012; 2013a; 2013b).

Since the first notice of a calf delivery from a completely IVP embryo (*in vitro* maturation, fertilization and culture; Lu *et al*., 1987) the *in vitro* production systems still have challenging outcomes. Despite 80 to 90% of oocytes submitted to *in vitro* maturation have the germinal vesicle breaks down from prophase I to metaphase II, and 80% cleave after fertilization, only 20 to 40% of the oocytes develop to the blastocyst stage, and 50% of transferred IVP blastocysts establish pregnancy, i.e., just only 10 to 20 % of the recovered oocytes submitted to *in vitro* production will result in pregnancy (Lonergan *et al*., 2016, 2006; Rizos *et al*., 2008; Wrenzycki and Stinshoff, 2013; Sudano *et al*., 2019).

In vivo, oocyte maturation occurs during follicle development, a period that oocytes produce and storage mRNAs and protein molecules necessary to supply the first embryonic activities (Brevini Gandolfi and Gandolfi, 2001). *In vitro*, the maturation medium and *in vitro* process are responsible to provide conditions to the oocytes undergo nuclear (reach metaphase II of meiosis), cytoplasmic (re-distribution of organelles such as the mitochondria and the cortical granules) and molecular (accumulation of specific molecules, largely unidentified, which prepare the oocyte for post-fertilization events) maturation (Sirard *et al*., 2006). However, the *in vitro* maturation conditions are still not so much efficient. It was reported that blastocyst production from oocytes matured *in vitro* is lower than *in vivo* (39.2% vs. 58.2%, respectively; Rizos *et al*., 2002). Even if different supplements like grown factors and hormones were supplemented during IVM, the blastocyst development rate was not higher compared with the serum-supplemented medium highlighting the importance of the initial status of the oocyte (Ward *et al*., 2002; Hoelker *et al*., 2014).

In this context, there are some investigative approach studies describing a greater gene expression pattern correspondent to cytoplasmic maturation involved genes in oocytes with higher blastocyst production (Räty *et al*., 2011) and also a greater expression for apoptosis activating related genes to non-competent structures (Warzych *et al*., 2007). This kind of works and a better understanding of *in vivo* oocyte maturation could improve the development of the maturation medium that produces viable embryos (Wrenzycki and Stinshoff, 2013).

In the literature, there is a consensus that improvements in oocyte quality is crucial for the proportion of oocytes developing to blastocysts stage, whereas the post-fertilization environment period is the major aspect determining the blastocyst quality and competence, including post-cryopreservation survival (Rizos *et al*., 2002; Lonergan *et al*., 2003; Hoelker *et al*., 2014).

In vivo, fertilization and early embryonic development take place at the oviduct due to the ability of this microenvironment to support embryogenesis, providing nutrients, growth factors, antioxidants, sex

hormones, proteases, and other regulatory molecules of gametes and embryos. Furthermore, the cellular structure of this reproductive organ allows the transport of embryos to the uterus (Li and Winuthayanon, 2017; Rizos *et al*., 2008, 2002). Mimic *in vivo* environment is very difficult and somehow we generally failed in this task. Unbalanced *in vitro* culture conditions itself impacts directly embryo competence, since it has a significant effect on the cellular metabolism (D'Souza *et al*., 2018; Khurana and Niemann, 2000; Rizos *et al*., 2002) quality and quantity of lipids (Sudano *et al*., 2016a), gene expression patterns (Lonergan *et al*., 2006), and on modifications in epigenetic markers, which can continue after birth (Ramos-Ibeas *et al*., 2019).

However, despite the great diversity of culture media and systems available and used for IVPE with research purpose and commercially, they generally are associated with a relative good developmental potential which sheds light to the embryo plasticity, i.e., the capacity to fetch survival and adapt to adverse conditions even in an environment that do not supply or exacerbate their physiological needs (Khurana and Niemann, 2000; Lonergan *et al*., 2006). For example, the capacity of switching energy consumption source according nutrient availability. In mice, this plasticity was represented by the significant increase of the consumption of pyruvate, due to lack of glucose in the used medium (Gardner and Leese, 1988).

At molecular level, the great plasticity and tolerance to distinct culture conditions/systems including temperature, gas atmosphere, medium composition, embryo density per drop, and many other situations, are associated with an exacerbate gene expression pattern on IVP embryos compared with the *in vivo* counterparts (Corcoran *et al*., 2006; Côté *et al*., 2011; Sudano *et al*., 2013a).

However, this embryo plasticity can cause some severe damage to its competence and offspring; e.g. the large offspring syndrome in cattle (McEvoy *et al*., 2000; Rizos *et al*., 2008) and the overweighting newborns in humans (Li and Winuthayanon, 2017). In bovines, this anomaly is associated with a protein source used in the culture media, the fetal calf serum (FCS), characterized by abnormal fetal and placental size, increased myogenesis, dystocia, abnormal neonatal lung activity, and increased post-neonatal mortality (McEvoy *et al*., 2000; Walker *et al*., 1996). In addition, as previously cited in this review, the FCS supplementation was also associated with increased cytoplasmic lipid deposit and reduced cryosurvival of the embryos (Abe *et al*., 2002; Sudano., 2011). In a previous work of our group, the cytoplasmic lipid droplets fluctuation observed on IVP embryos was closed related with FCS concentration supplemented to the culture media (Sudano *et al*., 2011) and with the mRNA levels for *ACSL3* (Sudano *et al*. 2016a).

Taking all these studies together, culture conditions dramatically affect embryo developmental potential, quality, and further ability to survive after cryopreservation, and establish and maintain pregnancy. Despite many knowledge of the *in vitro* production systems have been produced over the past decades, efficiency improvements in the biotechnology process of the bovine IVPE is still needed in order to increase blastocyst yield of an exceptional quality.

Lipids and other determining molecules for embryo competence

The morphological transitions performed by embryos during the early development until implantation are accompanied by changes in the substrate requirements due to the embryo metabolism demand during this period. *In vivo*, at the reproductive tract, where embryo development takes place, sources of amino acids, proteins, lactate, pyruvate, glucose, antioxidants, ions, growth factors, hormones, and lipids are dynamically available with variations in concentration according to species, estrous cycle period and location (Di Paolo and De Camilli, 2006).

The measurement of embryonic metabolism can be used as an important tool in the analysis of molecular and cellular competence (Thompson *et al*., 2016). Hamatani *et al*. (2004) described two different physiological profiles of blastocysts (dormancy and activation) according to the global expression of genes involved in cell cycle control, cell signaling, and energy metabolism. In a total of 18 genes associated with metabolism, 13 were expressed in blastocysts on activation and 5 in blastocysts on dormancy. These metabolism variations contributed to the further hypothesis of "quiet embryos", which describes the metabolic efficiency of competent embryos with the lower need of substrate for development as a result of a lower percentage of cell damage based in the comparison of IVD and IVP embryos (Leese *et al*., 2008, 2007).

When analyzing the energy metabolism during early embryonic development, energy consumption is relatively low until pre-compaction stage and the main source of ATP is derived from pyruvate through oxidative phosphorylation, whereas with blastocyst formation and cavitation process a significant increase on energy demand and consumption of glucose, pyruvate and oxygen are expected. Additionally, after formation of the blastocyst, other sources of energy are required in greater proportion, including: glucose, amino acids and lipids (D'Souza *et al*., 2018; Hu and Yu, 2017).

Because of the various formulations of medium used by IVF laboratories, the understanding of metabolic spent during IVPE helps in directing compositions that structure viable embryos to the establishment of pregnancy (Thompson *et al*., 2016). Recent studies showed a higher energetic requirement of pyruvate and lactate, during culture, for non-competent embryos (D'Souza *et al*., 2018). The influence of the culture medium on the embryonic

development and the production of healthy animals is already well established, so research that identifies the potential of energy supplements used in the bovine IVPE prevents the multiplication of inappropriate phenotypes (De Souza *et al*., 2015).

Additionally, lipids are an important source of ATP for cell development and actively participates in embryonic metabolic pathways (Hu and Yu, 2017). Lipids are responsible for the cross-talk between embryo, oviduct and uterus. This event is necessary in the synchronization of molecular and cellular events during the pregnancy establishment.

Studies demonstrated the lipid importance in signaling and coordination of biological events by mediating lipids such as phosphatidylinositols, sphingolipids, and eicosanoids (Di Paolo and De Camilli, 2006; Wang and Dey, 2005). Lipids are essential cellular biomolecules for plasma membrane and membranes of several organelles (Di Paolo and De Camilli, 2006). In bovine embryos, a stage-specific dynamic lipid fluctuation was observed during early embryo development (Sudano *et al*., 2016a). In addition, our group identified an increase in the mRNA levels for *ELOVL5* and *ELOVL6* (two transcripts of the ELOVLs family, responsible for fatty acids elongation) at the morula stage preceding an increase in membrane phospholipids containing elongated saturated, monosaturated and polysaturated fatty acids (16, 18 and 20 carbons) at the blastocyst stage (Sudano *et al*., 2016a).

Cells can synthesize simple fatty acids due to their biological functions. In this sense, lipid elongation facilitates the formation of complex biomolecules for specific activities (Guillou *et al*., 2010). We have recently observed the involvement of the ELOVL5 in the lipid metabolism of IVP embryos by regulating the cytoplasmic lipid deposit and the abundance of phosphatidylcholines, phosphatidylethanolamines and triacylglycerol (data not published).

All these findings demonstrate the importance of understanding the mechanisms involved in energy metabolism and embryo lipids composition in order to optimize *in vitro* culture conditions to allow the production of embryos with superior quality and greater cryosurvival.

Embryo cryopreservation

Embryo cryopreservation is a nitrogen (N_2) based conservation biotechnology that aims to maintain the cells in quiescent state, prolong the viability for long periods of time and enable their use in a timely manner (Fig. 3 3). In the area of assisted reproduction, primordial germ cells, gametes and embryos can be cryopreserved. It is considered a strategy to overcome logistical problems associated with the transfer of a large number of fresh embryos and mainly for the expansion of the commercialization of embryos between countries (Sudano *et al*., 2013b). Among the advantages are the optimization of reproductive biotechnologies such as IVPE and transfer of embryos in order to preserve surplus embryos, conservation of the genetic material of endangered species, prevention of problems arising from the transport of live animals and adaptations of the calving season.

The first success report on the survival of embryos (mice) subjected to freezing/thawing was recorded by Whittingham *et al*. (1972). In the following years, the technique was also applied successfully in different species and embryo cryopreservation started to be applied commercially. In Brazil, the majority of the IVP embryos are still transferred fresh, but the percentage of frozen (33.7%) follows a growth trend, and among the embryos produced in *vivo*, the slow freezing is the preferred option (62.1% of the total) (Viana, 2018). The principle of cryopreservation consists of maximum removal of water from the interior of the cells before freezing, avoiding the formation of large ice crystals that cause cell damage, which allows the resumption of cellular metabolism after storage at low temperatures. For this reason, cryopreservation strategies are based on two main factors: cooling/warming rates and choice of suitable cryoprotectants (CPA) (Vajta and Kuwayama, 2006).

According to Mazur (1980) there is a correlation between the cooling rate and the appearance of cell lesions, i.e. as the freezing rate increases, the survival after thawing also increases, until an ideal maximum index is reached from which the higher freezing speed also leads to greater damage and lower embryo survival. In addition to the freezing rate curve, the use of CPA is essential to provide cellular protection against temperature drop. Although CPA are absolutely necessary and widely used in cryopreservation protocols, the mechanisms that confer protection of the biological material as well as the toxicity and cellular metabolism of these agents are not fully elucidated and addressed in the literature (Castro *et al*., 2011). For review all cryobiology principles involved on embryo cryopreservation, please see Sudano *et al*. (2016b).

It is known, at least partially, that the CPA protective effect comes from the reduction of the solidification point of the solutions and the ability on intermediating intracellular water removal/restauration. According to their ability to penetrate cell membranes, CPA are divided into two groups: permeable (e.g. glycerol, ethylene glycol and propylene glycol) and impermeable (e.g. sucrose, galactose, polyethylene glycol and polyvinylpyrrolidone). In addition, there are two major cryopreservation methodology currently applied for bovine embryos, slow freezing and vitrification.

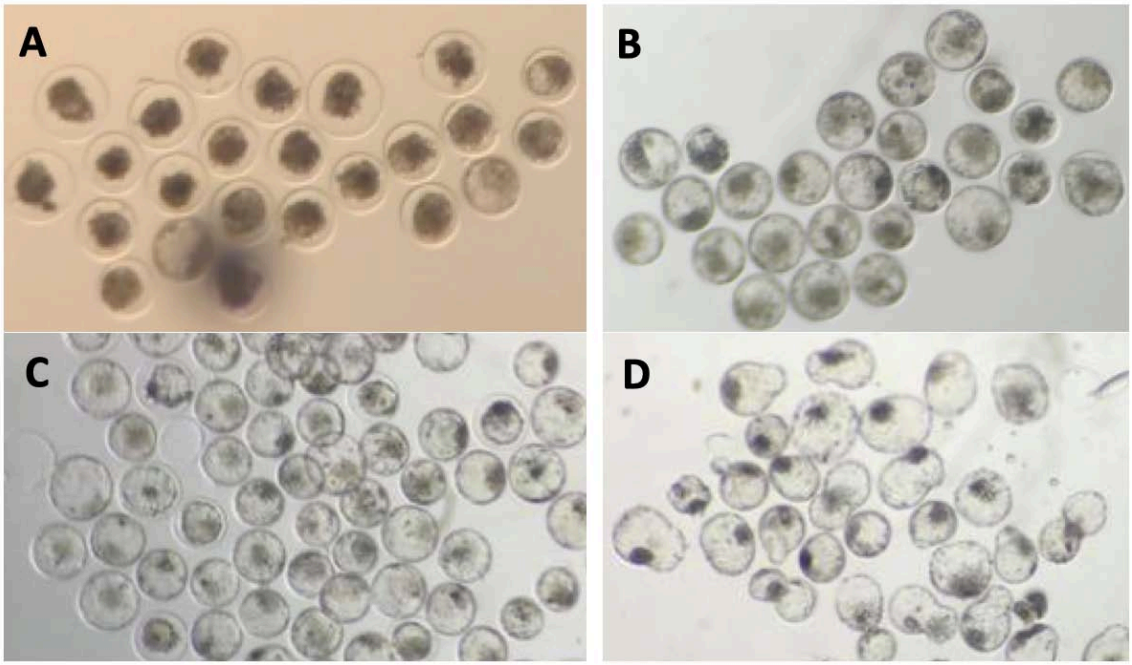


Figure 3. Vitrified *in vitro* produced bovine embryos immediately after warming (A), and following 6 h (B) and 12 h (re-expanded and hatched blastocysts, respectively for C and D) of re-culture.

Cryopreservation techniques

Slow or classic freezing is one of the most widespread embryo cryopreservation techniques and is based on the use of glycerol or ethylene glycol as a CPA. The main advantage of this technique is the reduced cellular toxicity through the use of low concentrations of CPA, however, slow freezing still allows the formation of ice crystals that could lead to cellular damage. After a period of previous CPA exposure, the embryos are loaded in straws together with the CPA solution, which will be allocated in programmable freezing devices for cooling. The temperature drop is controlled by keeping a constant curve until the temperature reaches -32°C, when the straws containing the embryos are immersed in the liquid nitrogen. After thawing, the embryos must be placed in petri dishes where the CPA solution will be diluted by successive washes in dilution solutions. Alternatively, Leibo (1983) developed the One-Step method where dilution of the CPA still occurs in the straw after thawing, letting the transfer to recipients be without previous microscopic evaluation.

Although slow freezing is the most widely used method for the cryopreservation of IVD embryos, vitrification has emerged as an alternative to IVP embryos. When comparing the cryosurvival and pregnancy results of both procedures considering the production method, IVD embryos present similar results, for both slow freezing and vitrification, whereas IVP embryos present more satisfactory results with vitrification (Tab. 1 and 2). However, slow-freezing also can be a good option to cryopreserve IVP embryos, Sanches *et al*. (2016) reported similar pregnancy rates on Day 60 after the transfer of IVP embryo cryopreserved by slow-freezing (34.7%, n = 311) or vitrification (31.2%, n = 234).

Vitrification eradicates the damage caused by the formation of ice crystals during the cooling process. The cryopreservation medium undergoes a direct passage from the liquid state to a vitrified and amorphous state without the crystallization of the medium occurring, which it is possible due to the high viscosity of the cryopreservation medium and the high freezing rate (>20.000°C/min) by direct immersion in N_2, from room temperature (Fahy *et al*., 1984). The technique is extremely fast and does not require the use of freezing devices; however, it requires more training and technical skills from the technician. Among the disadvantages are the difficulty of direct transfer and the need to use high concentrations of CPA that can cause cellular toxicity. As a result, several combinations with different CPA, concentrations and period of exposure have already been proposed. In order to ensure a rapid cooling rate, the use of the minimum volume of solution per sample is adopted in order to provide immediate contact with the N_2. For this purpose, specific tools have been developed for packaging the embryos into microdrops, such as *Open Pulled Straw* (OPS) (Vajta *et al*., 1998), *Cryoloop* (Lane *et al*., 1999) and *Cryotop* (Kuwayama, 2007). More recently, a new automated vitrification methodology has been proposed (Arav *et al*., 2018), which may facilitate and optimize the standardization of the technique.

Despite great results were achieved with enhancements of the cryopreservation methodology over the last decades; an embryo-focused approach to improve cryosurvival has been recommended.

Table 1. Post-cryopreservation survival of *in vitro* produced bovine embryos following slow-freezing and vitrification.

Reference	Slow-freezing (%)		Vitrification (%)		P-value
	Re-expansion	Hatching	Re-expansion	Hatching	
Dinnyés *et al*., 1996	62.0^{a} (n = 63)	81.0^{A} (n=63)	81.0^{b} (n = 64)	70.0^{A} (n = 64)	<or>0.05
O'Kearney-Flynn *et al*., 1998	58^{a} (n =73)	-	86^{b} (n = 64)	-	<0.05
Nedambale *et al*., 2004	40.0^{a} (n = 297)	22.0^{A} (n = 297)	64^{b} (n = 297)	54.0^{B} (n = 297)	<0.05
Mucci *et al*., 2006	16.7^{a} (n = 275)	19.6^{A} (n = 275)	52.1^{b} (n = 265)	51.3^{B} (n = 265)	<0.05
Barceló-Fimbres and Seidel, 2007	80.4 (n = 360)	19.1 (n = 360)	77.7 (n = 360)	16.0 (n = 360)	>0.05
Yu *et al*., 2010	46.9^{a} (n = 155)	24.7^{A} (n = 155)	58^{b} (n = 153)	36.2^{B} (n = 153)	<0.05
Inaba *et al*., 2011	88.6 (n = 44)	75.0^{A} (n = 44)	100 (n = 44)	93.2^{B} (n = 44)	<0.05

Re-expansion rate was evaluated 24h after thawing/warming and hatching/hatched rate was evaluate with 48 or 72 h after thawing/warming according the study methodology. Values without a common lowercase (comparisons between re-expansion rates) or uppercase (comparisons between hatching rates) letters differ ($P < 0.05$).

Table 2. Post-cryopreservation survival of *in vivo* produced bovine embryos following slow-freezing and vitrification.

Reference	Pregnancy rate (%)		P-value
	Slow-freezing	Vitrification	
Massip *et al*., 1987	51.8 (n = 27)	39.1 (n = 23)	>0.05
van Wagtendonk-de Leeuw *et al*., 1995	59 (n = 40)	43 (n = 34)	0.10
van Wagtendonk-de Leeuw *et al*., 1997	45.1 (n = 335)	44.5 (n = 393)	0.79
Mattos *et al*., 2010	19.5 (n = 79)	17.8 (n = 73)	>0.05
	29.8 (n = 102)	36.6 (n = 100)	>0.05
Inaba *et al*., 2011	45.2 (n = 62)	46.7 (n = 30)	>0.05

An embryo-focused approach to improve cryosurvival

The effect of cryopreservation on mammalian embryos reduces survival rates, leading to considerable morphological and functional damage. However, the extent of cryoinjury is highly variable and dependent on the species, stage of development and origin of the embryo (produced *in vivo* or *in vitro*) at the time of cryopreservation (Dalcin and Lucci, 2010).

There is a consensus in the literature that IVP embryos present lesser cryoresistance compared with IVD embryos. Ultrastructural and biochemical characteristics such as increased lipid content, reduced intercellular junctions, fewer mitochondria and microvilli, larger perivitelline space and more cell debris are generally associated with the reduced cryosurvival of IVP embryos (Fair *et al*., 2001; Abe *et al*., 2002, Rizos *et al*. 2002). Additional variables associated with embryo cryosurvival are presented on Table 3. Thus, some strategies can be used to increase the cryotolerance of IVP embryos, such as culture under a low oxygen atmosphere system to minimize oxidative stress, addition of antioxidants to the culture medium, and the use of apoptosis inhibitors (Lin *et al*., 2018; Pero *et al*., 2018).

Table 3. Features positively or negatively associated with cryosurvival evaluated so far by our group.

Variables	Associated Cryosurvival
Sire	Positively or Negatively
Genotype (*Bos Taurus taurus vs. Bos Taurus indicus*)	Positively or Negatively
Metabolic regulators and lipolytic molecule	Positively, Negatively or none
Embryo origin (IVP *vs.* IVD)	Positively or Negatively
Increased embryo quality	Positively
Increased fresh apoptosis rate	Negatively
Increased cytoplasmic lipid content	Negatively or positively
Membrane phospholipids profiles	Negatively or positively
Increased serum concentration	Negatively

As already mentioned, excessive lipid droplets accumulation of IVP embryos during development, especially of embryos cultured in serum-supplemented medium, is commonly associated with reduced cryosurvival and lower pregnancy rate (Rizos *et al*., 2008; Sudano *et al*., 2011). The exact mechanism for this increased lipid content on IVP embryos remains unknown. It seems that serum lipids could be absorbed by embryonic cells (Sata *et al*., 1999), altering the function of mitochondrial β-oxidation (Abe *et al*., 2002) and promoting the incorporation of saturated fatty acids and cholesterol in the cell membranes, which make them less permeable (Barceló-Fimbres and Seidel Jr, 2007). All these factors together also could explain the greater susceptibility of the IVP embryos to cryopreservation.

In fact, it is possible to produce embryos with a greater cryosurvival through manipulations of the culture medium. We have already demonstrated that only the reduction of the serum concentration in the developmental medium was capable of decrease the

lipid content and increase embryo survival after cryopreservation (Sudano *et al*., 2011). Recently, our group participate in the development of a serum-free culture medium of IVP embryos that reach similar results of embryo quality and pregnancy rate compared with a serum-supplemented medium (data not published).

Alternatively, chemical substances that act as a delipidant or lipolytic agents, such as Forskolin (Sanches *et al*., 2013; Paschoal *et al*., 2014), L-carnitine (Held-Hoelker *et al*., 2017), and 10t,12c-CLA (Pereira *et al*., 2007) have been commonly adopted in the attempt to reduce the embryonic lipid content and improve cryosurvival. In addition, embryo delipidation after centrifugation and subsequent micromanipulation has already been proposed (Ushijima *et al*., 1999), however, considering this is an invasive and time-consuming technique, this approach had become impracticable for large scale application and was limited for scientific purpose.

An additional strategy to improve cryosurvival of IVP embryos is the preincubation with grow-stimulating factors before cryopreservation. Beneficial effects of insulin-like growth factor 1 and leukaemia inhibitory factor were observed in the post-cryopreservation survival of bovine blastocysts (Kocyigit and Cevik, 2015).

Final considerations

Embryo developmental competence and quality are crucial for cryosurvival and pregnancy success. Many variables can impair embryo competence and cryosurvival such as genotype, oocyte quality and follicular microenvironment, *in vitro* production conditions, and lipids and other determining molecules. A great quality control of all steps of IPVE and the use of exceptional quality embryos are recommended to improve and achieve homogenous results. A promising scenario for the next few years is expected for the use of the IVP embryos in the reproductive management of beef and dairy cattle. However, it is essential that further research efforts focus on improvements of the efficiency of producing a greater amount of embryos of superior quality, per oocytes recovered and round of *in vitro* fertilization, to be cryopreserved following transferred in order to reduce costs and justify the activity economically.

Authors contributions

TVM: Writing – original draft; JC: Writing – original draft; RSV: Writing – original draft; MJS: Conceptualization, Funding acquisition, Writing – review & editing.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments and Funding disclosure statement

The present work was carried out with the support of CNPq, National Council of Scientific and Technological Development – Brazil - grants (MJS) 446647/2014-4; (RSV) 165496/2017-7; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - financial code 1 (TVM and JC).

References

Abe H, Yamashita S, Satoh T, Hoshi H. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod Dev*, 61:57-66.

Annes K, Müller DB, Vilela JAP, Valente RS, Caetano DP, Cibin FWS, Milazzotto MP, Mesquita FS, Belaz KRA, Eberlin MN, Sudano MJ. 2018. Influence of follicle size on bovine oocyte lipid composition, follicular metabolic and stress markers, embryo development and blastocyst lipid content. *Reprod Fertil Dev*, 31:462-472.

Arav A, Natan Y, Kalo D, Komsky-Elbaz A, Roth Z, Levi-Setti PE, Leong M, Patrizio P. 2018. A new, simple, automatic vitrification device: preliminary results with murine and bovine oocytes and embryos. *J Assist Reprod Genet*, 35:1161-1168.

Arreseigor CJ, Sisul A, Arreseigor AE, Stahringe RC. 1998. Effect of cryoprotectant, thawing method, embryo grade and breed on pregnancy rates of cryopreserved bovine embryos. *Theriogenology*, 49:160.

Báez F, Camargo A, Reyes AL, Márquez A, Paula-Lopes F, Viñolese C. 2019. Time-dependent effects of heat shock on the zona pellucida ultrastructure and *in vitro* developmental competence of bovine oocytes. *Reprod Biol*, 19:195-203.

Barceló-Fimbres M, Seidel Jr G. 2007. Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during in vitro culture of bovine embryos on embryonic development after cryopreservation. *Mol Reprod Dev*, 74:1395-1405.

Block J, Bonilla L, Hansen PJ. 2009. Effect of addition of hyaluronan to embryo culture medium on survival of bovine embryos in vitro following vitrification and establishment of pregnancy after transfer to recipients. *Theriogenology*, 71:1063-1071.

Bó G, Mapletoft RJ. 2013. Evaluation and classification of bovine embryos. *Anim Reprod*, 10:344-348.

Brackett BG, Zuelke KA. 1993. Analysis of factors involved in the in vitro production of bovine embryos. *Theriogenology*, 39:43-64.

Brevini Gandolfi TAL, Gandolfi F. 2001. The maternal legacy to the embryo: Cytoplasmic components and their effects on early development. *Theriogenology*, 55:1255-1276.

Castro S, de Andrade Carvalho A, Gomes da Silva CM, Rocha Faustino L, de Figueiredo JR, Ribeiro Rodrigues AP. 2011. Agentes crioprotetores intracelulares: características e utilização na criopreservação de tecido ovariano e oócitos. *Acta Scientiae Veterinariae*, 39:1-17.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, 113:643-655.

Chiaratti MR, Ferreira CR, Perecin F, Meo SC,

Sangalli JR, Mesquita LG, de Carvalho Balieiro JC, Smith LC, Garcia JM, Meirelles FV. 2011. Ooplast-mediated developmental rescue of bovine oocytes exposed to ethidium bromide. *Reprod Biomed Online*, 22:172-183.

Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans AC, Lonergan P. 2006. Suppressed expression of genes involved in transcription and translation in in vitro- compared with in vivo-cultured bovine embryos. *Reproduction*, 131:651-660.

Coskun S, Hellani A, Jaroudi K, Al-Mayman H, Al-Kabra M, Qeba M. 2003. Nucleolar precursor body distribution in pronuclei is correlated to chromosomal abnormalities in embryos. *Reprod Biomed Online*, 7:86-90.

Côté I, Vigneault C, Laflamme I, Laquerre J, Fournier É, Gilbert I, Scantland S, Gagné, D, Blondin P, Robert C. 2011. Comprehensive cross-production system assessment of the impact of in vitro microenvironment on the expression of messengers and long non-coding RNAs in the bovine blastocyst. *Reproduction*, 142:99-112.

D'Souza F, Uppangala S, Asampille G, Salian SR, Kalthur G, Talevi R, Atreya HS, Adiga SK. 2018. Spent embryo culture medium metabolites are related to the in vitro attachment ability of blastocysts. *Sci Rep*, 8:1-10.

Dalcin L, Lucci C. 2010. Criopreservação de embriões de animais de produção: princípios criobiológicos e estado atual. *Rev Bras Reprod Anim*, 34:149-159.

Dasiman R, Rahman NSA, Othman S, Mustafa MF, Mohd Yusoff NJ, Jusof WHW, Rajikin MH, Froemming GRA, Khan N. 2013. Cytoskeletal alterations in different developmental stages of in vivo cryopreserved preimplantation murine embryos. *Med Sci Monit Basic Res*, 19:258-266.

De Souza DK, Salles LP, Rosa E Silva A. 2015. Aspects of energetic substrate metabolism of in vitro and in vivo bovine embryos. *Braz J Med Biol Res*, 48:191-197.

Di Paolo G, De Camilli P. 2006. Phosphoinositides in cell regulation and membrane dynamics. *Nature*, 443:651-657

Dinnyés A, Carolan C, Lonergan P, Massip A, Mermillod P. 1996. Survival of frozen or vitrified bovine blastocysts produced in vitro in synthetic oviduct fluid. *Theriogenology*, 46:1425-1439.

Diskin MG, Sreenan JM. 1980. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *J Reprod Fertil*, 59:463-468.

El Shourbagy SH, Spikings EC, Freitas M, St John JC. 2006. Mitochondria directly influence fertilisation outcome in the pig. *Reproduction*, 131:233-245.

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D. 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. *Physiol Genomics*, 28:84-96.

Fahy GM, MacFarlane D, Angell C, Meryman H. 1984. Vitrification as an approach to cryopreservation. *Cryobiology*, 21:407-426.

Fair T, Lonergan P, Dinnyes A, Cottell DC, Hyttel P, Ward FA, Boland MP. 2001. Ultrastructure of bovine blastocysts following cryopreservation: effect of method of blastocyst production. *Mol Reprod Dev*, 58:186-195.

Fair T. 2003. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci*, 78:203-216.

Fair T. 2010. Mammalian oocyte development: checkpoints for competence. Reprod Fertil Dev, 22:13-20.

Galli C, Lazzari G. 1996. Practical aspects of IVM/IVF in cattle. *Anim Reprod Sci*, 42:371-379.

Gardner DK, Leese HJ. 1988. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. *Development*, 104:423-429.

Ghanem N, Salilew-Wondim D, Gad A, Tesfaye D, Phatsara C, Tholen E, Looft C, Schellander K, Hoelker M. 2011. Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments. *Reproduction*, 142:551-564.

Gomez E, Munoz M, Rodriguez A, Caamano JN, Facal N, Diez C. 2009. Vitrification of bovine blastocysts produced in vitro inflicts selective damage to the inner cell mass. *Reprod Domest Anim*, 44:194-199.

Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. 2014. Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proc Natl Acad Sci U S A*, 111:4139-4144.

Guillou H, Zadravec D, Martin PGP, Jacobsson A. 2010. The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog Lipid Res*, 49:186-199.

Hamatani T, Daikoku T, Wang H, Matsumoto H, Carter MG, Ko MS, Dey SK. 2004. Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. *Proc Natl Acad Sci*, 101:10326-31.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*, 43:141-152.

Hasler JF. 2000. In vitro culture of bovine embryos in Menezo's B2 medium with or without coculture and serum: the normalcy of pregnancies and calves resulting from transferred embryos. *Anim Reprod Sci*, 60-61:81-91.

Held-Hoelker E, Klein S, Rings F, Salilew-Wondim D, Saeed-Zidane M, Neuhoff C, Tesfaye D, Schellander K, Hoelker M. 2017. Cryosurvival of in vitro produced bovine embryos supplemented with l-Carnitine and concurrent reduction of fatty acids. *Theriogenology*, 96:145-152.

Hoelker M, Held E, Salilew-Wondim D, Schellander K, Tesfaye D. 2014. Molecular signatures of bovine embryo developmental competence. *Reprod Fertil Dev*, 26:22-36.

Hoelker M, Schmoll F, Schneider H, Rings F, Gilles M, Tesfaye D, Jennen D, Tholen E, Griese J, Schellander K. 2006. Bovine blastocyst diameter as a

morphological tool to predict embryo cell counts, embryo sex, hatching ability and developmental characteristics after transfer to recipients. *Reprod Fertil Dev*, 18:551-557.

Holm P, Booth PJ, Callesen H. 2002. Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction*, 123:553-565.

Hu K, Yu Y. 2017. Metabolite availability as a window to view the early embryo microenvironment in vivo. *Mol Reprod Dev*, 84:1027-1038.

Inaba Y, Aikawa Y, Hirai T, Hashiyada Y, Yamanouchi T, Misumi K, Ohtake M, Somfai T, Kobayashi S, Saito N, Matoba S, Konishi K, Imai K. 2011. In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. *J Reprod Dev*, 57:437-443

Jiang Z, Sun J, Dong H, Luo O, Zheng X, Obergfell C, Tang Y, Bi J, O'Neill R, Ruan Y, Chen J, Tian XC. 2014. Transcriptional profiles of bovine in vivo pre-implantation development. *BMC Genomics*, 15:1-15.

Khurana NK, Niemann H. 2000. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod*, 62:847-856.

Kocyigit A, Cevik M. 2015. Effects of leukemia inhibitory factor and insulin-like growth factor-I on the cell allocation and cryotolerance of bovine blastocysts. *Cryobiology*, 71:64-69.

Kuwayama M. 2007. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology*, 67:73-80.

Labrecque R, Fournier E, Sirard MA. 2016. Transcriptome analysis of bovine oocytes from distinct follicle sizes: Insights from correlation network analysis. *Mol Reprod Dev*, 83:558-569.

Lane M, Schoolcraft WB, Gardner DK, Phil D. 1999. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertility and sterility*, 72:1073-1078.

Latham KE. 2015. Endoplasmic reticulum stress signaling in mammalian oocytes and embryos: Life in the balance. *Int Rev Cell Mol Biol*, 316:227-265.

Lee HS, Ma H, Juanes RC, Tachibana M, Sparman M, Woodward J, Ramsey C, Xu J, Kang EJ, Amato P, Mair G, Steinborn R, Mitalipov S. 2012. Rapid mitochondrial DNA segregation in primate preimplantation embryos precedes somatic and germline bottleneck. *Cell Rep*, 1:506-515.

Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. 2008. Metabolism of the viable mammalian embryo: Quietness revisited. *Mol Hum Reprod*, 14:667-672.

Leese HJ, Donnay I, Thompson JG. 1998. Human assisted conception: a cautionary tale. Lessons from domestic animals. *Hum Reprod*, 13:184-202.

Leese HJ, Sturmey RG, Baumann CG, McEvoy TG. 2007. Embryo viability and metabolism: Obeying the quiet rules. *Hum Reprod*, 22:3047-3050.

Leibo S. 1983. A one-step in situ dilution method for frozen thawed bovine embryos. *Cryo-letters*, 4:387-400.

Li S, Winuthayanon W. 2017. Oviduct: Roles in fertilization and early embryo development. *J Endocrinol*, 232:1-26.

Lin T, Lee JE, Kang JW, Oqani RK, Cho ES, Kim SB, Il Jin D. 2018. Melatonin supplementation during prolonged in vitro maturation improves the quality and development of poor-quality porcine oocytes via anti-oxidative and anti-apoptotic effects. *Mol Reprod Dev*, 85:665-681.

Lindner GM, Wright RW, Jr. 1983. Bovine embryo morphology and evaluation. *Theriogenology*, 20:407-416.

Liu L, Trimarchi JR, Keefe DL. 2000. Involvement of mitochondria in oxidative stress-induced cell death in mouse zygotes. *Biol Reprod*, 62:1745-1753.

Lledo B, Ortiz JA, Morales R, García-Hernández E, Ten J, Bernabeu A, Llácer J, Bernabeu R. 2018. Comprehensive mitochondrial DNA analysis and IVF outcome. *Hum Reprod Open*, 4:1-9.

Lonergan P, Fair T, Corcoran D, Evans ACO. 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology*, 65:137-152.

Lonergan P, Fair T, Forde N, Rizos D. 2016. Embryo development in dairy cattle. *Theriogenology*, 86:270-277.

Lonergan P, Rizos D, Gutierrez-Adan A, Fair T, Boland MP. 2003. Oocyte and embryo quality: Effect of origin, culture conditions and gene expression patterns. *Reprod Domest Anim*, 38:259-267.

Lu KH, Gordon I, Gallagher M, Mcgovern H. 1987. Pregnancy established in cattle by transfer of embryos derived from invitro fertilization of oocytes matured in vitro. *Vet Rec*, 121:259-260.

Lundqvist M, Johansson U, Lundkvist O, Milton K, Westin C, Simberg N. 2001. Does pronuclear morphology and/or early cleavage rate predict embryo implantation potential? *Reprod Biomed Online*, 2:12-16.

Luo S, Mao C, Lee B, Lee AS. 2006. GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. *Mol Cell Biol*, 26:5688-5697.

Mahmoudzadeh AR, Van Soom A, Bols P, Ysebaert MT, de Kruif A. 1995. Optimization of a simple vitrification procedure for bovine embryos produced in vitro: effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. *J Reprod Fertil*, 103:33-39.

Mao L, Lou H, Lou Y, Wang N, Jin F. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. *Reprod Biomed Online*, 28:284-299.

Massip A. 1987. Recent progress in cryopreservation of cattle embryos. *Theriogenology*, 27:69-79.

Mattos MCC. Vitrificação e congelação de mórulas e blastocistos produzidos in vivo em bos taurus e bos indicus. [In portuguese]. Botucatu, Brazil: Universidade Estadual Paulista "Júlio de Mesquita Filho". Dissertation.

May-Panloup P, Chretien MF, Jacques C, Vasseur C, Malthiery Y, Reynier P. 2005. Low oocyte mitochondrial DNA content in ovarian insufficiency.

Hum Reprod, 20:593-597.

Mazur P. 1980. Fundamental aspects of the freezing of cells, with emphasis on mammalian ova and embryos. In: International Congress on Animal Reproduction and Artificial Insemination. *Proceedings of International Congress of Animal Reproduction and Artificial Insemination*, pp.99-114.

McEvoy TG, Sinclair KD, Young LE, Wilmut I, Robinson JJ. 2000. Large offspring syndrome and other consequences of ruminant embryo culture in vitro: Relevance to blastocyst culture in human ART. *Hum Fertil*, 3:238-246.

Merton S, 2002. Morphological Evaluation of Embryos in Domestic Species. In: **Van Soom A, Boerjan M**. (Ed.) Assessment of Mammalian Embryo Quality. Springer Science+Business Media Dordrecht, pp429.

Miller DJ, Eckert JJ, Lazzari G, Duranthon-Richoux V, Sreenan J, Morris D, Galli C, Renard JP, Fleming TP. 2003. Tight junction messenger RNA expression levels in bovine embryos are dependent upon the ability to compact and in vitro culture methods. *Biol Reprod*, 68:1394-1402.

Misirlioglu M, Page GP, Sagirkaya H, Kaya A, Parrish JJ, First NL, Memili E. 2006. Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc Natl Acad Sci U S A*, 50:18905-18910.

Mogas T. 2019. Update on the vitrification of bovine oocytes and in vitro-produced embryos. *Reprod Fertil Dev*, 31:105-117.

Mondou E, Dufort I, Gohin M, Fournier E, Sirard MA. 2012. Analysis of microRNAs and their precursors in bovine early embryonic development. *Mol Hum Reprod*, 18:425-434.

Mucci N, Aller J, Kaiser GG, Hozbor F, Cabodevila J, Alberio RH. Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology*, 65:1551-1562.

Munoz M, Uyar A, Correia E, Diez C, Fernandez-Gonzalez A, Caamano JN, Martinez-Bello D, Trigal B, Humblot P, Ponsart C, Guyader-Joly C, Carrocera S, Martin D, Marquant Le Guienne B, Seli E, Gomez E. 2014. Prediction of pregnancy viability in bovine in vitro-produced embryos and recipient plasma with Fourier transform infrared spectroscopy. *J Dairy Sci*, 97:5497-5507.

Nagai K, Yanagawa Y, Katagiri S, Nagano M. 2015. Fertilizability of oocytes derived from Holstein cows having different antral follicle counts in ovaries. *Anim Reprod Sci*, 163:172-178.

Nagano M. 2019. Acquisition of developmental competence and *in vitro* growth culture of bovine oocytes. *J Reprod De*v, in press.

Nedambale TL, Dinnyés A, Groenc W, Dobrinsky JR, Tiana XC, Yanga X. 2004. Comparison on in vitro fertilized bovine embryoscultured in KSOM or SOF and cryopreservedby slow freezing or vitrification. *Theriogenology*, 62:437-449.

O'Kearney-Flynn M, Wade M, Duffy P, Gat V, Boland MP, Dobrinsky JR. 1998. Effect of cryopreservation on IVP cattle embryo development in vitro and in vivo. *Theriogenology*, 49:173

Overstrom EW, Duby RT, Dobrinsky JR, Robb JM, Baguisi A, Lonergan P. 1993. Cytoskeletal damage in vitrified or frozen bovine embryos. *Theriogenology*, 39:276.

Overström EW. 1996. In vitro assessment of embryo viability. *Theriogenology*, 45:3-16.

Paschoal DM, Sudano MJ, Guastali MD, Dias Maziero RR, Crocomo LF, Ona Magalhaes LC, da Silva Rascado T, Martins A, da Cruz Landim-Alvarenga F. 2014. Forskolin effect on the cryosurvival of in vitro-produced bovine embryos in the presence or absence of fetal calf serum. *Zygote* 22:146-157.

Pereira RM, Baptista MC, Vasques MI, Horta AE, Portugal PV, Bessa RJ, Silva JC, Pereira MS, Marques CC. 2007. Cryosurvival of bovine blastocysts is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t,12c CLA). *Anim Reprod Sci*, 98:293-301.

Pero ME, Zullo G, Esposito L, Iannuzzi A, Lombardi P, De Canditiis C, Neglia G, Gasparrini B. 2018. Inhibition of apoptosis by caspase inhibitor Z-VAD-FMK improves cryotolerance of in vitro derived bovine embryos. *Theriogenology*, 108:127-135.

Perry G. 2016 Statistics of embryo collection and transfer in domestic farm animals. International embryo technology society. 2017. Available from: https://www.iets.org/pdf/comm_data/IETS_Data_Retrieval_Report_2016_v2.pdf. Accessed in: May 10th. 2019.

Peterson AJ, Lee RS. 2003. Improving successful pregnancies after embryo transfer. *Theriogenology*, 59:687-97.

Phillips T. 2008. Non-coding RNA and Gene Expression. *Nature Education*, 1:115.

Ralston A, Rossant J. 2008. Cdx2 acts downstream of cell polarization to cell-autonomously promote trophectoderm fate in the early mouse embryo. *Dev Biol*, 313:614-629.

Ramos-Ibeas P, Heras S, Gómez-Redondo I, Planells B, Fernández-González R, Pericuesta E, Laguna-Barraza R, Pérez-Cerezales S, Gutiérrez-Adán A. 2019. Embryo responses to stress induced by assisted reproductive technologies. *Mol Reprod Dev*, 1-15.

Räty M, Ketoja E, Pitkänen T, Ahola V, Kananen K, Peippo J. 2011. In vitro maturation supplements affect developmental competence of bovine cumulus-oocyte complexes and embryo quality after vitrification. *Cryobiology*, 63:245-255.

Rizos D, Clemente M, Bermejo-Alvarez P, De La Fuente J, Lonergan P, Gutiérrez-Adán A. 2008. Consequences of in vitro culture conditions on embryo development and quality. *Reprod Domest Anim*, 43:44-50.

Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev*, 61:234-248.

Rodgaard T, Heegaard PM, Callesen H. 2015. Non-invasive assessment of in-vitro embryo quality to improve transfer success. *Reprod Biomed Online*, 31:585-592.

Salilew-Wondim D, Holker M, Rings F, Ghanem N, Ulas-Cinar M, Peippo J, Tholen E, Looft C, Schellander K, Tesfaye D. 2010. Bovine pretransfer endometrium and embryo transcriptome fingerprints as predictors of pregnancy success after transfer. *Physiological Genomics*, 42:201-218.

Sanches BV, Lunardelli PA, Tannura JH, Cardoso BL, Pereira MH, Gaitkoski D, Basso AC, Arnold DR, Seneda MM. 2016. A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, 85:1147-1151.

Sanches BV, Marinho LS, Filho BD, Pontes JH, Basso AC, Meirinhos ML, Silva-Santos KC, Ferreira CR, Seneda MM. 2013. Cryosurvival and pregnancy rates after exposure of IVF-derived bos indicus embryos to forskolin before vitrification. *Theriogenology*, 80:372-377.

Santos P, Chaveiro A, Simões N, Moreira da Silva F. 2008. Bovine oocyte quality in relation to ultrastructural characteristics of zona pellucida, polyspermic penetration and developmental competence. *Reprod Domest Anim*., 43:685-689.

Sata R, Tsujii H, Abe H, Yamashita S, Hoshi H. 1999. Fatty acid composition of bovine embryos cultured in serum-free and serum-containing medium during early embryonic development. *J Reprod Dev*, 45:97-103.

Schaefer AM, McFarland R, Blakely EL, He L, Whittaker RG, Taylor RW, Chinnery PF, Turnbull DM. 2008. Prevalence of mitochondrial DNA disease in adults. *Ann Neurol*, 63:35-39.

Schatten H, Sun QY, Prather R. 2014. The impact of mitochondrial function/dysfunction on IVF and new treatment possibilities for infertility. *Reprod Biol Endocrinol*, 12:111.

Schrode N, Xenopoulos P, Piliszek A, Frankenberg S, Plusa B, Hadjantonakis AK. 2013. Anatomy of a blastocyst: cell behaviors driving cell fate choice and morphogenesis in the early mouse embryo. *Genesis*, 51:219-233.

Sirard MA, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, 65:126-136.

Spikings EC, Alderson J, St John JC. 2006. Transmission of mitochondrial DNA following assisted reproduction and nuclear transfer. *Hum Reprod Update*, 12:401-415.

Spikings EC, Alderson J, St John JC. 2007. Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. *Biol Reprod*, 76:327-335.

St John JC, Facucho-Oliveira J, Jiang Y, Kelly R, Salah R. 2010. Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. *Hum Reprod Update*, 16:488-509.

Stephenson RO, Rossant J, Tam PP. 2012. Intercellular Interactions, Position, and Polarity in Establishing Blastocyst Cell Lineages and Embryonic Axes. *Cold Spring Harb Perspect Biol*, 4:a008235.

Sudano MJ, Caixeta ES, Paschoal DM, Martins A, Machado R, Buratini J, Landim-Alvarenga FDC. 2013a. Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts. *Reprod Fertil Dev*, 26:1129-1141.

Sudano MJ, Marinho LSR, Costa CB, Cancian R. 2016b. Cryopreservation of Bovine Embryos. In: Seneda MM, Silva-Santos KC, Marinho LSR (Ed.). Biotechnology of Animal Reproduction. Hauppauge, NY, USA: Nova Science Publishers. p.1-33

Sudano MJ, Marinho LSR, Silveira JC, Basso AC, Bohrer R, Lamberti GSE, Watanabe YF, Seneda MM, Meirelles FV. Evolução aplicada da produção in vitro de embriões bovinos. In **Gonçalves PBD, Figueiredo JR, Gasperin BG**. **(Ed.)** Biotécnicas aplicadas à reprodução animal, 2019, 3 ed. Grupo Gen, in press.

Sudano MJ, Paschoal DM, da Silva Rascado T, Magalhães LCO, Crocomo LF, de Lima-Neto JF, da Cruz Landim-Alvarenga F. 2011. Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. *Theriogenology*, 75:1211-1220.

Sudano MJ, Paschoal DM, Maziero RRD, Rascado TS, Guastali MD, Crocomo LF, Magalhães LCO, Monteiro BA, Martins Jr A, Machado R, Landim-Alvarenga FDC. 2013b. Improving postcryopreservation curvival capacity: an embryo-focused approach. *Anim Reprod*, 10:160-167

Sudano MJ, Rascado TD, Tata A, Belaz KR, Santos VG, Valente RS, Mesquita FS, Ferreira CR, Araujo JP, Eberlin MN, Landim-Alvarenga FD. 2016a. Lipidome signatures in early bovine embryo development. *Theriogenology*, 86:472-484.

Sudano MJ, Santos VG, Tata A, Ferreira CR, Paschoal DM, Machado R, Buratini J, Eberlin MN, Landim-Alvarenga FDC. 2012. Phosphatidylcholine and Sphingomyelin Profiles Vary in Bos taurus indicus and Bos taurus taurus In Vitro- and In Vivo-Produced Blastocysts. *Biol Reprod*, 87:1-11.

Tan K, An L, Wang SM, Wang XD, Zhang ZN, Miao K, Sui LL, He SZ, Nie JZ, Wu ZH, Tian JH. 2015. Actin Disorganization Plays a Vital Role in Impaired Embryonic Development of In Vitro-Produced Mouse Preimplantation Embryos. *PLoS One*, 10:e0130382.

Thompson JG, Brown HM, Sutton-McDowall ML. 2016. Measuring embryo metabolism to predict embryo quality. *Reprod Fertil Dev*, 28:41-50.

Thouas GA, Trounson AO, Jones GM. 2005. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biol Reprod*, 73:366-373.

Thouas GA, Trounson AO, Wolvetang EJ, Jones GM. 2004. Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest in vitro. *Biol Reprod*, 71:1936-1942.

Tripurani SK, Wee G, Lee KB, Smith GW, Wang L Jianboyao. 2013. MicroRNA-212 post-transcriptionally regulates oocyte-specific basic-helix-loop-helix transcription factor, factor in the germline alpha (FIGLA), during bovine early embryogenesis. *PLoS One*, 8:e76114.

Ushijima H, Yamakawa H, Nagashima H. 1999. Cryopreservation of bovine pre-morula-stage in vitro matured/in vitro fertilized embryos after delipidation and before use in nucleus transfer. *Biol Reprod*, 60:534-539.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev*, 51:53-58. **Vajta G, Hyttel P, Callesen H**. 1997. Morphological changes of in-vitro-produced bovine blastocysts after vitrification, in-straw direct rehydration, and culture. *Mol Reprod Dev*, 48, 9-17.

Vajta G, Kuwayama M. 2006. Improving cryopreservation systems. *Theriogenology*, 65:236-244.

Van Blerkom J. 2004. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. *Reproduction*, 128:269-280.

Van Meer G, Voelker DR, Feigenson GW. 2008. Membrane lipids: Where they are and how they behave. *Nat Rev Mol Cell Biol*, 9:112-124.

Van Soom A, Boerjan M, Ysebaert MT, De Kruif A. 1996. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. *Mol Reprod Dev*, 45:171-182.

Van Soom A, Mahmoudzadeh AR, Christophe A, Ysebaert MT, de Kruif A. 2001. Silicone oil used in microdrop culture can affect bovine embryonic development and freezability. *Reprod Domest Anim*, 36:169-176.

Van Soom A, Mateusen B, Leroy J, De Kruif A. 2003. Assessment of mammalian embryo quality: what can we learn from embryo morphology? *Reprod Biomed Online*, 7:664-670.

Van Soom A, Ysebaert MT, de Kruif A. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol Reprod Dev*, 47:47-56.

Van Wagtendonk-de Leeuw AM, den Daas JH, Rall WF. 1997. Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three step dilution. *Theriogenology*, 48:1071-1084.

Van Wagtendonk-De Leeuw AM1, Den Daas JH, Kruip TA, Rall WF. 1995. Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. *Theriogenology*, 32:157-167.

Viana JH. 2018. A produção de embriões bovinos em 2017: o mercado em stand-by. *Jornal o embrião*, 62:6-10.

Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA. 2010. The role of mitochondrial DNA copy number in mammalian fertility. *Biol Reprod*, 83:52-62.

Wakefield SL, Lane M, Mitchell M. 2011. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biol Reprod*, 84:572-580.

Walker SK, Hartwich KM, Seamark RF. 1996. The production of unusually large offspring following embryo manipulation: Concepts and challenges. *Theriogenology*, 45:111-120.

Wang H, Dey SK. 2005. Lipid signaling in embryo implantation. *Prostaglandins and Other Lipid Mediators*, 77:84-102.

Ward F, Enright B, Rizos D, Boland M, Lonergan P. 2002. Optimization of in vitro bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology*, 57:2105-2117.

Warzych E, Wrenzycki C, Peippo J, Lechniak D. 2007. Maturation medium supplements affect transcript level of apoptosis and cell survival related genes in bovine blastocysts produced in vitro. *Mol Reprod Dev*, 74:280-289.

Whittingham D, Leibo S, Mazur P. 1972. Survival of mouse embryos frozen to-196 and-269 C. *Science*, 178:411-414.

Wrenzycki C, Stinshoff H. 2013. Maturation environment and impact on subsequent developmental competence of bovine oocytes. *Reprod Domest Anim*, 48:38-43.

Xu J, Guo Z, Su L, Nedambale TL, Zhang J, Schenk J, Moreno JF, Dinnyes A, Ji W, Tian XC, Yang X, Du F. 2006. Developmental potential of vitrified holstein cattle embryos fertilized in vitro with sex-sorted sperm. *J Dairy Sci*, 89:2510-2518.

Yu XL, Deng W, Liu FJ, Li YH, Li XX, Zhang YL, Zan LS. 2010. Closed pulled straw vitrification of in vitro-produced and in vivo-produced bovine embryos. *Theriogenology*, 73:474-479.

DOI: 10.21451/1984-3143-AR2019-0087

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Contribution of the immune system to follicle differentiation, ovulation and early corpus luteum formation

Noof Abdulrahman, Trudee Fair*

School of Agriculture & Food Sciences, University College Dublin, Belfield, Dublin 4, Ireland.

Abstract

Much of what we know about the involvement of the immune system in periovulatory follicle differentiation, ovulation and subsequent formation of the corpus luteum in cattle is drawn from the findings of studies in several mammalian livestock species. By integrating published histological data from cattle, sheep and pigs and referring back to the more comprehensive knowledge bank that exists for mouse and humans we can sketch out the key cells of the immune system and the cytokines and growth factors that they produce that are involved in follicle differentiation and luteinization, ovulation and early follicle development. These contributions are reviewed and the key findings, discussed.

Keywords: bovine, ovulation, corpus luteum.

Introduction

Optimum fertility underlies all livestock production systems and by it's nature reflects the metabolic and immunological health status of the animal. Exposure to environmental insults such as heat stress, undernutrition and drought, metabolic and pathogenic disease have well-documented negative consequences for female fertility (Fair, 2010). In dairy cattle the period from 3 weeks pre-calving to 3 weeks post-calving, known as the transition period, has been the subject of much focus and there is substantial scientific evidence that it exerts a profound effect on the animal's metabolic, immune and endocrine systems. Transition dairy cows become immunosuppressed due to lower dry matter intake, increased exposure to bacteria, and increased non-esterified fatty acid, beta-hydroxybutarate, concentrations and therefore more susceptible to increased incidence of endometritis and metritis, generally associated with reduced productivity and poor fertility in the rebreeding period (Sheldon *et al*., 2009; Thatcher *et al*., 2010; Giuliodori *et al*., 2013). Oocyte quality is considered a major contributor to the low fertility of these animals (Fair, 2010; Leroy *et al*., 2015), but so too is corpus luteum (CL) function (Niswender *et al*., 1994) and the endometrial environment. If we consider the ontogeny of the CL and it's primary function when formed, it is obvious that these key contributory factors are intricately related. Moreover, numerous studies have outlined an integral role for immune cells in follicular development (Fukumatsu *et al*., 1992), steroidogenesis (Petrovská *et al*., 1996), ovulation (Brännström and Enskog, 2002) and CL formation and regression (Pate *et al*., 2010). Thus it is likely that as the immune and endocrine systems coordinate the normal development and functioning of these tissues (Hansen *et al*., 2010), their susceptibility to modulation by adverse metabolic and environmental environments will act as the primary conduit by which oocyte quality and CL function will be compromised. Taking this statement as our hypothesis, the aim of this manuscript is to review the molecular and cellular involvement of the cow's immune system in follicle differentiation, ovulation and corpus luteum formation.

Follicle differentiation and luteinization

Differentiation of the dominant follicle is associated with granulosa cell proliferation, increased intrafollicular concentration of estradiol (E2) and a switch from follicle stimulating hormone (FSH) to luteinizing hormone (LH)- responsiveness as they develop. Following the preovulatory gonadotropin surge, these estrogen-active follicles lose their capacity to produce E2, for detailed information see the excellent review by Ireland *et al*., (Ireland *et al*., 2000). The subsequent switch from E2 dominance to progesterone (P4) dominance in the follicular fluid of preovulatory follicles in the period between the LH surge and ovulation signals the onset of follicle luteinization (Dieleman *et al*., 1983). Pre-ovulatory follicle differentiation and luteinization appear to be characterized by an immune-cell specific temporal influx of leukocytes likely initiated in response to the high E2 concentration and various other chemoattractant cues produced by the developing follicle (Townson and Liptak, 2003). Histological analysis of dominant follicles from cattle, revealed that the first influx of cells is constituted by granular leukocytes, primarily mast cells, which infiltrate the theca layer of the follicle. Based on findings from sheep and pigs, it has been proposed that the mast cells in the theca layer become activated, likely in response to the LH surge and release the contents of their granules. Mast cell granules contain many factors, of which it is likely that tumour necrosis factor-alpha (TNF-α), recruits additional granular leukocytes such as eosinophils and neutrophils (Murdoch and Steadman, 1991; Standaert, *et al*., 1991). Following the peak in oestradiol concentration in the differentiated dominant follicle, the final phase of leukocyte infiltration, an influx of phagocytic monocytes occurs more or less in parallel with ovulation

*Corresponding author: Trudee.Fair@ucd.ie
orcid.org/0000-0002-5079-8589
Received: June 23, 2019
Accepted: July, 25

(Murdoch and Steadman, 1991; Standaert, *et al*., 1991).

At the molecular level, several reports have detailed the transcriptomic profile of ovarian follicle development in cattle: (Li *et al*., 2009; Gilbert *et al*., 2011; Walsh *et al*., 2012a; Christenson *et al*., 2013; Hatzirodos *et al*., 2014). Deep sequencing analysis of bovine follicular theca and granulosa tissue during pre-ovulatory follicle development, have revealed dynamic expression of many genes within immune-related pathways according to the stage of follicle development. Pathways associated with cell proliferation, tissue vascularization and angiogenesis were overpopulated during follicle differentiation (Walsh *et al*., 2012a), these processes are understood to be carried out by macrophages in the theca layer of the differentiating follicle (Fraser, 2006; Turner *et al*., 2011). Following the surge in the pituitary gonadotrophin LH, pre-ovulatory follicle development is directed away from differentiation and towards luteinization, initiating the earliest stages of CL development (Richards *et al*., 2008). In particular, the LH surge sharply increases the local production of the 2 angiogenic factors, basic fibroblast growth factor (FGF) 2 (Berisha *et al*., 2006) and vascular endothelial growth factor (VEGF) A (Schams *et al*., 2001), as the integrity of the basement membrane between the theca and granulosa-cell layers breaks down (Dieleman *et al*., 1983), the movement of leukocytes from the theca layer into the granulosa tissue is permitted and angiogenesis, required for CL formation is initiated.

Ovulation

The biochemical events of mammalian ovulation have been likened to an acute inflammatory response, owing to the participation of leucocytes, classical inflammatory mediators as well as proteolytic enzymes (Richards *et al*., 2008). Based on data from studies in mice it is accepted that the initiation of the ovulatory process occurs primarily in granulosa cells (Richards *et al*., 2002; 2008). Indeed, the gene expression profile of bovine peri-ovulatory granulosa-cells is enriched with factors involved in acute inflammation and immunosurveillance (Walsh *et al*., 2012b). The expression of these factors by the granulosa cells may activate the ovarian innate immune system (Spanel-Borowski, 2011), as evidenced by the detection of acute phase proteins, defensins, interleukins and prostaglandins (PGs) in the pre-ovulatory follicular tissue and fluid (Takeda, 2004; Angelucci *et al*., 2006; Poulsen *et al*., 2019). Based on findings in rats, it is proposed that PGs and leukotrienes stimulate the synthesis and activity of collagenases which act to promote the degradation of the follicle matrix (Murdoch and Gottsch, 2003). While some of these factors may be expressed or secreted by the immune cells immigrating from the theca layer, it is likely that the damaged granulosa cells actively or passively release tissue damage signals initiating a pre-ovulatory inflammatory cascade. Typically an inflammatory cascade involves the activation of endothelial cells and local leucocytes, leading to the recruitment and accumulation of additional leucocytes, vascular endothelial cells and plasma proteins. Within the peri-ovulatory follicle, the cytokines TNF-α and interleukin-1 (IL-1) promote inflammatory associated processes, such as an increase in intrafollicular pressure, the activation of proteolytic pathways, collagenases and initiation of angiogenesis and tissue destruction and repair. These processes lead to the reorganization of the follicular stroma, extensive remodelling of the extracellular matrix and loss of the follicle's surface epithelium, which result in a weakening in the follicle so that finally the follicle ruptures, expelling the cumulus enclosed mature oocyte and the follicle is transformed into a wound-like structure, the corpus hemorrhagica.

Oocyte maturation

Ovulation is initiated by the LH surge which acts directly on the theca and mural granulosa cells of the follicle. However, the preovulatory oocyte does not express the LH receptor, and expression in the surrounding cumulus cells is very low (Lawrence *et al*., 1980), therefore the propagation of the LH stimulus throughout the follicle to the oocyte is mediated by secondary molecules, which initiate oocyte maturation. Oocyte maturation comprises expansion and proliferation of the cumulus cell layers, oocyte nuclear maturation, i.e. dissolution of the nuclear membrane and the resumption of meiosis, reorganization of the oocyte cytoplasmic organelles and a dramatic increase in protein synthesis and activation of molecular pathways (Fair, 2003; 2010). The propagators of the LH signal in the preovulatory follicle have been identified as three members of the epidermal growth factor (EGF)-family, the EGF-like peptides amphiregullin (AREG), epiregulin (EREG) and beta-cellulin (BTC) (Park *et al*., 2004). The ovulatory LH surge induces an acute upregulation of the EGF signalling network in mural granulosa cells, which is transmitted to the cumulus cells. This leads to initiation of mural granulosa cell luteinisation, production of an extensive extracellular matrix by cumulus cells and the closure of gap junctional communication between the oocyte and the cumulus cells. At the same time, the meiotic inhibitory signalling network mediated by C-type natriuretic peptide (CNP) and cyclic guanosine monophosphate (cGMP) in mural granulosa and cumulus cells is downregulated, leading to oocyte meiotic maturation (see Richani and Gilchrist for review) (Richani and Gilchrist, 2018). It is noteworthy that the amplification and propagation of the EGF signal from the mural to cumulus cells is dependent on the LH-induced production of PGE_2 in mural granulosa cells (Shimada *et al*., 2006). Furthermore, oocytes are not directly responsive to EGF-like peptides (Conti *et al*., 2006; Park *et al*., 2004), therefore, the LH/EGF-peptide ovulatory signal is transmitted to the oocyte via the EGF receptor on cumulus cells. It has been hypothesized the acquisition of EGF signalling capabilities by the mural granulosa and cumulus cells is important and likely to represent a milestone in oocyte development and acquisition of competence (Ritter *et al*., 2015).

Corpus Luteum Formation

The corpus hemorrhagica arises from the collapsed post-ovulatory follicle. Morphologically it resembles a fresh wound, but it is actually heterogeneous in nature, composed of multiple, distinctive cell types including steroidogenic cells, large and small luteal cells, which originate from the granulosa and thecal cells of the follicle ruptured at ovulation, as well as resident and migrating vascular endothelial cells, fibroblasts and immune cells (Lobel and Levy, 1968; Lei, *et al*., 1991; Spanel-Borowski *et al*., 1997; Penny *et al*., 1999; Bauer, *et al*., 2001; Davis and Pate, 2007).

Immune system in the developing CL

Immediately after ovulation, in parallel with the onset of the differentiation of the follicular steroidogenic cells into luteal cells, resolution of the inflammation and consequential tissue damage must occur. This is initiated by immune cells recruited during ovulation (Murdoch and McCormick, 1993; Oakley *et al*., 2010; Watanabe *et al*., 1997; Gaytán *et al*., 1998). Chemoattractant cytokines (e.g., chemokines IL-8 and C-C motif ligand 5 and 2), produced by the endothelial, fibroblast and immune -cells, establish concentration gradients within the CL, which recruit and direct immune cell migration, primarily granulocytes, neutrophils and eosinophils that have originated in the spleen (Penny *et al*., 1999; Lobel and Levy, 1968; Spanel-Borowski *et al*., 1997; Jiemtaweeboon *et al*., 2011; Shirasuna *et al*., 2012). Immune cell migration is also enabled by the expression of ligands on immune cells, which interact with adhesion molecules on endothelial cells. Eosinophils are recognized as actors in the innate immune response to parasitic infections, asthma, and allergic conditions. Therefore, it is interesting to note that there is a rapid influx of eosinophils into the CL shortly after ovulation in cattle (Reibiger and Spanel-Borowski, 2000). The expression of P-selectin on endothelial cells appears to recruit eosinophils into the developing CL (Aust *et al*., 2000; Rohm *et al*., 2002). The arrival of these first responders, appears to be an important, but not essential, stimulus for angiogenesis during the early stages of CL development, as dexamethasone- induced eosinopenia resulted in lowered plasma P4 concentrations and reduced CL VEGFA protein production in cattle (Kliem *et al*., 2013). Moreover, the role of eosinophils appears to be restricted to the repair of the site of follicle rupture and early CL development as they are barely detectable later in the oestrous cycle when the CL is well established, or at the end of the cycle during CL regression (Reibiger and Spanel-Borowski, 2000; Rohm *et al*., 2002; Jiemtaweeboon *et al*., 2011).

Similar to eosinophils, neutrophils are important in the primary, nonspecific stages of acute inflammatory reactions and were also observed in large numbers, along with a high level of IL-8 (a potent neutrophilic chemoattractant), during the early luteal phase (d 1–4 of the estrous cycle) in the CL of cows (Jiemtaweeboon *et al*., 2011). They too appear to be integral to the resestablishment of the local microvasculature and the promotion of the acute inflammatory cascade, as both PMN and IL-8 were reported to induce angiogenesis in vivo (Koch *et al*., 1992; Komatsu *et al*., 2003) and in vitro (Schruefer *et al*., 2005). These findings were verified in bovine CL tissue in a series of in vitro experiments, where Jiemtaweeboon *et al*. (2011) demonstrated that supernatant from cultures of early CL tissue could induce PMN migration in vitro and increase PMN IL-8 production. Moreover, IL-8 stimulated endothelial cells of the CL to form capillary-like structures, indicating that IL-8 acts as a major PMN chemoattractant and a strong stimulator of angiogenesis in the early CL (Jiemtaweeboon *et al*., 2011). The concept of functional polarization of neutrophils (classic proinflammatory versus novel anti-inflammatory) has been proposed to explain their action in angiogenesis (Fridlender *et al*., 2009). Concomitant with vascular angiogenesis, macrophages and endothelial cells infiltrate the developing CL. The number of macrophages and monocytes in the CL increases during the early stages of development in cows, but they are substantially fewer in number compared to during CL regression (Penny *et al*., 1999; Lawler *et al*., 1999; Townson *et al*., 2002). In response to local cytokines and other signals, macrophages differentiate to acquire a functional phenotype that is specific to the requirements of the tissue. Within the developing CL, these cells produce and secrete various cytokines, such as TNF-α, interferon gamma, interleukins, PGs and angiogenic growth factors (Sakumoto *et al*., 2000; Townson and Liptak, 2003). The cytokine, TNF, is a potent stimulator of luteal PGs including PGF2α, PGE2 and PGI2 (Benyo and Pate, 1992; Sakumoto *et al*., 2000). Thus, TNF-α and TNF-induced PGE2 have been proposed as key regulators of CL vascularization (Okuda and Sakumoto, 2003; Korzekwa *et al*., 2008). A defined role for macrophages in promoting the vascularization of the developing CL is further substantiated by the findings of conditional macrophage ablation studies in mice, where it was shown that the ablation of macrophages in the early CL disrupted the ovarian vasculature and CL integrity (Turner *et al*., 2011).

There is very little evidence to suggest an essential involvement of T lymphocytes in the repair of the ovulatory site or the formation of the new CL. In fact, reports from several species, including bovine (Penny *et al*., 1999), buffalo (Ramadan *et al*., 2001), human (Best *et al*., 1996), pigs (Standaert *et al*., 1991) and sheep (Cavender and Murdoch, 1988), are equivacol in their descriptions of low numbers of $CD4^+$and $CD8^+$ T lymphocytes in early to late luteal phase CL tissue and infiltration of larger populations during CL regression.

While, the immediate response to follicle differentiation, E2 production, the LH surge and subsequent ovulation is characterized by large numbers of PMN and macrophages, a substantial influx of endothelial cells also occurs. Moreover, these endothelial cells form the greatest cohort of

proliferating cells in the early CL (Townson and Liptak, 2003; Reynolds and Redmer, 1999). Microvascular growth and development occur at an extremely rapid pace in female reproductive tissues and these tissues are highly vascular when mature. For example, most (~50–85%) of luteal cell proliferation occurs in the microvascular compartment (Reynolds *et al*., 1992; Reynolds *et al*, 1998). As a result, in the mature CL, microvascular pericytes and endothelial cells comprise 50–70% of the total cell population (Farin *et al*., 1986; Lei *et al*., 1991)

Angiogenesis in the CL

In the ovary, the re-establishment of the luteal tissue microvasculature from pre-existing capillaries is a complex process that is necessary for the delivery of adequate levels of hormones and lipoprotein bound cholesterol into and out of the CL and ovary (Cherry *et al*., 2008) and is regulated by a number of growth factors. In cattle, peak expression of VEGF, its receptor VEGFR-2, FGF2, insulin-like growth factor (IGF), angiopoietin (ANPT) and hypoxia-inducible factor (HIF) family members has been reported from Day 0 - 4 of the oestrous cycle (Berisha *et al*., 2016; 2017; Castilho *et al*., 2019). The upregulation of these particular factors implies their particular importance for angiogenesis and maintenance of capillary structures during final follicle maturation and early CL development (Berisha *et al*., 2016). Luteal expression of VEGF occurs primarily in steroidogenic cells (granulose-lutein cells) and is regulated primarily by oxygen (Tropea *et al*., 2006). Hypoxia strongly induces angiogenesis, most likely through the HIF1–VEGF signalling pathway. Nitric oxide (NO) is produced by endothelial cells of luteal arterioles and capillaries; it is a potent vasodilator and stimulates endothelial cell proliferation, VEGF production and angiogenesis (Reynolds *et al*., 2000; Reynolds and Redmer, 1999). The purpose of luteal arteriole and capillary vasodilation is to facilitate increased blood flow and consequently delivery of peripheral immune cells to this site of tissue repair, regeneration and proliferation in the ovary. It is suggested that both FGF2 and VEGF play complementary roles in luteal angiogenesis (Robinson *et al*., 2009) as, FGF2 has also been shown to promote endothelial cell proliferation and appears to be critical to the initiation of the formation of the endothelial network in the bovine CL (Woad *et al*., 2009; Robinson *et al*., 2009). Furthermore, the suppression of VEGFA or FGF2 expression during the early luteal phase in cattle reportedly inhibited endothelial cell proliferation and reduced plasma P4 concentration (Kuhnert *et al*., 2008; Yamashita *et al*., 2008). A body of evidence also exists for a role for prostaglandins, including the luteolytic prostaglandin (PG) F2α in promoting CL vascularization and supporting CL growth. Indeed PGF2α has been shown to positively affect VEGF, FGF2, and P4 secretion in the bovine CL (Zalman *et al*., 2012; Miyamoto *et al*., 2010).

Maintenance of the corpus luteum

The LH surge is the main trigger of ovulation and luteinization. Progesterone regulates the length of the estrus cycle by influencing the timing of the luteolytic PGF2α signal from the endometrium see review (Mishra and Palai, 2014). Furthermore, there is evidence to suggest that P4 may affect the secretory function of the bovine CL in a stage-dependent fashion, in an autocrine and paracrine manner that may be dependent on cell-to-cell contact and cellular makeup (Skarzynski and Okuda, 1999). For example, P4 affects the function of the early and mid CL in cattle (Skarzynski and Okuda, 1999; Duras *et al*., 2005), stimulating P4, oxytocin and prostaglandin secretion in the early CL, but later this is reversed as P4 inhibits PGF2a secretion in the mature CL. Recent studies have demonstrated that intra-luteal P4 is one of the most important factors supporting maintenance of the CL, acting to suppress apoptosis in bovine luteal cells through the inhibition of Fas and caspase-3 mRNA expression and inhibition of caspase-3 activation (Rueda *et al*., 2000; Okuda *et al*., 2004). Progesterone may also act to keep ovarian immune cells in check, by supressing T lymphocyte proliferation (Cannon *et al*., 2003).

Corpus Luteum Regression

In the absence of an embryo(s) in the uterus, the process of CL regression begins on day 16 of the oestrous cycle in cattle (McCracken *et al*. 1999). Apoptosis of luteal cells and CL vascular regression are regulated by many different factors, however, in most species, uterine prostaglandin alpha (PGF2α) acts as the principal trigger for luteolysis. Although, it should be pointed out that there has been some debate about its direct action within the CL (Skarzynski and Okuda, 1999; Pate, 2003; Arosh *et al*., 2016). Nevertheless, PGF2α has been proven to acutely decrease P4 secretion by inhibiting 3ß-Hydroxysteroid dehydrogenase (3ßHSD) and steroid acute regulatory protein (StAR) mRNA expression and other rate-limiting steroidogenic enzymes in vivo (Tsai and Wiltbank, 1998; Atli *et al*., 2012), The process of luteolysis are understood to proceed with PGF2α induced angiolysis and vasoconstriction which limits the oxygen and nutrient supply to the tissue during luteolysis. Corpus luteum expression of members of the endothelin-1 (EDN1) system (EDN1, EDN converting enzymes, and EDNA and EDNB receptors) is up-regulated by PGF2 α (Mamluk *et al*., 1999; Klipper *et al*., 2010) during luteal regression (Klipper *et al*. 2004; Choudhary *et al*., 2005; Rosiansky-Sultan *et al*., 2006). Meanwhile mediators of PGF2 α luteolysis, i.e., vasoactive peptides, i.e. angiotensin II and atrial natriuretic peptide, trigger the luteolytic cascade, decrease blood flow and consequently inhibit P4 secretion (Shirasuna *et al*., 2004). Endothelin 1 is believed to participate in luteal regression, by promoting leucocyte migration and stimulating macrophages to

release cytokines, e.g. TNF-α and interferon-gamma (IFN-γ) (Girsh, *et al*., 1996); for review see also Smith and Meidan, 2014. Reportedly, TNF, TNF death receptors (TNF-RI), Fas and IFNγ mRNA expression is significantly increased during luteolysis in bovine CL (Korzekwa *et al*., 2008). Because of the ability of these cytokines to induce apoptosis in CL endothelial cells, they have been proposed as key regulators of bovine luteolysis (Okuda *et al*., 1999; Hojo *et al*., 2010). Additionally, cytokine membrane receptors, second messengers, including calcium ions and regulatory proteins are involved in apoptosis of steroidogenic and endothelial CL cells (Petroff, and Pate, 2001).

Immune cells in CL regression

Luteal regression has been likened to an acute inflammatory process because of the short duration of luteolysis, the characteristic immune cell infiltration (neutrophils, macrophages, and T lymphocytes) and the dramatic change in vascular diameter and blood flow (Shirasuna *et al*., 2012). Many reports from several species describe an increase in lymphocytes or macrophages in the CL during luteolysis. Endothelial cell secretion of Monocyte chemoattractant protein 1 (also known as chemokine ligand 2 or CCL2), in direct response to TNF and IFN-γ stimulation, has been implicated in the recruitment of immune cells into the CL during luteal regression (Townson *et al*., 2002). In particular, macrophages and T lymphocytes, are proposed to play a central role in structural and functional CL regression (Best *et al*., 1996; Penny *et al*., 1999; Bauer *et al*., 2001; Townson, *et al*., 2002; Pate *et al*., 2010). The phenotypes of T lymphocytes resident in the bovine CL were previously quantified before and after the induction of luteal regression. Prior to regression, and in contrast to their ratio in peripheral blood, the proportion of $CD8^+$-resident T cells was greater than $CD4^+$-resident T cells, however there was no difference in the proportion of γδ+ lymphocytes in the CL compared to peripheral blood, nor was the proportion altered during luteal regression (Poole and Pate, 2012). The proportion of $CD4^+$ $Foxp3^+$ cells (i.e., T regulatory cells) was greater in a functional CL, compared to a CL induced to regress. This lead the Authors, to propose that $Foxp3^+$ cells may control the actions of activated resident T lymphocytes to prevent premature luteal regression, but once luteal regression is initiated, a decline in the proportion the $Foxp3^+$ cells weakens the inhibitory action on T lymphocytes, permitting their release of cytokines that may induce luteal cell death. In addition, the arrival of large numbers of monocytes, macrophages, and other cell types that create an inflammatory environment may augment the activity of the resident T lymphocytes.

It is truly remarkable that the activity of immune cells during luteolysis is confined to the CL and does not spread to the whole ovary, such tight control of inflammation ensures that CL tissue degradation remains localized with no effects on the surrounding tissue.

Conclusion

It is well recognized that cattle require an appropriately functioning immune system for a swift and healthy recovery from parturition. The extent to which the maternal immune system is involved in bovine fertility is somewhat overlooked, yet it's significant role in creating an appropriate microenvironment for final oocyte maturation, gamete transport and early embryo development reminds us that the immune system is intricately integrated in to the first stages of establishing pregnancy. Therefore, when seeking to optimize cow fertility, we should think first of the animal's immune system and try to maintain its integrity, particularly in husbandry situations that expose the animal to significant metabolic and physiological stress.

Author contributions

NAKA: Funding acquisition, Writing – original draft (Noof Abdulrahman K Al Rabiah); TF: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Conflict of interest

Authors declare no conflicts of interest.

Acknowledgments and Funding disclosure statement

NA is funded by the Saudi Arabian Culture Bureau.

References

Angelucci, S, Ciavardelli D, Di Giuseppe F, Eleuterio E, Sulpizio M, Tiboni GM, Giampietro F, Palumbo P, Di Ilio C. 2006. Proteome analysis of human follicular fluid. *Biochim Biophys Acta*, 1764(11):1775-1785.

Arosh, JA, Sakhila KB, McCracken JA. 2016. Novel concepts on the role of prostaglandins on luteal maintenance and maternal recognition and establishment of pregnancy in ruminants. *J Dairy Sci*, 99(7):5926-5940.

Atli MO, Bender RO, Mehta V, Bastos MR, Luo W, Vezina CM Wiltbank MC. 2012. Patterns of gene expression in the bovine corpus luteum following repeated intrauterine infusions of low doses of prostaglandin F2alpha1. *Biol Reprod*, 86(4):1-13.

Aust G, Heider SU, Hmeidan FA, Blumenauer V, Spanel-Borowski K. 2000. Eosinophils in the human corpus luteum: the role of RANTES and eotaxin in eosinophil attraction into periovulatory structures. *Mol Hum Reprod*, 6(12):1085-1091.

Bauer M, Reibiger I, Spanel-Borowski K. 2001. Leucocyte proliferation in the bovine corpus luteum. *Reproduction*, 121(2):297-305.

Benyo DF, Pate JL. 1992. Tumor necrosis factor-α alters bovine luteal cell synthetic capacity and viability. *Endocrinology*, 130:854-860.

Berisha B, Steffl M, Amselgruber W, Schams D.

2006. Changes in Fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation. *Reproduction* 131(2):319-329.

Berisha B, Schams D, Rodler D, Pfaffl MW. 2016. Angiogenesis in The Ovary - The most important regulatory event for follicle and corpus luteum development and function in cow - An Overview. *Anat Histol Embryol*, 45(2):124-130.

Berisha B, Schams D, Rodler D, Sinowatz F, Pfaffl MW. 2017. Expression pattern of HIF1alpha and vasohibins during follicle maturation and corpus luteum function in the bovine ovary. *Reprod Domest Anim*, 52(1):130-139.

Best CL, Pudney J, Welch WR, BurgerN Hill JA. 1996. Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. *Hum Reprod*, 11(4):790-797.

Brännström M, Enskog A. 2002. Leukocyte networks and ovulation. *J Reprod Immunol*, 57(1-2):47-60.

Cannon, MJ, Petroff MG, Pate JL. 2003. Effects of prostaglandin F2alpha and progesterone on the ability of bovine luteal cells to stimulate T lymphocyte proliferation. *Biol Reprod*, 69(2):695-700.

Castilho ACS, Dalanezi FM, Franchi FF, Price CA, Ferreira JCP, Trevisol E, Buratini J. 2019. expression of fibroblast growth factor 22 (FGF22) and Its receptor, FGFR1B, during development and regression of bovine corpus luteum. *Theriogenology*, 125:1-5.

Cavender JL, Murdoch WJ. 1988. Morphological studies of the microcirculatory system of periovulatory ovine follicles. *Biol Reprod*, 39(4):989-997.

Cherry JA, Hou X, Rueda BR, Davis JS, Townson DH. 2008. Microvascular endothelial cells of the bovine corpus luteum: a comparative examination of the estrous cycle and pregnancy. *J Reprod Dev*, 54(3):183-191.

Choudhary E, Sen A, Inskeep KE, Flores JA. 2005. Developmental sensitivity of the bovine corpus luteum to prostaglandin F2alpha (PGF2alpha) and endothelin-1 (ET-1): Is ET-1 a mediator of the luteolytic actions of PGF2alpha or a tonic inhibitor of progesterone secretion?. *Biol Reprod*, 72(3):633-642.

Christenson LK., Gunewardena S, Hong X, Spitschak M, Baufeld A, Vanselow J. 2013. research resource: preovulatory LH surge effects on follicular theca and granulosa transcriptomes. *Mol Endocrinol*, 27(7):1153-1171.

Conti M, Hsieh M, Park JY, Su YQ. 2006. Role of the epidermal growth factor network in ovarian follicles. *Mol Endocrinol*, 20(4):715-723.

Davis TL, Pate JL. 2007. bovine luteal cells stimulate proliferation of major histocompatibility nonrestricted gamma delta T cells. *Biol Reprod*, 77(6):914-922.

Dieleman SJ, Kruip TA, Fontijne P, de Jong WH, van der Weyden GC. 1983. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. *J Endocrinol*, 97(1):31-42.

Duras M, Brzósko E, Kotwica J. 2005. Influence of progesterone, pregnenolone and 17beta-hydroxyprogesterone on the function of bovine luteal cells treated with luteinizing hormone, noradrenaline and prostaglandin E2. *Pol J Vet Sci*, 8(2):113-119.

Fair T. 2003. follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci*, 78(3-4):203-216.

Fair T. 2010. Mammalian oocyte development: checkpoints for competence. *Reprod Fertil Dev*, 22(1):13-20.

Farin, CE, Moeller CL, Sawyer HR, Gamboni F, Niswender GD. 1986. Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. *Biol Reprod*, 35(5):1299-1308.

Fraser HM. 2006. Regulation of the ovarian follicular vasculature. *Reprod Biol Endocrinol*, 18(4):1477-7827.

Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Worthen GS, Albelda SM. 2009. polarization of tumor-associated neutrophil phenotype by TGF-Beta: "N1" versus "N2" TAN. *Cancer Cell*, 16(3):183-194.

Fukumatsu Y, Katabuchi H, Naito M, Takeya M, Takahashi K, Okamura K. 1992. Effect of macrophages on proliferation of granulosa cells in the ovary in rats. *J Reprod Fertil*, 96(1):241-249.

Gaytán F, Morales C. García-Pardo L, Reymundo C, Bellido C, Sánchez-Criado JE. 1998. Macrophages, cell proliferation, and cell death in the human menstrual corpus luteum. *Biol Reprod*, 59(2):417-425.

Gilbert I, Claude R, Dieleman S, Blondin P, Sirard MA. 2011. Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period. *Reproduction*, 141(2):193-205.

Girsh E, Milvae RA, Wang W, Meidan R. 1996. effect of endothelin-1 on bovine luteal cell function: role in prostaglandin F2alpha-induced antisteroidogenic action. *Endocrinology*, 137(4):1306-1312.

Giuliodori MJ, R. Magnasco P, Becu-Villalobos D, Lacau-Mengido IM, Risco CA, de la Sota RL. 2013. Clinical Endometritis in an argentinean herd of dairy cows: risk factors and reproductive efficiency. *J Dairy Sci*, 96(1):210-218.

Hansen TR, Henkes LK, Ashley RL, Bott RC, Antoniazzi AQ, Han H. 2010. Endocrine actions of interferon-tau in ruminants. *Soc Reprod Fertil Suppl*, 67:325-340.

Hatzirodos N, Irving-Rodgers HF, Hummitzsch K, Harland ML, Morris SE, Rodgers RJ. 2014. Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genomics*, 15(24):1471-2164.

Hojo T, Akihiro O, Lee SH, Acosta TJ, Okuda K. 2010. effects of tumor necrosis factor α and interferon γ on the viability and MRNA expression of TNF receptor type I in endothelial cells from the bovine corpus luteum. *J Reprod Dev*, 56(5):515-519.

Ireland JJ, Mihm M, Austin E, Diskin MG, Roche JF. 2000. Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements, and terms. *J Dairy Sc*i, 83(7):1648-1658.

Jiemtaweeboon S, Shirasuna K, Nitta A, Kobayashi A, Schuberth HJ, Shimizu T, Miyamoto A. 2011. Evidence That polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. *Reprod Biol Endocrinol*, *7*9(9):1477-7827.

Kliem H, Rodler D, Ulbrich SE, Sinowatz F, Berisha B, Meyer HHD, Schams D. 2013. Dexamethasone-induced eosinopenia is associated with lower progesterone production in cattle. *Reprod Domest Anim*, 48(1):137-148.

Klipper E, Gilboa T, Levy N, Kisliouk T, Spanel-Borowski K, Meidan R. 2004. Characterization of endothelin-1 and nitric oxide generating systems in corpus luteum-derived endothelial cells. *Reproduction*, 128(4):463-73.

Klipper E, Levit A, Mastich Y, Berisha B, Schams D, Meidan R. 2010. Induction of endothelin-2 expression by luteinizing hormone and hypoxia: possible role in bovine corpus luteum formation. *Endocrinology*, 151(4):1914-1922.

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM. 1992. Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science*, 258(5089):1798-1801.

Komatsu K, Manabe N, Kiso M, Shimabe M, Miyamoto H. 2003. Changes in localization of immune cells and cytokines in corpora lutea during luteolysis in murine ovaries. *J Exp Zool A Comp Exp Biol, Part A*, 296(2):152-159.

Korzekwa A, Murakami S, Wocławek-Potocka I, Bah MM, Okuda K, Skarzynski DJ. 2008. The influence of tumor necrosis factor α (TNF) on the secretory function of bovine corpus luteum: TNF and its receptors expression during the estrous cycle. *Reprod Biol*, 8(3):245-262.

Kuhnert F, Tam BYY, Sennino B, Gray JT, Yuan J, Jocson A, Nayak NR, Mulligan RC, McDonald D, Kuo CJ. 2008. Soluble receptor-mediated selective inhibition of vegfr and pdgfrbeta signaling during physiologic and tumor angiogenesis. *Proc Natl Acad Sci U S A*, 105(29):10185-10190.

Lawler DF, Hopkins J, Watson ED. 1999. Immune cell populations in the equine corpus luteum throughout the oestrous cycle and early pregnancy: an immunohistochemical and flow cytometric study. *J Reprod Fertil*, 117(2):281-290.

Lawrence TS., Dekel N, Beers WH. 1980. Binding of human chorionic gonadotropin by rat cumuli oophori and granulosa cells: a comparative study. *Endocrinology*, 106(4):1114-1118.

Lei ZM, Chegini N, Rao CV. 1991. Quantitative cell composition of human and bovine corpora lutea from various reproductive states. *Biol Reprod*, 44(6):1148-1156.

Leroy JLMR, Valckx SDM, Jordaens L, De Bie J, Desmet KLJ, Van Hoeck V, Britt JH, Marei WF, Bols PEJ. 2015. Nutrition and maternal metabolic health in relation to oocyte and embryo quality: critical views on what we learned from the dairy cow model. *Reprod Fertil Dev*, 27(4):693-703.

Li Q, Jimenez-Krassel F, Ireland JJ, and Smith GW. 2009. Gene expression profiling of bovine preovulatory follicles: gonadotropin surge and prostanoid-dependent up-regulation of genes potentially linked to the ovulatory process. *Reproduction*, 137(2):297-307.

Lobel BL, Levy E. 1968. Enzymic correlates of development, secretory function and regression of follicles and corpora lutea in the bovine ovary. *Acta Endocrinol (Copenh), Suppl*, 132:5-63.

Mamluk R, Levy N, Rueda B, Davis JS, Meidan R. 1999. Characterization and regulation of type a endothelin receptor gene expression in bovine luteal cell types. *Endocrinology*, 140(5):2110-2116.

Mishra S, Palai T. 2014. Steroidogenesis in luteal cell: a critical pathway for progesterone production. *J Invest Biochem*, 3(4):170-172.

Miyamoto A, Shirasuna K, Shimizu T, Bollwein H, Schams D. 2010. Regulation of corpus luteum development and maintenance: specific roles of angiogenesis and action of prostaglandin F2alpha. *Soc Reprod Fertil Suppl*, 67:289-304.

Murdoch WJ, Steadman LE. 1991. Investigations concerning the relationship of ovarian eosinophilia to ovulation and luteal function in the sheep. *Am J Reprod Immunol*, 25(2):81-87.

Murdoch WJ, McCormick RJ. 1993. Mechanisms and Physiological implications of leucocyte chemoattraction into periovulatory ovine follicles. *Reproduction*, 97(2):375-380.

Murdoch WJ, Gottsch ML. 2003. Proteolytic mechanisms in the ovulatory folliculo-luteal transformation. *Connect Tissue Res*, 44(1):50-57.

Niswender GD., Juengel JL, McGuire WJ, Belfiore CF, Wiltbank MC. 1994. Luteal function: the estrous cycle and early pregnancy. *Biol Reprod*, 50(2):239-247.

Oakley OR, Kim HY, El-Amouri I, Lin PCP, Cho J, Bani-Ahmad M, Ko C. 2010. periovulatory leukocyte infiltration in the rat ovary. *Endocrinology*, 151(9):4551-4559.

Okuda K, Sakumoto R, Uenoyama Y, Berisha B, Miyamoto A, Schams D. 1999. Tumor necrosis factor alpha receptors in microvascular endothelial cells from bovine corpus luteum. *Biolf Reprod*, 61(4):1017-1022.

Okuda K, Sakumoto R. 2003. Multiple roles of TNF super family members in corpus luteum function. *Reprod Biol Endocrinol*, 1:95.

Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-Potocka I, Skarzynski DJ. 2004. Progesterone is a suppressor of apoptosis in bovine luteal cells. *Biol of Reprod*, 71(6):2065-2071.

Park, JY, Su YQ, Ariga M, Law E, Jin CSL, Conti M. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science*, 303(5658):682-684.

Pate JL. 2003. Lives in the Balance: responsiveness of the corpus luteum to uterine and embryonic signals. *Reprod Suppl*, 61:207-217.

Pate JL, Toyokawa K, Walusimbi S, Brzezicka E. 2010. The interface of the immune and reproductive systems in the ovary: lessons learned from the corpus luteum of domestic animal models. *Am J Reprod Immunol*, 64(4):275-286.

Penny LA, Armstrong D, Bramley TA, Webb R, Collins RA, Watson ED. 1999. Immune cells and cytokine production in the bovine corpus luteum throughout the oestrous cycle and after induced luteolysis. *J Reprod Fertil*, 115(1):87-96.

Petroff MG, Petroff BK, Pate JL. 2001. Mechanisms of cytokine-induced death of cultured bovine luteal cells. *Reproduction*, 121(5):753-760.

Petrovská M, Dimitrov DG, Michael SD. 1996. Quantitative changes in macrophage distribution in normal mouse ovary over the course of the estrous cycle examined with an image analysis system. *Am J Reprod Immunol*, 36(3):175-783.

Poole DH, Pate JL. 2012. Luteal microenvironment directs resident T lymphocyte function in cows. *Biol Reprod*, 86(2):1-10.

Poulsen LC, Englund ALM, Wissing MLM, Yding Andersen C, Borup R, Grøndahl ML. 2019. Human granulosa cells function as innate immune cells executing an inflammatory reaction during ovulation: a microarray analysis. *Mol Cell Endocrinol*, 486:34-46.

Ramadan AA, Selim SA, Hassan HM, Wahba MA. 2001. Immune regulation of ovarian function in buffaloes (Bubalus Bubalus). *Theriogenology*, 55(2):661-669.

Reibiger I, Spanel-Borowski K. 2000. 'Difference in Localization of Eosinophils and Mast Cells in the Bovine Ovary'. *J Reprod Fertil*, 118(2):243-249.

Reynolds LP, Redmer DA. 1999. Growth and development of the corpus luteum. *J Reprod Fertil Suppl*, 54:181-191.

Reynolds LP, Grazul-Bilska AT, Redmer DA. 2000. Angiogenesis in the corpus luteum. *Endocrine*, 12(1):1-9.

Reynolds LP, Redmer DA. 1999. Growth and Development of the Corpus Luteum. *J Reprod Fertil Suppl*, 54:181-191.

Richani D, Gilchrist RB. 2018. The epidermal growth factor network: role in oocyte growth, maturation and developmental competence. *Hum Reprod Update*, 24(1):1-14.

Richards, JAS, Liu Z, Shimada M. 2008. Immune-like mechanisms in ovulation. Trends *Endocrinol Metab*, 19(6):191-196.

Richards JS, Russell DL, Ochsner S, Espey LL. 2002. Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol*, 64:69-92.

Ritter LJ, Sugimura S, Gilchrist RB. 2015. Oocyte induction of EGF responsiveness in somatic cells is associated with the acquisition of porcine oocyte developmental competence. *Endocrinology*, 156(6):2299-2312.

Robinson RS, Woad KJ, Hammond AJ, Laird M, Hunter MG, Mann GE. 2009. Angiogenesis and vascular function in the ovary. *Reproduction*, 138(6):869-881.

Rohm F, Spanel-Borowski K, Eichler W, Aust G. 2002. Correlation between expression of selectins and migration of eosinophils into the bovine ovary during the periovulatory period. *Cell and Tissue Res*, 309(2):313-322.

Rosiansky-Sultan M, Klipper E, Spanel-Borowski K, Meidan R. 2006. Inverse relationship between nitric oxide synthases and endothelin-1 synthesis in bovine corpus luteum: interactions at the level of luteal endothelial cell. *Endocrinology*, 147(11):5228-5235.

Rueda BR, Hendry IR, Hendry III WJ, Stormshak F, Slayden OD, Davis JS. 2000. Decreased Progesterone Levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells. *Biol Reprod*, 62(2):269-276.

Sakumoto R, Berisha B, Kawate N, Schams D, Okuda K. 2000. Tumor necrosis factor-alpha and its receptor in bovine corpus luteum throughout the estrous cycle. *Biol Reprod*, 62(1):192-199.

Schams D, Kosmann M, Berisha B, Amselgruber WM, Miyamoto A. 2001. Stimulatory and synergistic effects of luteinising hormone and insulin like growth factor 1 on the secretion of vascular endothelial growth factor and progesterone of cultured bovine granulosa cells. *Exp Clin Endocrinol Diabetes*, 109(3):155-162.

Schruefer R, Lutze N, Schymeinsky J, Walzog B. 2005. Human Neutrophils Promote angiogenesis by a paracrine feedforward mechanism involving endothelial interleukin-8. *Am J Physiol Heart Circ Physiol*, 288(3):H1186-1192.

Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. 2009. Mechanisms of Infertility associated with clinical and subclinical endometritis in high producing dairy cattle. *Reprod Domest Anim*, 3(Suppl 44):1-9.

Shimada M, Hernandez-Gonzalez I, Gonzalez-Robayna I, Richards JS. 2006. Paracrine and autocrine regulation of epidermal growth factor-like factors in cumulus oocyte complexes and granulosa cells: key roles for prostaglandin synthase 2 and progesterone receptor. *Mol Endocrinol*, 20(6):1352-1365.

Shirasuna K, Nitta A, Sineenard J, Shimizu T, Bollwein H, Miyamoto A. 2012a. Vascular and immune regulation of corpus luteum development, maintenance, and regression in the cow. *Domest Anim Endocrinol*, 43(2):198-211.

Shirasuna K, Jiemtaweeboon S, Raddatz S, Nitta A, Schuberth HJ, Bollwein H, Shimizu T, Miyamoto A. 2012b. Rapid Accumulation of polymorphonuclear neutrophils in the corpus luteum during prostaglandin F(2α)-induced luteolysis in the cow. *PloS One*, 7(1):e29054.

Shirasuna K, Asaoka A, Acosta TJ, Wijayagunawardane MPB, Ohtani M, Hayashi M, Matsui M, Miyamoto A. 2004. Real-time relationships in intraluteal release among prostaglandin F2alpha, endothelin-1, and angiotensin II during spontaneous luteolysis in the Cow. *Biol Reprod*, 71(5):1706-1711.

Skarzynski DJ, Okuda K. 1999. Sensitivity of bovine corpora lutea to prostaglandin F2alpha Is dependent on progesterone, oxytocin, and prostaglandins. *Biol Reprod*, 60:1292-1298.

Smith, GW, Meidan R. 2014. Ever-Changing Cell interactions during the life span of the corpus luteum: relevance to luteal regression. *Reprod Biol*, 14(2):75-82.

Spanel-Borowski K, Rahner P, Ricken AM. 1997. Immunolocalization of CD18-positive cells in the bovine Ovary. *J Reprod Fertil*, 111(2):197-205.

Spanel-Borowski K. 2011. Ovulation as danger signaling event of innate immunity. *Mol Cell Endocrinol*,

333(1):1-7.

Standaert FE, Zamora CS, Chew BP. 1991. Quantitative and qualitative changes in blood leukocytes in the porcine ovary. *Am J Reprod Immunol*, 25(4):163-168.

Takeda K. 2004. Toll-like receptors in innate immunity. *Int Immunol*, 17(1):1-14.

Thatcher WW, Santos JEP, Silvestre FT, Kim IH, Staples CR. 2010. Perspective on physiological/ endocrine and nutritional factors influencing fertility in post-partum dairy cows. *Reprod Domest Anim*, 45(3):2-14.

Townson DH, O'Connor CL, Pru JK. 2002. Expression of monocyte chemoattractant protein-1 and distribution of immune cell populations in the bovine corpus luteum throughout the estrous cycle. *Biol Reprod*, 66(2):361-366.

Townson DH, Liptak AR. 2003. Chemokines in the corpus luteum: implications of leukocyte chemotaxis. *Reprod Biol Endocrinol*, 94(1):1477-7827.

Tropea A, Miceli F, Minici F, Tiberi F, Orlando M, Gangale MF, Romani F, Catino S, Mancuso S, Navarra P, Lanzone A, Apa R. 2006. Regulation of vascular endothelial growth factor synthesis and release by human luteal cells *in vitro*. *Clin Endocrinol Metab*, 91(6):2303-2309.

Tsai SJ, Wiltbank MC. 1998. Prostaglandin F2α regulates distinct physiological changes in early and mid-cycle bovine corpora lutea1. *Biol Reprod*, 58(2):346-352.

Turner EC, Hughes J, Wilson H, Clay M, Mylonas KJ, Kipari T, Duncan CW, Fraser HM. 2011. Conditional ablation of macrophages disrupts ovarian vasculature. *Reproduction*, 141(6):821-831.

Walsh SW, Mehta JP, McGettigan PA, Browne JA, Forde N, Alibrahim RM, Mulligan FJ, Loftus B, Crowe MA, Matthews D, Diskin M, Mihm M, Evans AC. 2012a. Effect of the metabolic environment at key stages of follicle development in cattle: focus on steroid biosynthesis. *Physiol Genomics*, 44(9):504-517.

Walsh SW, Fair T, Browne JA, Evans ACO, McGettigan PA. 2012b. Physiological status alters immunological regulation of bovine follicle differentiation in dairy cattle. *J Reprod Immunol*, 96(1-2):34-44.

Watanabe H, Tatsumi K, Yokoi H, Higuchi T, Iwai M, Fukuoka M, Fujiwara H, Fujita K, Nakayama H, Mori T, Fujita J. 1997. Ovulation defect and its restoration by bone marrow transplantation in osteopetrotic mutant mice of MitfMi/MitfMi genotype1. *Biol Reprod*, 57(6):1394-1400.

Woad KJ, Hammond AJ, Hunter M, Mann GE, Hunter MG, Robinson RS. 2009. FGF2 Is crucial for the development of bovine luteal endothelial networks in vitro. *Reproduction* 138(3):581-588.

Yamashita H, Kamada D, Shirasuna K, Matsui M, Shimizu T, Kida K, Berisha B, Schams D, Miyamoto A. 2008. Effect of Local Neutralization of basic fibroblast growth factor or vascular endothelial growth factor by a specific antibody on the development of the corpus luteum in the cow. *Mol Reprod Dev*, 75(9):1449-1456.

Zalman Y, Klipper E, Farberov S, Mondal M, Wee G, Folger JK, Smith GW, Meidan R. 2012. Regulation of Angiogenesis-related prostaglandin f2alpha-induced genes in the bovine corpus luteum. *Biol Reprod*, 86(3):1-10.

DOI: 10.21451/1984-3143-AR2019-0038

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Folliculogenesis and acquisition of oocyte competence in cows

Marc-André Sirard*

Département des Sciences Animales, Faculté des sciences de l'agriculture et de l'alimentation, Université Laval, Québec, Canada.

Abstract

IVF success depends on hundreds of factors and details but the oocyte quality remains the most important and problematic issue. All antral follicles contain oocytes and all of them have that have reached their full size, can be aspirated, can mature and can be fertilized in vitro. But only a few will make it to embryo unless harvested at a very specific time/status. The conditions impacting the oocyte competence are essentially dependant on the follicular status. Growing follicles contains oocytes that have not completed their preparation, as they are still writing information (RNA), later, dominant follicles or follicles at the plateau phase, stop transcription and become candidates for development. Once in transcriptional arrest, the oocytes, if not ovulated in a short amount of time, do not always make good embryos. This window is affected by time and follicle size and looks like a bell curve. The following review further explain the physiological and molecular evidences that we have to illustrate the competence window and provides clues on how to optimize ovarian stimulation to maximise oocyte quality.

Keywords: follicle stimulation, oocyte quality, *in vitro* maturation.

Introduction

The issue of oocyte quality in large mammals has been a major research focus since the beginning of IVF in the early eighties. The first observation of a variable quality came when oocyte obtained after ovarian stimulation using laparoscopy (Sirard and Lambert, 1985) were compared to oocyte obtained from non-stimulated cows at the slaughterhouse (Sirard and First, 1988). Already at the end of that decade, fertilization rates and culture conditions were sufficiently good that we knew that the blastocyst rate was directly dependent on oocyte origin. Although it was believed that this quality could be enhanced by in vitro culture following artificial meiotic arrest (Sirard and First, 1988), we rapidly realised that it was more complicated than just allowing more time in vitro. Thirty years later there is still an incomplete understanding of the all changes that occurs in the follicles leading to the modification of oocyte capacity to develop to the blastocyst stage but we are getting closer. One reason it took so long comes from the fact that oocytes do not contain enough material for most biochemical analytic methods and possesses a distinct physiology from most somatic cells. Protein characterization to compare oocyte with known developmental competence lead to very limited indicators of where to start the quest for a mechanism of oocyte competence (Sirard *et al.*, 2003). Even new powerful protein profiling methods require ug of proteins and a complete known proteome which is only available for somatic cells of model species or human. To add to the problem, it is quite difficult to obtain very competent oocytes to compare them to incompetent as a basis for mechanistic analysis. Indeed, the most competent oocyte available are the ones just about to be ovulated in a natural cycle of an unprimed cow and this count as 1 where we need hundreds to make any serious analysis even with the power of genomics. Surely, we can do individual cell RNAseq but the coverage will be incomplete and the RNA level of any gene in oocyte is a poor indicator of the associated function since it may be in the "stored" format. In somatic cells, the RNA as a half-life of a few minutes/hours while in oocyte an important fraction of RNAs are de-adenylated and stored (Tremblay *et al.*, 2005; Gohin *et al.*, 2014) Therefore, when a complete RNA analysis (transcriptome) is done (Robert *et al.*, 2011) the results are completely masked by the ignorance of the timing of translation of RNAs into proteins (Gilbert *et al.*, 2010) notwithstanding the fact that many proteins need a post translational modification to be active and such transformation needs hundreds of oocytes to be observed at a chemical level. In oocytes, as somatic cells, we easily observe 12000-15000 different transcripts coding for different proteins and without the capacity to distinguish their dynamics, the complexity of the analysis becomes overwhelming (Labrecque *et al.*, 2013, 2016; Labrecque and Sirard, 2014). Studies to identify the translated RNAs has been done on very specific stages (GV and MII) using large amount of slaughterhouse oocytes (Gohin *et al.*, 2014) but not on highly competent compared to less competent ones.

Unfortunately, the oocyte competence is not a single instantaneously event but a progressive transformation that occurs in a matter of days in the last part of the follicular wave (Sirard, 2016). The use of ovarian stimulation has been instrumental to learn more about oocyte quality and to obtain more material to study these cells. The initial dissection of the late folliculogenesis effect on oocyte quality was done initially by giving a few days of FSH and then slaughtering the animals to recover the ovaries to dissect the follicles and obtain oocyte from specific conditions (Blondin *et al.*, 1996; 1997a). The first big surprise was the fact that actively growing follicles from 5 to 15 mm

*Corresponding author: Marc-Andre.Sirard@fsaa.ulaval.ca
orcid.org/0000-0001-8667-6682
Received: April 17, 2019
Accepted: June 3, 2019

contains oocyte of poor developmental competence when compared to slaughterhouse oocyte from ovaries obtained at random and using follicles from 2-6mm (Blondin and Sirard, 1995). This observation was key to launch the next series of investigations to explore how the time post FSH arrest is important to drive the differentiation program in the follicles and induce oocyte competence. The analysis of follicles according to their level of atresia also reveal a quite astonishing reality: healthy growing follicles (2-6mm) contains oocyte of lower quality than early atretic follicles, as measured by the blastocyst rates (Blondin *et al.*, 1997b) Not only follicle size is not in a linear relation with oocyte competence but the less actively growing follicle are better that the more actively growing at about any size. Another observation that was instrumental to build the following protocols is the fact that oocyte aspirated from ovaries obtained immediately after death contains oocyte with less competence than if a period of 4 hours is used to hold the ovaries at body temperature before harvesting the oocytes (Blondin *et al.*, 1997c). What may be happening post-mortem that improves oocyte quality? It remained a mystery for 20 years but recently we discovered that the cumulus cells would release important information in the form of specific RNAs that would be loaded into the transzonal projections during the post-mortem time (Macaulay *et al.*, 2016) and then release into the oocyte by an exosome-like system of communication (Macaulay *et al.*, 2014). As explained in a classic review paper (Sirard 2006), the oocyte competence can be divided in 3 different parts; the capacity to resume meiosis which is acquired by the early antral stage; the cytoplasmic maturation which is triggered by removing the oocyte from the follicle or by the LH surge in vivo and ; the molecular maturation which encompass the accumulation of specific information in the form of gene transcripts (RNA) for the management of the several days that the oocyte-early embryo will have to do before the activation of the embryonic genome at the eight cell stage (Vigneault *et al.*, 2004).

The understanding of the nature of cytoplasmic/molecular maturation has progressively changed the methods to control ovarian stimulation to take advantage of the effect of time (Blondin *et al.*, 2002; Sirard *et al.*, 2006). The success rate has continued to improve and we can now observe quite remarkable embryo rates following OPU, IVF and in vitro culture from cows or heifers (Landry *et al.*, 2016a; Morin-Doré *et al.*, 2017). Such improvement in the quality of embryo generated comes from a better understanding of the follicular dynamics and the basic physiological events taken place during the last few days of folliculogenesis. The following text will summarize the journey taken to improve egg quality in dairy cows.

Results and discussion

The concept of follicular coasting

In a natural oestrous cycle, the rise of FSH at the beginning of each follicular wave is followed by the recruitment of a dominant follicle and within a few days, the dominant follicle will increase the negative feedback through rising inhibin and estradiol levels will cause an acute decrease in circulating FSH. The dominant follicle will survive this decrease in FSH level by developing sensitive LH receptors that will maintain the growth and prevent atresia, compared to the subordinate follicles that will stop and progressively enter the atresia process within 1-2 days. This period of time during folliculogenesis seems important to prepare the granulosa cells for the major change that will occur at ovulation, the modification from an epithelial cell type into a mesenchymal cell type within the future corpus luteum (Khan *et al.*, 2016a). If the basal LH is removed by using GnRH antagonist, a different gene expression condition will emerge (Sirard, 2016) and oocyte quality will decrease rapidly as in atretic follicles (Nivet *et al.*, 2017). Other experiment testing the quality of oocyte have also confirmed the importance of follicular size to generate blastocysts post fertilization although the effect of early atresia or plateau phase seems more important than size for preparing the oocyte to develop post fertilization (Blondin and Sirard, 1995; Blondin *et al.*, 2002; Nivet *et al.*, 2012). The combination of these 2 sources of information: the different follicular condition under basal LH growth and the beneficial effect of the plateau phase in antral follicles, was instrumental in understanding the importance of reducing FSH for a define period before harvesting the oocytes. It seems that as long as FSH is driving the growth of the follicle, the oocyte does not reduce its transcriptional activity (see below) and does not begin the final preparation leading to ovulation. It is only when FSH decrease that the oocyte will either be programmed by a dominant follicle under basal LH or will start a pseudo-chromatin compaction leading to cell death (atresia) and resorption of the follicle which happens in most cases in large mammals ovulating only one follicle per cycle. We now have molecular evidence that there is a synchrony between the 3 inner follicle components, the oocyte, the cumulus and the granulosa cells during that basal LH period resulting in the activation of the 5 principal components of the differentiation: estradiol dependant genes, TGFB1, TP53, retinoic acid dependant genes and HNF4 which are instrumental to promote the changes leading to the epithelial-mesenchymal transition (Khan *et al.*, 2016b). The value of these last few days of differentiation are demonstrated by a paper where follicles were maintained in the growth phase with FSH and not allowed to go through this low FSH period (Blondin *et al.*, 1996) and showing a markedly reduced blastocyst rate. Finally, in the early years of this century, our laboratory has designed a FSH withdrawal period to improve oocyte quality after OPU-IVF (Blondin *et al.*, 2002). After several years or playing with the concept, the optimization of the low FSH period was set at 48-62 hours in adult animals and the average rate of blastocyst obtained reached 75% with some animal producing 100 % blastocysts with a complete cohort of follicles (Nivet *et al.*, 2012). Since then, thousands of calves have been produced by this approach which now allows the full

potential of ART to be developed in cows (Landry *et al.*, 2016b).

The phenomenon of chromatin preparation and condensation

This second part of the puzzle comes from the group of Alberto Luciano who made the observation that oocytes at the immature stage (GV) would show different configurations for their chromatin. In very small follicles, the chromatin is diffused and associated with an active transcription as the oocyte still accumulate transcripts to support the transcriptional arrest of 7 days from pre-ovulatory follicle to the 8 cells stage in the bovine (Sirard, 2010). As the follicle continues its growth, the chromatin starts to change and becomes more compact as GV-1, in clusters in GV-2 and as a very dense structure in GV3 oocytes (Dieci *et al.*, 2016a). These changes are associated with the decrease in transcription but also involves several changes in the histones themselves (replacement with some H3.3) (Labrecque *et al.*, 2016) and their post translational modifications (Lodde *et al.*, 2017). This transformation is similar but somewhat different than the change from the non-surrounded nucleolus (NSN) pattern to the more compact form (surrounded nucleolus SN) as the mouse acquires development competence once it reaches its full size and the SN configuration while in large mammals the process is multi-step and terminates with the pre-ovulatory period where the oocytes have a GV-2 configuration and are ready to complete meiosis rapidly. The other main difference with the mouse seems to be the fact that follicle size does not predict the GV status of the oocyte. Indeed, the distribution of GV-1-2-3 is equilibrated in the different categories of follicle size and corresponds to the growing, plateau and atretic phase of follicle development (Dieci *et al.*, 2016b).

The progressive change in the chromatin configuration is not essential for meiotic resumption as GV-1 stage are fully capable of reaching the metaphase II in culture, but the developmental competence acquisition (Sirard, 2001) is not yet completed indicating that other changes must occurs in the chromatin, or in the same period as the chromatin changes. The transcriptome analysis of the oocyte according to the GV status has been done and revealed numerous histone variants changes (Labrecque *et al.*, 2016) as well as hundreds of other modifications in the RNA content associated with genes that are stored or the ones that are translated during the maturation period (Gohin *et al.*, 2014). The difficulty with RNA analysis in oocytes is that they store numerous transcripts through de-adenylation (leaving around 25 As) and the amplification systems that are used in molecular biology normally prime on the poly A tail and does not differentiate the short (stored) vs the long polyA tails (>100 As) that are rapidly translated creating a doubt if the transcript is used during the transition between GV stages or stored for maturation or post fertilization events (Gohin *et al.*, 2014). Clearly the process is dynamic and the oocyte content is modified as the ovulation get closer. Surprisingly the changes leading to atresia and ovulation are partly similar both in the gene expression profiles and in the microscopic observation of the chromatin compactness during these 2 events (Labrecque *et al.*, 2016).

The concept of oocyte capacitation and limited lifespan

In a natural cycle, the period between the drop in FSH and ovulation varies between 4-5 days (Fig. 1). During this period, as mentioned above, basal LH maintain the growth of the follicle but also induces a different type of growth (more volume and less cell division) compared to the FSH response. The comparative analysis of gene expression from the FSH growth phase indicates that cell multiplication is reduced during the plateau (Nivet *et al.*, 2013; Douville and Sirard, 2014; Girard *et al.*, 2015) to permit progressive differentiation leading to cell secretion (ex: estradiol) and accumulation of follicular fluid to generate volume faster than tissue growth.

In bovine, the dominant follicle will regress if the progesterone remains high creating waves of follicular emergence (2 or 3 per cycle). The reason why there is a limited follicular lifespan is not completely clear as other species like human, in which the dominant normally goes to ovulation at each cycle as there is no high progesterone level to repress LH rise in response to estradiol. Nevertheless, using hormonal stimulation and antagonist blockers, the more advanced follicles in human also show a reduced oocyte quality (Nivet *et al.*, 2016). The possible explanation for the reduced lifespan is the oocyte transcriptional arrest that occurs when the chromatin compaction is completed as describe above. The ability of the oocyte to maintain homeostasis is necessarily limited with a marked reduction of the capacity to make new proteins from new RNAs. This short duration of quality may be matched by the duration of the estradiol response (high in pre-ovulatory) of the uterus which may not be sustained if no ovulation occurs. The role of estradiol in mating behavior is also important and the synchrony between follicular final growth and mating must be organized to insure the presence of sperm at or around ovulation.

Follicles changes in natural cycles

To illustrate the progression of chromatin condensation as the follicles go through recruitment, selection, dominance and pre-ovulatory, the hypothetical configuration of early follicular wave is shown in figure 2. If oocytes are obtained at random at the slaughterhouse, we may expect to see a distribution where about one third of oocytes will be in the plateau phase and in the GV2 status (Dieci *et al.*, 2016b). This distribution is strikingly similar if we look at the blastocyst rate of 25 -35 % for oocytes from such tissues origins.

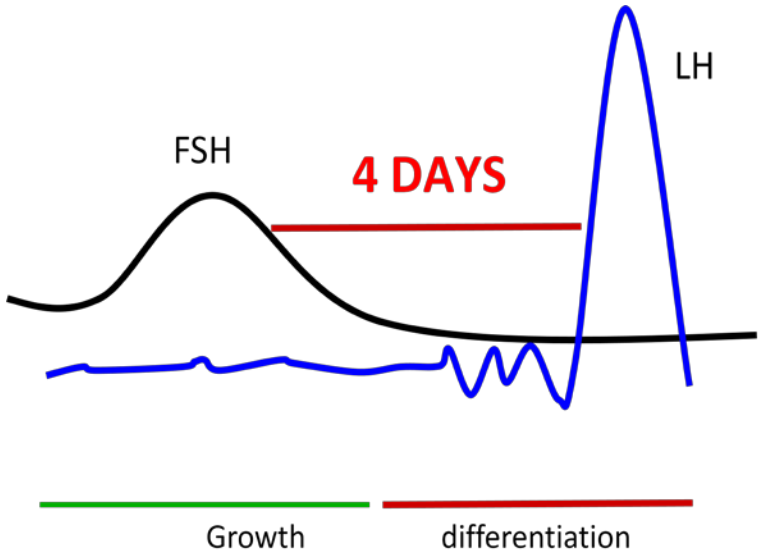


Figure 1. The rise and drop of FSH 4 days before ovulation

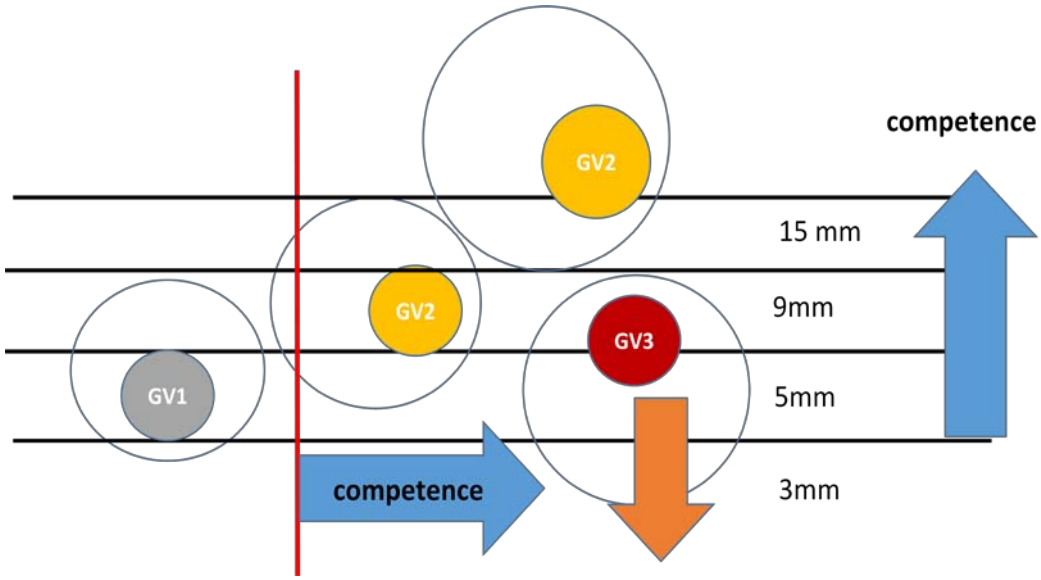


Figure 2. The progression of chromatin condensation in relation to follicle size and competence

Follicles changes in stimulated cycles

This phenomenon of chromatin compaction and limited lifespan of the oocyte is not often considered in superstimulation treatments. When cows are stimulated for embryo flushing at day 7, FSH is used to increase the number of growing follicles and although the removal of any dominant follicle 2 days before the beginning of FSH treatment results in better synchrony of growth and the follicular distribution remains large. If we follow the same kinetics as for the natural cycle, the exogenous FSH stimulated growth will bring many follicles to the dominance status while maintaining the GV status as GV1 (Fig. 2). In such stimulated cycles, when prostaglandins are used to trigger existing corpus luteum decay and ovulation, a large range of follicular sizes remains and rarely all the oocyte are ovulated (small growing follicles do not respond to LH) and such situation results in fewer day 7 embryos than follicle counted at ovulation and even fewer embryos than CL count at day 7 casting doubts on oocyte quality in this context of follicular heterogeneity.

Although the time between FSH arrest and ovulation is shorter (2-3 days) than the natural ovulation cycle, it seems sufficient for most follicles to go through the transformation leading to a mature oocyte and the formation of a corpus luteum. Therefore, when stimulation is used with exogenous FSH prior to OPU to obtain immature oocytes without CL removal, the process of creating a plateau phase where basal LH induces the required changes in follicle and oocyte differentiation becomes important otherwise the oocytes are incompetent (Blondin *et al.*, 1996; Nivet *et al.*, 2017b). In the stimulation context where all the follicles of the wave become dominant (acquire LH receptors), the mimicking of the low FSH basal LH becomes

important to increase the oocyte quality. Timeline studies comparing each animal to itself while using 1-2-3-or 4 days of coasting under inhibitory level of progesterone has shown and increase followed by a decrease of oocyte quality as measured by the blastocyst rate. The optimal coasting time was set at 44-72 hours for most adult animals indicating a limited window (28 hours) of opportunity to achieve the best results (Nivet *et al.*, 2012). This period can be compared to a natural cycle when another 24 hours is added for the response to an LH surge and a little more to include the rapid pulsing of LH before the surge preparing the follicle for the final transformation. The use of this coasting protocol has increased in dairy cows where the cost procedure is justified by the embryos value and where sexing the semen makes a real economic advantage (Landry *et al.*, 2016b). Now if we continue to mimic the timeline of follicular growth and differentiation we can observed that with the coasting of all dominant follicles, the majority of them are at the GV2 stage (unpublished) at the time of collection and accordingly their blastocyst rate is close to 75%.

Conclusion

From all these years of experimentation, it becomes clearer that the ovarian follicle is waiting for specific phases (recruitment-selection-dominance-preovulatory or atresia) to program the oocyte for transcriptional arrest and ovulation or resorption. The understanding of this process may be used to improve ovarian stimulation protocols and obtain high blastocyst rates after immature oocyte aspiration and IVP.

Author contributions

MAS: did the literature review and the conception of the paper and the writing.

Conflict of interest

MAS: has no conflict of interest with the information presented in this review

Acknowledgments and Funding disclosure statement

The funding fort his work has been obtained from NSERC (Natural Sciences and Engineering Research Council of Canada).

References

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev*, 41:54-62.

Blondin P, Coenen K, Guilbault LA, Sirard MA. 1996. Superovulation can reduce the developmental competence of bovine embryos. *Theriogenology*, 46:1191-1203.

Blondin P, Guilbault LA, **Sirard MA**. 1997a. The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology*, 48:803-813.

Blondin P, Coenen K, Guilbault LA, Sirard MA. 1997b. In vitro production of bovine embryos: developmental competence is acquired before maturation. *Theriogenology*, 47:1061-1075.

Blondin P, Guilbault LA, Sirard MA. 1997c. The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology*, 48:803-813.

Blondin P, Bousquet D, Twagiramungu H, Barnes F, Sirard M-A. 2002. Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biol Reprod*, 66:38-43.

Dieci C, Lodde V, Labreque R, Dufort I, Tessaro I, Sirard M-A, Luciano AM. 2016a. Differences in cumulus cell gene expression indicate the benefit of a pre-maturation step to improve in-vitro bovine embryo production. *Mol Hum Reprod*, 22:882-897.

Dieci C, Lodde V, Labreque R, Dufort I, Tessaro I, Sirard M-A, Luciano AM. 2016b. Differences in cumulus cell gene expression indicate the benefit of a pre-maturation step to improve in-vitro bovine embryo production. *Mol Hum Reprod*, 22:882-897.

Douville G, Sirard M-A. 2014. Changes in granulosa cells gene expression associated with growth, plateau and atretic phases in medium bovine follicles. *J Ovarian* Res, 7:50.

Gilbert I, Scantland S, Sylvestre E-L, Dufort I, Sirard M-A, Robert C. 2010. Providing a stable methodological basis for comparing transcript abundance of developing embryos using microarrays. *Mol Hum Reprod*, 16:601-616.

Girard A, Dufort I, Douville G, Sirard M-A. 2015. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod Biol Endocrinol*, 13:17.

Gohin M, Fournier E, Dufort I, Sirard M-A. 2014. Discovery, identification and sequence analysis of RNAs selected for very short or long poly A tail in immature bovine oocytes. *Mol Hum Reprod*, 20:127-138.

Khan DR, Landry DA, Fournier É, Vigneault C, Blondin P, Sirard M-A. 2016a. Transcriptome meta-analysis of three follicular compartments and its correlation with ovarian follicle maturity and oocyte developmental competence in cows. *Physiol Genomics*, 48:633-643.

Khan DR, Fournier É, Dufort I, Richard FJ, Singh J, Sirard M-A. 2016b. Meta-analysis of gene expression profiles in granulosa cells during folliculogenesis. *Reproduction*, 151:R103-110.

Labrecque R, Vigneault C, Blondin P, Sirard M-A. 2013. Gene expression analysis of bovine oocytes with high developmental competence obtained from FSH-stimulated animals. *Mol Reprod Dev*, 80:428-440.

Labrecque R, Sirard M-A. 2014. The study of mammalian oocyte competence by transcriptome analysis: progress and challenges. *Mol Hum Reprod*, 20:103-116.

Labrecque R, Fournier E, Sirard M-A. 2016. Transcriptome analysis of bovine oocytes from distinct follicle sizes: Insights from correlation network

analysis. *Mol Reprod Dev*, 83:558-569.

Landry DA, Bellefleur A-M, Labrecque R, Grand F-X, Vigneault C, Blondin P, **Sirard M-A**. 2016a. Effect of cow age on the in vitro developmental competence of oocytes obtained after FSH stimulation and coasting treatments. *Theriogenology*, 86:1240-1246.

Landry DA, Bellefleur A-M, Labrecque R, Grand F-X, Vigneault C, Blondin P, Sirard M-A. 2016b. Effect of cow age on the in vitro developmental competence of oocytes obtained after FSH stimulation and coasting treatments. *Theriogenology*, 86:1240-1246.

Lodde V, Luciano AM, Franciosi F, Labrecque R, Sirard MA. 2017. Accumulation of chromatin remodelling enzyme and histone transcripts in bovine oocytes. *Results Probl Cell Differ*, 63:223-255.

Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian ÉW, Richard FJ, Hyttel P, Robert C. 2014. The gametic synapse: RNA transfer to the bovine oocyte. *Biol Reprod*, 91:90.

Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HA, Gagné D, Sirard MA, Khandjian ÉW, Richard FJ, Hyttel P, Robert C. 2016. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biol Reprod*, 94:16.

Morin-Doré L, Blondin P, Vigneault C, Grand F-X, Labrecque R, Sirard M-A. 2017. Transcriptomic evaluation of bovine blastocysts obtained from peri-pubertal oocyte donors. *Theriogenology*, 93:111-123.

Nivet A-L, Bunel A, Labrecque R, Belanger J, Vigneault C, Blondin P, Sirard M-A. 2012. FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction*, 143:165-171.

Nivet A-L, Vigneault C, Blondin P, **Sirard M-A**. 2013. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. *Reproduction*, 145:555-565.

Nivet AL, Léveillé MC, Leader A, Sirard MA. 2016 Transcriptional characteristics of different sized follicles in relation to embryo transferability: potential role of hepatocyte growth factor signalling. *Mol Hum Reprod*, 22:475-484.

Nivet A-L, Vigneault C, Blondin P, Sirard M-A. 2017. Influence of luteinizing hormone support on granulosa cells transcriptome in cattle. *Anim Sci J*, 89:21-30

Robert C, Nieminen J, Dufort I, Gagné D, Grant JR, Cagnone G, Plourde D, Nivet AL, Fournier É, Paquet É, Blazejczyk M, Rigault P, Juge N, Sirard MA. 2011. Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays. *Mol Reprod Dev*, 78:651-664.

Sirard MA, Lambert RD. 1985. In vitro fertilization of bovine follicular oocytes obtained by laparoscopy. *Biol Reprod*, 33:487-494.

Sirard MA, First NL. 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. *Biol Reprod*, 39:229-234.

Sirard MA. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*, 55:1241-1254.

Sirard MA, Dufort I, Coenen K, Tremblay K, Massicotte L, Robert C. 2003. The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals. *Reproduction*, 61:117-129.

Sirard M-A, Richard F, Blondin P, Robert C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, 65:126-136.

Sirard MA. 2010. Activation of the embryonic genome. *Soc Reprod Fertil Suppl*, 67:145-158.

Sirard M-A. 2016. Somatic environment and germinal differentiation in antral follicle: The effect of FSH withdrawal and basal LH on oocyte competence acquisition in cattle. *Theriogenology*, 86:54-61.

Tremblay K, Vigneault C, McGraw S, Sirard M-A. 2005. Expression of cyclin B1 messenger RNA isoforms and initiation of cytoplasmic polyadenylation in the bovine oocyte. *Biol Reprod*, 72:1037-1044.

Vigneault C, McGraw S, Massicotte L, Sirard M-A. 2004. Transcription factor expression patterns in bovine in vitro-derived embryos prior to maternal-zygotic transition. *Biol Reprod*, 70:1701-1709.

DOI: 10.21451/1984-3143-AR2019-0063

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

Stress, strain, and pregnancy outcome in postpartum cows

Matthew C. Lucy*

Division of Animal Sciences, University of Missouri, Animal Science Research Center, Columbia, MO, USA.

Abstract

Stress affects the productivity and fertility of cattle. Stress causes strain and individual animals experience different amounts of strain in response to the same amount of stress. The amount of strain determines the impact of stress on fertility. Typical stresses experienced by cattle include environmental, disease, production, nutritional, and psychological. The effect of stress on the reproductive system is mediated by body temperature (heat stress), energy metabolites and metabolic hormones (production and nutritional stresses), the functionality of the hypothalamus-pituitary-gonadal (HPG) axis and (or) the activation of the hypothalamus-pituitary-adrenal (HPA) axis. The strain that occurs in response to stress affects uterine health, oocyte quality, ovarian function, and the developmental capacity of the conceptus. Cows that have less strain in response to a given stress will be more fertile. The goal for future management and genetic selection in farm animals is to reduce production stress, manage the remaining strain, and genetically select cattle with minimal strain in response to stress.

Key words: stress, strain, pregnancy, cattle.

Introduction

The correct definition of stress is important in any discussion of reproduction and pregnancy in cattle. A *stress* or *stressor* is a force external to a system that acts to displace the system. A stress condition can be quantified and applied equally across animals. A *strain* is the animal's response to stress (the magnitude of the displacement). The strain often represents a cost to the individual animal. As depicted in Figure 1, the level of strain in response to an equivalent stress can vary from animal to animal. There is production stress, for example, that places strain on the animal (Fig. 1). The strain in response to greater stress is minimally increased in cow A, moderately increased in cow B, and greatly increased in cow C (Fig. 1). The goal for future management and genetic selection in farm animals is to reduce production stress, manage the remaining strain and genetically select cattle with minimal strain in response to production stress (Cow A).

Stressors assume a variety of forms (Fig. 2). The stresses create strain that can affect many aspects of animal production including embryonic development and pregnancy outcome. Cows that have less strain in response to a stress will be more fertile (cow A). Less strain may be explained by lesser biological response to stress or a greater capacity to function (cope) in the presence of stress. Stresses, their mediators, and strains that affect pregnancy in cattle are reviewed in this paper. The stresses are described first, followed by the mediators of stress, the strains, and finally the outcome (effect on embryonic development and pregnancy outcome).

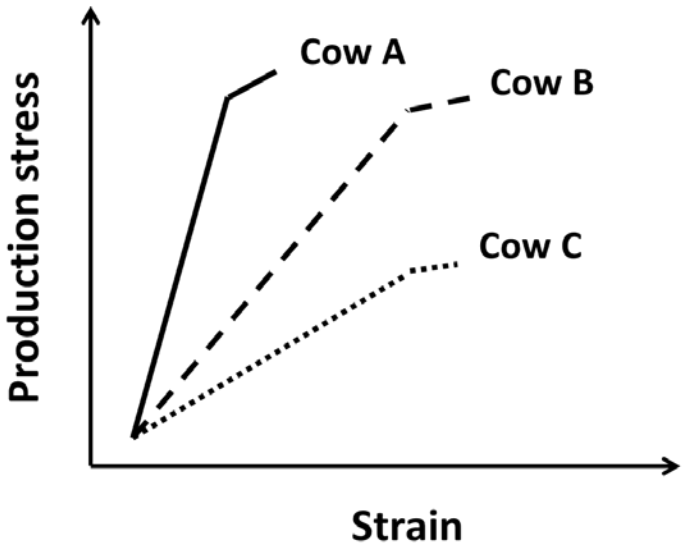


Figure 1. Graph depicting the relationship between production stress and associated strain. For cow A, an increase in production stress leads to the smallest increase in strain. Cows B and C have progressively greater production strain in response to production stress. The most desirable cow is A because there is the least strain in response to production stress.

*Corresponding author: lucym@missouri.edu
orcid.org/0000-0001-5771-9460
Received: May 15, 2019
Accepted: July 25, 2019

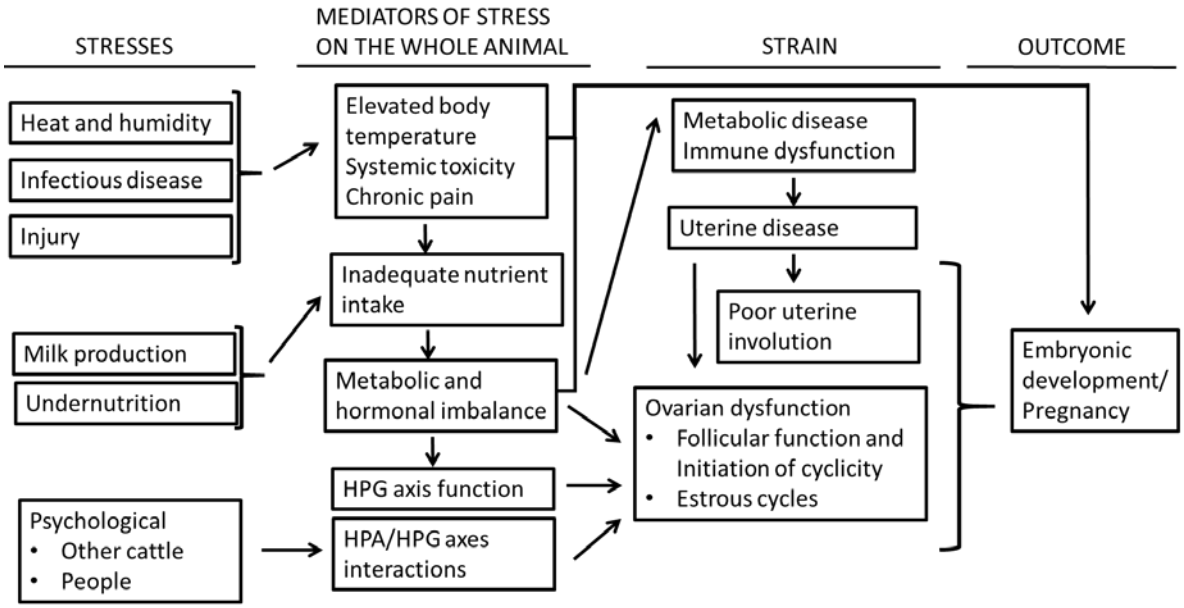


Figure 2. Diagram depicting stresses that affect cattle, the mediators of stress in the whole animal, and the associated strain that ultimately affects pregnancy outcome. HPG = hypothalamic-pituitary-gonadal; HPA = hypothalamic-pituitary-adrenal.

Stresses that affect pregnancy outcome in cattle

There are many well-known stresses that affect the productive and pregnancy outcome of cattle. The reader is referred to several recent review articles on stressors and the mechanisms through which stress can affect production and pregnancy outcome (Tab. 1). This review will attempt to integrate some of the difference stressors and their effects on pregnancy.

Table 1. Table 1. Sources of stress and recent review papers written on the topic.

Sources of stress	Review paper
Infectious disease of the reproductive tract	Bromfield *et al.*, 2015; Eckel and Ametaj, 2016; Sheldon *et al.*, 2018; Gilbert, 2019.
Injury	Funnell and Hilton, 2016; Jewell *et al.*, 2019; Kammel *et al.*, 2019.
Heat	Gwazdauskas, 1985; Hansen, 2007, 2009; Collier *et al.*, 2017; Polsky and von Keyserlingk, 2017; Roth, 2017, 2018a.
Metabolic imbalance postpartum	Lucy *et al.*, 2014; Baumgard *et al.*, 2017; Overton *et al.*, 2017; Lucy, 2019.
Social/psychological	Dobson and Smith, 2000; Karsch *et al.*, 2002; Breen and Karsch, 2006; Ralph *et al.*, 2016; von Keyserlingk and Weary, 2017.
Nutritional	Butler, 1998; Wilde, 2006; D'Occhio *et al.*, 2019.
Transportation	Hong *et al.*, 2019.

Heat and humidity

Environmental stress caused by heat and humidity (heat stress) is common in both beef and dairy cattle but the strain (elevated body temperature) associated with heat stress is greater in dairy cows (Collier *et al.*, 2017; Polsky and von Keyserlingk, 2017). Lactating dairy cows are particularly sensitive to heat stress because there is metabolic heat production associated with high milk production. In a study of Florida dairy cows, for example, summer infertility was greatest in high milk producing dairy cows (al-Katanani *et al.*, 1999). There is an additive negative effect, therefore, of heat stress and greater milk production on pregnancy outcome in dairy cows. The effects of environmental stress on lactating cows is explained partially by reduced feed intake but there are aspects of the metabolic response that are entirely unique to heat stress (Baumgard and Rhoads, 2013). Many breeds of European cattle are not adapted to production in hot and humid environments. Crossing European breeds with local indigenous breeds will yield lactating cattle that are resistant to heat stress and function well in local environments (Canaza-Cayo *et al.*, 2015).

Infectious disease and injury

Injury and disease are stresses that create strain that can affect production and pregnancy outcome. Routine vaccination programs control many of the

infectious diseases that negatively impact production and pregnancy (Newcomer and Givens, 2016). There are pathogens that affect postpartum reproduction that cannot be effectively controlled by vaccination (Gilbert, 2019). Infectious disease stress, therefore, is common in postpartum cows. Common diseases that affect postpartum dairy cows include metabolic diseases (ketosis and fatty liver) (Overton *et al.*, 2017), periparturient disorders (dystocia and retained placenta) (Funnell and Hilton, 2016), uterine diseases (metritis and endometritis) (Gilbert, 2019) and mastitis (Sordillo, 2018). Dystocia predisposes cattle to retained placenta and uterine infection (metritis) both of which are painful and are associated with reduced feed intake (Esposito *et al.*, 2014; Collier *et al.*, 2017; Shock *et al.*, 2018). Decreased feed intake can lead to metabolic and hormonal changes and associated weight loss (strain) that can affect pregnancy outcome. Likewise, cattle can become injured because of poor facility design on farm (for example, hock lesions and other abrasions in free stall barns) (Jewell *et al.*, 2019). Correct barn and stall design (including flooring) can reduce injury (a stress) and associated strain in lactating dairy cows (Cook, 2019; Kammel *et al.*, 2019).

High milk production

Genetic selection within the dairy industry has successfully increased milk production per cow (Miglior *et al.*, 2017; Lucy, 2019). For the purpose of this review, high milk production will be defined as a stress. There is a change in circulating hormones and metabolites associated with high milk production and this creates a strain within the animal (Lucy *et al.*, 2014). The strain affects the normal function of the hypothatlamic-pituitary-gonadal (HPG) axis (abnormal gonadotropin secretion) that leads to ovarian dysfunction (Butler, 2000, 2014). Physiological stress caused by high milk production also affects the immune systems to create immune dysfunction (a strain) and disease (LeBlanc, 2012; Lucy *et al.*, 2014; Gilbert, 2019).

Undernutrition

Under-feeding may arise from short or long-term feed shortages. Cows that are under-fed are stressed and undergo metabolic adaptation to the stress [lipid mobilization and non-esterified fatty acid (NEFA) release etc.] (D'Occhio *et al.*, 2019). There are similarities with respect to the hormonal and metabolic changes that occur in high milk-producing cows and underfed cows but there are differences as well. One important biological difference is that cows that are under-fed are mobilizing nutrients from tissue for survival. High producing cows that are fully-fed, however, are undergoing a genetically programmed homeorhetic process to support high milk production. Other nutritional stresses that include over-feeding protein (Butler, 1998) or under-feeding minerals (Wilde, 2006) can affect pregnancy outcome but will not be discussed further in this review.

Psychological

Psychological stress may occur on farms (Dobson and Smith, 2000; von Keyserlingk and Weary, 2017). The common forms of psychological stress include social interactions with other farm animals and people (Lima *et al.*, 2018). Cattle are social animals that live in groups with a dominance hierarchy. Mixing groups of cattle creates stress. Productivity is decreased when the hypothalamic-pituitary- adrenal (HPA) axis is activated (Hong *et al.*, 2019) and feed intake is reduced (O'Driscoll *et al.*, 2006). Cows also spend time and energy to re-establish the dominance structure within the group (Val-Laillet *et al.*, 2008) and displace one another at the feed bunk (Huzzey *et al.*, 2006). Displacement will affect feeding behavior in subordinate cows. Cows that have recently calved and younger cows may benefit from being housed in smaller groups with less competition (Jensen and Proudfoot, 2017). In addition to other animals, cattle may experience stress from interaction with humans depending on how animals are handled and the specific individuals involved (Lima *et al.*, 2018).

Mediators of stress in the whole animal

As stated in the introduction, a stress can be applied equally across animals. The strain is the response of the individual to the stress (Fig. 1). The amount of strain may be explained by the magnitude of the specific response (for example, how much NEFA is released postpartum) or the strain may be explained by the sensitivity of the animal to the specific response (for example, some cows have less strain because they are better able to metabolize NEFA). This section will describe some the mediators that link the stress to the strains that affect pregnancy outcome (Fig. 2).

Elevated body temperature, systemic toxicity with fever, and chronic pain

Many of the effects of heat stress on pregnancy outcome can be explained by the increase in body temperature in heat-stressed cows. Small increases in maternal body temperature will decrease pregnancy rates in cattle (Ulberg and Burfening, 1967). The increase in body temperature affects the reproductive tract and the early embryo. One possible mechanism involves the direct effect of elevated temperature on the embryo (Hansen, 2009). A second mechanism involves the effect of heat stress on the gut (leaky gut syndrome) that causes loss of intestinal barrier function and the release of endotoxin into the circulation and may affect animal productivity and pregnancy outcome (Baumgard and Rhoads, 2013; Abuajamieh *et al.*, 2018).

Fever presents a change in hypothalamic body temperature set point in response to disease (Collier *et al.*, 2017). Disease with fever and injury with chronic pain will affect a cow's motivation to eat or the capacity of the cow to reach the feed bunk (Aditya *et al.*, 2017). Poor intake causes a shift in hormones and metabolites toward a catabolic state (similar to that described in the

previous section). Disease releases endotoxins into the systemic circulation that can have direct effects on reproductive tissues themselves (Eckel and Ametaj, 2016) and also increase body temperature. For example, mastitis infection can cause endotoxin release, immune system activation, cytokine production, and body temperature elevation. The cumulative effects on the whole animal can damage the developing embryo and (or) cause regression of the corpus luteum and early embryonic loss (Hansen *et al.*, 2004; Kumar *et al.*, 2017).

Metabolic and hormonal imbalance caused by inadequate nutrient intake

A high production cow in early lactation will produce over 50 kg of milk per day. The milk production represents a type of stress. In response to the stress, the cow undergoes homeorhesis; a term that was originally defined as "the orchestrated or coordinated control in metabolism of body tissues necessary to support a physiological state" (Bauman and Currie, 1980). There is an increase in circulating growth hormone (GH) postpartum that stimulates hepatic gluconeogenesis and increases glucose supply (Lucy, 2004; Baumgard *et al*., 2017). Growth hormone also antagonizes insulin action and creates an insulin resistant state (preventing the utilization of glucose by liver, muscle or adipose tissue). The increase in GH stimulates lipolysis that mobilizes fatty acids (NEFA) from adipose tissue. The large mass of glucose created through gluconeogenesis and fatty acids created though lipolysis are used for milk synthesis.

The strain associated with production stress is explained by changes in circulating hormone and metabolites. Some cows experience a larger hormonal and metabolic change that can lead to disease (for example, ketosis) (White, 2015). Low blood glucose concentrations postpartum are associated with low blood insulin concentrations. Low blood insulin is associated with low liver GH receptor expression and low circulating IGF1 concentrations (Lucy, 2004). Inadequate glucose supply contributes to the incomplete oxidation of fatty acids (NEFA) which creates elevated beta-hydroxybutyrate (BHB) postpartum (ketosis). The metabolic and endocrine state of early lactation (high GH, low IGF1, low insulin, low glucose, high NEFA and high BHB) affects the ability of the cow to become pregnant.

Cows that eat poorly because of disease or under-feeding undergo many of the same metabolic changes. There is an uncoupling of the somatotropic axis when animals are not eating (Radcliff *et al.*, 2003). Uncoupling of the axis leads to less IGF1 and elevated GH concentrations. The increase in GH drives lipid mobilization to increase NEFA in the circulation. Ketosis may occur if there is insufficient glucose supply and incomplete oxidation of fatty acids. There is also reduced circulating insulin associated with depressed circulating glucose and insulin resistance associated with elevated GH.

HPG Axis function

Luteinizing hormone (LH) is a critical pituitary hormone for the resumption of normal estrous cycles in postpartum cows (Canfield and Butler, 1990, 1991). Greater frequency of LH pulses leads to maturation of preovulatory follicles and the initiation of cyclicity. Preovulatory follicles secrete estradiol that causes the hypothalamus to release a surge of GnRH to cause the LH surge for ovulation and formation of the corpus luteum. Stress can cause a strain on reproduction by slowing the pulsatile release of LH, decreasing follicular estradiol, and (or) blocking the LH surge (Karsch *et al.*, 2002; Breen and Karsch, 2006).

A variety of metabolites and metabolic signals can act at the level of the hypothalamus to affect GnRH and LH pulsatility. Glucose controls insulin secretion in the whole animal and ultimately controls hepatic IGF1 secretion via insulin release (Butler *et al.*, 2003). Circulating glucose and the insulin/IGF1 systems, therefore, are functionally linked in the whole animal (Lucy, 2004; Kawashima *et al.*, 2012). One study concluded that glucose and insulin were the most-likely molecules to exert a positive effect on hypothalamic GnRH and LH secretion in the postpartum dairy cow (Leroy *et al.*, 2008). The most important actions of insulin and IGF1 are observed when the hormone acts synergistically with the gonadotropins [stimulating hormone (FSH) or LH] (Lucy, 2011). There is a positive correlation between circulating concentrations of insulin and IGF1 and the interval to first postpartum ovulation (Velazquez *et al.*, 2008; Kawashima *et al.*, 2012). In a variety of species (including farm animals, humans, and laboratory animals) greater concentrations of blood IGF1 are found in young, well nourished, fertile, and healthy individuals (Thissen *et al.*, 1994). Animals that are old, diseased, infertile, or malnourished have low blood IGF1 concentrations.

HPA/HPG Interactions

The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to stress (Minton, 1994). The reproductive system can be affected by the HPA axis through interactions with the HPG axis (Collier *et al.*, 2017). Neurons within the hypothalamus secrete corticotropin releasing hormone (CRH) into the median eminence. The CRH travels through the hypothalamic-pituitary portal system and causes the release of ACTH from pituitary corticotroph cells. Adrenocorticotrophic hormone causes the adrenal gland to synthesize and secrete glucocorticoid (cortisol). The stressors that cause activation of the HPA axis may cause infertility by inhibiting LH secretion (Breen and Karsch, 2006). Some CRH neurons within the hypothalamus terminate on the cell bodies of GnRH neurons (Wade and Jones, 2004). When CRH neurons are stimulated and release CRH, GnRH release from GnRH neurons may be blocked. There are inhibitory effects of glucocorticoids on GnRH and LH release but these effects are not directly mediated by glucocorticoids because GnRH neurons do not possess the type II glucocorticoid

receptor. The KNDy cells within the hypothalamus that express kisspeptin, neurokinin B, and dynorphin do possess glucocorticoid receptor and may transmit the glucocorticoid signal to the GnRH neurons (Ralph *et al.*, 2016; Scott *et al.*, 2019) .

Mechanisms through which stress and strain affect pregnancy outcome

Stress and strain affect pregnancy outcome. In some cases, the stress can act directly on the pregnancy itself. For example when elevated body temperature from heat stress affects ovarian function, developmental capacity of the oocyte, or early pregnancy development. There are other examples where a strain affects pregnancy outcome. An example would be when hormonal and metabolic imbalance postpartum causes immune dysfunction that leads to uterine disease and infertility. Mechanisms through which stress and strain affect pregnancy outcome are discussed in this section.

Elevated body temperature (heat stress)

Many investigators have reported reduced estrogenic capacity of the ovarian follicle in response to heat stress (Gwazdauskas, 1985; Wolfenson *et al.*, 1997; Wilson *et al.*, 1998). The somatic cells within the follicle (theca and granulosa cells), therefore, can be damaged when cows have elevated body temperature caused by heat stress. Whether or not heat stress affects the corpus luteum is less clear (Hansen and Aréchiga, 1999). The cells of the corpus luteum differentiate from the cells of the follicle. If heat stress decreases blood progesterone then the decrease could arise from the effects of heat stress on the follicle which ultimately forms the corpus luteum.

There are large and consistent effects of heat stress on the oocyte and developing embryo (Putney *et al.*, 1989; Ealy *et al.*, 1993; Hansen, 2009; Roth, 2018a, b). The period of greatest susceptibility of the oocyte/embryo is immediately after the onset of estrus and early during the post-breeding period (Sakatani, 2017). Embryonic development was impaired in heifers subjected to heat stress for 10 hours after the onset of estrus (Putney *et al.*, 1989). Heat stress on day 1 after breeding also decreased subsequent embryonic development. Heat stress on days 3, 5, or 7 after breeding, however, did not affect embryonic development (Ealy *et al.*, 1993). The period of embryonic sensitivity to heat stress, therefore, begins early during the development of the follicle and continues until about 1 day after breeding. By 3 days after breeding, embryos have apparently developed resistance to the effects of heat stress. Several investigators have demonstrated that embryo transfer nearly doubled conception rates when compared with dairy cows inseminated artificially at estrus (Hansen, 2007). It is possible, therefore, to by-pass early embryonic stages and improve conception rates during heat stress.

Metabolic imbalance postpartum

The associations between postpartum hormones and metabolites and subsequent reproduction are found early postpartum when the most-extreme homeorhetic states are known to occur. The early postpartum metabolic profile, therefore, may have the capacity to imprint ovarian tissue either through permanent effects on the genome (epigenetic mechanisms) or by changing the chemical composition of the cells themselves. The oocyte rests in a quiescent state within the ovary until approximately 2 months before ovulation. At that time, it initiates growth along with the surrounding granulosa cells. The metabolic environment within which the oocyte develops can affect its capacity for fertilization and further development (Leroy *et al.*, 2008, 2011; Berlinguer *et al.*, 2012). One theory is that the long development program of the oocyte before ovulation enables an irreversible imprinting of the metabolome on the oocyte itself. If this imprint is negative then this may explain why cows with metabolic disease early postpartum have infertility several months later.

Glucose is an important energy source for ATP production through mitochondrial oxidative phosphorylation. In the uterus and placenta, however, the bulk of the glucose is used to supply carbons for the synthesis of cellular components (nucleotides, amino acids, lipids, etc.). This latter phenomenon is known as the “Warburg effect” and typifies proliferating cells (Vander Heiden *et al.*, 2009). In a study designed to test the effects of glucose on the pregnancy, cows were either milked normally or dried off (not milked) immediately after calving (Green *et al.*, 2012). Milking or not milking postpartum created treatment groups with either low or high circulating glucose concentrations, respectively. The fetus and placenta from the milked (lactating) cows were smaller (weighed less) than the fetus and placenta from nonlactating cows. There was less glucose reaching the fetus in lactating compared with nonlactating cows (Lucy *et al.*, 2012). The reduction in glucose reaching the pregnancy could potentially affect how the pregnancy develops because the pregnancy depends on glucose as a substrate for tissue synthesis and metabolic energy (Battaglia and Meschia, 1978). In the horse, delayed development of the embryonic vesicle generally leads to embryonic loss (Carnevale *et al.*, 2000). Several recent studies in the bovine have demonstrated that pregnant cows that undergo pregnancy loss have lesser blood concentrations of pregnancy associated glycoproteins (PAG) leading up to the time that the pregnancy is aborted (Pohler *et al.*, 2016). The lesser blood PAG concentration may indicate that the cow is pregnant with a small embryo or fetus. Low concentrations of glucose in postpartum cows, therefore, may predispose the cow to pregnancy loss if the placenta does not have adequate substrate for the creation of new cells and the pregnancy grows too slowly (Lucy *et al.*, 2014).

Immune dysfunction

The strain of an abnormal metabolic and hormonal environment postpartum creates dysfunction within the innate immune system through its effects on polymorphonuclear neutrophils (PMN) (Lucy, 2004; Graugnard *et al.*, 2012; LeBlanc, 2012). For example, glucose is the primary metabolic fuel that PMN use to generate the oxidative burst that leads to killing activity. Glycogen concentrations in PMN within the postpartum cow decrease in a manner that is similar to the decrease in blood glucose postpartum (Galvão *et al.*, 2010). Galvão *et al.* (2010) concluded that the lesser glycogen reserve reduced the capacity for oxidative burst in PMN and predisposed the cow to uterine disease.

Epidemiological evidence indicates that an abnormal metabolic profile during the periparturient period predisposes the cow to uterine disease during the early postpartum period and infertility later postpartum (Chapinal *et al.*, 2012; Wathes, 2012; Gilbert, 2019). Cows that had uterine infection early postpartum have more inflammation in the pregnant uterus (Lucy *et al.*, 2016). Inflammation and the presence of lymphocytic foci within the pregnant uterus were associated with a smaller placenta and embryonic loss (Lucy *et al.*, 2016).

Ovarian dysfunction

Stressors that affect ovarian function in dairy cattle commonly do so by interfering with LH release. For example, negative energy balance in dairy cattle (a stress) will cause a decrease in the frequency of LH pulses (a strain) (Canfield and Butler, 1990, 1991). The exact mechanisms through which undernutrition slows the frequency of LH pulses are poorly understood but a variety of mechanisms are probably involved.

Follicular growth and steroidogenesis in postpartum cattle depends on the combined effects of gonadotropins (LH and FSH), systemic hormones (insulin and IGF1) and metabolites (glucose) whose concentrations are highly correlated (Lucy, 2004). The magnitude and duration of the decrease in circulating insulin and IGF1 depends on the depth of negative energy balance postpartum (Beam and Butler, 1999). Cattle in poor body condition or cows failing to increase body condition during lactation have an extended period of low blood insulin and IGF1 and elevated blood GH. There is a positive correlation between blood insulin and IGF1 concentrations and ovarian function in postpartum cows (Wathes *et al.*, 2007). Greater LH pulsatility leads to increased follicular growth that decreases the interval to first ovulation. Both insulin and IGF1 may control the activity of the GnRH neurons in the hypothalamus and (or) the LH release from gonadotrophs (Veldhuis *et al.*, 2006).

Patterns of estrous cyclicity for lactating cows are less regular when compared with estrous cycle of nulliparous heifers (Lucy, 2019). The same hormones that control when the cow begins to cycle (insulin, IGF1, and LH) also have an effect on cyclicity which relates to the functionality of the follicle and corpus luteum. The hormonal environment created by lactation (in this example low blood glucose, insulin and IGF1 concentrations) may potentially affect the capacity for ovarian cells to respond to gonadotropins. In the cycling cow, this could potentially affect estradiol production by the follicle as well as progesterone production by the corpus luteum. Low blood glucose could potentially compromise a variety of essential metabolic processes in ovarian cells including the oocyte that depends on glucose for energy (Berlinguer *et al.*, 2012). There is also the potential for greater steroid metabolism in lactating compared with nonlactating cows that can be explained by greater dry matter intake in cows that are lactating (Wiltbank *et al.*, 2011). Lesser circulating estradiol from the preovulatory follicle can lead to abnormal patterns of follicular growth, anovulatory conditions, multiple ovulation and also reduced estrous expression (Lucy, 2019).

Several authors have recently reviewed the mechanisms assoicated with subnormal luteal development and early embryonic loss (Spencer *et al.*, 2016; Forde and Lonergan, 2017). Low progesterone during the first weeks after insemination may be caused by the stress of high milk production (Lonergan, 2011; Wiltbank *et al.*, 2011). Progesterone stimulates uterine histotroph secretion and lesser uterine histotroph secretion (caused by low progresteorne) may lead to slower embyronic development. The slowly developing embryos may fail to reach adequate size to generate an adequate interferon-tau (IFNT) signal to the mother (Hansen *et al.*, 2017). The pregnancy is lost because the mother fails to recognize the pregnancy and undergoes luteal regression as if she is not pregnant.

Immune dysfunction postpartum may be associated with a high incidence of mastitis in early postpartum cows (Sordillo, 2018). Mastitis may not directly affect reproductive tissues but secondary responses of the cow to the disease can disrupt estrous cycles and cause embryonic loss. Several authors have found that a mastitis event during breeding was associated with lower fertility (Fuenzalida *et al.*, 2015). Cytokines and other hormones released by the inflamed mammary tissue can circulate throughout the cow and block ovulation or cause premature regression of the corpus luteum (Sheldon, 2015).

Psychological stress

Cows interact with other cows in the herd and also the people that care for them. The mechanisms linking changes in social status within the herd to reproductive efficiency are not clear but may involve activation of the HPA axis and subsequent inhibition of the HPG axis in animals that are subjected to aggression from other cows. A recent study of dairy cows showed that dairy cows losing social status during the breeding period had a longer interval from calving to conception and required more inseminations per conception (Dobson and Smith, 2000). The activation of the HPA axis that occurs in response to social stress can inhibit the pituitary release of LH (Karsch *et al.*, 2002). Furthermore, cortisol may decrease responsiveness of ovarian follicles to LH.

Dairy cattle can recognize individual people and have better performance when handled by gentle people compared with aggressive people (Munksgaard *et al.*, 1997; Lindahl *et al.*, 2016). Conception rate was positively correlated with positive human-animal interactions in one study of 66 commercial farms (Hemsworth *et al.*, 2000). It is possible that some of the variation in inseminator conception rate could be explained by handling of the animals before and during insemination. Aggressive handling may activate the HPA axis and disrupt normal processes that precede ovulation and affect fertility.

Conclusions

Stress and associated strain are important topics because they affect the ability of farm animals to become pregnant. Stresses arise from a variety of sources that reside outside or within the individual animal. Outside sources of stress include the physical environment (ambient temperature and humidity), physical surroundings (facilities), other cows (social interactions), people (human-cattle interactions) and microbial (disease). To some extent, the strain from outside sources can be mitigated by reducing the stress itself. For example, the physical environment can be improved by cooling, facilities can be improved by replacement or renovation, cows can be housed in smaller groups of similar-sized cattle, aggressive cow handlers can be re-trained so that they use appropriate techniques, and disease can be reduced through vaccination, cleanliness, and antibiotic treatment. Stress can also come from within the animal (abnormal thermal, hormonal or metabolic profile that creates immunological and ovarian dysfunction, uterine disease, poor oocyte quality and embryonic loss). The strain from the internal stress response can be managed through programs such as timed AI that control ovarian function and the time of breeding (Carvalho *et al*., 2018) or embryo transfer that circumvents periods of embryo sensitivity (Hansen, 2007). Genetic selection of animals that are resistant to stress and have less strain is an additional method to improve productivity. The goal for future of management and genetic selection programs in farm animals should be to reduce production stress, manage the remaining strain using technologies like timed AI and embryo transfer and also genetically select cattle with minimal strain in response to stress.

Author contributions

MCL: Conceptualization, Writing – original draft, Writing - review & editing.

Conflict of interest

The author has no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials presented in this manuscript.

Acknowledgments and Funding disclosure statement

The author would like to thank the University of Missouri (Columbia Missouri, USA) for providing author salary and office support during the preparation of this manuscript.

References

Abuajamieh M, Kvidera SK, Mayorga EJ, Kaiser A, Lei S, Seibert JT, Horst EA, Sanz Fernandez MV, Ross JW, Selsby JT 2018. The effect of recovery from heat stress on circulating bioenergetics and inflammatory biomarkers. *J Anim Sci*, 96:4599-4610.

Aditya S, Humer E, Pourazad P, Khiaosa-Ard R, Huber J, Zebeli Q. 2017. Intramammary infusion of Escherichia coli lipopolysaccharide negatively affects feed intake, chewing, and clinical variables, but some effects are stronger in cows experiencing subacute rumen acidosis. *J Dairy Sci*, 100:363-1377.

Battaglia FC, Meschia G. 1978. Principal substrates of fetal metabolism. *Physiol Rev*, 58:499-527.

Bauman DE, Currie WB. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. *J Dairy Sci*, 63:1514-1529.

Baumgard LH, Rhoads RP. 2013. Effects of heat stress on postabsorptive metabolism and energetics. *Ann Rev Anim Biosci*, 1:311-337.

Baumgard LH, Collier RJ, Bauman DE. 2017. A 100-Year Review: Regulation of nutrient partitioning to support lactation. *J Dairy Sci*, 100:10353-10366.

Beam SW, Butler WR. 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *J Reprod Fertil Suppl*, 54:411-424.

Berlinguer F, Gonzalez-Bulnes A, Contreras-Solis I, Spezzigu A, Torres-Rovira L, Succu S, Naitana S, Leoni GG. 2012. Glucogenic supply increases oocyte developmental competence in sheep. *Reprod Fertil Dev*, 24:1055-1062.

Breen KM, Karsch FJ. 2006. New insights regarding glucocorticoids, stress and gonadotropin suppression. *Front Neuroendocrinol*, 27:233-245.

Bromfield JJ, Santos JEP, Block J, Williams RS, Sheldon IM. 2015. Physiology and Endocrinology Symposium: Uterine infection: linking infection and innate immunity with infertility in the high-producing dairy cow. *J Anim Sci*, 93:2021-2033.

Butler WR. 1998. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *J Dairy Sci*, 81:2533-2539.

Butler WR. 2000. Nutritional interactions with reproductive performance in dairy cattle. *Anim Reprod Sci*, 60-61:449-457.

Butler ST. 2014. Nutritional management to optimize fertility of dairy cows in pasture-based systems. *Animal*, 8(Suppl 1):15-26.

Butler ST, Marr AL, Pelton SH, Radcliff RP, Lucy MC, Butler WR. 2003. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. *J Endocrinol*, 176:205-217.

Canaza-Cayo AW, Lopes PS, da Silva MVGB, de Almeida Torres R, Martins MF, Arbex WA, Cobuci JA. 2015. Genetic parameters for milk yield and lactation persistency using random regression models in girolando cattle. *Asian-Austr J Anim Sci*, 28:1407-1418.

Canfield RW, Butler WR. 1990. Energy balance and pulsatile LH secretion in early postpartum dairy cattle. *Domest Anim Endocrinol*, 7:323-330.

Canfield RW, Butler WR. 1991. Energy balance, first ovulation and the effects of naloxone on LH secretion in early postpartum dairy cows. *J Anim Sci*, 69:740-746.

Carnevale EM, Ramirez RJ, Squires EL, Alvarenga MA, Vanderwall DK, McCue PM. 2000. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. *Theriogenology*, 54:965-979.

Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke PM 2018. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology*, 114:165-172.

Chapinal N, Leblanc SJ, Carson ME, Leslie KE, Godden S, Capel M, Santos JEP, Overton MW, Duffield TF. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. *J Dairy Sci*, 95:5676-5682.

Collier RJ, Renquist BJ, Xiao Y. 2017. A 100-Year Review: Stress physiology including heat stress. *J Dairy Sci*, 100:10367-10380.

Cook NB. 2019. Designing facilities for the adult Dairy cow during the nonlactation and early lactation period. *Vet Clin N Am Food Anim Prac*, 35:125-138.

Dobson H, Smith RF. 2000. What is stress, and how does it affect reproduction? *Anim Reprod Sci*, 60-61:743-752.

D'Occhio MJ, Baruselli PS, Campanile G. 2019. Influence of nutrition, body condition, and metabolic status on reproduction in female beef cattle: A review. *Theriogenology*, 125:277-284.

Ealy AD, Drost M, Hansen PJ. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci*, 76:2899-2905.

Eckel EF, Ametaj BN. 2016. Invited review: Role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. *J Dairy Sci*, 99:5967-5990.

Esposito G, Irons PC, Webb EC, Chapwanya A. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Anim Reprod Sci*, 144:60-71.

Forde N, Lonergan P. 2017. Interferon-tau and fertility in ruminants. *Reproduction*, 154:F33-F43.

Fuenzalida MJ, Fricke PM, Ruegg PL. 2015. The association between occurrence and severity of subclinical and clinical mastitis on pregnancies per artificial insemination at first service of Holstein cows. *J Dairy Sci*, 98:3791-3805.

Funnell BJ, Hilton WM. 2016. Management and prevention of dystocia. *Vet Clin N Am Food Anim Prac*, 32:511-522.

Galvão KN, Flaminio MJBF, Brittin SB, Sper R, Fraga M, Caixeta L, Ricci A, Guard CL, Butler WR, Gilbert RO. 2010. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. *J Dairy Sci*, 93:2926-2937.

Gilbert RO. 2019. Symposium review: Mechanisms of disruption of fertility by infectious diseases of the reproductive tract. *J Dairy Sci*, 102:3754-3765.

Graugnard DE, Bionaz M, Trevisi E, Moyes KM, Salak-Johnson JL, Wallace RL, Drackley JK, Bertoni G, Loor JJ. 2012. Blood immunometabolic indices and polymorphonuclear neutrophil function in peripartum dairy cows are altered by level of dietary energy prepartum. *J Dairy Sci*, 95:1749-1758.

Green JC, Meyer JP, Williams AM, Newsom EM, Keisler DH, Lucy MC. 2012. Pregnancy development from day 28 to 42 of gestation in postpartum Holstein cows that were either milked (lactating) or not milked (not lactating) after calving. *Reproduction*, 143:699-711.

Gwazdauskas FC. 1985. Effects of climate on reproduction in cattle. *J Dairy Sci*, 68:1568-1578.

Hansen PJ. 2007. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology*, 68 (Suppl 1): S242-249.

Hansen PJ. 2009. Effects of heat stress on mammalian reproduction. *Phil Trans Royal Soc London. Series B, Biol Sci*, 364:3341-3350.

Hansen PJ, Aréchiga CF. 1999. Strategies for managing reproduction in the heat-stressed dairy cow. *J Anim Sci*, 77(Suppl 2):36-50.

Hansen PJ, Soto P, Natzke RP. 2004. Mastitis and fertility in cattle - possible involvement of inflammation or immune activation in embryonic mortality. *Am J Reprod Immunol*, 51:294-301.

Hansen TR, Sinedino LDP, Spencer TE. 2017. Paracrine and endocrine actions of interferon tau (IFNT). *Reproduction*, 154:F45-F59.

Hemsworth PH, Coleman GJ, Barnett JL, Borg S. 2000. Relationships between human-animal interactions and productivity of commercial dairy cows. *J Anim Sci*, 78:2821-2831.

Hong H, Lee E, Lee IH, Lee S-R. 2019. Effects of transport stress on physiological responses and milk production in lactating dairy cows. *Asian-Austral J Anim Sci*, 32:442-451.

Huzzey JM, DeVries TJ, Valois P, von Keyserlingk M a G. 2006. Stocking density and feed barrier design affect the feeding and social behavior of dairy cattle. *J Dairy Sci*, 89:126-133.

Jensen MB, Proudfoot KL. 2017. Effect of group size and health status on behavior and feed intake of multiparous dairy cows in early lactation. *J Dairy Sci*, 100:9759-9768.

Jewell MT, Cameron M, Spears J, McKenna SL, Cockram MS, Sanchez J, Keefe GP. 2019. Prevalence of hock, knee, and neck skin lesions and associated risk

factors in dairy herds in the Maritime Provinces of Canada. *J Dairy Sci*, 102:3376-3391.

Kammel DW, Burgi K, Lewis J. 2019. Design and management of proper handling systems for dairy cows. *Vet Clin N Am Food Anim Prac*, 35:195-227.

Karsch FJ, Battaglia DF, Breen KM, Debus N, Harris TG. 2002. Mechanisms for ovarian cycle disruption by immune/inflammatory stress. *Stress*, 5:101-112.

al-Katanani YM, Webb DW, Hansen PJ. 1999. Factors affecting seasonal variation in 90-day nonreturn rate to first service in lactating Holstein cows in a hot climate. *J Dairy Sci*, 82:2611-2616.

Kawashima C, Matsui M, Shimizu T, Kida K, Miyamoto A. 2012. Nutritional factors that regulate ovulation of the dominant follicle during the first follicular wave postpartum in high-producing dairy cows. *J Reprod Dev*, 58:10-16.

von Keyserlingk MAG, Weary DM. 2017. A 100-Year Review: Animal welfare in the J Dairy Sci,-The first 100 years. *J Dairy Sci*, 100:10432-10444.

Kumar N, Manimaran A, Kumaresan A, Jeyakumar S, Sreela L, Mooventhan P, Sivaram M. 2017. Mastitis effects on reproductive performance in dairy cattle: a review. *Trop Anim Health Prod*, 49:663-673.

LeBlanc SJ. 2012. Interactions of metabolism, inflammation, and reproductive tract health in the postpartum period in dairy cattle. *Reprod Domest Anim*, 47 (Suppl 5):18-30.

Leroy JLMR, Vanholder T, Van Knegsel ATM, Garcia-Ispierto I, Bols PEJ. 2008. Nutrient prioritization in dairy cows early postpartum: mismatch between metabolism and fertility? *Reprod Domest Anim*, 43 (Suppl 2):96-103.

Leroy JLMR, Rizos D, Sturmey R, Bossaert P, Gutierrez-Adan A, Van Hoeck V, Valckx S, Bols PEJ. 2011. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. *Reprod Fertil Dev*, 24:1-12.

Lima MLP, Negrão JA, de Paz CCP, Grandin T. 2018. Minor corral changes and adoption of good handling practices can improve the behavior and reduce cortisol release in Nellore cows. *Trop Anim Health Prod*, 50:525-530.

Lindahl C, Pinzke S, Herlin A, Keeling LJ. 2016. Human-animal interactions and safety during dairy cattle handling--Comparing moving cows to milking and hoof trimming. *J Dairy Sci*, 99:2131-2141.

Lonergan P. 2011. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology*, 76:1594-1601.

Lucy MC. 2004. Mechanisms linking the somatotropic axis with insulin: Lessons from the postpartum dairy cow. *Proc NZ Soc Anim Prod*, 64:19-23.

Lucy MC. 2011. Growth hormone regulation of follicular growth. *Reprod Fertil Dev*, 24:19-28.

Lucy MC. 2019. Symposium review: Selection for fertility in the modern dairy cow-Current status and future direction for genetic selection. *J Dairy Sci*, 102:3706-3721.

Lucy MC, Green JC, Meyer JP, Williams AM, Newsom EM, Keisler DH. 2012. Short communication: glucose and fructose concentrations and expression of glucose transporters in 4- to 6-week pregnancies collected from Holstein cows that were either lactating or not lactating. *J Dairy Sci*, 95:5095-5101.

Lucy MC, Butler ST, Garverick HA. 2014. Endocrine and metabolic mechanisms linking postpartum glucose with early embryonic and foetal development in dairy cows. *Animal*, 8:82-90.

Lucy MC, Evans TJ, Poock SE. 2016. Lymphocytic foci in the endometrium of pregnant dairy cows: Characterization and association with reduced placental weight and embryonic loss. *Theriogenology*, 86:1711-1719.

Miglior F, Fleming A, Malchiodi F, Brito LF, Martin P, Baes CF. 2017. A 100-Year Review: Identification and genetic selection of economically important traits in dairy cattle. *J Dairy Sci*, 100:10251-10271.

Minton JE. 1994. Function of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system in models of acute stress in domestic farm animals. *J Anim Sci*, 72:1891-1898.

Munksgaard L, De Passillé AM, Rushen J, Thodberg K, Jensen MB. 1997. Discrimination of people by dairy cows based on handling. *J Dairy Sci*, 80:1106-1112.

Newcomer BW, Givens D. 2016. Diagnosis and Control of Viral Diseases of Reproductive Importance: Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhea. *Vet Clin N Amer Food Anim Prac*, 32:425-441.

O'Driscoll K, von Keyserlingk MAG, Weary DM. 2006. Effects of mixing on drinking and competitive behavior of dairy calves. *J Dairy Sci*, 89:229-233.

Overton TR, McArt JAA, Nydam DV. 2017. A 100-Year Review: Metabolic health indicators and management of dairy cattle. *J Dairy Sci*, 100:10398-10417.

Pohler KG, Pereira MHC, Lopes FR, Lawrence JC, Keisler DH, Smith MF, Vasconcelos JLM, Green JA. 2016. Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. *J Dairy Sci*, 99:1584-1594.

Polsky L, von Keyserlingk MAG. 2017. Invited review: Effects of heat stress on dairy cattle welfare. *J Dairy Sci*, 100:8645-8657.

Putney DJ, Drost M, Thatcher WW. 1989. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology*, 31:765-778.

Radcliff RP, McCormack BL, Crooker BA, Lucy MC. 2003. Growth hormone (GH. binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. *J Dairy Sci*, 86:3933-3940.

Ralph CR, Lehman MN, Goodman RL, Tilbrook AJ 2016. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? Reproduction, 152:R1-R14.

Roth Z. 2017. Effect of Heat Stress on Reproduction in Dairy Cows: Insights into the Cellular and Molecular Responses of the Oocyte. *Ann Rev Animl Biosci* 5:151-170.

Roth Z. 2018a. Stress-induced alterations in oocyte transcripts are further expressed in the developing blastocyst. *Mol Reprod Dev*, 85:821-835.

Roth Z. 2018b. Symposium review: Reduction in oocyte developmental competence by stress is associated with alterations in mitochondrial function. *J Dairy Sci*, 101:3642-3654.

Sakatani M. 2017. Effects of heat stress on bovine preimplantation embryos produced in vitro. *J Reprod Dev*, 63:347-352.

Scott CJ, Rose JL, Gunn AJ,, McGrath BM 2019. Kisspeptin and the regulation of the reproductive axis in domestic animals. *J Endocrinol*, 240:R1-R16.

Sheldon IM. 2015. Milk somatic cell counts and pregnancy rates in dairy cattle. *Vet Rec*, 176:409-410.

Sheldon IM, Cronin JG, Pospiech M, Turner ML. 2018. Symposium review: Mechanisms linking metabolic stress with innate immunity in the endometrium. *J Dairy Sci*, 101:3655-3664.

Shock DA, Renaud DL, Roche SM, Poliquin R, Thomson R, Olson ME. 2018. Evaluating the impact of meloxicam oral suspension administered at parturition on subsequent production, health, and culling in dairy cows: A randomized clinical field trial. *PloS One*, 13:e0209236.

Sordillo LM. 2018. Mammary Gland Immunobiology and Resistance to Mastitis. *Vet Clin N Amer Food Anim Prac*, 34:507-523.

Spencer TE, Forde N, Lonergan P. 2016. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy Sci*, 99:5941-5950.

Thissen JP, Ketelslegers JM, Underwood LE. 1994. Nutritional regulation of the insulin-like growth factors. *Endocrine Rev*, 15:80-101.

Ulberg LC, Burfening PJ. 1967. Embryo death resulting from adverse environment on spermatozoa or ova. *J Anim Sci*, 26:571-577.

Val-Laillet D, Veira DM, von Keyserlingk M a G. 2008. Short communication: dominance in free-stall-housed dairy cattle is dependent upon resource. *J Dairy Sci*, 91:3922-3926.

Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science (New York, N.Y.)* 324:1029-1033.

Velazquez MA, Spicer LJ, Wathes DC. 2008. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. *Domest Anim Endocrinol*, 35:325-342.

Veldhuis JD, Roemmich JN, Richmond EJ, Bowers CY. 2006. Somatotropic and gonadotropic axes linkages in infancy, childhood, and the puberty-adult transition. *Endocrine Rev* 27:101-140.

Wade GN, Jones JE. 2004. Neuroendocrinology of nutritional infertility. *Am J Physiol Reg Integrat Comp Physiol*, 287:R1277-1296.

Wathes DC. 2012. Mechanisms linking metabolic status and disease with reproductive outcome in the dairy cow. *Reprod Dom Anim*, 47 (Suppl 4):304-312.

Wathes DC, Cheng Z, Bourne N, Taylor VJ, Coffey MP, Brotherstone S. 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. *Domest Anim Endocrinol*, 33:203-225.

White HM. 2015. The Role of TCA Cycle Anaplerosis in ketosis and fatty liver in periparturient dairy cows. *Animals*, 5:793-802.

Wilde D. 2006. Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. *Anim Reprod Sci*, 96:240-249.

Wilson SJ, Marion RS, Spain JN, Spiers DE, Keisler DH, Lucy MC. 1998. Effects of controlled heat stress on ovarian function of dairy cattle. 1. Lactating cows. *J Dairy Sci*, 81:2124-2131.

Wiltbank MC, Sartori R, Herlihy MM, Vasconcelos JLM, Nascimento AB, Souza AH, Ayres H, Cunha AP, Keskin A, Guenther JN 2011. Managing the dominant follicle in lactating dairy cows. *Theriogenology*, 76:1568-1582.

Wolfenson D, Lew BJ, Thatcher WW, Graber Y, Meidan R. 1997. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. *Anim Reprod Sci*, 47:9-19.

DOI: 10.21451/1984-3143-AR2019-0062

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

DNA methylation, environmental exposures and early embryo development

Mélanie Breton-Larrivée[1,], Elizabeth Elder[1,**], Serge McGraw[1,2,*]**

[1]Department of Biochemistry and Molecular Medicine, Université de Montréal, Research Center of the CHU Sainte-Justine. Montreal, Canada.
[2]Department of Obstetrics & Gynecology, Université de Montréal, Research Center of the CHU Sainte-Justine, Montréal, Canada.

Abstract

The first crucial step in the developmental program occurs during pre-implantation, the time after the oocyte has been fertilized and before the embryo implants in the uterus. This period represents a vulnerable window as the epigenome undergoes dynamic changes in DNA methylation profiles. Alterations in the early embryonic reprogramming wave can impair DNA methylation patterns and induce permanent changes to the developmental program, leading to the onset of adverse health outcomes in offspring. Although there is an increasing body of evidence indicating that harmful exposures during pre-implantation embryo development can trigger lasting epigenetic alterations in offspring, the mechanisms are still not fully understood. Since physiological or pathological changes in DNA methylation can occur as a response to environmental cues, proper environmental milieu plays a critical role in the success of embryonic development. In this review, we depict the mechanisms behind the embryonic epigenetic reprogramming of DNA methylation and highlight how maternal environmental stressors (e.g., alcohol, heat stress, nutrient availability) during pre-implantation and assisted reproductive technology procedures affect development and DNA methylation marks.

Keywords: epigenetics, DNA methylation, pre-implantation embryos, prenatal exposures, developmental programming.

Introduction

The rapidly emerging field of epigenetics studies genome modifications that regulate gene expression without altering the content of the genetic sequence. DNA and histones —*the structural proteins of the chromatin*— can possess a layer of reversible epigenetic modifications that contribute to how genes are expressed and how they interact within a cell. Epigenetic modifications are chemical tags, such as phosphate, methyl and acetyl groups, affixed to the histone proteins and DNA by a highly dynamic and synergic network of nuclear enzymes that modulate chromatin availability thereby regulating gene expression (Jenuwein and Allis, 2001; Gibney and Nolan, 2010). The epigenome is of utmost importance and comprehensively susceptible to environmental factors, (Marsit, 2015; Legault *et al*., 2018; Norouzitallab *et al*., 2019) particularly during the early stages of embryo development as its epigenetic regulation is concomitant with proper cell fate determination (Morey *et al*., 2015; Ohbo and Tomizawa, 2015; Vougiouklakis *et al*., 2017). The most notable epigenetic mechanism during mammalian pre-implantation is the epigenetic reprogramming of DNA methylation that triggers embryonic genome activation, a pivotal step for proper embryo development. While these processes are similar between species, they differ in regards to the rate and timing of events and sex-specific variations. Although many studies have shown the highly significant physiological roles of the epigenome in mammalian development, it is still considerably misunderstood and insufficiently studied during pre-implantation, partially due to technological limitations as a consequence of the very small number of cells and the short duration of this stage of development. This review will depict the mechanisms behind the embryonic epigenetic reprogramming of DNA methylation and will assess the epigenetic consequences of various assisted reproductive technology (ART) procedures as well as how environmental stressors during pre-implantation will affect short-term and long-term development, focussing specifically on the maternal environment.

DNA Methylation

DNA methylation is the most widely understood epigenetic modification as a mechanism for gene expression mediation and is involved in many key physiological processes such as genomic imprinting, transposable elements silencing, X-chromosome inactivation and aging (Bird, 2002; Smith and Meissner, 2013). In mammals, DNA methylation occurs mainly on the cytosines of cytosine-guanine dinucleotides known as CpG sites (CpGs) (Razin and Cedar, 1991; Weber and Schubeler, 2007), though non-CpG (i.e. CpA, CpT, CpC) methylation can also be found at specific stages of cellular development, primarily in stem cells and brain tissues (Lister *et al*., 2013; Patil *et al*., 2014). The enzymes directly responsible for the methylation of DNA are DNA methyltransferases (DNMTs). DNMT3A and DNMT3B add *de novo* methylation thus they have been identified to be involved in the establishment of methylation patterns required for cell lineage determination during development (Okano *et al*., 1999;

*Corresponding author: serge.mcgraw@umontreal.ca
orcid.org/0000-0002-3504-8253
**Contributed equally
Received: May 13, 2019
Accepted: June 12, 2019

Li, 2002). For optimal activity, DNMT3A and DNMT3B require the accessory protein DNMT3L (DNA methyltransferase-like), a protein similar to DNMTs but lacking methyltransferase activity (Suetake *et al*., 2004). In contrast, DNMT1 carries out heritable DNA methylation pattern maintenance during cellular division due to its preference for substrates with hemi-methylated CpGs, *i.e.* only one methylated DNA strand, which would naturally occur during semi-conservative DNA replication (Leonhardt *et al*., 1992; Lei *et al*., 1996; Pradhan *et al*., 1999). Although DNA methylation patterns are heritable from cell to cell, it remains strikingly dynamic in nature. Physiological or pathological changes in DNA methylation can occur as a response to environmental cues, therefore demethylation is conjointly a greatly relevant process. Contrary to enzyme-mediated methylation, demethylation can occur passively or actively (Kishikawa *et al*., 2003). Passive genome demethylation is replication-dependant, caused by DNMT1 reduced activity resulting in an unspecific progressive dilution of DNA methylation over multiple consecutive cell divisions (Kagiwada *et al*., 2013; Wu and Zhang, 2014). Conversely, active demethylation is specific and is catalytically directed by ten-eleven translocation enzymes (TET1, TET2, TET3) (Tahiliani *et al*., 2009; Ito *et al*., 2010). The addition and erasure of DNA methylation and other epigenetic marks are the driving forces behind embryo development, as they dictate how, when and at what level genes are expressed.

CpGs are present all across the mammalian genome but their methylation will bear different consequences depending upon their location (e.g. promoter regions, gene bodies, enhancers) and upon their level of enrichment. CpG methylation located in gene bodies has been shown to promote high levels of gene expression whereas when located in promoter regions, it is associated with transcriptional silencing, which coordinates cellular differentiation (Goll and Bestor, 2005; Jones, 2012). In mammalian genomes, promoters comprised of highly CpG dense sequences, known as CpG islands (CGIs), control approximately 60-80% of genes depending on the species (Antequera and Bird, 1993; Saxonov *et al*., 2006). Methylation of CGIs of a promoter accompanied with repressive histone modifications (H3K9me3, H3K27me3) induces nucleosome compaction and prevents transcription factor (TF) binding, causing the repression of gene transcription. On the other hand, the promoter regions of transcribed genes have CGIs devoid of methylation along with active histone modifications (H3K4me2/3, H3K9ac) (Barski *et al*., 2007; Koch *et al*., 2007; Henikoff and Shilatifard, 2011; Severin *et al*., 2011), thus ensuring the open chromatin configuration that allows for TF binding and gene activation. However, recent studies have started to refute this general rule suggesting that, in some cases, the loss of methylation can be a consequence of TF binding as opposed to the cause of action, leading some to believe that gene activation may not always be methylation driven (Zhu *et al*., 2016a; Pacis *et al*., 2019).

A particularly important role of DNA methylation in mammalian development is genomic imprinting. A small cohort of genes called imprinted genes possesses germline differentially methylated regions (gDMRs). gDMRs acquire monoallelic genomic methylation in a parent-of-origin manner causing only one allele to be expressed (Reik *et al*., 2001; Ferguson-Smith, 2011). A more specific type of gDMR is imprinting control regions (ICRs) that are directly implicated in the binding of TFs and regulate the expression of multiple imprinted genes at a time (e.g., *H19* and *Igf2; Insulin-Like Growth Factor 2*) (Thorvaldsen *et al*., 1998; Fitzpatrick *et al*., 2002; Ideraabdullah *et al*., 2008). These genomic imprints are determined prior to fertilization in growing diplotene oocytes and in perinatal prospermatogonia and must be maintained throughout the entire lifespan of the new generation (Stoger *et al*., 1993; Kono *et al*., 1996; Davis *et al*., 2000; Ueda *et al*., 2000).

Embryonic Epigenetic Reprogramming

In early embryogenesis, the embryo undergoes a reprogramming wave of DNA methylation during which the global methylation profiles, with the exception of gDMRs, are remodeled. Shortly after fertilization, the zygotic genome remains separated into two distinct paternal and maternal pronuclei which must sustain extensive global demethylation to erase the germ cell-specific methylation profiles and implement totipotency prior to implantation of the embryo (Seisenberger *et al*., 2013). The demethylation mechanisms are known to be distinct for both pronuclei, but have not been fully characterized thus far. Another perplexing part of the reprogramming process is the maintenance of gDMR methylation patterns as it is a major requirement for normal mammalian development. In fact, the loss of genomic imprints during embryo development causes permanent damage to cellular functions since the embryo is unable to restore them (Howell *et al*., 1998; Howell *et al*., 2001; McGraw *et al*., 2013; McGraw *et al*., 2015). Since only one allele is inherently active, imprinted gene expression is hypersensitive to changes in regulation, which can cause dramatic effects on development as many imprinted genes have growth regulatory functions (Plasschaert and Bartolomei, 2014). Many studies in mice have demonstrated the prevalent involvement of DNMT1 variants (DNMT1o; DNMT1-oocyte, DNMT1s; somatic) in the maintenance of genomic imprints throughout embryonic epigenetic reprograming (Bostick *et al*., 2007; Arita *et al*., 2008; Avvakumov *et al*., 2008). How DNMT1 specifically recognizes and maintains gDMRs but does not maintain global methylation remains mostly unclear. However, *Dnmt1*$^{-/-}$ mice are embryonic lethal as the absence of *Dnmt1* causes the exhaustive loss of genomic imprints and does not allow for *de novo* methylation to be properly maintained during remethylation (Li *et al*., 1992). Moreover, we observed that the loss of *Dnmt1o* caused sex-specific placental defects in female embryos as well as perturbed imprinted X-inactivation (McGraw *et al*., 2013). These data highlight how a brief perturbation in the DNA methylation maintenance process of early stage embryos

can influence development, and further emphasize the importance of studying the impact of the maternal environment and sex-specific alterations during this critical period.

The loss of global DNA methylation during reprogramming initiates the embryonic genome activation, vital for proper development. The thoroughly timed expression of genes in embryonic genome activation is controlled by chromatin structural changes. A schematic representation of the mouse embryonic epigenetic reprogramming of DNA methylation is depicted in Figure 1, showing the dynamics of DNA methylation from the fertilization of the zygote to the maturation of embryonic and placental tissues. After global demethylation, the inner cell mass and the trophoblast gain *de novo* methylation catalyzed by DNMT3A and DNMT3B to implement the epigenetic patterns for the development of the embryo and the placenta (Red-Horse *et al*., 2004; Marikawa and Alarcon, 2009). Studies conducted in mice have indicated separate specific phenotypes in *Dnmt3a*$^{-/-}$ versus *Dnmt3b*$^{-/-}$. *Dnmt3a*$^{-/-}$ mice make it to term, albeit severely runted and die shortly thereafter, whereas *Dnmt3b*$^{-/-}$ are embryonic lethal (Niakan *et al*., 2012). The altered expression of these key enzymes is unmistakably symptomatic of epigenetic developmental disturbances, but it is becoming more and more evident that the regulation of the epigenome is also staggeringly sensitive to embryonic environment, particularly throughout the pre-implantation period.

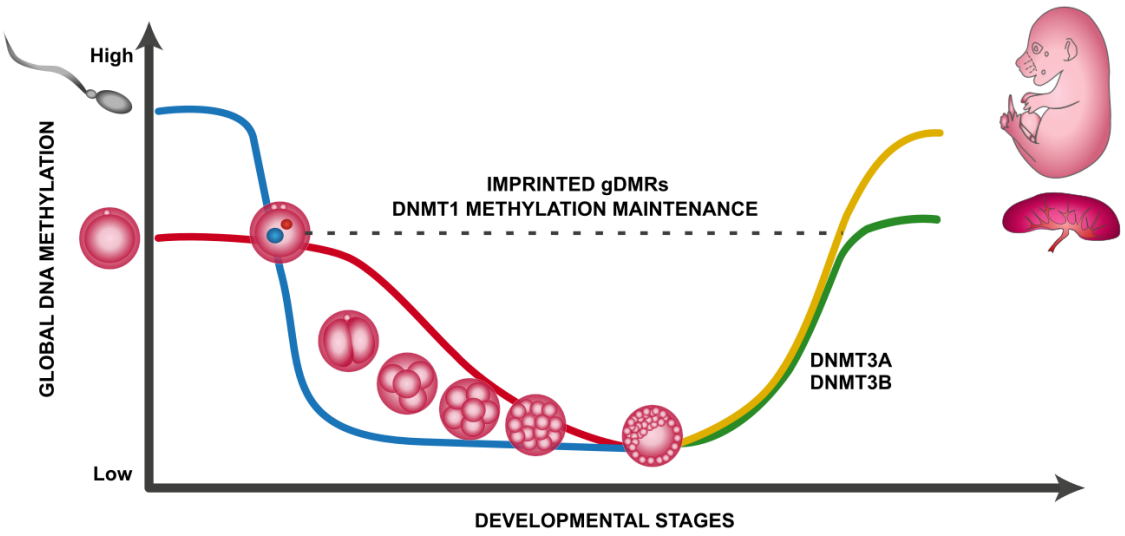


Figure 1. Global DNA demethylation and remethylation during the epigenetic reprogramming of early embryogenesis in mice. Soon after fertilization, the zygotic paternal and maternal pronuclei undergo global demethylation during the pre-implantation stages, except for gDMRs which are maintained via DNMT1 activity. The paternal genome (blue) is initially actively demethylated by the TET3 enzyme followed by passive demethylation, whereas the maternal genome (red) demethylation is solely passive due to DNMT1 inactivity, hence the sharper demethylation slope for the paternal curve. After implantation, the blastocyst acquires *de novo* methylation patterns catalyzed by DNMT3A and DNMT3B to establish the embryonic and placental programs imperative for development initiation.

Early embryonic environment and impact on epigenetic reprogramming events

A considerable amount of evidence has begun to show how epigenetic programming is susceptible to early embryonic environment, such as nutrient availability and toxin exposures, prior to implantation of the blastocyst. The dynamics of the embryonic wave of DNA methylation – proper erasure and *de novo* methylation or methylation maintenance – that is crucial to trigger the developmental program can become disturbed in response to these environmental cues leading to changes in gene expression and growth defects. Many have investigated the effects of the environment on embryo development using mainly *in vitro* models. However, the direct epigenetic impacts of the embryonic environment and the lasting effects on long-term development have been poorly studied, due to technological barriers and limited number of cells during pre-implantation stages. When studying early embryonic development exposures, one must be cautious when comparing *in utero* and *in vitro* models being that *in vitro* culture technologies have yet to accurately reproduce the maternal tract conditions. We have thus divided the following section describing the effects of environmental factors during early embryonic development in two parts: firstly, the influence of *in vitro* reproductive technologies of mammalian embryos, and secondly, the intrauterine exposures during pregnancy.

Assisted Reproductive Technology (ART)

ART is an umbrella term used to describe the assortment of medical procedures and approaches (e.g., superovulation, *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo *in vitro* culture (IVC)) that can be performed to achieve

pregnancy. Such procedures are now an integral part of human infertility treatment, as the number of children born by ART is estimated at more than 8 million worldwide (Weinerman, 2018). ART also plays a major role in animal reproduction to increase reproductive efficiency and genetic improvement in livestock, as well as to conserve endangered species. The outcomes of human pregnancies produced by ART have undergone intense scrutiny and while most children conceived using ART are healthy, these procedures have been associated with an increased risk of preeclampsia, intrauterine growth restriction, birth defects (Qin *et al*., 2016; Zhu *et al*., 2016b; Choux *et al*., 2018; Choufani *et al*., 2019) and imprinting disorders (Market-Velker *et al*., 2010; White *et al*., 2015). Although various studies have shown that ART may lead to epigenetic perturbations (El Hajj and Haaf, 2013; Urrego *et al*., 2014; Duranthon and Chavatte-Palmer, 2018), the etiology associated with ART and increased risk of perinatal complications is still poorly understood. However, the dynamic epigenome reprogramming during germ cell development and the pre-implantation period, especially of DNA methylation patterns, are processes that are prone to being affected by approaches used in ART and could provide biological plausibility.

ART procedures

Although it is unclear which ART procedure has the greatest influence, we know that the dramatic changes in embryo environment can induce long-term effects on the epigenome. In ovarian superstimulation, various studies suggest that the acquisition of imprinting patterns in the oocyte might be perturbed and lead to abnormal allelic expression in later embryo and placenta development (reviewed in Anckaert *et al*., 2013; McGraw and Trasler, 2013). However, studies show that superovulation treatments do not alter normal imprinted methylation acquisition in oocytes, but rather disrupt maternal-effect gene products that are required during pre-implantation for imprint maintenance (Denomme *et al*., 2011; Uysal *et al*., 2018). We also showed that some of these induced errors of imprinted gene expression (*H19*, *Igf2*) present in mid-gestation mouse placenta are no longer apparent at the end of the gestation (Fortier *et al*., 2008; Fortier *et al*., 2014). This suggests that even though superovulation produces abnormal oocytes that initiate altered expression of imprinted genes in embryos, compensatory mechanisms regulating imprinted gene networks are able to restore proper levels of gene expression during development. Although, as highlighted across the literature, the alterations in DNA methylation following ART procedures are not always striking and vary between studies (reviewed in Berntsen *et al*., 2019), in part because of distinctions in treatments used. It was recently reported that human placenta, but not cord blood, from IVF/ICSI showed decreased DNA methylation levels for imprinted loci *H19/IGF2* and *KCNQ1OT1*, as well as for specific repetitive elements (Choux *et al*., 2018), whereas in another recent study, no obvious overall differences in genome-wide DNA methylation differences in placental tissues were associated with ART (Choufani *et al*., 2019). Yet, a subset of ART pregnancies associated with ICSI showed marked decrease in placental DNA methylation levels at imprinted loci (*GNAS*, *SGCE*, *KCNQ1OT1* and *NNAT*). Not only do these studies reveal that ICSI generates distinct DNA methylation alterations in specific tissues compared to controls as opposed to less invasive ART procedures, they highlight the importance of carefully pairing and comparing equivalent ART procedures when designing epigenetic studies.

Another ART procedure that is routine practice in commercial and clinical settings is cryopreservation of oocytes and embryos. Flash-freezing cryopreservation protocols (i.e., vitrification) have been linked to epigenetic alterations. Selective loss of DNA methylation of imprinted loci was observed in blastocysts subsequent to fertilization of vitrified bovine and mouse oocytes (Chen, Zhang *et al*., 2016; Cheng *et al*., 2014), whereas others found no effect on DNA methylation levels at the *H19/IGF2* ICR loci at embryonic day 3 in human ICSI blastocysts following vitrification (Derakhshan-Horeh *et al*., 2016). When vitrification of mouse embryo at E2.5 (8-cell stage) was paired with IVC, transferred embryos revealed increased levels of global DNA methylation in both E9.5 fetus and placenta compared to IVC, but interestingly were similar to naturally mated derived samples (Ma *et al*., 2019). The long-term effect of vitrification was further observed in the fetus with increased DNA methylation levels at the imprinted *KvDMR1* loci and significant gene expression increase of *Dnmt1* and *Dnmt3b* compared to the IVC and natural mating groups. Together, the body of work on vitrification suggests that such exposures could influence the epigenome and lead to abnormal expression of imprinted genes. However, it is difficult to make any definitive conclusions regarding the influence of vitrification as most of these studies only assessed a limited number of loci for DNA methylation analyses, which are mostly restricted to imprinted genes, and did not investigate the long-term impact on postnatal development.

ART culture environment

As previously mentioned, although the vast majority of ART-conceived offspring are healthy, they have a higher frequency of birth defects suggesting epigenetic costs. A large body of research now supports that the *in vitro* culture environment has both long-lasting and significant repercussions on DNA methylation reprogramming events and embryonic development, but the exact mechanisms remain unclear. In humans, an increased prevalence of Beckwith-Wiedemann syndrome has been associated with ART procedures. This overgrowth disorder has similar adverse phenotypes and epigenetic profiles (e.g., loss of imprinting) as the large offspring syndrome in ruminants, (Chen *et al*., 2015) for which the incidence has been linked to the presence of serum in the culture media. (Young *et al*., 1998; Chen *et al*., 2013). As such,

the ART field has mostly limited the use of serum and has designed various serum-free and chemically defined media for livestock, mice, and humans.

Various commercial and custom culture systems exist but are not as complex and dynamic as the oviduct fluid. They may present a lack or excess of different key factors and metabolites when compared to the maternal reproductive environment (Morbeck *et al*., 2014a; Morbeck *et al*., 2014b; Morbeck *et al*., 2017). A number of different studies have investigated the impact of culture systems on epigenetic profiles in humans and other animals, but a direct correlation between results is challenging as additional associated parameters (e.g., culture conditions, protocols, ART-procedures) may introduce a range of confounding and unpredictable variables. To circumvent these effects, Market-Velker *et al*. (2010) undertook a direct side-by-side comparison between naturally mated mouse embryos cultured from the 2-cell stage to the blastocyst stage in commercial systems and *in vivo*-derived blastocyst. They uncovered that all commercial media compromised the early embryo's proficiency in maintaining genomic imprinting profiles of *H19*, *Peg3*, and *Snrpn* to a variable extent. Although some media systems appeared to be more suitable for maintaining DNA methylation levels on these imprinted loci, we cannot know how the rest of the genome behaves under these conditions because of the narrow epigenetic analyses that were performed. Interestingly, a recent study tested the addition of natural reproductive fluids in the culture system to safeguard the embryo's epigenome. They showed that by using natural reproductive fluids they could produce IVF-blastocysts with reduced morphological, epigenetic and transcriptomic anomalies when compared to porcine blastocysts produced from unsupplemented IVF protocols (Canovas *et al*., 2017). Furthermore, by using both whole-genome DNA methylation and RNA-seq approaches of single blastocysts, they were able to demonstrate that the addition of oviductal tract fluid compensated for the lack of specific factors in standard culture medium required for proper development. Since this strategy has been successful so far in improving the ART procedures in mice, humans and other livestock animals, it shows great potential for rescuing troubled early embryo development and future negative impacts in offspring.

Maternal and environmental influences

It is now well established that the maternal environment (e.g., nutrition, stress, toxicants) can create an adverse *in utero* milieu that affects the fetal developmental program and increase disease susceptibility in adulthood (aka. Developmental Origins of Health and Disease; DoHaD hypothesis). Since the *all-or-none* phenomenon once presumed that exposure that occurs on early stage embryos results in either death or in no adverse outcome, little research on the impact of harmful maternal environment on pre-implantation embryos was done in the past. However, this once pervasive tenet is now being revisited as several studies demonstrate that the direct contact of pre-implantation embryo with the cells of the mother's reproductive tract can influence future development via interference with epigenetic mechanisms (Adam, 2012). Here, we will underline how adverse *in uterine* conditions triggered by the maternal environment (alcohol, heat stress) can have deleterious effects on the early embryonic epigenome.

Adverse stressors

Alcohol has teratogenic and neurotoxic effects on numerous potential mechanisms such as folate metabolism and DNMTs activity (Garro *et al*., 1991; Bielawski *et al*., 2002; Bonsch *et al*., 2006; Varela-Rey *et al*., 2013). We know that an exposure to alcohol during pregnancy can lead to abnormal brain development and cause fetal alcohol spectrum disorders (FASD), with symptoms ranging from craniofacial abnormalities to intellectual deficiency, behavioral difficulties and learning disabilities (Chudley *et al*., 2005; Cook *et al*., 2016; Legault *et al*., 2018). Although pioneer work demonstrated that early embryonic alcohol exposure can negatively influence development (Checiu and Sandor, 1986; Fazakas-Todea *et al*., 1986; Wiebold and Becker, 1987; Padmanabhan and Hameed, 1988), we still don't fully understand how alcohol directly impacts the early embryo, especially its epigenome. A recent report shows that porcine zygotes exposed to alcohol *in vitro* have a lower rate of blastocyst formation, with blastocysts having increased mitochondrial dysfunctions and abnormal gene expression (Page-Lariviere *et al*., 2017). Haycock and Ramsay (2009) did show in a mouse model that alcohol exposure at E1.5 and E2.5 was associated with loss of *H19* imprinted DNA methylation in the placenta at E10.5 and growth restriction (Haycock and Ramsay, 2009). In early stage embryos, ethanol exposure seems to have a lasting impact on *Dnmt1* by reducing its expression, whereas *Dnmt3a* and *Dnmt3b* expression levels remained the same (Dasmahapatra and Khan, 2015). Since *Dnmt1* is required for the maintenance of DNA methylation profiles, especially imprinted gene methylation during the early embryonic reprogramming wave (Hirasawa *et al*., 2008; McGraw *et al*., 2013), alcohol exposure might compromise proper *Dnmt1* function and lead to altered epigenetic phenotypes. Although prenatal cigarette and recreational drugs (e.g., cocaine, cannabis) exposure have been linked to lasting behavioral and neurodevelopmental impairments, low birth weight, preterm birth, poor intrauterine growth and even infant death (Wehby *et al*., 2011), as well as alterations in DNA methylation and epigenetic profiles (Novikova *et al*., 2008; Toro *et al*., 2008; Breton *et al*., 2009; Guerrero-Preston *et al*., 2010; Suter *et al*., 2010; Toledo-Rodriguez *et al*., 2010; DiNieri *et al*., 2011), none of the prenatal expositions were done on pre-implantation embryos. As such, preclinical animal models of early embryonic exposure are needed to determine the deleterious consequence of cigarette smoking and recreational drugs on development, epigenome and gene expression. By being aware of the deleterious effects of cigarette and drug expositions on

the embryo during the first days of gestation days, women wanting to conceive might stop their consumption as preventive measures to protect their embryo.

Livestock does not have comparable environmental stressors, however, they are exposed to changing environmental conditions that affect their fertility. For example, livestock fertility, especially in dairy cows, is particularly vulnerable to higher temperature and humidity. Heat stress disrupts many metabolism processes (e.g., microtubules and microfilaments reorganization, reactive oxygen species production, DNA fragmentation and apoptosis) in embryos, leading to disrupted embryo development and increased embryonic mortality (Zhu *et al*., 2008; Koyama *et al*., 2012; de Barros and Paula-Lopes, 2018). Heat stress has a greater impact on pre-implantation embryos since heat resistance mechanisms are not fully developed at this stage. Embryos at 2-cell or 4-cell stage will be more affected since the acquisition of these processes overlaps with zygotic genome activation (ZGA) and early embryos do not respond to proapoptotic signals (de Barros and Paula-Lopes, 2018). One study suggests that the epigenetic changes seem to predominantly impact paternal imprinting genes, as the paternal genome is demethylated faster in the first days of embryo development compared to the maternal genome (Zhu *et al*., 2008). They reported that blastocysts resulting from mouse zygotes exposed to a 1 hour 40°C heat shock prior to IVC, showed loss of DNA methylation for paternally imprinted genes *H19* and *Igf-2r*, but normal DNA methylation for maternally imprinted genes *Peg1* and *Peg3*. However, since these embryos were only treated and cultured *in vitro*, it would be pertinent to retrieve oocytes, zygotes or embryos from livestock animals exposed to heat stress to define how genome-wide DNA methylation profiles are disturbed.

Conclusion

Epigenetic modifications, specifically DNA methylation, play a crucial role in embryo development and are vulnerable to prenatal environmental factors and exposures occurring during the pre-implantation period. So far, a handful of *in vitro* studies have explored the effects of assisted reproductive technologies as well as prenatal environmental conditions and exposures, such as alcohol consumption and heat stress, during pre-implantation looking at short-term effects of severe epigenetic disturbances causing early manifestation of serious developmental phenotypes. Though mild impacts during pre-implantation are hugely understudied and may cause latent long-term effects on postnatal development. Therefore, there is a dire need to study the impacts of early embryo *in vitro* exposures past the blastocyst-stage using embryo transfer experiments, as well as early embryo *in vivo* exposure models, while also taking into consideration the importance of sex-specific variations and timing of exposure. Moreover, very few studies have been able to establish the direct link between DNA methylation alterations and observed phenotypes, mainly because of the limitations in studying the methylome in the early stages of development. Cutting-edge adaptations of standard whole-genome and reduced bisulfite genome sequencing technologies are now rapidly emerging, permitting high-resolution low-input single-cell methylation analyses. Thanks to these technological and intellectual advancements, as well as the integrative analysis of multi-omics layers, a promising future lies ahead for the study of pre-implantation epigenetics.

Author contributions

MBL: Writing – original draft; EE: Writing – original draft, Writing – review & editing; SM: Conceptualization, Writing – review & editing, Supervision.

Conflict of interest

Authors declare that they have no conflict of interest

Acknowledgments and Funding disclosure statement

MBL and SM are supported by Fonds de Recherche du Québec en Santé (FRQS). MBL is also supported by Réseau Québécois en Reproduction (RQR), and EE is supported by Fonds de Recherche du Québec en Nature et Technologies (FRQNT). We also thank Karine Doiron for figure design and editing.

References

Adam MP. 2012. The all-or-none phenomenon revisited. *Birth Defects Res A Clin Mol Teratol*, 94:664-669.

Anckaert E, De Rycke M, Smitz J. 2013. Culture of oocytes and risk of imprinting defects. *Hum Reprod Update*, 19:52-66.

Antequera F, Bird A. 1993. Number of CpG islands and genes in human and mouse. *Proc Natl Acad Sci U S A*, 90:11995-11999.

Arita K, Ariyoshi M, Tochio H, Nakamura Y, Shirakawa M. 2008. Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism. *Nature*, 455:818-821.

Avvakumov GV, Walker JR, Xue S, Li Y, Duan S, Bronner C, Arrowsmith CH, Dhe-Paganon S. 2008. Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. *Nature*, 455:822-825.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. 2007. High-resolution profiling of histone methylations in the human genome. *Cell*, 129:823-837.

Berntsen S, Soderstrom-Anttila V, Wennerholm UB, Laivuori H, Loft A, Oldereid NB, Romundstad LB, Bergh C, Pinborg A. 2019. The health of children conceived by ART: 'the chicken or the egg?'. *Hum Reprod Update*, 25:137-158.

Bielawski DM, Zaher FM, Svinarich DM, Abel EL.

2002. Paternal alcohol exposure affects sperm cytosine methyltransferase messenger RNA levels. *Alcohol Clin Exp Res*, 26:347-351.

Bird A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev*, 16:6-21.

Bonsch D, Lenz B, Fiszer R, Frieling H, Kornhuber J, Bleich S. 2006. Lowered DNA methyltransferase (DNMT-3b) mRNA expression is associated with genomic DNA hypermethylation in patients with chronic alcoholism. *J Neural Transm (Vienna)*, 113:1299-1304.

Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. 2007. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science*, 317:1760-1764.

Breton CV, Byun HM, Wenten M, Pan F, Yang A, Gilliland FD. 2009. Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med*, 180:462-467.

Canovas S, Ivanova E, Romar R, Garcia-Martinez S, Soriano-Ubeda C, Garcia-Vazquez FA, Saadeh H, Andrews S, Kelsey G, Coy P. 2017. DNA methylation and gene expression changes derived from assisted reproductive technologies can be decreased by reproductive fluids. *Elife*, 6.

Checiu M, Sandor S. 1986. The effect of ethanol upon early development in mice and rats. IX. Late effect of acute preimplantation intoxication in mice. *Morphol Embryol (Bucur)*, 32:5-11.

Chen Z, Hagen DE, Elsik CG, Ji T, Morris CJ, Moon LE, Rivera RM. 2015. Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction. *Proc Natl Acad Sci U S A*, 112:4618-4623.

Chen Z, Robbins KM, Wells KD, Rivera RM. 2013. Large offspring syndrome: a bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann. *Epigenetics*, 8:591-601.

Choufani S, Turinsky AL, Melamed N, Greenblatt E, Brudno M, Berard A, Fraser WD, Weksberg R, Trasler J, Monnier P. 2019. Impact of assisted reproduction, infertility, sex and paternal factors on the placental DNA methylome. *Hum Mol Genet*, 28:372-385.

Choux C, Binquet C, Carmignac V, Bruno C, Chapusot C, Barberet J, Lamotte M, Sagot P, Bourc'his D, Fauque P. 2018. The epigenetic control of transposable elements and imprinted genes in newborns is affected by the mode of conception: ART versus spontaneous conception without underlying infertility. *Hum Reprod*, 33:331-340.

Chudley AE, Conry J, Cook JL, Loock C, Rosales T, LeBlanc N. 2005. Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis. *Cmaj*, 172:S1-s21.

Cook JL, Green CR, Lilley CM, Anderson SM, Baldwin ME, Chudley AE, Conry JL, LeBlanc N, Loock CA, Lutke J, Mallon BF, McFarlane AA, Temple VK, Rosales T. 2016. Fetal alcohol spectrum disorder: a guideline for diagnosis across the lifespan. *Cmaj*, 188:191-197.

Dasmahapatra AK, Khan IA. 2015. DNA methyltransferase expressions in Japanese rice fish (Oryzias latipes) embryogenesis is developmentally regulated and modulated by ethanol and 5-azacytidine. *Comp Biochem Physiol C Toxicol Pharmacol*, 176-177:1-9.

Davis TL, Yang GJ, McCarrey JR, Bartolomei MS. 2000. The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum Mol Genet*, 9:2885-2894.

de Barros FRO, Paula-Lopes FF. 2018. Cellular and epigenetic changes induced by heat stress in bovine preimplantation embryos. *Mol Reprod Dev*, 85:810-820.

Denomme MM, Zhang L, Mann MR. 2011. Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition. *Fertil Steril*, 96:734-738.e732.

Derakhshan-Horeh M, Abolhassani F, Jafarpour F, Moini A, Karbalaie K, Hosseini SM, Nasr-Esfahani MH. 2016. Vitrification at Day3 stage appears not to affect the methylation status of H19/IGF2 differentially methylated region of in vitro produced human blastocysts. *Cryobiology*, 73:168-174.

DiNieri JA, Wang X, Szutorisz H, Spano SM, Kaur J, Casaccia P, Dow-Edwards D, Hurd YL. 2011. Maternal cannabis use alters ventral striatal dopamine D2 gene regulation in the offspring. *Biol Psychiatry*, 70:763-769.

Duranthon V, Chavatte-Palmer P. 2018. Long term effects of ART: What do animals tell us? *Mol Reprod Dev*, 85:348-368.

El Hajj N, Haaf T. 2013. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. *Fertil Steril*, 99:632-641.

Fazakas-Todea I, Sandor S, Hateganu M, Perta D, Checiu I, Stefanescu S. 1986. The effect of ethanol upon early development in mice and rats. X. The effect of acute intoxication with some alcoholic beverages upon preimplantation development in rats. *Morphol Embryol (Bucur)*, 32:159-164.

Ferguson-Smith AC. 2011. Genomic imprinting: the emergence of an epigenetic paradigm. *Nat Rev Genet*, 12:565-575.

Fitzpatrick GV, Soloway PD, Higgins MJ. 2002. Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. *Nat Genet*, 32:426-431.

Fortier AL, Lopes FL, Darricarrere N, Martel J, Trasler JM. 2008. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet*, 17:1653-1665.

Fortier AL, McGraw S, Lopes FL, Niles KM, Landry M, Trasler JM. 2014. Modulation of imprinted gene expression following superovulation. *Mol Cell Endocrinol*, 388:51-57.

Garro AJ, McBeth DL, Lima V, Lieber CS. 1991. Ethanol consumption inhibits fetal DNA methylation in mice: implications for the fetal alcohol syndrome. *Alcohol Clin Exp Res*, 15:395-398.

Gibney ER, Nolan CM. 2010. Epigenetics and gene expression. *Heredity (Edinb)*, 105:4-13.

Goll MG, Bestor TH. 2005. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*, 74:481-514.

Guerrero-Preston R, Goldman LR, Brebi-Mieville P,

Ili-Gangas C, Lebron C, Witter FR, Apelberg BJ, Hernandez-Roystacher M, Jaffe A, Halden RU, Sidransky D. 2010. Global DNA hypomethylation is associated with in utero exposure to cotinine and perfluorinated alkyl compounds. *Epigenetics*, 5:539-546.

Haycock PC, Ramsay M. 2009. Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol Reprod*, 81:618-627.

Henikoff S, Shilatifard A. 2011. Histone modification: cause or cog? *Trends Genet*, 27:389-396.

Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, Sasaki H. 2008. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev*, 22:1607-1616.

Howell CY, Bestor TH, Ding F, Latham KE, Mertineit C, Trasler JM, Chaillet JR. 2001. Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. *Cell*, 104:829-838.

Howell CY, Steptoe AL, Miller MW, Chaillet JR. 1998. cis-Acting signal for inheritance of imprinted DNA methylation patterns in the preimplantation mouse embryo. *Mol Cell Biol*, 18:4149-4156.

Ideraabdullah FY, Vigneau S, Bartolomei MS. 2008. Genomic imprinting mechanisms in mammals. *Mutat Res*, 647:77-85.

Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. 2010. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*, 466:1129-1133.

Jenuwein T, Allis CD. 2001. Translating the histone code. *Science*, 293:1074-1080.

Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet*, 13:484-492.

Kagiwada S, Kurimoto K, Hirota T, Yamaji M, Saitou M. 2013. Replication-coupled passive DNA demethylation for the erasure of genome imprints in mice. *Embo j*, 32:340-353.

Kishikawa S, Murata T, Ugai H, Yamazaki T, Yokoyama KK. 2003. Control elements of Dnmt1 gene are regulated in cell-cycle dependent manner. *Nucleic Acids Res Suppl*:307-308.

Koch CM, Andrews RM, Flicek P, Dillon SC, Karaoz U, Clelland GK, Wilcox S, Beare DM, Fowler JC, Couttet P, James KD, Lefebvre GC, Bruce AW, Dovey OM, Ellis PD, Dhami P, Langford CF, Weng Z, Birney E, Carter NP, Vetrie D, Dunham I. 2007. The landscape of histone modifications across 1% of the human genome in five human cell lines. *Genome Res*, 17:691-707.

Kono T, Obata Y, Yoshimzu T, Nakahara T, Carroll J. 1996. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nat Genet*, 13:91-94.

Koyama H, Ikeda S, Sugimoto M, Kume S. 2012. Effects of Folic Acid on the Development and Oxidative Stress of Mouse Embryos Exposed to Heat Stress. *Reproduction in Domestic Animals*, 47:921-927.

Legault LM, Bertrand-Lehouillier V, McGraw S. 2018. Pre-implantation alcohol exposure and developmental programming of FASD: an epigenetic perspective. *Biochem Cell Biol*, 96:117-130.

Lei H, Oh SP, Okano M, Juttermann R, Goss KA, Jaenisch R, Li E. 1996. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development*, 122:3195-3205.

Leonhardt H, Page AW, Weier HU, Bestor TH. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell*, 71:865-873.

Li E, Bestor TH, Jaenisch R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell*, 69:915-926.

Li E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet*, 3:662-673.

Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, Lucero J, Huang Y, Dwork AJ, Schultz MD, Yu M, Tonti-Filippini J, Heyn H, Hu S, Wu JC, Rao A, Esteller M, He C, Haghighi FG, Sejnowski TJ, Behrens MM, Ecker JR. 2013. Global epigenomic reconfiguration during mammalian brain development. *Science*, 341:1237905.

Ma Y, Ma Y, Wen L, Lei H, Chen S, Wang X. 2019. Changes in DNA methylation and imprinting disorders in E9.5 mouse fetuses and placentas derived from vitrified eight-cell embryos. *Mol Reprod Dev*, 86:404-415.

Marikawa Y, Alarcon VB. 2009. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. *Mol Reprod Dev*, 76:1019-1032.

Market-Velker BA, Fernandes AD, Mann MR. 2010. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. *Biol Reprod*, 83:938-950.

Marsit CJ. 2015. Influence of environmental exposure on human epigenetic regulation. *J Exp Biol*, 218:71-79.

McGraw S, Oakes CC, Martel J, Cirio MC, de Zeeuw P, Mak W, Plass C, Bartolomei MS, Chaillet JR, Trasler JM. 2013. Loss of DNMT1o disrupts imprinted X chromosome inactivation and accentuates placental defects in females. *PLoS Genet*, 9:e1003873.

McGraw S, Trasler J. 2013. Oocyte epigenetics and the risks for imprinting disorders associated with assisted reproduction. . *Biology & Pathology of the Oocyte 2nd Edition*, Cambridge University Press.

McGraw S, Zhang JX, Farag M, Chan D, Caron M, Konermann C, Oakes CC, Mohan KN, Plass C, Pastinen T, Bourque G, Chaillet JR, Trasler JM. 2015. Transient DNMT1 suppression reveals hidden heritable marks in the genome. *Nucleic Acids Res*, 43:1485-1497.

Morbeck DE, Baumann NA, Oglesbee D. 2017. Composition of single-step media used for human embryo culture. *Fertil Steril*, 107:1055-1060.e1051.

Morbeck DE, Krisher RL, Herrick JR, Baumann NA, Matern D, Moyer T. 2014a. Composition of commercial media used for human embryo culture. *Fertil Steril*, 102:759-766.e759.

Morbeck DE, Paczkowski M, Fredrickson JR,

Krisher RL, Hoff HS, Baumann NA, Moyer T, Matern D. 2014b. Composition of protein supplements used for human embryo culture. *J Assist Reprod Genet*, 31:1703-1711.

Morey L, Santanach A, Di Croce L. 2015. Pluripotency and Epigenetic Factors in Mouse Embryonic Stem Cell Fate Regulation. *Mol Cell Biol*, 35:2716-2728.

Niakan KK, Han J, Pedersen RA, Simon C, Pera RA. 2012. Human pre-implantation embryo development. *Development*, 139:829-841.

Norouzitallab P, Baruah K, Vanrompay D, Bossier P. 2019. Can epigenetics translate environmental cues into phenotypes? *Sci Total Environ*, 647:1281-1293.

Novikova SI, He F, Bai J, Cutrufello NJ, Lidow MS, Undieh AS. 2008. Maternal cocaine administration in mice alters DNA methylation and gene expression in hippocampal neurons of neonatal and prepubertal offspring. *PLoS One*, 3:e1919.

Ohbo K, Tomizawa S. 2015. Epigenetic regulation in stem cell development, cell fate conversion, and reprogramming. *Biomol Concepts*, 6:1-9.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*, 99:247-257.

Pacis A, Mailhot-Leonard F, Tailleux L, Randolph HE, Yotova V, Dumaine A, Grenier JC, Barreiro LB. 2019. Gene activation precedes DNA demethylation in response to infection in human dendritic cells. *Proc Natl Acad Sci U S A*, 116:6938-6943.

Padmanabhan R, Hameed MS. 1988. Effects of acute doses of ethanol administered at pre-implantation stages on fetal development in the mouse. *Drug Alcohol Depend*, 22:91-100.

Page-Lariviere F, Campagna C, Sirard MA. 2017. Mechanisms Involved in Porcine Early Embryo Survival following Ethanol Exposure. *Toxicol Sci*, 156:289-299.

Patil V, Ward RL, Hesson LB. 2014. The evidence for functional non-CpG methylation in mammalian cells. *Epigenetics*, 9:823-828.

Plasschaert RN, Bartolomei MS. 2014. Genomic imprinting in development, growth, behavior and stem cells. *Development*, 141:1805-1813.

Pradhan S, Bacolla A, Wells RD, Roberts RJ. 1999. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. *J Biol Chem*, 274:33002-33010.

Qin J, Liu X, Sheng X, Wang H, Gao S. 2016. Assisted reproductive technology and the risk of pregnancy-related complications and adverse pregnancy outcomes in singleton pregnancies: a meta-analysis of cohort studies. *Fertil Steril*, 105:73-85.e71-76.

Razin A, Cedar H. 1991. DNA methylation and gene expression. *Microbiol Rev*, 55:451-458.

Red-Horse K, Zhou Y, Genbacev O, Prakobphol A, Foulk R, McMaster M, Fisher SJ. 2004. Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J Clin Invest*, 114:744-754.

Reik W, Dean W, Walter J. 2001. Epigenetic reprogramming in mammalian development. *Science*, 293:1089-1093.

Saxonov S, Berg P, Brutlag DL. 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A*, 103:1412-1417.

Seisenberger S, Peat JR, Hore TA, Santos F, Dean W, Reik W. 2013. Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers. *Philos Trans R Soc Lond B Biol Sci*, 368:20110330.

Severin PM, Zou X, Gaub HE, Schulten K. 2011. Cytosine methylation alters DNA mechanical properties. *Nucleic Acids Res*, 39:8740-8751.

Smith ZD, Meissner A. 2013. DNA methylation: roles in mammalian development. *Nat Rev Genet*, 14:204-220.

Stoger R, Kubicka P, Liu CG, Kafri T, Razin A, Cedar H, Barlow DP. 1993. Maternal-specific methylation of the imprinted mouse Igf2r locus identifies the expressed locus as carrying the imprinting signal. *Cell*, 73:61-71.

Suetake I, Shinozaki F, Miyagawa J, Takeshima H, Tajima S. 2004. DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *J Biol Chem*, 279:27816-27823.

Suter M, Abramovici A, Showalter L, Hu M, Shope CD, Varner M, Aagaard-Tillery K. 2010. In utero tobacco exposure epigenetically modifies placental CYP1A1 expression. *Metabolism*, 59:1481-1490.

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*, 324:930-935.

Thorvaldsen JL, Duran KL, Bartolomei MS. 1998. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev*, 12:3693-3702.

Toledo-Rodriguez M, Lotfipour S, Leonard G, Perron M, Richer L, Veillette S, Pausova Z, Paus T. 2010. Maternal smoking during pregnancy is associated with epigenetic modifications of the brain-derived neurotrophic factor-6 exon in adolescent offspring. *Am J Med Genet B Neuropsychiatr Genet*, 153b:1350-1354.

Toro R, Leonard G, Lerner JV, Lerner RM, Perron M, Pike GB, Richer L, Veillette S, Pausova Z, Paus T. 2008. Prenatal exposure to maternal cigarette smoking and the adolescent cerebral cortex. *Neuropsychopharmacology*, 33:1019-1027.

Ueda T, Abe K, Miura A, Yuzuriha M, Zubair M, Noguchi M, Niwa K, Kawase Y, Kono T, Matsuda Y, Fujimoto H, Shibata H, Hayashizaki Y, Sasaki H. 2000. The paternal methylation imprint of the mouse H19 locus is acquired in the gonocyte stage during foetal testis development. *Genes Cells*, 5:649-659.

Urrego R, Rodriguez-Osorio N, Niemann H. 2014. Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle. *Epigenetics*, 9:803-815.

Uysal F, Ozturk S, Akkoyunlu G. 2018. Superovulation alters DNA methyltransferase protein expression in mouse oocytes and early embryos. *J Assist Reprod Genet*, 35:503-513.

Varela-Rey M, Woodhoo A, Martinez-Chantar ML, Mato JM, Lu SC. 2013. Alcohol, DNA methylation, and cancer. *Alcohol Res*, 35:25-35.

Vougiouklakis T, Nakamura Y, Saloura V. 2017. Critical roles of protein methyltransferases and demethylases in the regulation of embryonic stem cell fate. *Epigenetics*, 12:1015-1027.

Weber M, Schubeler D. 2007. Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr Opin Cell Biol*, 19:273-280.

Wehby GL, Prater K, McCarthy AM, Castilla EE, Murray JC. 2011. The Impact of Maternal Smoking during Pregnancy on Early Child Neurodevelopment. *J Hum Cap*, 5:207-254.

Weinerman R. 2018. In vitro fertilization (IVF): Where are we now? *Birth Defects Res*, 110:623-624.

White CR, Denomme MM, Tekpetey FR, Feyles V, Power SG, Mann MR. 2015. High Frequency of Imprinted Methylation Errors in Human Preimplantation Embryos. *Sci Rep*, 5:17311.

Wiebold JL, Becker WC. 1987. In-vivo and in-vitro effects of ethanol on mouse preimplantation embryos. *J Reprod Fertil*, 80:49-57.

Wu H, Zhang Y. 2014. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell*, 156:45-68.

Young LE, Sinclair KD, Wilmut I. 1998. Large offspring syndrome in cattle and sheep. *Rev Reprod*, 3:155-163.

Zhu H, Wang G, Qian J. 2016a. Transcription factors as readers and effectors of DNA methylation. *Nat Rev Genet*, 17:551-565.

Zhu JQ, Liu JH, Liang XW, Xu BZ, Hou Y, Zhao XX, Sun QY. 2008. Heat stress causes aberrant DNA methylation of H19 and Igf-2r in mouse blastocysts. *Mol Cells*, 25:211-215.

Zhu L, Zhang Y, Liu Y, Zhang R, Wu Y, Huang Y, Liu F, Li M, Sun S, Xing L, Zhu Y, Chen Y, Xu L, Zhou L, Huang H, Zhang D. 2016b. Maternal and Live-birth Outcomes of Pregnancies following Assisted Reproductive Technology: A Retrospective Cohort Study. *Sci Rep*, 6:35141.

DOI: 10.21451/1984-3143-AR2019-0043

Proceedings of the 33rd Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Ilha de Comandatuba, BA, Brazil, August 15th to 19th, 2019.

New tools for cell reprogramming and conversion: Possible applications to livestock

Fulvio Gandolfi[1,*], Sharon Arcuri[2], Georgia Pennarossa[2], Tiziana A.L. Brevini[2]

[1]Department of Agricultural and Environmental Sciences - Production, Landscape, Agroenergy, University of Milan, Italy.
[2]Department of Health, Animal Science and Food Safety, University of Milan, Italy.

Abstract

Somatic cell nuclear transfer and iPS are both forms of radical cell reprogramming able to transform a fully differentiated cell type into a totipotent or pluripotent cell. Both processes, however, are hampered by low efficiency and, in the case of iPS, the application to livestock species is uncertain.

Epigenetic manipulation has recently emerged as an efficient and robust alternative method for cell reprogramming. It is based upon the use of small molecules that are able to modify the levels of DNA methylation with 5-azacitidyne as one of the most widely used. Among a number of advantages, it includes the fact that it can be applied to domestic species including pig, dog and cat.

Treated cells undergo a widespread demethylation which is followed by a renewed methylation pattern induced by specific chemical stimuli that lead to the desired phenotype. A detailed study of the mechanisms of epigenetic manipulation revealed that cell plasticity is achieved through the combined action of a reduced DNA methyl transferase activity with an active demethylation driven by the TET protein family. Surprisingly the same combination of molecular processes leads to the transformation of fibroblasts into iPS and regulate the epigenetic changes that take place during early development and, hence, during reprogramming following SCNT.

Finally, it has recently emerged that mechanic stimuli in the form of a 3D cell rearrangement can significantly enhance the efficiency of epigenetic reprogramming as well as of maintenance of pluripotency. Interestingly these mechanic stimuli act on the same mechanisms both in epigenetic cell conversion with 5-Aza-CR and in iPS.

We suggest that the balanced combination of epigenetic erasing, 3D cell rearrangement and chemical induction can go a long way to obtain ad hoc cell types that can fully exploit the current exiting development brought by gene editing and animal cloning in livestock production.

Keywords: cell reprogramming, epigenetic erasing, mechanosensing.

Introduction

After many decades of heated debate, the birth of Dolly proved, beyond any doubt, that each cell of our organism retains all the information originally present in the zygote. Somatic cell nuclear transfer is indeed the ultimate form of cell conversion since it enables the birth of a new individual from a single differentiated cell. Consequently, this also implies that differentiation does not permanently silence any of this information and that, providing the right stimuli, such information can be restored and become functional again.

A slightly less dramatic form of somatic cell reprogramming is achieved through the transfection of four master genes to obtain what are universally known as induced pluripotent stem (iPS) cells. In this case instead of a whole individual it is possible to generate several of its parts. As indicated by their name, iPS cells can potentially generate each of the approximately 200 cell types that constitute a human or an animal body. These properties are not exclusive of iPS but were first described in embryonic stem cells (ESC) that are derived from pre-implantation embryos. In both cases, cells are characterised by a stable pluripotency which is not found in any cell neither in the embryo nor in any other phase of life. It can rather be considered a cell culture artefact that captures a physiological stage naturally transient in early development.

As we summarised in a previous paper (Gandolfi *et al*., 2012), the ability to transform such transient stage into a permanent property is limited to a very small number of species (mostly mice and primates including humans) while it has proved very elusive in livestock species. The reason for such difference is still unclear. One hypothesis linked the problematic derivation of bona fide ESC in domestic species to the prolonged pre-implantation period typical of domestic animals. However, this looks unlikely because also the derivation of bona fide iPS cells has proved to be elusive in these species.

Since stable pluripotency does not exist in nature, living organisms utilise different mechanisms for the day to day replacement of worn or damaged cells. Differentiation is physiologically associated with cell proliferation and, on the contrary, fully differentiated cells lose their ability to proliferate. For this reason, maintaining tissue homeostasis is the functional task of the so-called somatic stem cells. These small groups of dedicated cells reside in well-defined niches which modulate their proliferative capacity in response to the functional requirements of the organism. Stem cells proliferate into intermediate, partially differentiated populations that cease to replicate once they become fully matured and ready to perform their specific function (Gandolfi and Brevini, 2018).

In some circumstances we know that such process can be reverted, and a fully differentiated cell

*Corresponding author: fulvio.gandolfi@unimi.it
orcid.org/0000-0002-3246-2985
Received: April 19, 2019
Accepted: June 19, 2019

may resume its proliferative ability and give rise to a different cell type in response to specific stimuli (Brevini and Gandolfi, 2013).

Understanding what stimulates and regulates the transformation of a differentiated cell into a different type of cell is a fascinating topic of study and being able to harness this process has a wide range of medical and commercial applications.

Aim of this short review is to provide the reader with an overall perspective of the most recent concepts about the relationships that exist between stem cells, cell conversion and cloning in livestock.

Pluripotency *in vivo* and *in vitro*

In the early phases of mammalian embryonic development, three germ layers, the endoderm, mesoderm and ectoderm are formed; each one gives rise to a different set of tissue types and contributes to specific organs. Stem cells are classified according to their potency that can span from unipotency when only a single cell type can be generated, to multipotency, when a stem cell can originate to all or many cells of a single germ layer.

When a stem cell can differentiate into cells that arise from all three germ layers, is defined as pluripotent. In nature, pluripotency is limited to the epiblast, a transient tissue that exists only for a brief stage period of embryonic development, before giving origin to the three germ layers. Therefore, the epiblast is not a kind of stem cell because it lacks the property of asymmetric division and stable pluripotent cells are not a physiological component of the body but are created only *in vitro* (Smith, 2001).

In vitro, it has been possible to transform pluripotency from a transient state into a permanent property of stable cell lines. These can be derived directly from early embryos generating the embryonic stem cells (ESC) or can be obtained through the transfection of 4 transcription factors into somatic cells, generating the induced pluripotent stem cells (iPS). Both kind of pluripotent stem cells can be readily derived in mouse and human but it has proved much more challenging if not outright impossible, to derive pluripotent cells in livestock species, as detailed in some recent reviews (Brevini *et al*., 2010; Koh and Piedrahita, 2014; Kumar *et al*., 2015; Soto and Ross, 2016). Pluripotent cell lines in these species are defined as ES-like since they show several major deficiencies, ranging from a short life in culture to the lack of controlled pluripotency or of the ability to form chimeras (Talbot and Blomberg le, 2008). Despite the extensive research activity, it is still unclear why it is not possible to derive truly pluripotent ESC or iPS from these species.

Since ESC originate from the epiblast the question arises if the lack of domestic animals ESC is due to the lack of appropriate culture conditions or the epiblast from these species is inherently different so that "suspending" it's properties *in vitro* may not be possible.

The process of epiblast formation in mouse is known in great detail (Rossant and Tam, 2009). During the first embryonic divisions, all blastomeres are totipotent and all express the transcription factor Octamer Binding Protein 4 (OCT4). The first differentiation process consists in the generation of trophectoderm (TE) and inner cell mass (ICM) cells from their unique totipotent blastomere precursors. This is marked by the restriction of OCT4 expression to ICM cells, which is caused by its repression by caudal type homeobox 2 (CDX2). The result is that TE cells express CDX2 and ICM cells express OCT4. ICM cells will then undergo a further differentiation leading to the formation of the hypoblast, that will lose OCT4 expression, and of the epiblast that will retain it.

Mouse epiblast differentiation and restriction of Oct4 expression to this tissue is completed by E3.5. By E5.5 mouse embryos are embedded into the uterine wall. Human embryos go through the same changes but at a slower pace with OCT4 restriction to the epiblast completed by E6 and implantation taking place at E7-9 (Rossant, 2015).

When we examined the distribution of OCT4 in bovine embryos we soon realized that it is not as tightly restricted to ICM as described in mouse and human embryos but it was ubiquitously expressed also in expanded blastocysts (Van Eijk *et al*., 1999). When observations were extended to later stage embryos it was determined that OCT4 restriction to the epiblast is completed only by E11 in bovine (Berg *et al*., 2011) and E8-9 in pig (Hall *et al*., 2009) embryos.

Based on this different timing, attempts have been performed using day 10-12,5 elongated pig blastocysts, using the knowledge that late, or so-called "primed", epiblast responds better to FGF2 than to LIF (Alberio *et al*., 2010). Indeed, results were encouraging with cell lines showing a robust self-renewal and the ability to differentiate into precursor cells derived from all three germ layers as well as into trophectoderm and germ cell precursors. However it is possible to obtain similar results with day 6 blastocysts using both LIF and FGF2 (Brevini *et al*., 2010).

At present, culture conditions are still far from being elucidated. Telugu *et al*. (2011) derived LIF-dependent, so-called naive, pluripotent stem cells from the ICM of porcine blastocysts by up-regulating expression of KLF4 and POU5F1 with lentivirus vector. Haraguchi *et al*., (2012) generated porcine ES-like cells from the ICM of porcine embryos by using inhibitors, CH99021 and PD184352. Recent results showed that the combination of bFGF, EGF, Activin-a, ITS, and KO Serum is also effective to promote attachment, outgrowth and expansion of porcine ICMs and generate ESC-like cells (Hou *et al*., 2016).

Given the possibility that the specific morphological and functional characteristic of domestic ungulate pre-implantation embryos may have a profound influence on the possibility to derive ESC lines, it was interesting to see whether the forced induction of pluripotency achieved with the iPS technology made it possible to obtain ungulates bona fide pluripotent stem cells bypassing the embryo as a starting material.

Indeed iPS have been obtained in wide range

domestic ungulates, but in some instances, expression of the exogenous pluripotency genes was not down regulated or was artificially maintained (Gandolfi *et al*., 2012). In the first case, this made it difficult to induce teratoma formation. In the latter, the absence of expression induced a rapid differentiation in pig (Esteban *et al*., 2009; Wu *et al*., 2009), sheep (Li *et al*., 2011) and cow (Sumer *et al*., 2011) cell lines. More importantly, the ability of livestock iPSCs to generate chimeras was very low and even lower was their ability to contribute to the germ line (West *et al*., 2011). The results are consistent with the fact that most of these cell lines show the characteristics of the primed type.

The recent developments of new media were able to convert pig primed cell lines into the naïve type and to confer higher clonal properties to primed lines renewing our hopes that further developments may be achieved in livestock species able to generate a chimera in the near future (Ma *et al*., 2018).

At present, however, available data suggest that true LIF-dependent naïve/ESC equivalent to those of mouse cannot be obtained in ungulates, possibly due to some inherent characteristic of their epiblast.

Whether or not in the future will be worthwhile to pursue this line of research in domestic or in other species is open to debate. A large body of evidence shows that the differentiation of pluripotent stem cells, both embryonic or induced, is difficult to control and it is dangerously similar to neoplastic transformation. On the contrary new approaches have been developed that enable the study of the differentiation process and, at the same time, look much safer for clinical applications, as described in the next section.

Epigenetic cell conversion

Following on from the pioneering work of Taylor and Jones (1979), many groups have reported that it is possible to use small molecules and epigenetic modifiers in order to directly convert an adult cell into an alternative differentiated cell type (Brevini *et al*., 2014; Chandrakanthan *et al*., 2016; Manzoni *et al*., 2016; Pennarossa *et al*., 2013). Several protocols using epigenetic modifiers have been developed that can push cells to a transient 'less committed state', increasing cell plasticity for a short time, sufficient to redirect them towards a different cell type (Brevini *et al*., 2014; Chandrakanthan *et al*., 2016; Harris *et al*., 2011; Mirakhori *et al*., 2015; Pennarossa *et al*., 2014, 2013). The general concept of these experiments is that DNA methylation plays a fundamental role during cell differentiation during early embryonic development and cell lineage specification. For this reason, 5-azacytidine (5-aza-CR), a well-characterised DNA methyltransferase inhibitor, has often been used to remove the epigenetic 'blocks' that are responsible for tissue specification (Brevini *et al*., 2014; Chandrakanthan *et al*., 2016; Pennarossa *et al*., 2013). Because of its powerful effects, 5-aza-CR induces global DNA hypomethylation (Christman, 2002) and gene reactivation (Jones, 1985) facilitating somatic cells switching from one phenotype to another (Glover *et al*., 1986; Harris *et al*., 2011; Taylor and Jones, 1979). A brief exposure to 5-aza-CR can convert adult skin fibroblasts and granulosa cells into different cell types (Brevini *et al*., 2016, 2014; Pennarossa *et al*., 2017, 2014, 2013). Such fate switch is not limited to cells belonging to the same embryonic layer but can also occur between cells belonging to different embryonic layers.

After a 18 h-exposure to 5-aza-CR, cells acquire a 'highly permissive state' with significant changes in their phenotype and gene expression pattern accompanied by a decrease in global DNA methylation. Most surprisingly following exposure to this demethylating agent, cells acquire the morphological features distinctive of ESCs, iPSCs and pluripotent cells described by Tamada *et al*. (2006). These include reduced dimensions with large nuclei, global chromatin decondensation, as well as expression of pluripotency-related genes such as OCT4, NANOG, ZFP42 zinc finger protein (REX1) and SRY (sex determining region Y)-box 2 (SOX2). This was achieved not only with human and mouse but also with pig and dog fibroblasts (Brevini *et al*., 2016, 2014; Pennarossa *et al*., 2017, 2014, 2013) and the high efficiency and robustness of the process makes it the best option for working in domestic species (Gandolfi and Brevini, 2018).

The mechanisms at work during epigenetic reprogramming are very similar to, or even the same, that regulate early embryonic development and the transformation of a somatic cell into an iPS (Fig.1). Pluripotent cells, either ESC or iPS, show a global cytosine demethylation (Leitch *et al*., 2013) which is crucial for maintaining the naive state and antagonising the self-activating differentiation signal, resetting the epigenome and re-establishing the pluripotency network (Grabole *et al*., 2013). In addition, downregulation of DNA methyl transferase enzymes (DNMT) is correlated with boosting symmetry in cell division (Jasnos *et al*., 2013), further supporting the idea that demethylation plays a major role in promoting self-renewal and maintaining cells in their most naive state. In agreement with these observations, cell fate restriction and subsequent differentiation is accompanied by a progressive build-up of DNA methylation. Indeed, it has been demonstrated that lineage specification is supported by dynamic epigenetic changes and genome-wide redistribution of DNA methylation that silence pluripotency genes and establish a phenotype-specific methylation pattern (Berdasco and Esteller, 2011; Oda *et al*., 2013). During cell fate commitment, pluripotency genes such as octamer-binding transcription factor 4 (Oct4) and Nanog undergo silencing and de novo DNA methylation in their promoter and enhancer regions. This hypermethylated state is then maintained in differentiated somatic cells (Epsztejn-Litman *et al*., 2008; Li *et al*., 2007).

As described above, cell phenotype can be reversed by transferring a somatic cell nucleus into an enucleated oocyte and, similarly, somatic cells transfected with specific reprogramming factors are converted into iPSCs. On the other hand, the identity of a differentiated cell is guaranteed by a unique methylation profile that maintains its lineage definition

and prevents free transition among different cell types. Therefore, methylation must be removed in order to allow a switch in phenotype. For example, demethylation of pluripotency genes is a hallmark of somatic cell reprogramming into a pluripotent state (Gurdon and Melton, 2008; Takahashi and Yamanaka, 2006). Recently, studies have shown that experimental reprogramming requires active demethylation by the TET (ten-eleven-translocation) family of enzymes, which recently were identified to catalyse the conversion of cytosine-5 methylation to 5-hydroxymethyl-cytosine, an intermediate form potentially involved in demethylation (Mohr *et al*., 2011), leading to activation of epigenetically silenced pluripotency genes. In agreement with these observations, it has been reported that oocyte TET enzymes exhibited reprogramming activity for pluripotency gene reactivation during early embryonic development, after nuclear transfer and natural fertilisation (Gu *et al*., 2011). Together, these findings point to the possibility that TET enzymes play a key role in cell reprogramming as well as in mesenchymal to epithelial transition (MET) that characterise iPS formation. This hypothesis finds further support in experiments performed in mouse embryonic fibroblasts (MEFs), in which TET genes were inactivated, resulting in cell failure to undergo MET and a complete block of their reprogramming potential (Hu *et al*., 2014). These observations indicate that TET enzymes are indispensable for factor-driven reprogramming of somatic cells to iPSCs. Interestingly, the same authors showed that TET-deficient MEFs failed to reactivate microRNAs, such as miR-200 s, miR-200a and miR-200b, which play a critical role in MET and are upregulated in cells undergoing reprogramming. Indeed, Hu *et al*., (2014) showed that the expression of the miR-200 family diminished in TET-deficient MEFs, and this was accompanied by the reprogramming block. However, ectopic expression of miR-200s was able to restore the MET process and rescue up to 80% of the reprogramming efficiency of wild-type fibroblasts (Hu *et al*., 2014).

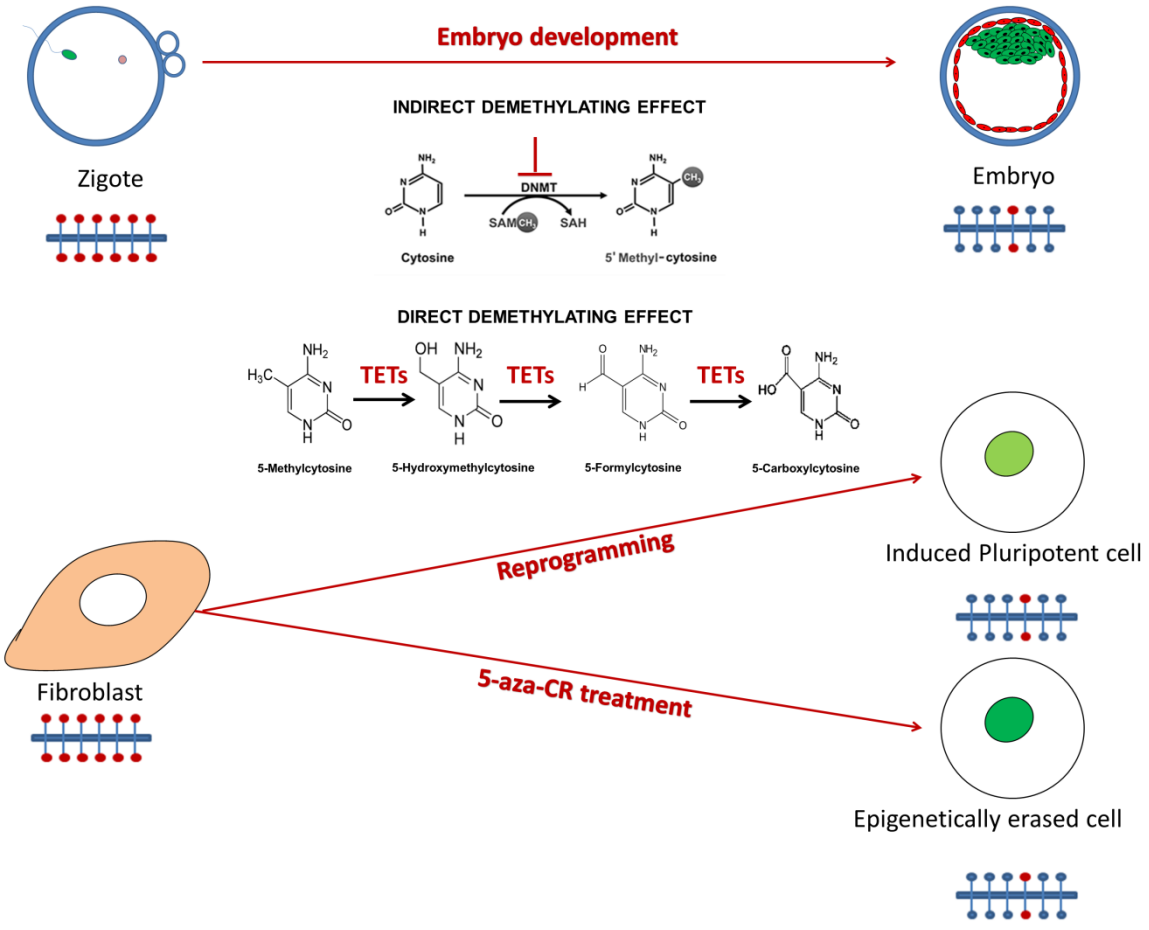


Figure 1. Inhibition of DNA methyl transferase (DNMT) enzymes combined with the activation of ten eleven translocation (TET) enzymes are at work in pluripotent and highly plastic cells. This indicates that cell plasticity is achieved and maintained through a common mechanism.

DNA methylation has also been shown to promote the adequate and proper regulation of gene expression, ensuring both temporal activation and spatial restriction, allowing cells to acquire distinct differentiation traits, stabilising the terminal cell phenotype and maintaining the established patterns by copying them onto daughter DNA strands during cell replication and division (Oda *et al*., 2013). Consistently, studies performed recently using culture media supplemented with two small kinase inhibitor (PD0325901 and CHIR99021) report the derivation of ESC lines with a level of hypomethylation higher than those derived using conventional media (Habibi *et al*., 2013; Leitch *et al*., 2013). In particular, also in this case, TET enzymes are involved since the two inhibitors increases their activity, boosting TET-mediated conversion of 5-mC to 5-hmC, which synergise with the simultaneous DNMT-related passive effect, easing cells into a 'naive state' in which the genome becomes hypomethylated and reminiscent of early blastomeres seen *in vivo* (Hu *et al*., 2014).

Epigenetic reprogramming can unleash the full differentiation potential of any cell type with none of the limits that constrain embryonic or induced pluripotent stem cells. It represents a step forward since it works through natural pathways instead of inducing artificial states. Therefore, in the near future we are likely to see a rapid expansion of this approach both in basic and in clinical research.

Cell spatial arrangement in a 3D microenvironment

From all of the above we learned that manipulation of the epigenetic status of a somatic cell enables the quick and substantial increase of its plasticity that can be readily exploited for changing its fate and remodelling it according to our wishes. This is a very efficient and safe process because the raise of cell plasticity is temporary and reversible avoiding the danger linked to a permanent pluripotent state that severely limit the possible clinical use of iPS and ESC (Brevini *et al*., 2018).

However, in some circumstances the availability of more stable pluripotent cells may be of interest. One of such cases could be the use of pluripotent cells as nuclear donors for improving the currently low efficiency of somatic cell nuclear transfer.

As described in detail in a recent review numerous attempts have been performed to use epigenetic modifiers to improve SCNT efficiency (Curcio *et al*., 2017). However, at present, the possibility to significantly improve offspring production is controversial at best.

In this context, we studied the possibility to stabilize the high plasticity status obtained *in vitro* by the epigenetic reprogramming through the addition of a 3D microenvironment. In particular we used polytetrafluoroethylene (PTFE) micro-bioreactors to induce cells to self-assemble and form multicellular spheroids, displaying a uniform size geometry (Pennarossa *et al*., 2019). This stems from previous studies indicating that PTFE is able to efficiently encourage cell aggregation, facilitating the formation of embryoid bodies from murine ESC (Sarvi *et al*., 2013) or the establishment of olfactory ensheathing cell spheroid structures (Vadivelu *et al*., 2015). Our results demonstrate the 3D cell rearrangement, obtained within the microbioreactors induced global DNA demethylation and elevated transcription of pluripotency markers. Ultrastructural analysis demonstrated that cells in the 3D spherical structures showed significant intercellular spaces, high nucleus to cytoplasm ratio, nuclei containing euchromatin and large reticulated nucleoli. Cytoplasm was characterized by the presence of free ribosomes, polyribosomes, elongated tubular mitochondria, well-developed rough endoplasmic reticulum, Golgi complexes, few reticulum cisternae and lipid droplets. All these features resemble the morphology typical of undifferentiated cells like ESC and iPS, and remind of the inner cell mass (ICM) of blastocysts (Courtot *et al*., 2014; Efroni *et al*., 2008; Lai *et al*., 2015; Liang and Zhang, 2013; Meshorer *et al*., 2006; Meshorer and Misteli, 2006; Sathananthan *et al*., 2002). These observations suggest that the use of PTFE microbioreactors encourages cell aggregation and boosts the induction and stable maintenance of morphological properties typical of pluripotent cells.

Molecular analysis showed that PTFE encapsulated cells remained significantly hypomethylated for the entire length of the experiments. Furthermore, our results showed that epigenetic erasing led to an increased expression of the ten-eleven translocation family member TET2, accompanied by the onset of the pluripotency-related genes, OCT4, NANOG, REX1 and SOX2, as well as the up-regulation of EPCAM, and CDH1 genes, confirming and expanding previous studies carried out in our laboratory (Brevini *et al*., 2014; Manzoni *et al*., 2016; Pennarossa *et al*., 2014, 2013). As we described above, this is the same mechanism taking place in epigenetic reprogramming which, in turn, replicates the methylation changes taking place during iPS reprogramming with the combined effect of reduced DNMT activity with the active demethylation controlled by TET proteins (Hysolli *et al*., 2016). The two play an essential role in pluripotency maintenance and the acquisition of a high plasticity phenotype (Ito *et al*., 2010; Tahiliani *et al*., 2009), resulting in the decrease of fibroblast-specific marker (THY1), the onset of pluripotency-related genes (OCT4, NANOG, REX1, and SOX2), and the upregulation of key MET markers (EPCAM, CDH1).

Interestingly, these changes were promoted and stably maintained by the use of the PTFE microbioreactor, suggesting that 3D cell confinement boosts pluripotency gene transcription and maintains long-term cell plasticity. These morphological and molecular changes were accompanied by the activation of the Hippo-signalling pathway with distinctive modifications in the transcriptional cofactor TAZ localization. In particular, the 3D cell confinement encouraged TAZ nuclear retention, that was stably maintained for the entire length of the experiments. TAZ localization was mirrored by a parallel nuclear

accumulation of signal transducer SMAD2 (Fig.2). This evidence is in line with previous reports that indicate a direct interaction between TAZ and SMAD proteins, where TAZ defines a hierarchical system, regulating SMAD complexes shuttling and coupling to the transcriptional machinery (Ohgushi *et al*., 2015; Varelas *et al*., 2008). These observations are even more intriguing, given the fact that the Hippo signalling pathway and its activators are highly expressed in the mammalian embryo and have been recently shown to contribute to and to improve early embryonic development (Yu *et al*., 2016). Mechanosensing-related activation of such pathway is therefore likely to enhance epigenetic reprogramming and plasticity.

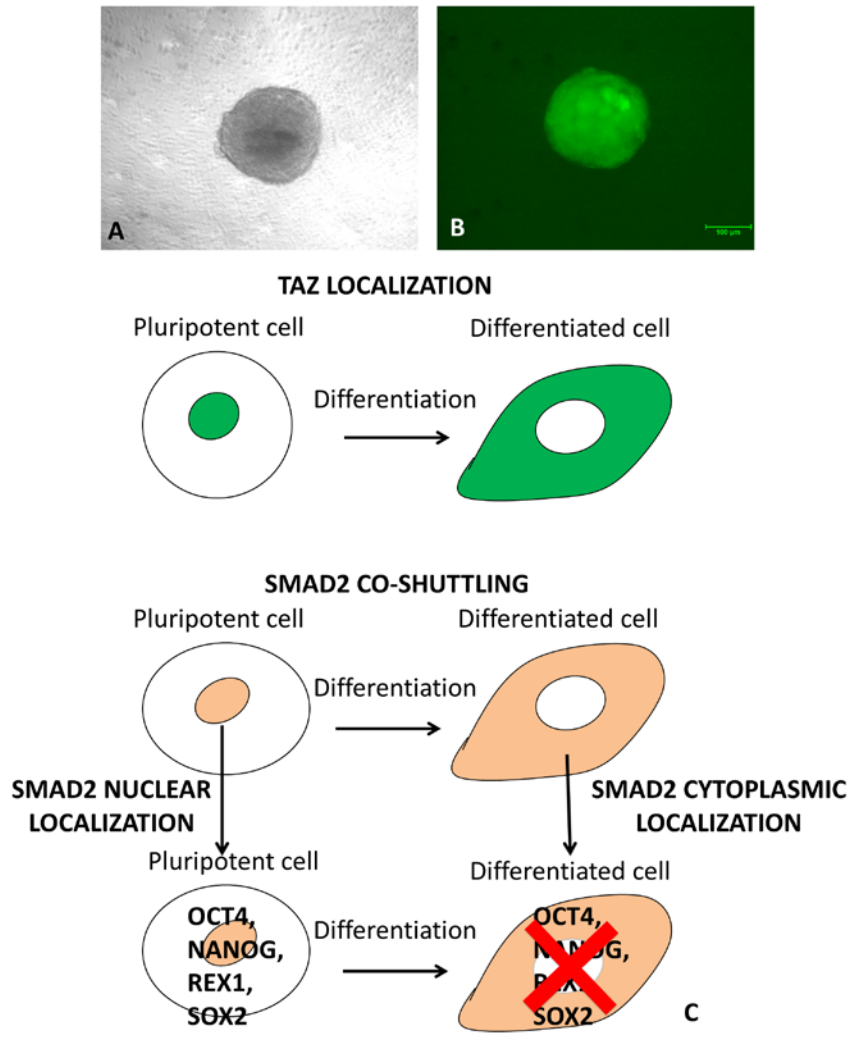


Figure 2. When cells are allowed to grow in a 3D arrangement, obtained within PTFE microbioreactors (panel A), DNA undergoes a global demethylation and transcription of pluripotency markers increases (e.g. Oct4 as shown in panel B). This is achieved by the nuclear translocation of TAZ protein that drives SMAD2 into the nucleus that, in turn, activates the transcription of Oct4, Nanog, Rex1 and Sox2.

Conclusions

Careful modulation of the epigenetic make-up provides an efficient and safe way to change the state of any somatic cells. The increased plasticity is reversible and transient, making it much more physiological than the permanent pluripotency of ESC and iPS.

Recent developments revealed a surprising overlap among the molecular mechanisms that control cell reprogramming, even if it is achieved through different techniques, and the regulatory pathways acting in the early embryo.

In the near future it will be interesting to see if it is possible to harness the full potential of these mechanisms to achieve an accurate epigenetic resetting. For instance, we know that the low efficiency of SCNT is largely due to the short time available for the nucleus to undergo the extensive epigenetic reprogramming that takes place after fertilization. Even the use of ESC or iPS has been unable to significantly improve it, possibly because pluripotent cells are more similar to the epiblast than to the zygotic nucleus. We can hypothesise that the balanced combination of epigenetic erasing, 3D cell rearrangement and chemical induction can transform the epigenetic status of the somatic nucleus into that found in the zygote, in practice, reprogramming it before its

transfer, so that it can follow the physiological evolution leading to the complete development (Fig. 3). This may prove to be a novel tool to obtain cell nuclei much more amenable to a correct reprogramming within the short time frame provided by SCNT. The resulting improvement may enable to fully exploit the exiting developments promised by gene editing in livestock production.

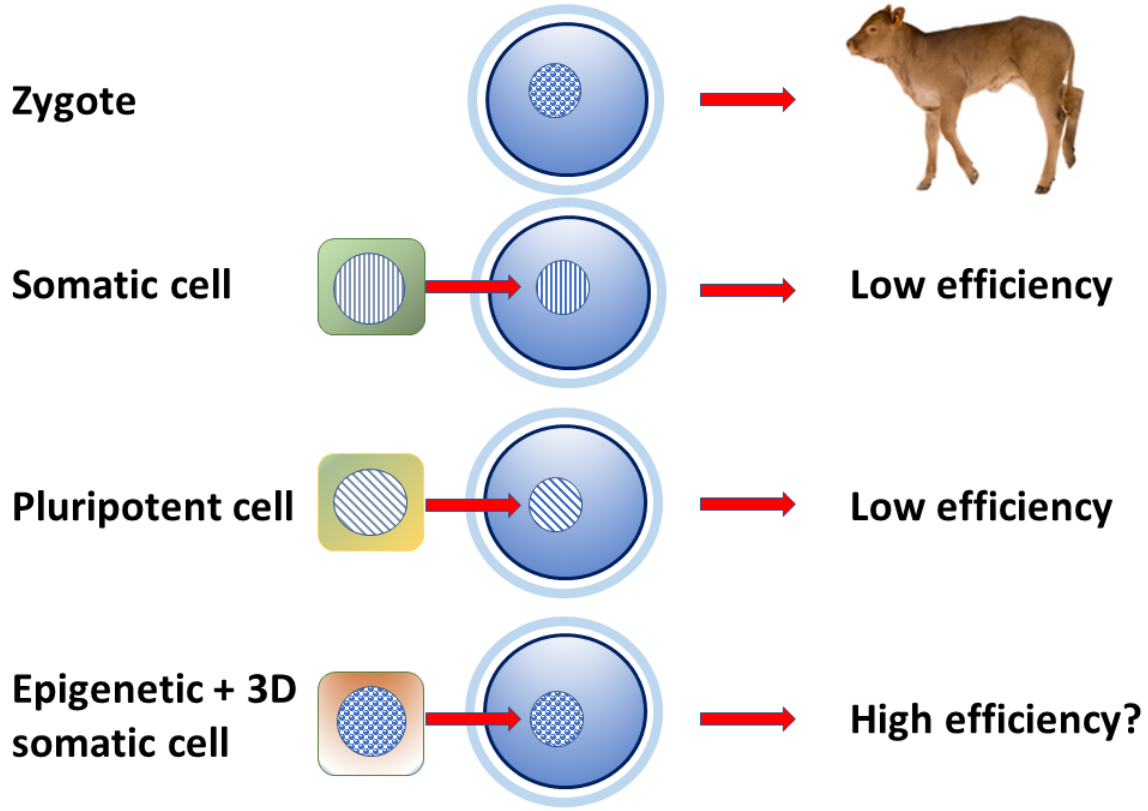


Figure 3. Efficiency of somatic cell nuclear transfer has remained low though the years. It is thought that the different epigenetic status between the donor cells and the zygote nucleus may be the main reason. The use of ESC of iPS pluripotent cells has brought only small improvements if any. We hypothesise that the understanding of the molecular mechanisms common to different reprogramming methods may lead to an accurate control of the nuclear epigenetic status that will resemble that of the zygote, thereby significantly increasing SCNT efficiency and unleashing the full potential of genome editing in livestock species.

Author contributions

FG: Conceptualization, Funding acquisition, Supervision, Writing; SA Data curation, Formal analysis; GP: Data curation, Formal analysis; TALB Conceptualization, Funding acquisition, Supervision, Writing.

Conflict of interest

The Authors declare no conflict of interest.

Acknowledgments and Funding disclosure statement

FG, SA, GP, TALB were supported with funds by Carraresi Fundation project "Innovative strategies to ensure a fast translation process from the laboratory bench to the patient bedsite and implement the community quality of life" and University of Milan Grant PSR 2017.

References

Alberio R, Croxall N, Allegrucci C. 2010. Pig epiblast stem cells depend on activin/nodal signaling for pluripotency and self-renewal. *Stem Cells Dev*, 19:1627-1636. Doi.org/10.1089/scd.2010.0012

Berdasco M, Esteller M. 2011. DNA methylation in stem cell renewal and multipotency. *Stem Cell Res*, Ther. 2:42. Doi.org/10.1186/scrt83.

Berg DK, Smith CS, Pearton DJ, Wells DN, Broadhurst R, Donnison M, Pfeffer PL. 2011. Trophectoderm lineage determination in cattle. *Dev Cell*, 20:244-255. Doi.org/10.1016/j.devcel.2011.01.003.

Brevini TAL, Pennarossa G, Attanasio L, Vanelli A, Gasparrini B, Gandolfi F. 2010. Culture conditions and signalling networks promoting the establishment of cell lines from parthenogenetic and biparental pig embryos. *Stem Cell Rev. Reports*, 6:484-495. Doi.org/10.1007/s12015-010-9153-2.

Brevini TAL, Gandolfi F. 2013. Pluripotency in domestic animal embryos, In: Pluripotency in

Domestic Animal Cells. SpringerBriefs in Stem Cells. Springer, New York, NY. Doi.org/10.1007/978-1-4899-8053-3_2.

Brevini TAL, Pennarossa G, Rahman MM, Paffoni A, Antonini S, Ragni G, deEguileor M, Tettamanti G, Gandolfi F. 2014. Morphological and molecular changes of human granulosa cells exposed to 5-azacytidine and addressed toward muscular differentiation. *Stem Cell Rev*, 10:633-42. Doi.org/ 10.1007/s12015-014-9521-4.

Brevini TAL, Pennarossa G, Acocella F, Brizzola S, Zenobi A, Gandolfi F. 2016. Epigenetic conversion of adult dog skin fibroblasts into insulin-secreting cells. *Vet J*, 211:52-56. Doi.org/10.1016/j.tvjl.2016.02.014.

Brevini TAL, Manzoni EFM, Gandolfi F. 2018. Methylation mechanisms and biomechanical effectors controlling cell fate. *Reprod Fertil Dev*, 30:64-72. Doi.org/10.1071/RD17348.

Chandrakanthan V, Yeola A, Kwan JC, Oliver RA, Qiao Q, Kang YC, Zarzour P, Beck D, Boelen L, Unnikrishnan A, Villanueva JE, Nunez AC, Knezevic K, Palu C, Nasrallah R, Carnell M, Macmillan A, Whan R, Yu Y, Hardy P, Grey ST, Gladbach A, Delerue F, Ittner L, Mobbs R, Walkley CR, Purton LE, Ward RL, Wong JW, Hesson LB, Walsh W, Pimanda JE. 2016. PDGF-AB and 5-Azacytidine induce conversion of somatic cells into tissue-regenerative multipotent stem cells. *Proc Natl Acad Sci USA*, 113:E2306-15. Doi.org/10.1073/pnas.1518244113.

Christman JK. 2002. 5-Azacytidine and 5-aza-2[prime]-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene, 21:5483-5495.

Courtot AM, Magniez A, Oudrhiri N, Feraud O, Bacci J, Gobbo E, Proust S, Turhan AG, Bennaceur-Griscelli A. 2014. Morphological analysis of human induced pluripotent stem cells during induced differentiation and reverse programming. *Biores Open Access*, 3:206-216. Doi.org/10.1089/biores.2014.0028

Curcio AG, Bressan FF, Meirelles FV, Dias AJB, Curcio AG, Bressan FF, Meirelles FV, Dias AJB. 2017. Achievements and perspectives in cloned and transgenic cattle production by nuclear transfer: influence of cell type, epigenetic status and new technology. *Anim Reprod*, 14:1003-1013. Doi.org/10.21451/1984-3143-AR853.

Efroni S, Duttagupta R, Cheng J, Dehghani H, Hoeppner DJ, Dash C, Bazett-Jones DP, Le Grice S, McKay RD, Buetow KH, Gingeras TR, Misteli T, Meshorer E. 2008. Global transcription in pluripotent embryonic stem cells. *Cell Stem Cell*, 2:437-447. Doi.org/10.1016/j.stem.2008.03.021.

Epsztejn-Litman S, Feldman N, Abu-Remaileh M, Shufaro Y, Gerson A, Ueda J, Deplus R, Fuks F, Shinkai Y, Cedar H, Bergman Y. 2008. De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nat Struct Mol Biol*, 15:1176-1183. Doi.org/10.1038/nsmb.1476.

Esteban MA, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D. 2009. Generation of induced pluripotent stem cell lines from tibetan miniature Pig. *J Biol Chem*, 284:17634–17640. Doi.org/10.1074/jbc.M109.008938.

Gandolfi F, Pennarossa G, Maffei S, Brevini T. 2012. Why is it so difficult to derive pluripotent stem cells in domestic ungulates? *Reprod Domest Anim*, 47:11-17. Doi.org/10.1111/j.1439-0531.2012.02106.x.

Gandolfi F, Brevini TAL. 2018. Stem cells and cell conversion in livestock. In: Niemann H., Wrenzycki C. (eds) Animal Biotechnology 2. Springer, Cham. Doi.org/10.1007/978-3-319-92348-2_10.

Glover TW, Coyle-Morris J, Pearce-Birge L, Berger C, Gemmill RM. 1986. DNA demethylation induced by 5-azacytidine does not affect fragile X expression. *Am J Hum Genet*, 38:309-318.

Grabole N, Tischler J, Hackett JA, Kim S, Tang F, Leitch HG, Magnusdottir E, Surani MA. 2013. Prdm14 promotes germline fate and naive pluripotency by repressing FGF signalling and DNA methylation. *EMBO Rep*, 14:629-637.Doi.org/10.1038/embor.2013.67.

Gu T-P; Guo F; Yang H; Wu H-P; Xu G-F; Liu W; Xie Z-G; Shi L; He X; Jin S; Iqbal K; Shi YG; Deng Z; Szabo PE; Pfeifer GP; Li J; Xu G-L. 2011. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. *Nature*, 477:606-610.

Gurdon JB, Melton DA. 2008. Nuclear reprogramming in cells. *Science*, 322:1811-1815.

Habibi E, Brinkman AB, Arand J, Kroeze LI, Kerstens HHD, Matarese F, Lepikhov K, Gut M, Brun-Heath I, Hubner NC, Benedetti R, Altucci L, Jansen JH, Walter J, Gut IG, Marks H, Stunnenberg HG. 2013. Whole-genome bisulfite sequencing of two distinct interconvertible DNA methylomes of mouse embryonic stem cells. *Cell Stem Cell*, 13:360-369. Doi.org/10.1016/J.STEM.2013.06.002.

Hall VJ, Christensen J, Gao Y, Schmidt MH, Hyttel P. 2009. Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. *Dev Dyn*, 238:2014-2024. Doi.org/10.1002/ dvdy.22027.

Haraguchi S, Kikuchi K, Nakai M, Tokunaga T. 2012. Establishment of self-renewing porcine embryonic stem cell-like cells by signal inhibition. *J Reprod Dev*, 58:707-716. Doi.org/10.1262/jrd.2012-008.

Harris DM, Hazan-Haley I, Coombes K, Bueso-Ramos C, Liu J, Liu Z, Li P, Ravoori M, Abruzzo L, Han L, Singh S, Sun M, Kundra V, Kurzrock R, Estrov Z. 2011. Transformation of human mesenchymal cells and skin fibroblasts into hematopoietic cells. *PLoS One*, 6:e21250. Doi.org/10.1371/journal.pone.0021250.

Hou D-R, Jin Y, Nie X-W, Zhang M-L, Ta N, Zhao L-H, Yang N, Chen Y, Wu Z-Q, Jiang H-B, Li Y-R, Sun Q-Y, Dai Y-F, Li R-F. 2016. Derivation of porcine embryonic stem-Like cells from *in vitro*-produced blastocyst-stage embryos. *Sci Rep*, 6:25838. Doi.org/10.1038/srep25838.

Hu X, Zhang L, Mao SQ, Li Z, Chen J, Zhang RR, Wu HP, Gao J, Guo F, Liu W, Xu GF, Dai HQ, Shi YG, Li X, Hu B, Tang F, Pei D, Xu GL. 2014. Tet and TDG mediate DNA demethylation essential for mesenchymal-to-epithelial transition in somatic cell reprogramming. *Cell Stem Cell*, 14:512-522. Doi.org/10.1016/j.stem.2014.01.001

Hysolli E, Tanaka Y, Su J, Kim KY, Zhong T, Janknecht R, Zhou XL, Geng L, Qiu C, Pan X, Jung YW, Cheng J, Lu J, Zhong M, Weissman SM, Park IH. 2016. Regulation of the DNA methylation landscape in human somatic cell reprogramming by the miR-29 family. *Stem Cell Reports*, 7:43-54. Doi.org/10.1016/j.stemcr.2016.05.014.

Ito S, D/'Alessio AC, Taranova O V Hong K, Sowers LC, Zhang Y. 2010. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature*, 466:1129-1133.

Jasnos L, Aksoy FB, Hersi HM, Wantuch S, Sawado T. 2013. Identifying division symmetry of mouse embryonic stem cells: negative impact of dna methyltransferases on symmetric self-renewal. *Stem Cell Reports*, 1:360-369. Doi.org/10.1016/J.STEMCR.2013.08.005.

Jones PA. 1985. Effects of 5-azacytidine and its 2'-deoxyderivative on cell differentiation and DNA methylation. *Pharmacol Ther*, 28:17-27. Doi.org/0163-7258(85)90080-4 [pii].

Koh S, Piedrahita JA. 2014. From "ES-like" cells to induced pluripotent stem cells: a historical perspective in domestic animals. *Theriogenology*, 81:103-111. Doi.org/10.1016/j.theriogenology.2013.09.009.

Kumar D, Talluri TR, Anand T, Kues WA. 2015. Induced pluripotent stem cells: Mechanisms, achievements and perspectives in farm animals. World J Stem Cells, 7:315-328. Doi.org/10.4252/wjsc.v7.i2.315.

Lai D, Wang Y, Sun J, Chen Y, Li T, Wu Y, Guo L, Wei C. 2015. Derivation and characterization of human embryonic stem cells on human amnion epithelial cells. *Sci Rep*, 5:10014. Doi.org/10.1038/srep10014

Leitch HG, McEwen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, Surani MA, Hajkova P. 2013. Naive pluripotency is associated with global DNA hypomethylation. *Nat Struct Mol Biol*, 20:311-316. Doi.org/10.1038/nsmb.2510.

Li J-Y, Pu M-T, Hirasawa R, Li B-Z, Huang Y-N, Zeng R, Jing N-H, Chen T, Li E, Sasaki H, Xu G-L. 2007. Synergistic function of DNA methyltransferases Dnmt3a and Dnmt3b in the methylation of Oct4 and Nanog. *Mol Cell Biol*, 27:8748-8759. Doi.org/10.1128/MCB.01380-07.

Li Y, Cang M, Lee AS, Zhang K, Liu D. **2011**. Reprogramming of sheep fibroblasts into pluripotency under a drug-inducible expression of mouse-derived defined factors. *PLoS One*, 6:e15947. Doi.org/10.1371/journal.pone.0015947.

Liang G, Zhang Y. 2013. Embryonic stem cell and induced pluripotent stem cell: an epigenetic perspective. *Cell Res*, 23:49-69. Doi.org/10.1038/cr.2012.175.

Ma Y, Yu T, Cai Y, Wang H. 2018. Preserving self-renewal of porcine pluripotent stem cells in serum-free 3i culture condition and independent of LIF and b-FGF cytokines. *Cell Death Discov*, 4:21. Doi.org/10.1038/s41420-017-0015-4.

Manzoni EFM, Pennarossa G, Deeguileor M, Tettamanti G, Gandolfi F, Brevini TAL. 2016. 5-azacytidine affects TET2 and histone transcription and reshapes morphology of human skin fibroblasts. *Sci Rep*, 6:37017. Doi.org/10.1038/srep37017.

Meshorer E, Misteli T. 2006. Chromatin in pluripotent embryonic stem cells and differentiation. *Nat Rev Mol Cell Biol*, 7:540-546. Doi.org/10.1038/nrm1938.

Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T. 2006. Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev Cell*, 10:105-116. Doi.org/10.1016/j.devcel.2005.10.017.

Mirakhori F, Zeynali B, Kiani S, Baharvand H. 2015. Brief azacytidine step allows the conversion of suspension human fibroblasts into neural progenitor-like cells. *Cell J*, 17:153-158.

Mohr F, Döhner K, Buske C, Rawat VPS. 2011. TET Genes: new players in DNA demethylation and important determinants for stemness. *Exp Hematol*, 39:272-281. Doi.org/10.1016/J.EXPHEM.2010.12.004.

Oda M, Kumaki Y, Shigeta M, Jakt LM, Matsuoka C, Yamagiwa A, Niwa H, Okano M. 2013. DNA methylation restricts lineage-specific functions of transcription factor Gata4 during embryonic stem cell differentiation. *PLoS Genet*, 9:e1003574. Doi.org/10.1371/journal.pgen.1003574.

Ohgushi M, Minaguchi M, Sasai Y. 2015. Rho-signaling-directed YAP/TAZ activity underlies the Long-term survival and expansion of human embryonic stem cells. *Cell Stem Cell*, 17:448-461. Doi.org/10.1016/J.STEM.2015.07.009.

Pennarossa G, Maffei S, Campagnol M, Tarantini L, Gandolfi F, Brevini TAL. 2013. Brief demethylation step allows the conversion of adult human skin fibroblasts into insulin-secreting cells. *Proc Natl Acad Sci USA*, 110:8948-8953. Doi.org/10.1073/pnas.1220637110.

Pennarossa G, Maffei S, Campagnol M, Rahman MM, Brevini TAL, Gandolfi F. 2014. Reprogramming of pig dermal fibroblast into insulin secreting cells by a brief exposure to 5-aza-cytidine. *Stem Cell Rev*, 10:31-43. Doi.org/10.1007/s12015-013-9477-9

Pennarossa G, Santoro R, Manzoni EFM, Pesce M, Gandolfi F, Brevini TAL. 2017. Epigenetic erasing and pancreatic differentiation of dermal fibroblasts into insulin-producing cells are boosted by the use of low-stiffness substrate. *Stem Cell Rev*, 14:398-411. Doi.org/10.1007/s12015-017-9799-0.

Pennarossa G, Manzoni EFM, Ledda S, deEguileor M, Gandolfi F, Brevini TAL. 2019. Use of a PTFE Micro-Bioreactor to Promote 3D Cell rearrangement and maintain high plasticity in epigenetically erased fibroblasts. *Stem Cell Rev*, 15:82-92. Doi.org/10.1007/s12015-018-9862-5.

Rossant J, Tam PPL. 2009. Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development*, 136:701-713. Doi.org/10.1242/dev.017178.

Rossant J. 2015. Mouse and human blastocyst-derived stem cells: vive les differences. *Development*, 142:9-12. Doi.org/10.1242/dev.115451.

Sarvi F, Arbatan T, Chan PPY, Shen WA. 2013. A novel technique for the formation of embryoid bodies inside liquid marbles. *RSC Adv*, 3:14501-14508. Doi.org/10.1039/C3RA40364E.

Sathananthan H, Pera M, Trounson A. 2002. The fine structure of human embryonic stem cells. *Reprod Biomed Online*, 4:56-61.

Smith AG. 2001. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol*, 17:435-462.

Soto DA, Ross PJ. 2016. Pluripotent stem cells and livestock genetic engineering. *Transgenic Res*, 25:289-306. Doi.org/10.1007/s11248-016-9929-5.

Sumer H, Liu J, Malaver-Ortega LF, Lim ML, Khodadadi K, Verma PJ. 2011. NANOG is a key factor for induction of pluripotency in bovine adult fibroblasts. *J Anim Sci*, 89:2708-2716.Doi.org/10.2527/jas.2010-3666.

Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*, 324:930-935. Doi.org/10.1126/science.1170116.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126:663-676. Doi.org/10.1016/j.cell.2006.07.024.

Talbot NC, Blomberg le A. 2008. The pursuit of ES cell lines of domesticated ungulates. *Stem Cell Rev*, 4:235-254. Doi.org/10.1007/s12015-008-9026-0.

Tamada H, Van Thuan N, Reed P, Nelson D, Katoku-Kikyo N, Wudel J, Wakayama T, Kikyo N. 2006. Chromatin decondensation and nuclear reprogramming by nucleoplasmin. *Mol Cell Biol*, 26:1259-1271. Doi.org/10.1128/MCB.26.4.1259-1271.2006.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell*, 17:771-779. Doi.org/0092-8674(79)90317-9 [pii].

Telugu BPVL, Ezashi T, Sinha S, Alexenko AP, Spate L, Prather RS, Roberts RM. 2011. Leukemia inhibitory factor (LIF)-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. *J Biol Chem*, 286:28948-28953. Doi.org/ 10.1074/ JBC.M111.229468

Vadivelu RK, Ooi CH, Yao RQ, Tello Velasquez J, Pastrana E, Diaz-Nido J, Lim F, Ekberg JA, Nguyen NT, St John JA. 2015. Generation of three-dimensional multiple spheroid model of olfactory ensheathing cells using floating liquid marbles. *Sci Rep*, 5:15083. Doi.org/10.1038/srep15083.

Van Eijk MJT, Van Rooijen MA, Modina S, Scesi L, Folkers G, Van Tol HTA, Bevers MM, Fisher SR, Lewin HA, Rakacolli D, Galli C, De Vaureix C, Trounson AO, Mummery CL, Gandolfi F. 1999. Molecular cloning, genetic mapping, and developmental expression of bovine POU5F1. *Biol Reprod*, 60:1093-1103.

Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, Yaffe MB, Zandstra PW, Wrana JL. 2008. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. *Nat Cell Biol*, 10:837-848. Doi.org/10.1038/ncb1748.

West FD, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, Gallegos-Cardenas A, Pratt SL, Stice SL. 2011. Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. *Stem Cells*, 29:1640-1643.

Wu Z, Chen J, Ren J, Bao L, Liao J, Cui C, Rao L, Li H, Gu Y, Dai H, Zhu H, Teng X, Cheng L, Xiao L. 2009. Generation of pig induced pluripotent stem cells with a drug-inducible system. *J Mol Cell Biol*, 1:46-54. Doi.org/mjp003 [pii]10.1093/jmcb/mjp003.

Yu C, Ji SY, Dang YJ, Sha QQ, Yuan YF, Zhou JJ, Yan LY, Qiao J, Tang F, Fan HY. 2016. Oocyte-expressed yes-associated protein is a key activator of the early zygotic genome in mouse. *Cell Res*, 26:275-287. Doi.org/10.1038/cr.2016.20.

DOI: 10.21451/1984-3143-AR2019-0051

Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Intrafollicular barriers and cellular interactions during ovarian follicle development

Gabriella Mamede Andrade, Maite del Collado, Flávio Vieira Meirelles, Juliano Coelho da Silveira, Felipe Perecin*

[1]Faculty of Animal Sciences and Food Engineering, Department of Veterinary Medicine, University of São Paulo, Pirassununga, São Paulo, Brazil.

Abstract

Follicles are composed of different interdependent cell types including oocytes, cumulus, granulosa, and theca cells. Follicular cells and oocytes exchange signaling molecules from the beginning of the development of the primordial follicles until the moment of ovulation. The follicular structure transforms during folliculogenesis; barriers form between the germ and the somatic follicular cells, and between the somatic follicular cells. As such, communication systems need to adapt to maintain the exchange of signaling molecules. Two critical barriers are established at different stages of development: the zona pellucida, separating the oocyte and the cumulus cells limiting the communication through specific connections, and the antrum, separating subpopulations of follicular cells. In both situations, communication is maintained either by the development of specialized connections as transzonal projections or by paracrine signaling and trafficking of extracellular vesicles through the follicular fluid. The bidirectional communication between the oocytes and the follicle cells is vital for driving folliculogenesis and oogenesis. These communication systems are associated with essential functions related to follicular development, oocyte competence, and embryonic quality. Here, we discuss the formation of the zona pellucida and antrum during folliculogenesis, and their importance in follicle and oocyte development. Moreover, this review discusses the current knowledge on the cellular mechanisms such as the movement of molecules via transzonal projections, and the exchange of extracellular vesicles by follicular cells to overcome these barriers to support female gamete development. Finally, we highlight the undiscovered aspects related to intrafollicular communication among the germ and somatic cells, and between the somatic follicular cells and give our perspective on manipulating the above-mentioned cellular communication to improve reproductive technologies.

Keywords: cellular communication, extracellular vesicles, granulosa cells, oocyte, ovarian follicle, transzonal projections

Introduction: Follicle development

The ovarian follicle development starts long before birth during the intra-uterine period (Russe, 1983). The primordial germ cells migrate to the genital ridge, colonize, and proliferate. After this highly proliferative period, a human female fetus has approximately 6-7 million germ cells around the 20th week of gestation, however a vast majority of these germ cells are lost and approximately 1 to 2 million oocytes remain viable at birth (Motta *et al*., 1997; Sun *et al*., 2017). In bovines, the maximum number of germ cells is around 2.5 million at about the 15th week of gestation (Erickson, 1966) and thirteen days after birth bovine germ cells number decrease approximately to 68 thousand. This dramatic loss of germ cells close after birth occurs in most female mammals (Paulini *et al*., 2014).

Once mitotic proliferation stops, these germ cells arrest at meiotic prophase I to form the germ cell nests (Buehr, 1997; Tilly, 2001; Sun *et al*., 2017). Close to birth, breakdown of the germ cell nests occurs with the formation of the primordial follicle. Two cell types characterize this primordial follicle: a primary oocyte surrounded by a single layer of pre-granulosa cells (Fortune, 1994; BrawTal and Yossefi, 1997; Eppig, 2001). The primordial follicle population in the ovary serves as a reservoir for developing follicles and oocytes throughout the female reproductive life (Zuckerman, 1951; Kerr *et al*., 2013). After puberty, groups of primordial follicles are periodically recruited to initiate folliculogenesis.

Although the precise mechanisms that regulate germline nest breakdown and primordial follicle formation are mostly unknown (Wang *et al*., 2017), several growth factors and hormones play essential roles in primordial follicle formation (Pepling, 2012), for example estradiol-17β (E2) and members of the transforming growth factor beta (TGF-β) superfamily (Knight and Glister, 2006; Wang and Roy, 2007; Chakraborty and Roy, 2017). The TGF-β family members are secreted by the oocyte and include bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), which act via autocrine and paracrine mechanisms, regulating follicle growth and differentiation, as well as granulosa and thecal cell function during follicular development (Dong *et al*., 1996; Eppig *et al*., 1997; Gilchrist *et al*., 2004; Sanfins *et al*., 2018). By secreting these members of TGF-β family the oocyte is the main responsible for activating primordial follicles (Eppig, 2001).

Ovarian follicle development is a continuous process that has two different phases: the preantral and antral. The first phase, preantral, is gonadotropin-independent and relies on local growth factors. As folliculogenesis progresses, the follicle becomes

*Corresponding author: fperecin@usp.br
orcid.org/0000-0003-2009-5863
Received: July 30, 2019
Accepted: May3, 2019

gonadotropin-responsive and develops until secondary follicles. The second phase, antral, is gonadotropin-dependent and is characterized by the presence of the tertiary follicles, which has the presence the antrum, a cavity filled with follicular fluid (Dvořák and Tesařík, 1980; Erickson and Shumichi, 2001). This entire process of growth and differentiation of the follicle is accompanied by the oocyte growth and acquisition of competence (El-Hayek and Clarke, 2015; Monniaux, 2016).

Stimulation by the locally secreted factors activates the primordial follicles initiating the preantral growth phase for development into a primary follicle. Factors responsible for primary follicle development are not fully known; however, it is known that granulosa cell-derived anti-Mullerian hormone and activins participate in the regulation of this process (reviewed by Matzuk *et al*., 2002). The primary follicles are characterized by the presence of an oocyte covered with a single layer of cuboidal granulosa cells. As the oocyte grows, the granulosa cells proliferate to envelop the surface of the expanding oocyte (vandenHurk *et al*., 1997).

Continuous granulosa cell proliferation results in multiple layers of cells surrounding the oocyte and the follicles are referred to as secondary follicles. At this stage, the formation of the theca cell layer starts, separated from the granulosa by a basement membrane (BrawTal and Yossefi, 1997). At the same time, oocytes undergo alterations as the formation of cortical granules in the cytoplasm (Fair *et al*., 1997) and the beginning of mRNA synthesis (McLaughlin *et al*., 2010). At this stage, the formation of the zona pellucida (ZP) around the oocyte starts, to form the first significant barrier between the oocyte and the somatic granulosa cells (BrawTal and Yossefi, 1997; Clarke, 2018) (Fig. 1A).

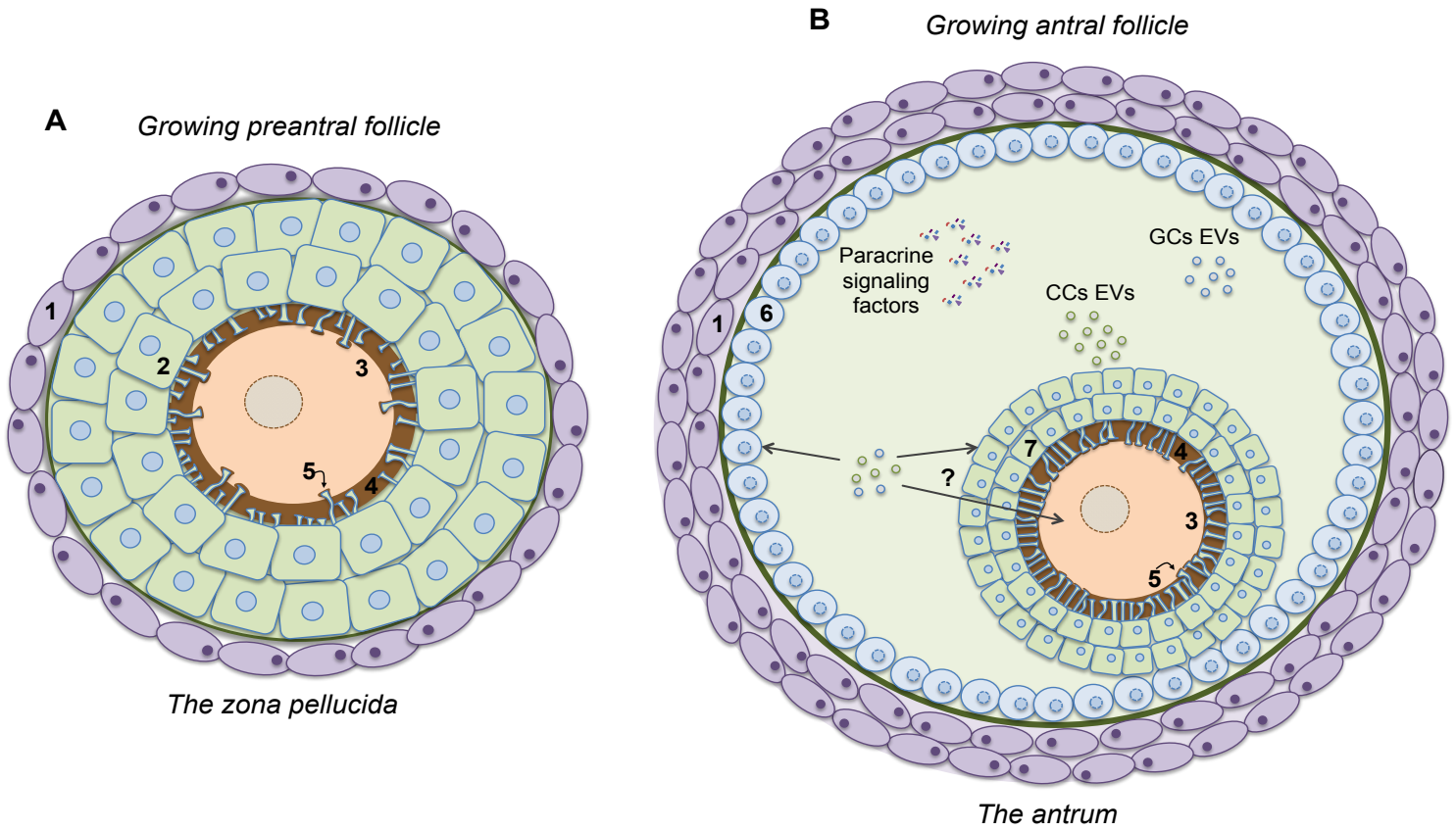
As the secondary follicle develops, more layers of granulosa cells form, and an antral cavity filled with follicular fluid develops between them. With the initiation of the antral phase of follicular growth, the follicle is now a tertiary follicle. During the transition of the secondary to tertiary follicle the second significant barrier between follicular cells is formed (Fig. 1B). Indeed, the antrum induces the differentiation of granulosa subpopulations, the original granulosa cells present in the outer wall of the follicle and that specialized granulosa cells, now cumulus cells, that directly surround the oocyte during further development. Mural granulosa cells and cumulus cells became exposed to opposing gradients of follicle-stimulated hormone (FSH) and oocyte-secreted factors (OSF) (Fortune, 1994; Eppig, 2001; Wigglesworth *et al*., 2015). In this phase of intense follicle growth, the oocyte slows down or even stops its growth, while stromal cells form two layers of cells external to the basement membrane, the internal and external theca cell layers (Fair *et al*., 1997; Hyttel *et al*., 1997; Guo *et al*., 2016).

In bovine, from the beginning of antral phase until a diameter of approximately 8 mm, follicle growth is stimulated by FSH secreted by the pituitary gland. Follicles develops by the rapid proliferation of granulosa and theca cells that contribute to the further enlargement of the antrum and the follicle itself. From a diameter of 8 mm onwards, the follicle develops mainly by the trophic stimulation of LH, and eventually, after the LH surge, will be termed preovulatory follicles (Eppig *et al*., 1997).

At the end of its growth, the dominant follicle reaches a plateau phase of non-exponential growth with fewer cell divisions and slower diameter increase (Girard *et al*., 2015). Following the preovulatory gonadotropin surge, follicular cells initiate morphological, endocrine, and biochemical changes associated with luteinization process (Smith *et al*., 1994; Revelli *et al*., 2009). In monovulatory species, only one follicle continues its growth to become an ovulatory follicle, while the remaining antral follicles regress and undergo atresia (Hennet and Combelles, 2012).

For the follicle formation and its steady growth during the whole folliculogenesis process, the bidirectional communication within the follicle environment is essential for the complete development of the follicle as well as the oocyte. The crosstalk between the oocyte and somatic follicular cells and between the somatic follicular cells occurs through the interactions mediated by paracrine signaling factors, by gap junctions and, as recently described, by extracellular vesicles. The paracrine signaling occurs through the secretion of factors from the oocyte or from the somatic cells. The gap junctions are structures formed by connexins that allow the transport of molecules of low molecular weight (<1 kDa) as ions, metabolites and amino acids between granulosa cells and cumulus cells, and between cumulus and oocyte cells. These junctions connect neighbor follicular cells or germ and somatic cell, at the bulk end of transzonal projections. The extracellular vesicles consist in a communication system mediated by vesicles secreted by cells. These vesicles, may have proteins, miRNAs and mRNAs as cargo, and are secrete and uptake by follicular cells (reviewed by Del Collado *et al*., 2018).

Hence, there are two physical barriers existing in the follicular environment, the ZP and the antrum. In both cases, cellular communication mechanism overcomes these barriers to maintain the exchange of messages via transzonal projections (TZPs) and extracellular vesicles (EVs; Fig. 2). These barriers, the communication mechanisms within, and the importance of such communication for the follicle and oocyte development are discussed in the following part of the review.



Legend: **1 –** Theca cells; **2 –** Granulosa cells; **3 –** Oocyte; **4 –** Zona pellucida; **5 –** Transzonal projections; **6 –** Mural granulosa cells; **7 –** Cumulus cells.

Figure 1. *Physical barriers to cell-to-cell communication in the ovarian follicle are established during folliculogenesis.* During preantral growth, zona pellucida, the first significant barrier between the oocyte and follicular cells, is formed. This barrier between the germ and somatic cells results from the deposition of glycoproteins by the oocyte. For continuous maintenance of a cytoplasmic bridge between germ and somatic cells, the oocyte stimulates the granulosa cells to generate specialized cytoplasmic filaments connecting both cells – the transzonal projections **(A)**. In antral growing follicles, a second significant barrier among follicular cells is formed – the antrum. Bilateral communication is maintained by paracrine signaling and extracellular vesicle traffic. Paracrine signaling of oocyte-secreted factors and transactivation of the EGF receptor by LH signaling drives follicle development and ovulation. Extracellular vesicles are secreted into the follicular fluid and are taken up by different cells types by a cargo delivery mechanism. The direct transfer of EVs-cargo from the follicular fluid to the oocyte remains elusive **(B)**. CCs – cumulus cells; EVs – extracellular vesicles; GCs – granulosa cells; Theca – theca cells.

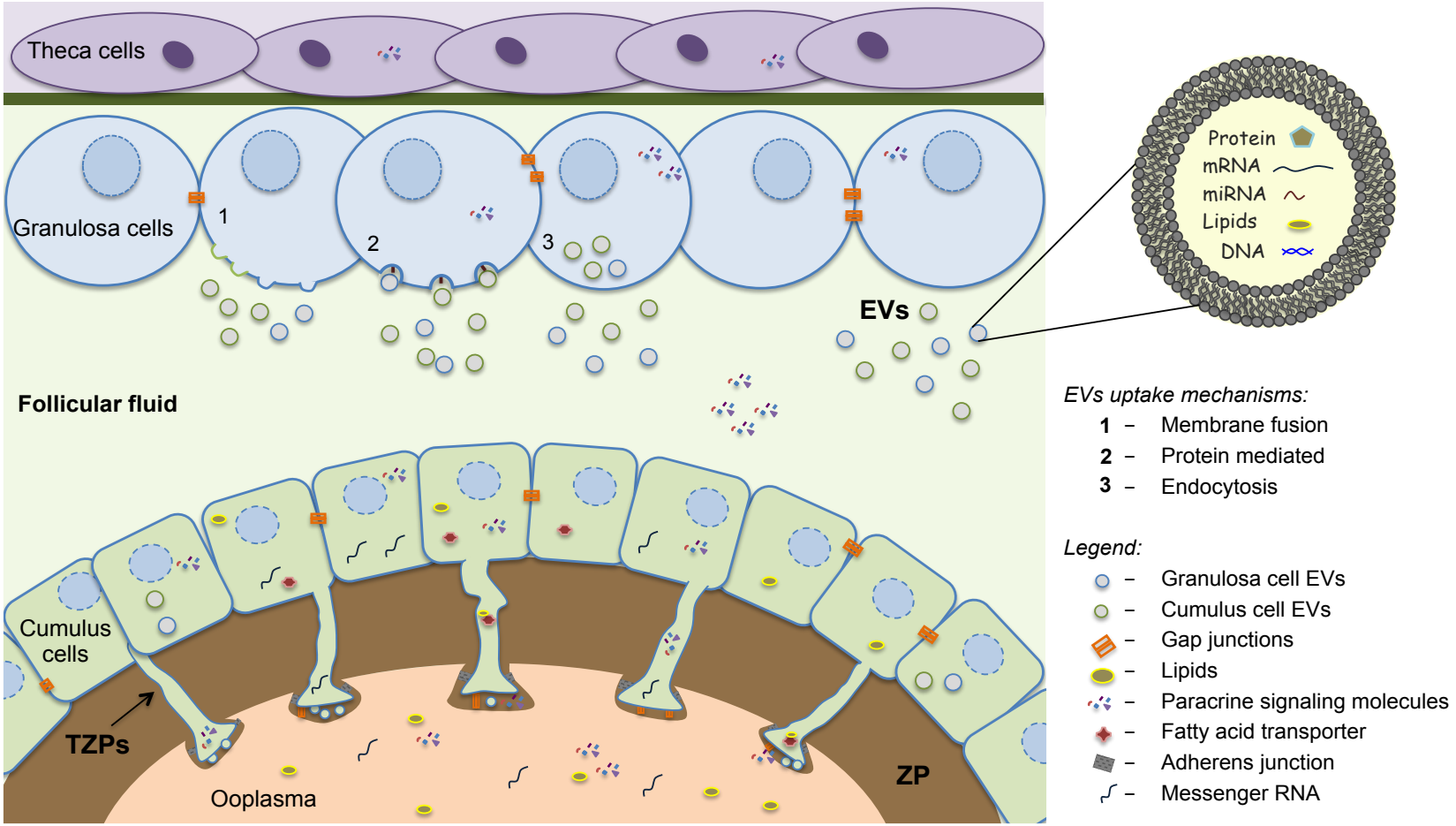


Figure 2. *Bidirectional communication within the ovarian follicle microenvironment.* The bidirectional crosstalk between cells that compose the follicle is associated with follicular development and acquisition of oocyte competence. Cellular crosstalk between germ-somatic cells and between somatic cells is mediated by the secretion of paracrine factors, by the communication through transzonal projections (TZPs) via gap junctions, and via extracellular vesicles (EVs) trafficking from the bulk end of TZPs to the oocyte, or trafficking into the follicular fluid. TZPs are specialized cytoplasmic projections that extend across zona pellucida (ZP) and allow the exchange of small molecules such as sugars, pyruvate, amino acids, and nucleotides, and large molecules such as mRNAs, lipids, and small organelles. Follicular fluid EVs are lipid bilayer vesicles loaded with proteins, mRNAs, microRNAs, lipids, and DNA and are taken up through endocytosis, protein recognition, and membrane fusion by distinct cell types within the ovarian follicle allowing communication with distant cells.

The first barrier

ZP formation and the germ-soma barrier

The ZP is a relatively thick extracellular coat that surrounds all mammalian oocytes. It is formed in the preantral phase of follicular development during the formation of secondary follicles, when the oocytes are arrested in the late diplotene stage and undergoing active growth (Wassarman and Litscher, 2012). This porous extracellular coat is formed by three or four glycoproteins depending on the species (Bleil and Wassarman, 1980; reviewed by Gupta, 2015). The ZP has essential functions during oogenesis, fertilization, and pre-implantation development (Wassarman and Litscher, 2012). For example, during oocyte development, the ZP integrity is important to maintain the communication between the oocyte and the cumulus cells (Wang *et al*., 2019). During fertilization the ZP play fundamental roles, as the block of non-specific fertilization, the block of polyspermia (Wassarman, 1999; Florman and Ducibella, 2006). And during early embryo development the ZP permit that cleavage stage embryos move freely along the oviduct and protect the growing embryo until implantation (Gupta *et al*., 2015) so that the ZP presence is necessary for normal early development in uterus (Modliński, 1970).

As an oocyte increases in diameter, its ZP increases in thickness (Wassarman and Litscher, 2013) and separate the oocytes and the surrounding cumulus cells. Depending on the species, the ZP ranges in thickness from less than 1 mm to more than 25 mm (Keefe *et al*., 1997). Despite this physical separation, the oocytes and cumulus cells maintain contact by the formation of TZPs (Albertini *et al*., 2001; Eppig, 2018). TZPs mainly originate from the cumulus cells in the layer immediately adjacent to the oocyte, but they are also shown to arise from cumulus cells positioned in oocyte more distant layers (Jaffe and Egbert, 2017). Since the number of TZPs present in the ZP of a developing oocyte is quite high, it is likely that each somatic cell surrounding the oocyte emits multiple projections towards the oocyte. Also, some projections extend from a single point of origin at the granulosa cell membrane and subsequently dividing into several TZPs towards the ooplasm (El-Hayek *et al*., 2018).

There are two hypotheses for the formation of TZPs: by "stretching" or "pushing." In the "stretching" model, the adhesion sites between the oocyte and granulosa cells are already present before ZP formation and remain during the ZP deposition process, to become stretched cytoplasmic filaments called TZPs. The second hypothesis, known as "pushing," proposes that the TZPs are elaborated from the granulosa cells after the deposition of the ZP and grow towards the oocyte where they establish contact with their plasma membrane (Clarke, 2017).

The growing oocyte induces somatic cells to generate the TZPs (El-Hayek *et al*., 2018). Factors secreted by the oocyte, such as GDF9 and FSH, correlate with the development of TZPs. Recent studies show that GDF9 produced by the oocyte acts via the SMAD signaling pathway to stimulate neighboring granulosa cells to generate TZP structures (El-Hayek *et al*., 2018). Besides that, recent functional studies verified that GDF9 maintains stable mRNAs that encode TZPs structural components (El-Hayek *et al*., 2018) and the absence of oocyte GDF9 leads to morphologically abnormal TZPs in mice (Dong *et al*., 1996; Carabatsos *et al*., 1998). FSH induces the retraction of TZPs, but the specific pathways by which this happens are still unclear (Combelles *et al*., 2004).

Oocyte and soma interactions through TZPs

The TZPs form concomitantly with the ZP and are specialized filopodia characterized as communication channels of approximately 2 um in diameter without the fusion of membranes (Macaulay *et al*., 2014). These channels originate from the cytoplasmic filaments of actin or tubulin, and their function depends on the composition. TZPs formed by tubulin filaments are related to cell adhesion while actin TZPs are involved in cell communication, and the latter are prevalent in oocyte ZP (Li and Albertini, 2013).

These projections allow communication between the oocyte and somatic cells. As the TZPs are free-ended structures, the exchange of small molecules occurs at the bulk end of the projections by gap junctions and intermediate junctions (zonula adherens-like junctions) that keep the cytoplasmic membranes of both cells in close contact (Hyttel *et al*., 1997; Albertini and Barrett, 2004). Additionally, at the bulk end of TZPs, a cleft is formed between the plasma membrane of the TZP and the oolemma. Extracellular vesicles were identified at this cleft (Macaulay *et al*., 2014) and are involved in potential mechanisms by which cargo transfer occurs from somatic cells to oocyte.

Some molecules, such as mRNAs, lipids, pyruvate and cGMP, are shown to be transported through TZPs, suggesting the importance of these communication mechanisms between the oocyte and the surrounding cumulus cells. TZPs have distinct roles, for example: i) in mRNA accumulation, as evidenced by passage of polyadenylated transcripts (Macaulay *et al*., 2014; Macaulay *et al*., 2016), ii) in metabolic and nutritional cooperation, due to continuous exchange of small molecule ions, cyclic nucleotides, and amino acids (Thomas *et al*., 2004; Lodde *et al*., 2013), iii) and providing energy substrates such as pyruvate, lactate (Scantland *et al*., 2014), and other metabolites. Recent results show that TZPs also have a role in lipid transport from cumulus cells to oocyte. The TZPs lipid transport was proved by co-localization of fatty acid binding protein 3 (FABP3), a protein responsible for carrying lipids, with TZPs within zona pellucida and by the increase of oocyte lipid droplets dependent on the presence of TZPs, indicating that TZPs might be involved in the oocyte lipid accumulation during maturation (del Collado *et al*., 2017).

The communication through TZPs have fundamental role in the oocyte meiosis control and oocyte maturation, since the transport of essential molecules as cAMP, is mediated by TZPs from somatic

cells to the oocyte (Eppig *et al*., 2005; Gilchrist *et al*., 2016). *In vitro* studies with bovine cumulus-oocyte complex revealed that the communication is maintained until the resumption of meiosis and onset of detachment within 9 h of maturation, and gradually decreases up to 22 h, when it eventually comes to a stop (Macaulay *et al*., 2014). A recent study pointed out that inclusion of the pre-IVM phase with a combination of cAMP modulators, resulted in maintenance of the density of TZPs after 20 h of *in vitro* maturation, resulting in improvement in the cumulus-oocyte communication leading to enhanced oocyte developmental competence (Soto-Heras *et al*., 2019). In aging females, the ability of somatic cells to respond to oocyte signals is reduced, resulting in lower formation of TZPs. Consequently, the reduced oocyte-somatic cell communication is the presumed cause for the reduced fertility in aged females (El-Hayek *et al*., 2018).

Interestingly, most of the time the TZPs are not in contact with the oocyte, they subdivide and form gap junctions between each other (Baena and Terasaki, 2019), a sign that these projections also have other essential functions such as communication between somatic cells. The TZPs are involved in essential processes for oocyte and consequently, embryo development. Studies investigating the transport mechanisms present in TZPs and how the *in vitro* environment influences these projections are still ongoing. This knowledge will probably be helpful to prevent lipid accumulation, aging consequences, and to improve *in vitro* oocyte maturation, with broad implications for animal and human assisted reproduction technologies.

The second barrier

Antrum formation and the cumulus-granulosa barrier

The antral follicles are characterized by the formation of a cavity filled with the follicular fluid. The follicular fluid originates from two sources, the bloodstream of thecal capillaries present in the ovary cortical region, and the components secreted by follicular cell layers, especially the granulosa cells and the fluid production, which intensifies with the enlargement of the follicles (Rodgers and Irving-Rodgers, 2010; Hennet and Combelles, 2012). The main hypothesis on follicular fluid formation suggests that an osmotic gradient is generated by granulosa cells production of hyaluronan and the chondroitin sulfate proteoglycan versican. This gradient generates influx of fluid derived from the thecal vasculature (Rodgers and Irving-Rodgers, 2010).

The follicular fluid contains a complex mixture of ions, proteins, metabolites, hormones, lipids, energy substrates, and reactive oxygen species (Leroy *et al*., 2004; Meeker *et al*., 2009; Ambekar *et al*., 2013). It serves as a source of regulatory molecules, such as gonadotrophins, steroids, growth factors, enzymes, proteoglycans, and lipoproteins (Revelli *et al*., 2009). This diverse array of molecules suggest that the follicular fluid is more than a reservoir and also supports intense metabolic activity, with substantial impact on follicular cells (Freitas *et al*., 2017) and oocyte. Roles of the follicular fluid were already reported, such as the participation in oocyte's acquisition of developmental competence (Fayezi *et al*., 2014; O'Gorman *et al*., 2013; Wallace *et al*., 2012) and in oocyte meiosis (Byskov *et al*., 1995; Mendoza *et al*., 2002). For example, hormone level in the follicular fluid, such as FSH (Suchanek *et al*., 1988), hCG (Ellsworth *et al*., 1984; Enien *et al*., 1998) and LH (Cha *et al*., 1986) have been reported to promote oocyte maturation and to increase chances of fertilization. The gonadotropins induce granulosa cells to secret hyaluronic acid (Mendoza *et al*., 2002) affecting oocyte development; they also act synergistically with estradiol (E2) enhancing cytoplasmatic maturation and controlling oocyte meiosis via cAMP secretion (Mendoza *et al*., 2002; Revelli *et al*., 2009). Another example is the fatty acids found in the follicular fluid that are incorporated by the oocyte and that have influence on oocyte maturation and quality. Excess of fatty acids were reported to negatively impact fertility outcomes (Shaaker *et al*., 2012).

The signaling mechanism for the formation of the antrum is not well understood; however, it was shown that FSH and type 1 insulin-like epidermal growth factors promote the formation of the antrum in cultured follicles *in vitro* (Gutierrez *et al*., 2000; Hillier, 2009). The growth of the antral follicles in bovines occurs in two distinct phases. The first is the slow phase where the follicles take approximately 30 days to advance from 0.3 mm in diameter to the stage of small antral follicles, which are about 3 mm in diameter. In this period of follicular growth, the oocyte reaches its final growth, approximately 110 µm in diameter, which relates to the acquisition of competence for development (Fair *et al*., 1997; Rodriguez and Farin, 2004). The second phase is the active phase when small follicles, approximately 3 mm in diameter, take from five to seven days to become dominant follicles, more than 8 mm in diameter. This phase is followed by a variable period of dominance, culminating in the development of the preovulatory follicle and ovulation (Bleach *et al*., 2001; Mihm and Bleach, 2003).

Given that antrum formation separates follicular cells and gametes, the need to maintain the communication between these cells is accomplished mainly through paracrine signaling. An example of paracrine signaling is the oocyte-secreted factors (GDF9 and BMP15) that interact with molecules such as FSH, IGF1 and androgens to promote mural granulosa proliferation and cumulus cells differentiation (Gilchrist *et al*., 2004).

Other examples of critical paracrine factors are found in preovulatory follicle. The signaling cascade triggered by the pre-ovulatory LH peak propagate through the ovulatory follicle via paracrine factors and stimulates the release of epidermal growth factor (EGF) ligands from the mural granulosa cells, that move across the follicular fluid to reach the cumulus cells. In these target cells induce changes in gene expression that will decrease the cGMP concentration in the cumulus cells

and consequently in the oocyte, resulting in cumulus cells expansion and meiosis resumption (Conti *et al*., 2012). The OSF also play vital roles regulating extracellular matrix stability, leading to ovulation (Gilchrist *et al*., 2004). Thus, this cascade is essential for the induction of gene expression required for follicle rupture, oocyte maturation and ovulation.

A novel communication system in the antrum

While paracrine signaling communication in follicular fluid have been described for many years, a novel communication mechanism, mediated by extracellular vesicles, has recently been described. Extracellular vesicles are phospholipid bilayer vesicles that transport biomolecules such as proteins, microRNAs, mRNAs, DNA, and lipids (Taylor and Gercel-Taylor, 2013; Di Pietro, 2016; Ávila *et al*., 2019). Their content varies and reflects the cell of origin (Akers *et al*., 2013). It was shown that the secretory cells actively select the number and the cargo of EVs depending on specific physiological and environmental conditions, such as diseases, nutritional status and stress (van Niel *et al*., 2018).

EVs are classified into microvesicles (MVs) and exosomes (Exos) according to their characteristics such as size, shape, membrane proteins, structural lipids, and their origin. The MVs are big with a diameter ranging from 500-1000 nm, have an irregular shape, and originate from the rupture of the cellular plasma membrane, which makes MVs a more heterogeneous population. On the other hand, the Exos have an approximate diameter of 50-100 nm (Crescitelli *et al*., 2013) and appear in electron microscopy as a cup-shaped form, depending on the preparation method. Importantly, EVs originate from the late endosomes, also called multivesicular bodies and are released into the extracellular space by fusion of the multivesicular body membrane with the plasma cell membrane (Taylor and Gercel-Taylor, 2013).

There are three modes of interaction between the EV and their target cells; i) the first is through direct interaction between membrane proteins of the EV with receptors on the target cell membrane, ii) The second by membrane cleavage of the EV proteins by proteases present in the extracellular space, and the release of products which act on the receptors of the target cell, and iii) the third by direct fusion of the EV membranes with the cell membrane, releasing the EV content in the cell and incorporating proteins and receptors into the cell membrane (Mathivanan *et al*., 2010).

EVs are present in several body fluids and were first described in the follicular fluid a few years ago (da Silveira *et al*., 2012). Follicular cells secrete these vesicles into the follicular environment (Andrade *et al*., 2017a) and the EVs are taken up by the granulosa and cumulus cells, in *in vivo* and *in vitro* systems (da Silveira *et al*., 2012; Di Pietro, 2016). Additionally, the oocyte surrounding cumulus cells could provide an entry point to deliver to oocyte the molecules that cannot pass through gap junctions, such as RNAs, miRNAs, proteins and lipids (Macaulay *et al*., 2014; Macaulay *et al*., 2016). Interestingly, *in vitro* studies showed that follicular fluid EVs alter transcript levels in oocytes (Dalanezi *et al*., 2017) and enhance oocyte competence to develop until the blastocyst stage (da Silveira *et al*., 2017).

The follicular fluid undergoes dynamic changes over late stages of folliculogenesis and its EVs content modify as consequence. As an example, the follicular fluid EVs from different size follicles have distinct concentrations and miRNA content (Navakanitworakul *et al*., 2016), and it has been described that they can differentially stimulate granulosa cells proliferation *in vitro* (Hung *et al*., 2017). Additionally, female age (Diez-Fraile *et al*., 2014; da Silveira *et al*., 2015a), and endocrine environment (da Silveira *et al*., 2015a) are important factors that can alter EVs content. Regarding female age, the miRNAs content of EVs from follicular fluid varies according to age. In old mares compare to young, a group of highly expressed miRNAs negatively modulates TGF-β, resulting in compromised maturation of oocytes (Da Silveira *et al*., 2015b). Also, the miR-23a, highly expressed in old mares (Da Silveira *et al*., 2015b), is correlated with human granulosa cells apoptosis pathway by inhibition of X-linked inhibitor of apoptosis protein (XIAP) and an increase in caspase 3 protein levels (Yang *et al*., 2012; Mobarak *et al*., 2019).

The bidirectional communication through EVs is associated with follicular development, oocyte growth, and quality, in humans and domestic animals (da Silveira *et al*., 2012; Sang *et al*., 2013; Sohel *et al*., 2013; Hung *et al*., 2015). During the maturation process, the EVs induce cumulus cell expansion and alter expression of genes related to the expansion process, when used as a supplement on cumulus-oocyte complexes (COCs) maturation medium (Hung *et al*., 2015). In another study, EVs stimulated granulosa cell proliferation by modulating Src, Pi3K/Akt and mitogen-activated protein kinase (MAPK) pathways, and interestingly, the EVs from small follicles were preferentially taken up by granulosa cells (Hung *et al*., 2017). Further, using EVs as supplements for embryo maturation in culture media, partially altered genes related to metabolism and development as well as miRNA and global DNA methylation and hydroxymethylation of bovine embryos produced *in vitro* (da Silveira *et al*., 2017). Another study observed a positive effect of follicular fluid EVs during *in vitro* maturation; they protected COCs from the harmful effects of heat shock stress (Rodrigues *et al*., 2019).

Although EVs carry different molecules, many studies show that the effect of EVs on cells are related to miRNAs and their regulatory effects, mainly because they are very stable and show resistance to degradation. The miRNAs in the follicular fluid EVs were associated with fertilization and embryo quality (Machtinger *et al*., 2017). Some studies, in humans and animals, have shown the role of miRNAs present in the follicular fluid, regulating follicular growth and development, cellular signaling, oocyte meiosis, and ovarian function (Martinez *et al*., 2018). EVs miRNAs modulate such important reproduction processes by the regulation of

pathways as insulin, epidermal growth factor receptor (ErbB), MAPK, Wnt signaling, TGF-β and PI3K-Akt signaling among others (da Silveira *et al*., 2012; Santonocito *et al*., 2014; Andrade *et al*., 2017b). Due to the importance of cellular communication in this environment and the immense potential of EVs, efforts are dedicated to better understand its functions and importance during oocyte maturation and early embryo development *in vitro*.

Conclusions and perspectives

Normal oocyte development depends on a finely regulated, constant, and reciprocal cell-to-cell communication between the follicle components. The crosstalk may occur by paracrine signaling and exchange of small molecules via gap-junctions; these are well-studied mechanisms. However, novel mechanisms of communication in the follicle microenvironment have been recently identified. These mechanisms allow the exchange of large molecules, such as nucleic acids, proteins, and lipids between follicular compartments, and are mediated by the trafficking of vesicles from the bulk-end of TZPs to the oolemma, or by the transit of EVs in the follicular fluid.

The importance of these novel communication mechanisms is exemplified by circumstances for which association with communication mediated by TZP or EVs has already been demonstrated, such as the accumulation of maternal transcripts in the oocyte, the acquisition of oocyte competence, and the decline in oocyte developmental potential associated with aging. Moreover, there is increasing evidence that assisted reproductive technologies disturbs the intrafollicular interactions, but their short and long-term effects are yet to be studied.

Studies in this field are limited, and in addition to the lack of knowledge about the effects of disrupting such communication mechanisms, there are plenty of unanswered questions in the subject area. Among other questions, it is unknown how the passage of molecules into the oocytes is regulated, and whether EVs in the follicular fluid can directly or indirectly modulate the oocyte. A thorough understanding of the biology of TZPs and EVs-mediated communication will potentiate the advancement of assisted reproductive technologies. These possibilities include modulation of the function of TZPs, regulation of EVs-cargo, and its use in *in vitro* culture conditions.

Author contributions

GMA: Conceptualization, Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing; MC: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing; FVM: Funding acquisition, Resources, Writing – review & editing; JCS: Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing; FP: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review& editing.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments and Funding disclosure statement

The authors would like to thank Sao Paulo Research Foundation (FAPESP) – grants (GMA) 2018/01431-9; (MDC) 2017/19825-0; (FMV) 2013/08135-2; (JCS) 2014/22887-0; (FP) 2018/13155-6; and National Council for Scientific and Technological Development (CNPq) – grant (FP) 306349/2017-5.

References

Akers JC, Gonda D, Kim R, Carter BS, Chen CC. 2013. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*, 113:1-11.

Albertini DF, Barrett SL. 2004. The developmental origins of mammalian oocyte polarity. *Semin Cell Dev Biol*, 15, 599-606.

Albertini DF, Combelles CMH, Benecchi E, Carabatsos MJ. 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*, 121:647-653.

Ambekar AS, Nirujogi RS, Srikanth SM, Chavan S, Kelkar DS, Hinduja I, Zaveri K, Prasad TSK, Harsha HC, Pandey A, Mukherjee S. 2013. Proteomic analysis of human follicular fluid: A new perspective towards understanding folliculogenesis. *J Proteomics*, 87:68-77.

Andrade G, Meirelles F, Perecin F, Silveira J. 2017a. Cellular and extracellular vesicular origins of miRNAs within the bovine ovarian follicle. *Reprod Domest Anim*, 52(6):1036-1045.

Andrade GM, da Silveira JC, Perrini C, Del Collado M, Gebremedhn S, Tesfaye D, Meirelles FV, Perecin F. 2017b. The role of the PI3K-Akt signaling pathway in the developmental competence of bovine oocytes. *Plos One*, 12.

Ávila ACFM, Andrade GM, Bridi A, Gimenes LU, Meirelles FV, Perecin F, da Silveira JC. 2019. Extracellular vesicles and its advances in female reproduction. *Anim Reprod*,16:31-38.

Baena V, Terasaki M. 2019. Three-dimensional organization of transzonal projections and other cytoplasmic extensions in the mouse ovarian follicle. *Sci Rep*, 9(1):1262.

Bleach ECL, Glencross RG, Feist SA, Groome NP, Knight PG. 2001. Plasma inhibin A in heifers: Relationship with follicle dynamics, gonadotropins, and steroids during the estrous cycle and after treatment with bovine follicular fluid. *Biol Reprod*, 64:743-752.

Bleil JD, Wassarman PM. 1980. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Dev Biol*, 76:185-202.

BrawTal R, Yossefi S. 1997. Studies *in vivo* and *in ivtro* on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil*, 109:165-171.

Buehr M. 1997. The primordial germ cells of mammals: Some current perspectives. *Exp Cell Res*, 232:194-207.

Byskov AG, Andersen CY, Nordholm L, Thogersen H, Xia GL, Wassmann O, Andersen JV, Guddal E, Roed T. 1995. Chemical-structure of sterols that activate oocyte meiosis. *Nature*, 374:559-562.

Carabatsos MJ, Elvin J, Matzuk MM, Albertini DF. 1998. Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol*, 204:373-384.

Cha K-Y, Barnes RB, Marrs RP, Lobo RA. 1986. Correlation of the bioactivity of luteinizing hormone in follicular fluid with oocyte maturity in the spontaneous cycle. *Fertil Steril*, 45:338-341.

Chakraborty P, Roy SK. 2017. Stimulation of primordial follicle assembly by estradiol-17 beta requires the action of bone morphogenetic protein-2 (BMP2). *Sci Rep*, 7(1):15581.

Clarke HJ. 2017. Regulation of germ cell development by intercellular signaling in the mammalian ovarian follicle. Wiley Interdisciplinary Reviews: *Dev Biol*, 7:e294. Doi: 10.1002/wdev.294.

Clarke HJ. 2018. History, origin, and function of transzonal projections: the bridges of communication between the oocyte and its environment. *Anim Reprod*, 15:215-223.

Combelles CMH, Carabatsos MJ, Kumar TR, Matzuk MM, Albertini DF. 2004. Hormonal control of somatic cell oocyte interactions during ovarian follicle development. *Mol Reprod Dev*, 69:347-355.

Conti M, Hsieh M, Zamah AM, Oh JS. 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Mol Cell Endocrinol*, 356:65-73.

Crescitelli R, Lässer C, Szabo TG, Kittel A, Eldh M, Dianzani I, Buzás EI, Lötvall J. 2013. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *J Extracell Vesicles*, 2: eCollection 2013. Doi: 10.3402/jev.v2i0.20677.

da Silveira JC, Andrade GM, del Collado M, Sampaio RV, Sangalli JR, Silva LA, Pinaffi FVL, Jardim IB, Cesar MC, Nogueira MFG, Cesar ASM, Coutinho LL, Pereira RW, Perecin F, Meirelles FV. 2017. Supplementation with small-extracellular vesicles from ovarian follicular fluid during *in ivtro* production modulates bovine embryo development. *Plos One*, 12(6):e0179451. Doi: 10.1371/journal.pone.0179451.

da Silveira JC, de Andrade GM, Nogueira MFG, Meirelles FV, Perecin F. 2015a. Involvement of mirnas and cell-secreted vesicles in mammalian ovarian antral follicle development. *Reprod Sci*, 22:1474-1483.

da Silveira JC, Veeramachaneni DNR, Winger QA, Carnevale EM, Bouma GJ. 2012. Cell-Secreted Vesicles in Equine Ovarian Follicular Fluid Contain miRNAs and Proteins: A Possible New Form of Cell Communication Within the Ovarian Follicle. *Biol Reprod*, 86(3):71. Doi: 10.1095/biolreprod.111.093252.

da Silveira JC, Winger QA, Bouma GJ, Carnevale EM. 2015b. Effects of age on follicular fluid exosomal microRNAs and granulosa cell transforming growth factor-beta signalling during follicle development in the mare. *Reprod Fertil Dev*, 27:897-905.

Dalanezi F, Destro F, Ferrazza R, García HM, Franchi F, Fontes P, Castilho A, Sartori R, Ferreira J. 2017. 183 Gene expression of *in ivtro*-maturated oocytes can be modulated by follicle exosomes from cows kept under thermoneutral or heat stress conditions. *Reprod Fertil Dev*, 29:200-200.

del Collado M, Andrade GM, Meirelles FV, da Silveira JC, Perecin F. 2018. Contributions from the ovarian follicular environment to oocyte function. *Anim Reprod*, 15:261-270.

del Collado M, da Silveira JC, Sangalli JR, Andrade GM, Sousa L, Silva LA, Meirelles FV, Perecin F. 2017. Fatty acid binding protein 3 and transzonal projections are involved in lipid accumulation during *in ivtro* maturation of bovine oocytes. *Sci Rep*, 7:2645. Doi:10.1038/s41598-017-02467-9

Di Pietro C. 2016. Exosome-mediated communication in the ovarian follicle. Journal of Assisted Reproduction and *Genetics*, 33:303-311.

Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter P, Benoit Y, Espeel M, D'Herde K. 2014. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of *in ivtro* fertilization. *Hum Fertil*, 17:90-98.

Dong JW, Albertini DF, Nishimori K, Kumar TR, Lu NF, Matzuk MM. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, 383:531-535.

Dvořák M, Tesařík J. 1980. Ultrastructure of human ovarian follicles, Biology of the Ovary, Springer, pp.121-137.

El-Hayek S, Clarke HJ. 2015. Follicle-stimulating hormone increases gap junctional communication between somatic and germ-line follicular compartments during murine oogenesis. *Biol Reprod*, 93(2):47. Doi: 10.1095/biolreprod.115.129569.

El-Hayek S, Yang Q, Abbassi L, FitzHarris G, Clarke HJ. 2018. Mammalian oocytes locally remodel follicular architecture to provide the foundation for germline-soma communication. *Curr Biol*, 28(7):1124-1131.

Ellsworth L, Balmaceda J, Schenken RS, Silverman A, Prihoda T, Asch R. 1984. Human chorionic gonadotropin and steroid concentrations in human follicular fluid in relation to follicle size and oocyte maturity in stimulated ovarian cycles. *Acta Eur Fertil*, 15:343-346.

Enien W, Chantler E, Seif M, Elstein M. 1998. Human ovarian granulosa cells and follicular fluid indices: the relationship to oocyte maturity and fertilization *in ivtro*. *Human Reprod*, 13:1303-1306.

Eppig JJ. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*, 122:829-838.

Eppig JJ. 2018. Reproduction: oocytes call, granulosa cells connect. *Curr Biol*, 28:R354-R356.

Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. 2005. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: Amino acid

transport. *Biol Reprod*, 73:351-357.

Eppig JJ, Wigglesworth K, Pendola F, Hirao Y, 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod*, 56:976-984.

Erickson B. 1966. Development and senescence of the postnatal bovine ovary. *J Anim Sci*, 25:800-805.

Erickson GF, Shimasaki S. 2001. The physiology of folliculogenesis: the role of novel growth factors. *Fertil Steril*, 76:943-949.

Fair T, Hulshof SCJ, Hyttel P, Greve T, Boland M. 1997. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anat Embryol*, 195:327-336.

Fayezi S, Darabi M, Nouri M, Rahimipour A, Mehdizadeh A. 2014. Analysis of follicular fluid total phospholipids in women undergoing in-vitro fertilisation. *J Obstet Gynaecol*, 34:259-262. Florman HM, Ducibella T. 2006. Fertilization in mammals. *Knobil and Neill's physiology of Reproduction*, 3:55-112.

Fortune JE. 1994. Ovarian follicular-growth and development in mammals. *Biol Reprod*, 50:225-232.

Freitas C, Neto AC, Matos L, Silva E, Ribeiro A, Silva-Carvalho JL, Almeida H. 2017. Follicular Fluid redox involvement for ovarian follicle growth. *J Ovarian Res*, 10(1):44. Doi: 10.1186/s13048-017-0342-3.

Gilchrist RB, Luciano AM, Richani D, Zeng HT, Wang X, De Vos M, Sugimura S, Smitz J, Richard FJ, Thompson JG. 2016. Oocyte maturation and quality: role of cyclic nucleotides. *Reproduction*, 152:R143-R157.

Gilchrist RB, Ritter LJ, Armstrong DT. 2004. Oocyte-somatic cell interactions during follicle development in mammals. *Anim Reprod Sci*, 82-3:431-446.

Girard A, Dufort I, Douville G, Sirard MA. 2015. Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod Biol Endocrinol*, 13:17. Doi: 10.1186/s12958-015-0010-7.

Guo J, Shi LY, Gong XH, Jiang MJ, Yin YX, Zhang XY, Yin H, Li H, Emori C, Sugiura K, Eppig JJ, Su YQ. 2016. Oocyte-dependent activation of MTOR in cumulus cells controls the development and survival of cumulus-oocyte complexes. *J Cell Sci*, 129:3091-3103.

Gupta SK. 2015. Role of zona pellucida glycoproteins during fertilization in humans. *J Reprod Immunol*, 108:90-97.

Gutierrez CG, Ralph JH, Telfer EE, Wilmut I, Webb R. 2000. Growth and antrum formation of bovine preantral follicles in long-term culture *in ivtro*. *Biol Reprod*, 62:1322-1328.

Hennet ML, Combelles CMH. 2012. The antral follicle: a microenvironment for oocyte differentiation. *Int J Dev Biol*, 56:819-831.

Hillier SG. 2009. Paracrine support of ovarian stimulation. *Mol Hum Reprod*, 15:843-850.

Hung W-T, Hong X, Christenson LK, McGinnis LK. 2015. Extracellular vesicles from bovine follicular fluid support cumulus expansion. *Biol Reprod*, 93(5):117. Doi: 10.1095/biolreprod.115.13297.

Hung W-T, Navakanitworakul R, Khan T, Zhang P, Davis JS, McGinnis LK, Christenson LK. 2017. Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. *Biol Reprod*, 97(4):644-655.

Hyttel P, Fair T, Callesen H, Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, 47:23-32.

Jaffe LA, Egbert JR. 2017. Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Ann Rev Physiol*, 79:237-260.

Keefe D, Tran P, Pellegrini C, Oldenbourg R. 1997. Polarized light microscopy and digital image processing identify a multilaminar structure of the hamster zona pellucida. *Hum Reprod*, 12:1250-1252.

Kerr JB, Myers M, Anderson RA. 2013. The dynamics of the primordial follicle reserve. *Reproduction*, 146:R205-R215.

Knight PG, Glister, C, 2006. TGF-beta superfamily members and ovarian follicle development. *Reproduction*, 132:191-206.

Leroy J, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, de Kruif A. 2004. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Anim Reprod Sci*, 80:201-211.

Li R, Albertini DF. 2013. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol*, 14:141-152.

Lodde V, Franciosi F, Tessaro I, Modina SC, Luciano AM. 2013. Role of gap junction-mediated communications in regulating large-scale chromatin configuration remodeling and embryonic developmental competence acquisition in fully grown bovine oocyte. *J Assist Reprod Genet*, 30:1219-1226.

Macaulay AD, Gilbert I, Caballero J, Barreto R, Fournier E, Tossou P, Sirard MA, Clarke HJ, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2014. The gametic synapse: rna transfer to the bovine oocyte. Biol Reprod, 91(4):90. doi: 10.1095/biolreprod.114.119867.

Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HAS, Gagne D, Sirard MA, Khandjian EW, Richard FJ, Hyttel P, Robert C. 2016. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biol Reprod*, 94(1):16. Doi: 10.1095/biolreprod.114.127571.

Machtinger R, Rodosthenous RS, Adir M, Mansour A, Racowsky C, Baccarelli AA, Hauser R. 2017. Extracellular microRNAs in follicular fluid and their potential association with oocyte fertilization and embryo quality: an exploratory study. *J Assist Reprod Genet*, 34:525-533.

Martinez RM, Liang LM, Racowsky C, Dioni L, Mansur A, Adir M, Bollati V, Baccarelli AA, Hauser R, Machtinger R. 2018. Extracellular microRNAs profile in human follicular fluid and IVF outcomes.*Sci Rep*, 8(1):17036. Doi: 10.1038/s41598-018-35379-3.

Mathivanan S, Ji H, Simpson RJ. 2010. Exosomes: Extracellular organelles important in intercellular communication. *J Proteomics*,73:1907-1920.

Matzuk MM, Burns KH, Viveiros MM, Eppig JJ.

2002. Intercellular communication in the mammalian ovary: Oocytes carry the conversation. *Science*, 296:2178-2180.

McLaughlin M, Bromfield JJ, Albertini DF, Telfer EE. 2010. Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. *Mol Hum Reprod*, 16:644-653.

Meeker JD, Missmer SA, Altshul L, Vitonis AF, Ryan L, Cramer DW, Hauser R. 2009. Serum and follicular fluid organochlorine concentrations among women undergoing assisted reproduction technologies. *Environ Health*, 8:32. Doi: 10.1186/1476-069X-8-32.

Mendoza C, Ruiz-Requena E, Ortega E, Cremades N, Martinez F, Bernabeu R, Greco E, Tesarik J. 2002. Follicular fluid markers of oocyte developmental potential. *Hum Reprod*, 17:1017-1022.

Mihm A, Bleach ECL. 2003. Endocrine regulation of ovarian antral follicle development in cattle. *Anim Reprod Sci*, 78:217-237.

Mobarak H, Heidarpour M, Lolicato F, Nouri M, Rahbarghazi R, Mahdipour M. 2019. Physiological impact of extracellular vesicles on female reproductive system; highlights to possible restorative effects on female age-related fertility. *Biofactors*, 45(3):293-303.

Modliński JA. 1970. The role of the zona pellucida in the development of mouse eggs *in vivo*. *J Embryol Exp Morphol*, 23:539-547.

Monniaux D. 2016. Driving folliculogenesis by the oocyte-somatic cell dialog: Lessons from genetic models. *Theriogenology*, 86:41-53.

Motta PM, Makabe S, Nottola SA. 1997. The ultrastructure of human reproduction .1. The natural history of the female germ cell: origin, migration and differentiation inside the developing ovary. *Hum Reprod Update*, 3:281-295.

Navakanitworakul R, Hung WT, Gunewardena S, Davis JS, Chotigeat W, Christenson LK. 2016. characterization and small RNA content of extracellular vesicles in follicular fluid of developing bovine antral follicles. *Sci Rep*, 6:25486. Doi: 10.1038/srep25486.

O'Gorman A, Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. 2013. Metabolic profiling of human follicular fluid identifies potential biomarkers of oocyte developmental competence. *Reproduction*, 146:389-395.

Paulini F, Silva RC, Rolo J, Lucci CM. 2014. Ultrastructural changes in oocytes during folliculogenesis in domestic mammals. *J Ovarian Res*, 7:102. Doi: 10.1186/s13048-014-0102-6.

Pepling ME. 2012. Follicular assembly: mechanisms of action. *Reproduction*, 143:139-149.

Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. 2009. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol*, 7:40. Doi: 10.1186/1477-7827-7-40.

Rodgers RJ, Irving-Rodgers HF. 2010. Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod*, 82:1021-1029.

Rodrigues TA, Tuna KM, Alli AA, Tribulo P, Hansen P, Koh J, Paula-Lopes F. 2019. Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survival to heat shock. *Reprod Fertil Dev*, 31(5):888-897. Doi: 10.1071/RD18450.

Rodriguez KF, Farin CE. 2004. Developmental capacity of bovine cumulus oocyte complexes after transcriptional inhibition of germinal vesicle breakdown. *Theriogenology*, 61:1499-1511.

Russe I. 1983. Oogenesis in cattle and sheep. *Bibl Anat*, 24:77-92.

Sanfins A, Rodrigues P, Albertini DF. 2018. GDF-9 and BMP-15 direct the follicle symphony. *J Assist Reprod Genet*, 35:1741-1750.

Sang Q, Yao ZY, Wang H, Feng RZ, Wang HJ, Zhao XZ, Xing QH, Jin L, He L, Wu LQ, Wang L. 2013. Identification of microRNAs in human follicular fluid: characterization of microRNAs that govern steroidogenesis *in ivtro* and are associated with polycystic ovary syndrome *in vivo*. *J Clin Endocrinol Metab*, 98:3068-3079.

Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzi P, Rizzari S, Maugeri M, Scollo P, Tatone C, Valadi H, Purrello M, Di Pietro C. 2014. Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. *Fertil Steril*, 102:1751-U1590.

Scantland S, Tessaro I, Macabelli CH, Macaulay AD, Cagnone G, Fournier E, Luciano AM, Robert C. 2014. The adenosine salvage pathway as an alternative to mitochondrial production of ATP in maturing mammalian oocytes. *Biol Reprod*, 91(3):75. Doi: 10.1095/biolreprod.114.120931.

Shaaker M, Rahimipour A, Nouri M, Khanaki K, Darabi M, Farzadi L, Shahnazi V, Mehdizadeh A. 2012. Fatty acid composition of human follicular fluid phospholipids and fertilization rate in assisted reproductive techniques. *Iran Biomed J*, 16:162-168.

S**mith MF, McIntush EW, Smith GW**, 1994. Mechanisms Associated With Corpus-Luteum Development. *J Anim Sci*, 72:1857-1872.

Sohel MMH, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K, Tesfaye D. 2013. Exosomal and non-exosomal transport of extra-cellular micrornas in follicular fluid: implications for bovine oocyte developmental competence. *Plos One*, 8(11):e78505. Doi: 10.1371/journal.pone.0078505.

Soto-Heras S, Paramio M-T, Thompson JG. 2019. Effect of pre-maturation with C-type natriuretic peptide and 3-isobutyl-1-methylxanthine on cumulus-oocyte communication and oocyte developmental competence in cattle. *Anim Reprod Sci*, 202:49-57.

Suchanek E, Simunic V, Macas E, Kopjar B, Grizelj V. 1988. Prostaglandin F2α, progesterone and estradiol concentrations in human follicular fluid and their relation to success of *in ivtro* fertilization. *Eur J Obstet Gynecol Reprod Biol*, 28:331-339.

Sun YC, Sun XF, Dyce PW, Shen W, Chen H. 2017. The role of germ cell loss during primordial follicle assembly: a review of current advances. *Int J Biol Sci*, 13:449-457.

Taylor DD, Gercel-Taylor C. 2013. The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids. *Front Genet*, 4:142. Doi: 10.3389/fgene.2013.00142.

Thomas RE, Armstrong DT, Gilchrist RB. 2004. Bovine cumulus cell-oocyte gap junctional communication during *in ivtro* maturation in response to manipulation of cell-specific cyclic adenosine 3 ',5 '-monophosophate levels. *Biol Reprod*, 70:548-556.

Tilly JL. 2001. Commuting the death sentence: How oocytes strive to survive. *Nat Rev Mol Cell Biol*, 2:838-848.

van Niel G, D'Angelo G, Raposo G. 2018. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*, 19:213-228.

vandenHurk R, Bevers MM, Beckers JF. 1997. In-vivo and in-vitro development of preantral follicles. *Theriogenology*, 47:73-82.

Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L. 2012. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. *Fertil Steril*, 97(5):1078-1084.e1-8. Doi: 10.1016/j.fertnstert.2012.01.122.

Wang C, Roy SK. 2007. Development of primordial follicles in the hamster: Role of estradiol-17 beta. *Endocrinology*, 148:1707-1716.

Wang Y, Lv C, Huang H-L, Zeng M-H, Yi D-J, Tan H-J, Peng T-L, Yu W-X, Deng H-W, Xiao H-M. 2019. Influence of mouse defective zona pellucida in folliculogenesis on apoptosis of granulosa cells and developmental competence of oocytes. *Biol Reprod*. Doi: 10.1093/biolre/ioz093.

Wang YY, Sun YC, Sun XF, Cheng SF, Li B, Zhang XF, De Felici M, Shen W. 2017. Starvation at birth impairs germ cell cyst breakdown and increases autophagy and apoptosis in mouse oocytes. *Cell Death Dis*, 8(2):e2613. Doi: 10.1038/cddis.2017.3.

Wassarman PM. 1999. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*, 96:175-183.

Wassarman PM, Litscher ES. 2012. Influence of the zona pellucida of the mouse egg on folliculogenesis and fertility. Int J Dev Biol, 56(10-12):833-9. Doi: 10.1387/ijdb.120136pw.

Wassarman PM, Litscher ES. 2013. Biogenesis of the Mouse Egg's Extracellular Coat, the Zona Pellucida. *Gametogenesis*, 102:243-266.

Wigglesworth K, Lee KB, Emori C, Sugiura K, Eppig JJ. 2015. Transcriptomic diversification of developing cumulus and mural granulosa cells in mouse ovarian follicles. *Biol Reprod*, 92(1):23. Doi: 10.1095/biolreprod.114.121756.

Yang XK, Zhou Y, Peng S, Wu L, Lin HY, Wang SY, Wang HM. 2012. Differentially expressed plasma microRNAs in premature ovarian failure patients and the potential regulatory function of mir-23a in granulosa cell apoptosis. *Reproduction*, 144:235-244.

Zuckerman S. 1951. The number of oocytes in the mature ovary. *Recent Prog Horm Res*, 6:63-109.

DOI: 10.21451/1984-3143-AR2019-0053

Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Reproductive physiology of the heat-stressed dairy cow: implications for fertility and assisted reproduction

Peter J. Hansen*

Department of Animal Sciences, D. H. Barron Reproductive and Perinatal Biology Research Program and Genetics Institute, University of Florida, Gainesville, Florida, USA.

Abstract

Heat stress causes a large decline in pregnancy success per insemination during warm times of the year. Improvements in fertility are possible by exploiting knowledge about how heat stress affects the reproductive process. The oocyte can be damaged by heat stress at the earliest stages of folliculogenesis and remains sensitive to heat stress in the peri-ovulatory period. Changes in oocyte quality due to heat stress are the result of altered patterns of folliculogenesis and, possibly, direct effects of elevated body temperature on the oocyte. While adverse effects of elevated temperature on the oocyte have been observed *in vitro*, local cooling of the ovary and protective effects of follicular fluid may limit these actions *in vivo*. Heat stress can also compromise fertilization rate. The first seven days of embryonic development are very susceptible to disruption by heat stress. During these seven days, the embryo undergoes a rapid change in sensitivity to heat stress from being very sensitive (2- to 4-cell stage) to largely resistant (by the morulae stage). Direct actions of elevated temperature on the embryo are likely to be an important mechanism for reduction in embryonic survival caused by heat stress. An effective way to avoid effects of heat stress on the oocyte, fertilization, and early embryo is to bypass the effects through embryo transfer because embryos are typically transferred into females after acquisition of thermal resistance. There may be some opportunity to mitigate effects of heat stress by feeding antioxidants or regulating the endocrine environment of the cow but neither approach has been reduced to practice. The best long-term solution to the problem of heat stress may be to increase genetic resistance of cows to heat stress. Thermotolerance genes exist within dairy breeds and additional genes can be introgressed from other breeds by traditional means or gene editing.

Keywords: heat stress, lactating cow, reproduction, fertility, embryo, oocyte.

Introduction

The overall reproductive function of a herd of dairy cows is often estimated by calculating pregnancy rate, i.e., the product of estrus detection rate (how many cows in estrus are detected in estrus by farm personnel) and conception rate (a misnomer but a measure of how many cows that are inseminated are diagnosed as pregnant). A pregnancy rate of 100% would mean that every cow eligible to be pregnant in a 21-day period becomes pregnant in that time. By this measure, the reproductive function of the heat-stressed dairy cow can be very low indeed. Data in Figure 1 illustrate how heat stress can affect characteristics of estrous activity; only 19% of estrus periods were detected by farm workers in the summer in one study in Florida (Thatcher *et al*., 1986). Fertility after artificial insemination (AI) can also be low during heat stress. In a survey of dairy herds in Israel, less than 20% of inseminations resulted in pregnancies in the summer and pregnancy per AI (P/AI) in the worst herds (those with milk low production and a moderate amount of cooling) was only 3% (Fig. 2; Flamenbaum and Galon, 2010). In another study, P/AI at day 32 after insemination for lactating cows in Minais Gerais, Brazil was 17% when cows experienced two of more occurrences of a morning rectal temperature greater than 39.1°C at days -3, -2, 0 and 7 relative to timed AI *vs* 25% for cows with one occurrence and 37% for cows with no occurrence (Pereira *et al*., 2013).

Fortunately, the situation is not always so bleak. For example, P/AI at day 36 after insemination for multiparous cows in Oklahoma and Kansas during hot weather was 25-27% (Voelz *et al*., 2016). In a study in Florida, P/AI for multiparous cows in the summer on a free-stall dairy in Florida with fans and sprinkler was 32% (Zolini *et al*., 2019). Higher pregnancy rates during heat stress can be ascribed to superior cooling systems and implementation of timed AI programs. Not only do protocols for timed AI eliminate the need for estrus detection but, for some protocols, they can increase cow fertility (Carvalho *et al*., 2018).

Further improvements in fertility during the summer are possible by exploiting knowledge about how heat stress affects the reproductive process. Here, the impact of heat stress on follicular development, oocyte quality, fertilization, and embryonic development will be briefly outlined and the consequences of those changes for strategies to improve fertility will be discussed. Since AI using frozen semen can bypass effects on the bull, and timed AI bypasses effects on estrus behavior, the focus will be on biological processes important for establishing a high level of fertility. Keep in mind that there are effects of heat stress after establishment of pregnancy, most notably in late gestation when heat stress can affect milk yield of the cow after calving and the epigenetic program, growth and milk yield of the resulting calf (Dahl *et al*., 2017; Skiebiel *et al*., 2018). However, this important aspect of actions of heat stress on the pregnant cow is beyond the scope of the current review.

*Corresponding author: pjhansen@ufl.edu
orcid.org/0000-0003-3061-9333
Received: May 7, 2019
Accepted: July 25, 2019

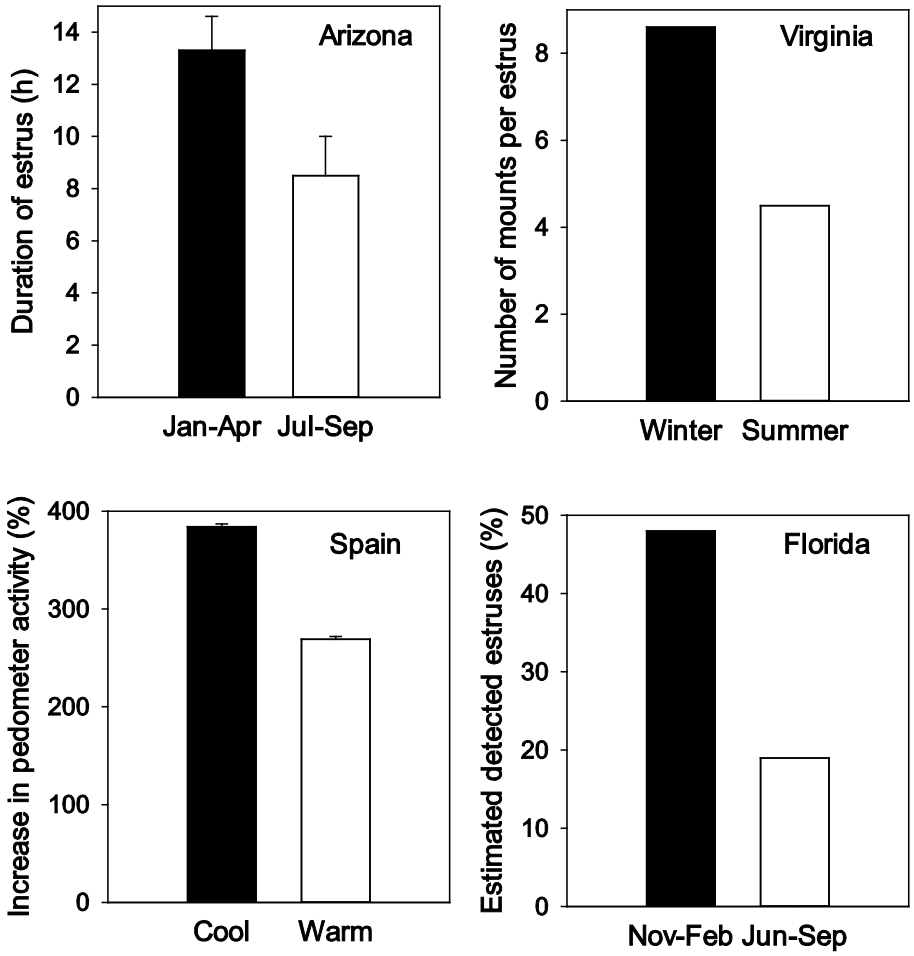


Figure 1. Seasonal variation in characteristics of estrus in lactating cows. Shown are data on duration of estrus in Arizona (Wolff and Monty, 1974), number of mounts per estrus in Virginia (Nebel *et al*., 1997), the increase in pedometer activity at estrus in Spain (López-Gatius *et al*., 2005a) and estimated percent of estrus periods detected by farm personnel in Florida (Thatcher *et al*., 1986). The figure is reproduced from Hansen (2017) with permission of the American Dairy Science Association.

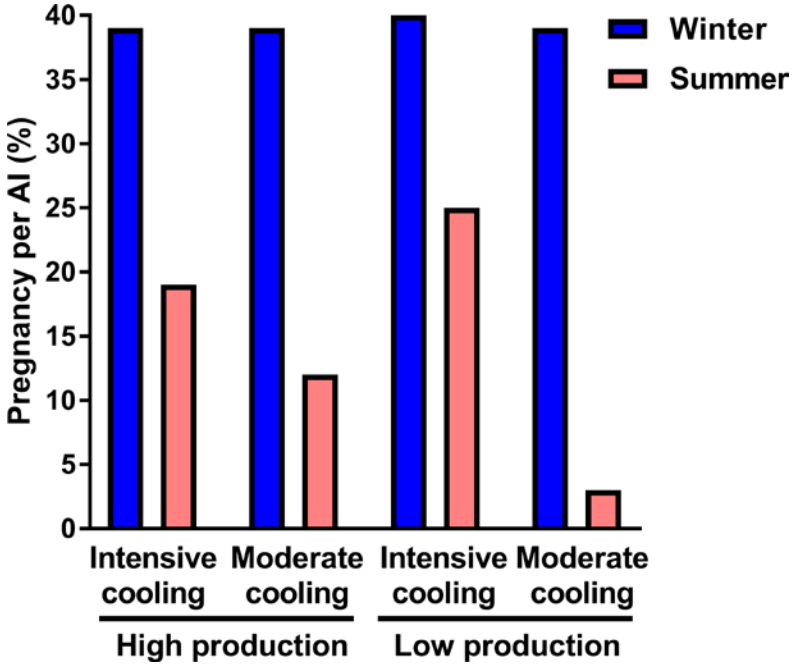


Figure 2. Pregnancies per artificial insemination of herds surveyed in Israel (Flamenbaum and Galon, 2010). Herds were classified based on the overall level of milk production (high *vs* low) and on the degree of cooling that cows receive (intensive *vs* moderate).

Reproductive Events Susceptible to Heat Stress

There have been attempts to reduce the impact of heat stress by cooling lactating cows for a limited period of time around ovulation, fertilization and early development but the improvement in fertility has been slight (Stott and Wiersma, 1976; Her *et al*., 1988; Ealy *et al*., 1994). This is because there is a broad window of time in which the reproductive process of the cow can be disrupted by heat stress. An experiment with Gir cattle indicates that heat stress can compromise development of the oocyte at the earliest stages of folliculogenesis. In particular, competence of oocytes to develop to the blastocyst stage after *in vitro* fertilization was reduced by heat stress occurring as early as 105 to 133 days before oocyte retrieval (Torres-Júnior *et al*., 2008). In an experiment with lactating Holsteins, secretion of androstenedione from cultured thecal cells and estradiol from cultured granulosa cells from medi,um-sized follicles was reduced by exposure of donor cows to heat stress 20 to 26 days earlier (Roth *et al*., 2001a). Additional evidence that heat stress affects the oocyte early in folliculogenesis are observations that oocyte competence for supporting embryonic development after *in vitro* activation was restored only gradually after the end of summer (see review by Hansen, 2013a; Roth, 2017). Moreover, treatments to increase follicular turnover, including multiple follicular aspirations (Roth *et al*., 2001b), follicle stimulating hormone (Roth *et al*., 2002) or somatotropin (Roth *et al*., 2002), hastened restoration of oocyte competence.

The oocyte remains sensitive to heat stress in the peri-ovulatory period. Treatment of superovulated cows with heat stress for 10 hours beginning at the onset of estrus reduced the percent of embryos recovered at day 7 after estrus that were classified as having normal morphology and increased the percent of embryos that were retarded with development (Putney *et al*., 1989a).

Heat stress can compromise fertilization rate (Sartori *et al*., 2002; Hackbart *et al*., 2010). For example, fertilization rate in lactating cows bred by AI was lowered from 88% in winter to 55% in summer (Sartori *et al*., 2002). Low fertilization rates could reflect damage to the oocyte, sperm deposited in the uterus, or disruption of the fertilization process itself. Effects on the sperm or fertilization process may be a more important cause than effects on the oocyte. Competence of the oocyte to be fertilized *in vitro* was not reduced in the summer although there was a reduction in development of cleaved embryos to the blastocyst stage (Ferreira *et al*., 2011). Further, indirect evidence for an effect of heat stress on the sperm is the observation of Girolando cows that insemination in the morning was associated with a slight but significant increase in P/AI (Rocha de Zouza *et al*., 2016).

The first 7 days of embryonic development are very susceptible to disruption by heat stress. Experimental treatment of superovulated cows with heat stress during this time reduced the development of embryos at day 7 (Putney *et al*, 1988a). Moreover, among single-ovulating lactating cows, there was a large reduction in the percent of recovered embryos classified as viable during periods of heat stress as compared to periods of no heat stress (Sartori *et al*., 2002). During these 7 days, the embryo undergoes a rapid change in sensitivity to heat stress. Exposure of superovulated cows to heat stress on day 1 after estrus reduced the percent of embryos that were blastocysts at day 8 (Ealy *et al*., 1993). However, heat stress at day 3, 5 or 7 had no effect on embryonic development. Thus, the resistance of the embryo to heat stress increases greatly in just a few days of development.

There is little known about sensitivity of the bovine embryo to heat stress after day 7. There is one report in beef cattle that heat stress from day 8 to 16 can reduce conceptus weight at day 16 (Biggers *et al*., 1987). However, the effectiveness of embryo transfer as a tool for improving fertility during heat stress (see discussion further in this paper) is indicative that embryo survival is not dependent to any large extent on the occurrence of maternal heat stress after day 7.

A proportion of cows initially diagnosed as pregnant around day 28-60 of pregnancy subsequently lose the pregnancy. There is some evidence that the frequency of this loss can be increased by heat stress (García-Ispierto *et al*., 2006; Santolaria *et al*., 2010; El-Tarabany and El-Tarabany, 2015). For example, pregnancy loss between days 34 to 45 of gestation and day 90 of gestation were 2% for cows in the cool season *vs* 12% for cows in the warm season (García-Ispierto *et al*., 2006). Attempts have been made to understand the crucial period in the reproductive process during which heat stress acts to increase late embryonic and fetal mortality by relating indices of heat stress at specific phases in the reproductive process to pregnancy loss. Such an approach is difficult to decipher because environmental conditions at one period are often highly correlated with environmental conditions at another period.

Physiological Causes of Effects of Heat Stress on the Oocyte and Embryo

Effects of heat stress are related to the inability of the affected cow to maintain its body temperature within the regulated range. As mentioned, P/AI is related to rectal temperature (Pereira *et al*., 2013). It has been estimated that fertility begins to decline when uterine temperature rises about 0.5°C above normal (Gwazdauskas *et al*., 1973). One reason why lactating cows are more susceptible to the negative effects of heat stress on fertility than heifers (Badinga *et al*., 1985) is because the metabolic heat production associated with lactation makes it more difficult for cows to regulate body temperature during heat stress than non-lactating heifers (Sartori *et al*., 2002). Effects of heat stress on the ovary, oviduct, uterus, and embryo could result from either physiological changes caused by heat stress or by the direct effects of elevated temperature on cells involved in reproduction.

Alterations in oocyte quality due to heat stress probably involve deviations in patterns of folliculogenesis. Follicular dominance is reduced in cows exposed to heat stress so that there is an increase in number of large follicles on the ovary, prolonged

period of dominance of the ovulatory follicle, increased circulating concentrations of follicle stimulating hormone (FSH) and reduced concentrations of estradiol-17β and inhibin (Wolfenson *et al*., 1995; Roth *et al*., 2000; Trout *et al*., 1998; Wilson *et al*., 1998). Heat stress can also dampen the preovulatory surge of luteinizing hormone and estradiol-17β (Gwazdauskas *et al*., 1981; Gilad *et al*., 1993; Armengol-Gelonch *et al*., 2017). Indeed, heat stress can increase the proportion of cows that fail to ovulate after administration of GnRH. Ovulation failure was 12% during the warm period *vs* 3% during the cool period (López-Gatius *et al*., 2005b). Use of more active analogs of gonadotropin releasing hormone (GnRH) can reduce the incidence of ovulation failure (García-Ispierto *et al*., 2019).

There are direct effects of elevated temperature (i.e., heat shock) on the competence of the oocyte undergoing maturation to develop into a blastocyst following fertilization or artificial activation (see Roth, 2017 for review). Possible local cooling of the ovary and protective effects of follicular fluid may limit these actions *in vivo*. Work by López-Gatius and Hunter (2017, 2019a,b) has revealed that the ovary experiences a cooler temperature than that measured in the rectum or on the surface of the uterus. Additionally, culture of maturing oocytes in a medium containing follicular fluid or follicular fluid exosomes reduced the negative effect of elevated temperature on oocyte competence for cleavage and blastocyst development after fertilization (Rodrigues *et al*., 2019). Direct effects of elevated temperature on the follicle may be important in some circumstances, however. Cows in which follicular temperature was lower than rectal temperature were more likely to ovulate and achieve pregnancy than cows in which the gradient between follicular and rectal temperature was low (López-Gatius and Hunter, 2019ab).

Direct actions of elevated temperature on the embryo are likely to be an important mechanism for reduction in embryonic survival caused by heat stress after ovulation. Indeed, the changes in embryonic resistance to maternal heat stress observed *in vivo* (Ealy *et al*., 1993) are also seen with effects of heat shock on cultured embryos. Exposure of the zygote and 2-cell embryo causes a large reduction in percent of embryos developing to the blastocyst stage (Edwards and Hansen, 1997; Sakatani *et al*., 2012; Ortega *et al*., 2016). Embryos at the 4- and 8-cell stage are also susceptible to heat shock but the magnitude of the deleterious effect is reduced as compared to that for the 2-cell embryo (Edwards and Hansen, 1997). Physiologically-relevant heat shock has little effect on development of morula-stage embryos (Edwards and Hansen, 1997; Eberhardt *et al*., 2009; Sakatani *et al*., 2012). Mechanisms responsible for acquisition of thermotolerance are not known but probably involve activation of the embryonic genome at the 8-cell stage (Graf *et al*., 2014) so that the full range of cellular adaptations to heat shock can be employed.

It is also possible that changes in circulating concentrations of steroid hormones induced by heat stress could alter the oviductal or uterine environment and thereby affect embryonic development. As stated previously, heat stress can reduce plasma concentrations of estradiol-17β (Gwazdauskas *et al*., 1981; Wolfenson *et al*., 1995; Wilson *et al*., 1998). Short-term exposure to heat stress either had no effect on plasma concentrations of progesterone (Roth *et al*., 2000) or caused an increase (Trout *et al*., 1998; Wilson *et al*., 1998). Long-term exposure to heat stress may lead to reduced progesterone concentrations, however, because luteal concentrations of the hormone during the luteal phase have been reported to be lower in summer than winter (Howell *et al*., 1994). Additionally, cooling cows during the summer increased circulating concentrations of progesterone (Wolfenson *et al*., 1988). Some effects of heat stress on peripheral blood concentrations of hormones could be the result of changes in water balance during heat stress and reduced hematocrit (Richards, 1985; Lamp *et al*., 2015).

Embryo Transfer: The Most Effective Mechanism for Maximizing Fertility During Heat Stress

One way to avoid consequences of heat stress on the oocyte, fertilization, and early embryo is to bypass its effects through implementation of an embryo transfer program. Embryos are typically transferred into females at day 7 after estrus. By that time, embryos have gained resistance to effects of heat stress. Embryo transfer can be coupled with ovulation synchronization programs to allow timed embryo transfer and avoid the need for estrus detection.

One way to demonstrate the effectiveness of embryo transfer for improving fertility during heat stress is to compare pregnancy outcomes for embryo transfer as compared to AI. As summarized in Figure 3, pregnancy rates during heat stress have been consistently higher for cows receiving an embryo than for cows submitted to AI. The only exception is when embryos were produced *in vitro* and cryopreserved before transfer. Thus, there was either no improvement in fertility as compared to AI when vitrified embryos produced *in vitro* were transferred (Drost *et al*., 1999; Fig. 3B) or the improvement was less than if fresh embryos were transferred (Stewart *et al*., 2011; Fig. 3C). These results are the consequence of poor cryopreservation of *in vitro* produced embryos (Hansen and Block, 2004).

Another way to demonstrate how embryo transfer reduces the impact of heat stress is to examine seasonal variation in pregnancy success after embryo transfer. Of eight studies in which seasonal variation in pregnancy rates were evaluated, there were only two cases where there was a large difference in pregnancy rate between hot and cool conditions including an experiment in Florida with fresh embryos produced *in vitro* (Block *et al*., 2007; Fig. 4B) and an experiment in South Dakota with vitrified embryos produced *in vitro* (Chebel *et al*., 2008; Fig. 4C). There was no difference between seasons for embryos produced by superovulation in the southwest United States (Putney *et al*., 1988b; Fig. 4A) or for fresh embryos produced *in vitro* in Florida (Loureiro *et al*., 2009; Fig. 4B) or South Dakota (Chebel *et al*., 2008; Fig. 4C). In the largest trials, there was a slight reduction in pregnancy per

embryo transfer in the hottest months (Ferraz *et al*., 2016; Vasconcelos *et al*., 2011; Baruselli *et al*., 2011) but the difference in pregnancy outcomes between the coolest and warmest times were only 3 to 4% (Fig. 4C, 4E and 4F). Seasonal variation of that magnitude is much less than what would be the case for AI.

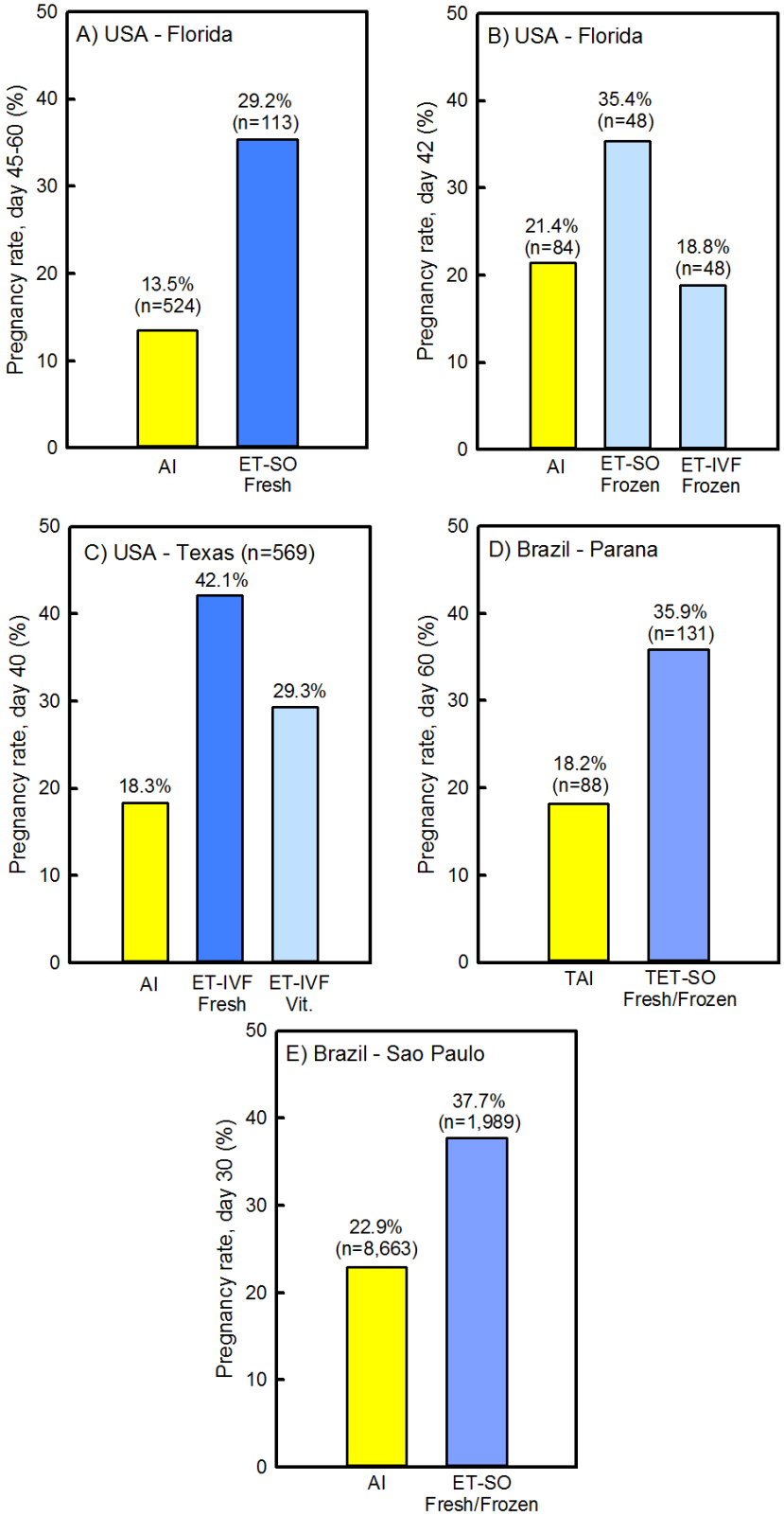


Figure 3. Comparisons of pregnancy success for artificial insemination *vs* embryo transfer in the summer. Data are from Putney *et al*. (1989b) (A), Drost *et al*. (1999) (B), Stewart *et al*. (2011) (C), Vasconcelos *et al*. (2011) (D) and Baruselli *et al*. (2011) (E). Abbreviations are as follows: AI, artificial insemination, ET, embryo transfer; IVF, *in vitro* fertilized; SO, superovulation; TAI, timed AI; TET, timed embryo transfer; Vit., vitrified. The figure is modified from a technical bulletin by Vetoquinol and is reproduced with permission.

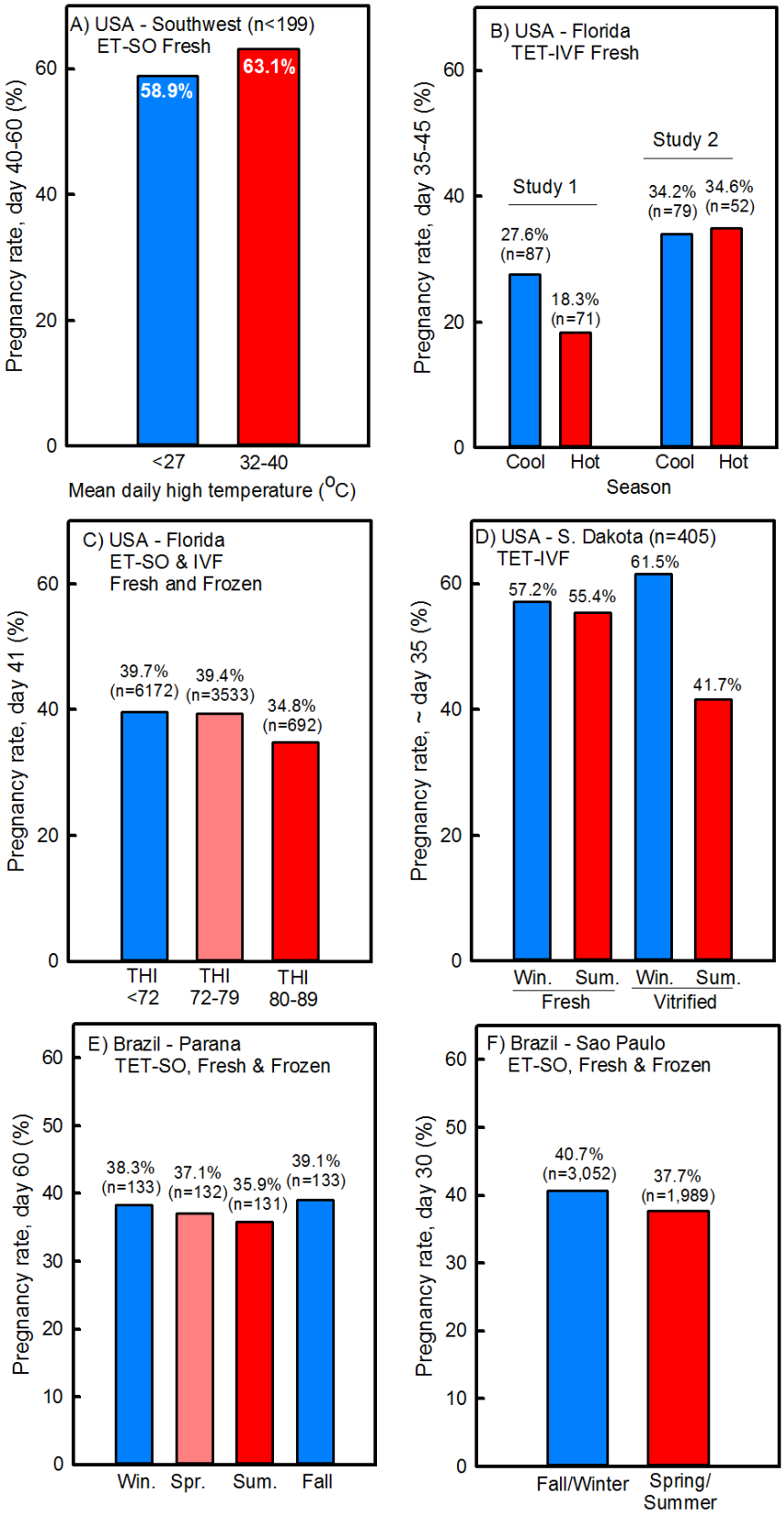


Figure 4. Comparisons of percent cows pregnant following embryo transfer in cool or hot weather. Data are from Putney *et al*. (1988b) (A), Block *et al*. (2007) and Loureiro *et al*. (2009) (B), Ferraz *et al*. (2016) (C), Chebel *et al*. (2008) (D), Vasconcelos *et al*. (2011) (E) and Baruselli *et al*. (2011) (F). Abbreviations are as follows: ET, embryo transfer; IVF, *in vitro* fertilized; S. Dakota, South Dakota; Spr., spring; SO, superovulation; Sum., summer; TET, timed embryo transfer; THI, temperature-humidity index; Win., winter. The figure is modified from a technical bulletin by Vetoquinol and is reproduced with permission.

Antioxidants

Exposure to elevated temperature can increase production of reaction oxygen species in oocytes (Nabenishi *et al*., 2012; Cavallari de Castro *et al*., 2019) and embryos (Sakatani *et al*., 2004; Sakatani *et al*., 2008; Ortega *et al*., 2016). *In vitro*, effects of heat shock on oocyte maturation can be reduced by provision of antioxidants (Lawrence *et al*., 2004; Nabenishi *et al*., 2012; Ispada *et al*., 2018; Cavallari de Castro *et al*., 2019). In the embryo, however, thermoprotective benefits of antioxidants *in vitro* have been inconsistent. There was no protective effect of dithiothreitol, glutathione, melatonin, taurine, or vitamin E (Ealy *et al*., 1995; Paula-Lopes *et al*., 2003a; de Castro *et al*., 2008; Ortega *et al*., 2016) but β-mercaptoethanol was protective (Sakatani *et al*., 2008). One interpretation of these data is that reactive oxygen species are a more important mediator of the embryotoxic actions of heat shock for the oocyte than the embryo.

Efforts to improve fertility of lactating cows exposed to heat stress by delivering antioxidants have generally not yielded positive effects (see review by Hansen, 2013b and Roth, 2017). There are two reports of beneficial effects of antioxidant administration, however. In the first, Aréchiga et al. (1998) found that a higher proportion of cows fed supplemental β-carotene from about day 15 after calving were pregnant at 90 d postpartum than control cows when the experiment was performed during the summer but not when the experiment was performed during the winter. Feeding supplemental β-carotene did not increase pregnancy per AI at first service so the effect on the proportion pregnant at 90 d reflects either alterations in fertility after first service or estrus detection. In the second report, administration of long-acting melatonin implants beginning at 220 d of gestation to cows during the summer shortened the interval to conception in the subsequent postpartum period and decreased the incidence of cows experiencing > 3 breedings per conception (García-Ispierto *et al*., 2013). The peak concentration of melatonin in the blood of cows receiving implants was low (i..e, 260-300 pM) and it might be that melatonin was acting as a hormone rather than as an antioxidant. Further studies are needed with both β-carotene and melatonin to evaluate efficacy of their administration for improving fertility during heat stress.

Hormonal Treatments

Much work continues on optimization of timed AI protocols in general and under the specific conditions of heat stress. Few studies have been performed to compare whether a specific improvement in a timed AI protocol works better for heat-stressed cows than cows not subjected to heat stress. One exception is for induction of ovulation. As already discussed, ovulation failure is more frequent during periods of heat stress and administration of more active analogs of GnRH can reduce the incidence of ovulation failure (García-Ispierto *et al*., 2019). In another study, Shabankareh *et al*. (2010) evaluated the summer-winter differences in P/AI at first service for cows bred at spontaneous estrus or following timed AI using either GnRH (Ovsynch) or estradiol cypionate (Heatsynch) to induce ovulation. There was no difference in P/AI (32, 30 and 30% for OvSynch, Heatsynch and spontaneous estrus) in the summer while P/AI in the winter was highest for spontaneous estrus (51%), intermediate in Ovsynch (40%) and lowest for Heatsynch (35%).

Several experiments have been conducted to evaluate effects of increasing circulating progesterone concentrations on fertility of heat-stressed cows. Results have been inconsistent and often dependent on the subset of cows treated. Administration of progesterone using a CIDR device from day 5 to 18 after insemination did not cause an overall increase in P/AI but there were positive effects of the treatment in cows with low body condition or postpartum uterine disorders (Friedman *et al*., 2012). In the study of Shabankareh *et al*. (2010), treatment with human chorionic gonadotropin (hCG) on day 5 after insemination increased P/AI in both summer (24 *vs* 38% for saline and hCG) and winter (35 *vs* 47%). Treatment with hCG at day 5 also increased pregnancy rate in cows during summer but the effect was seen only for primiparous cows (Zolini *et al*., 2019). Treatment with GnRH at AI or at both AI and day 12 of the estrous cycle increased P/AI in an experiment by López-Gatius *et al*. (2005c). In another research trial, there was no beneficial effect of treatment at day 0 on P/AI whereas treatment with GnRH at either day 5 or both day 0 and 5 increased P/AI but only for cows in third or greater lactation (Mendonça *et al*., 2017).

The idea that ovarian follicles can be compromised by heat stress at early stages of folliculogenesis has lead to the idea that fertility can be improved in the autumn by hastening the removal of damaged follicles from the ovary. Improved oocyte competence in the autumn, as measured by *in vitro* development to the blastocyst stage, has been achieved using several treatments to increase follicular turnover, including multiple follicular aspirations (Roth *et al*., 2001b) or treatment with FSH (Roth *et al*., 2002) or somatotropin (Roth *et al*., 2002). In addition, generation of three consecutive 9-day follicular waves by treatment with GnRH and prostaglandin $F_{2\alpha}$ has been reported to have some positive effects on fertility of lactating cows in the summer and autumn (Friedman *et al*., 2011). Treatment effects were seen for primiparous cows (37% *vs* 53% for control and treated cows) but not for multiparous cows (27 *vs* 29%).

Genetic Selection

Heritability estimates in Holsteins for body temperature during heat stress is 0.17 (Dikmen *et al*., 2012) and for the decline in milk yield during heat stress is 0.19 (Nguyen *et al*., 2016). Thus, it should be possible to reduce the impact of heat stress on reproduction by selecting genetically for thermoregulation. Data from Australia indicate that cows that are more thermotolerant genetically also have higher breeding values for fertility (Nguyen *et al*.,

2016). Unfortunately, they also have a lower genetic ability for milk yield so genetic strategies must be developed to allow selection for genes that confer superior thermotolerance without compromising milk yield.

One option is to introgress genes from thermotolerant breeds into dairy breeds using crossbreeding or gene editing. The prolactin receptor gene is one gene that has been mutated in a manner that leads to a slick hair phenotype characterized by a sleek, short hair coat and increased capacity for regulating body temperature (Dikmen *et al*., 2014). Arising in criollo breeds of cattle, several mutations in the gene exist that result in a truncated version of the protein to be synthesized (Porto-Neto *et al*., 2018). The gene has been introduced into Holsteins and is associated with reduced milk yield depression in the summer (Dikmen *et al*., 2014). Data from Puerto Rico indicate that slick-haired Holsteins are more fertile than Holsteins without the mutation (Ortiz-Colón *et al*., 2018).

There are also genetic effects on cellular resistance to elevated temperature. Embryos from *Bos indicus* breeds or the Romosinuano, a criollo breed, are more resistant to deleterious effects of heat shock on development of cultured embryos (Paula-Lopes *et al*., 2003b; Hernández-Cerón *et al*., 2004; Eberhardt *et al*., 2009; Silva *et al*., 2013). Fertility of cows in the summer was higher when inseminations were performed with Gyr semen than when Holstein semen was used (Pegorer *et al*., 2007). One gene that contains mutations that increases cellular resistance to heat shock is *HSPA1L*, as indicated by studies with lymphocytes (Basiricò *et al*., 2011) and embryos (Ortega *et al*., 2016).

Final Note

The decision as to which strategies to implement to reduce effects of heat stress on fertility is not a simple one. Embryo transfer, for example, while effective at minimizing the summer decline in fertility, is also expensive and may not be economically-effective unless the cost is constrained. In addition, getting cows pregnant in the summer can have long-term negative consequences for the resultant calf. Pinedo and De Vries (2017) have demonstrated that cows conceived in summer were older at first calving, had lower odds of surviving for a second calving, longer intervals from calving to first breeding and conception, and lower milk yield than cows conceived in winter. Thus, in some cases, non-uniform or seasonal calving may be the most profitable strategy. Genetic strategies that increase thermotolerance of the cow population are also desirable because, among other reasons, effects are permanent for that animal and extend to its offspring.

Author contributions

PJH: wrote and finalized the paper.

Conflicts of interest

The author declares no conflicts of interest.

Funding

Recent research by the author on this topic was funded by the Southeast Milk Checkoff Program and by the L.E. "Red" Larson Endowment.

References

Armengol-Gelonch R, Mallo J M, Ponté D, Jimenez A, Valenza A, Souza AH. 2017. Impact of phase of the estrous cycle and season on LH surge profile and fertility in dairy cows treated with different GnRH analogs (gonadorelin vs. buserelin). *Theriogenology*, 91:121-126.

Aréchiga CF, Staples CR, McDowell LR, Hansen PJ. 1998. Effects of timed insemination and supplemental β-carotene on reproduction and milk yield of dairy cows under heat stress. *J Dairy Sci*, 81**:**390-402.

Baruselli P S, Ferreira R M, Sales J N, Gimenes L U, Sá Filho MF, Martins C M, Rodrigues C A, Bó G A. 2011. Timed embryo transfer programs for management of donor and recipient cattle. *Theriogenology*, 76:1583-1593.

Badinga L, Collier R J, Thatcher WW, Wilcox C J. 1985 Effects of climatic and management factors on conception rate of dairy cattle in subtropical environment. *J Dairy Sci*, 68:78-85.

Basiricò L, Morera P, Primi V, Lacetera N, Nardone A, Bernabucci U . 2011. Cellular thermotolerance is associated with heat shock protein 70.1 genetic polymorphisms in Holstein lactating cows. *Cell Stress Chaperones*, 16:441-448.

Biggers B G, Geisert R D, Wetteman R P, Buchanan DS. 1987. Effect of heat stress on early embryonic development in the beef cow. *J Anim Sci*, 64:1512-1518.

Block J, Hansen PJ. 2007. Interaction between season and culture with insulin-like growth factor-1 on survival of *in vitro* produced embryos following transfer to lactating dairy cows. *Theriogenology*, 67:1518-1529.

Carvalho PD, Santos VG, Giordano JO, Wiltbank MC, Fricke P M. 2018. Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology*, 114:165-172.

Cavallari de Castro F, Leal, CLV, Roth, Z, Hansen PJ. 2019. Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during *in vitro* maturation. *Zygote*, 27:180-186.

Chebel RC, Demétrio DG, Metzger J. 2008. Factors affecting success of embryo collection and transfer in large dairy herds. *Theriogenology*, 69:98-106.

Dahl GE, Tao S, Laporta J. 2017. Late gestation heat stress of dairy cattle programs dam and daughter milk production. *J Anim Sci*, 95:5701-5710.

de Castro e Paula LA, Hansen PJ. 2008. Modification of actions of heat shock on development and apoptosis of cultured preimplantation bovine embryos by oxygen concentration and dithiothreitol. *Mol Reprod Dev*, 75:1338-1350.

Dikmen S, Cole J B, Null DJ, Hansen PJ. 2012. Heritability of rectal temperature and genetic correlations with production and reproduction traits in dairy cattle. *J Dairy Sci*, 95:3401-3405.

Dikmen S, Khan F A, Huson H J, Sonstegard T S,

Moss JI, Dahl GE, Hansen PJ. 2014. The *SLICK* hair locus derived from Senepol cattle confers thermotolerance to intensively managed lactating Holstein cows. *J Dairy Sci*, 97:5508-5520.

Drost M, Ambrose JD, Thatcher MJ, Cantrell CK, Wolfsdorf KE, Hasler JF, Thatcher WW. 1999. Conception rates after artificial insemination or embryo transfer in lactating dairy cows during summer in Florida. *Theriogenology*, 52:1161-1167.

Ealy AD, Drost M, Hansen PJ. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci*, 76:2899-2905.

Ealy AD, Aréchiga CF, Bray DR, Risco CA, Hansen PJ. 1994. Effectiveness of short-term cooling and vitamin E for alleviation of infertility induced by heat stress in dairy cows. *J Dairy Sci*, 77:3601-3607.

Ealy AD, Howell JL, Monterroso VH, Arechiga CF, Hansen PJ. 1995. Developmental changes in sensitivity of bovine embryos to heat shock and use of antioxidants as thermoprotectants. *J Anim Sci*, 73:1401 -1407.

Eberhardt BG, Satrapa RA, Capinzaiki CR, Trinca LA, Barros CM. 2009. Influence of the breed of bull (*Bos taurus indicus* vs. *Bos taurus taurus*) and the breed of cow (*Bos taurus indicus*, *Bos taurus taurus* and crossbred) on the resistance of bovine embryos to heat. *Anim Reprod Sci*, 114:54-61.

Edwards JL, Ealy AD, Monterroso VH, Hansen PJ. 1997. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos, *Mol Reprod Dev*, 48: 25-33.

El-Tarabany MS, El-Tarabany AA. 2015. Impact of maternal heat stress at insemination on the subsequent reproductive performance of Holstein, Brown Swiss, and their crosses. *Theriogenology*, 84:1523-1529.

Ferraz PA, Burnley C, Karanja J, Viera-Neto A, Santos JE, Chebel RC, Galvão KN. 2016. Factors affecting the success of a large embryo transfer program in Holstein cattle in a commercial herd in the southeast region of the United States. *Theriogenology*, 86:1834-1841.

Ferreira RM, Ayres H, Chiaratti MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, Vireque AA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS. 2011. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J Dairy Sci*, 94:2383-2392.

Flamenbaum I, Galon N. 2010. Management of heat stress to improve fertility in dairy cows in Israel. *J Reprod Dev*, 56 (Suppl):S36-41.

Friedman E, Voet H, Reznikov D, Dagoni I, Roth Z. 2011. Induction of successive follicular waves by gonadotropin-releasing hormone and prostaglandin $F_{2\alpha}$ to improve fertility of high-producing cows during the summer and autumn. *J Dairy Sci*, 94:2393-2402.

Friedman E, Roth Z, Voet H, Lavon Y, Wolfenson D. 2012. Progesterone supplementation postinsemination improves fertility of cooled dairy cows during the summer. *J Dairy Sci*, 95:3092-3099.

García-Ispierto I, Abdelfatah A, López-Gatius F. 2013. Melatonin treatment at dry-off improves reproductive performance postpartum in high-producing dairy cows under heat stress conditions. *Reprod Domest Anim*, 48: 577-583.

García-Ispierto I, López-Gatius F, Santolaria P, Yániz JL, Nogareda C, López-Béjar M, De Rensis F. 2006. Relationship between heat stress during the peri-implantation period and early fetal loss in dairy cattle. *Theriogenology*, 65:799-807.

García-Ispierto I, De Rensis F, Pérez-Salas JA, Nunes JM, Pradés B, Serrano-Pérez B, López-Gatius F. 2019. The GnRH analogue dephereline given in a fixed-time AI protocol improves ovulation and embryo survival in dairy cows. *Res Vet Sci*, 122:170-174.

Gilad E, Meidan R, Berman A, Graber Y, Wolfenson D. 1993. Effect of tonic and GnRH-induced gonadotrophin secretion in relation to concentration of oestradiol in plasma of cyclic cows. *J Reprod Fertil*, 99:315-321.

Graf A, Krebs S, Zakhartchenko V, Schwalb B, Blum H, Wolf E. 2014. Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proc Natl Acad Sci USA*, 111:4139-4144.

Gwazdauskas FC, Thatcher WW, Wilcox CJ. 1973. Physiological, environmental, and hormonal factors at insemination which may affect conception. *J Dairy Sci*, 56:873-877.

Gwazdauskas FC, Thatcher WW, Kiddy CA, Paape MJ, Wilcox CJ. 1981. Hormonal patterns during heat stress following PGF2α-tham salt induced luteal regression in heifers. *Theriogenology*, 16:271-285.

Hackbart KS, Ferreira RM, Dietsche AA, Socha MT, Shaver RD, Wiltbank MC, Fricke PM. 2010. Effect of dietary organic zinc, manganese, copper, and cobalt supplementation on milk production, follicular growth, embryo quality, and tissue mineral concentrations in dairy cows. *J Anim Sci*, 88:3856-3870.

Hansen PJ, Block J. 2004. Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. *Reprod Fertil Dev*, 16:1-14.

Hansen PJ. 2013a. Antecedents of mammalian fertility: Lessons from the heat-stressed cow regarding the importance of oocyte competence for fertilization and embryonic development. *Anim Frontiers*, 3: 34-28.

Hansen PJ. 2013b. Cellular and molecular basis of therapies to ameliorate effects of heat stress on embryonic development in cattle. *Anim Reprod*, 10: 322-333.

Hansen PJ. 2017. Physiological approaches to improving fertility during heat stress. In *Large Herd Dairy Management*, 3rd. ed., Beede, D.K. (ed.), American Dairy Science Assn, Champaign-Urbana, Illinois, chapter 7-24.

Her E, Wolfenson D, Flamenbaum I, Folman Y, Kaim M, Berman A. 1988. Thermal, productive, and reproductive responses of high yielding cows exposed to short-term cooling in summer. *J Dairy Sci*, 71:1085 - 1092.

Hernández-Cerón J, Chase CC Jr, Hansen PJ. 2004. Differences in heat tolerance between preimplantation embryos from Brahman, Romosinuano, and Angus Breeds. *J Dairy Sci*, 87:53-58

Howell JL, Fuquay JW, Smith AE. 1994. Corpus luteum growth and function in lactating Holstein cows during spring and summer. *J Dairy Sci*, 77:735-739.

Ispada J, Rodrigues TA, Risolia PHB, Lima RS, Gonçalves DR, Rettori D, Nichi M, Feitosa WB,

Paula-Lopes FF. 2018. Astaxanthin counteracts the effects of heat shock on the maturation of bovine oocytes. *Reprod Fertil Dev*, 30:1169-1179.

Lamp O, Derno M, Otten W, Mielenz M, Nürnberg G, Kuhla B. 2015. Metabolic heat stress adaption in transition cows: differences in macronutrient oxidation between late-gestating and early-lactating German Holstein dairy cows. *PLoS One*, 10:e0125264.

Lawrence JL, Payton RR, Godkin JD, Saxton AM, Schrick FN, Edwards JL. 2004. Retinol improves development of bovine oocytes compromised by heat stress during maturation. *J Dairy Sci*, 87:2449-2454.

López-Gatius F, Hunter R. 2017. Clinical relevance of pre-ovulatory follicular temperature in heat-stressed lactating dairy cows. *Reprod Domest Anim*, 52:366-370.

López-Gatius F, Hunter RHF. 2019a. Pre-ovulatory follicular cooling correlates positively with the potential for pregnancy in dairy cows: Implications for human IVF. *J Gynecol Obstet Hum Reprod*, 48:419-422.

López-Gatius F, Hunter RHF. 2019b. Pre-ovulatory follicular temperature in bi-ovular cows. *J Reprod Dev*, 65:191-194.

López-Gatius F, López-Béjar M, Fenech M, Hunter RH. 2005b. Ovulation failure and double ovulation in dairy cattle: risk factors and effects. *Theriogenology*, 63:1298-307.

López-Gatius F, Santolaria P, Martino A, Delétang F, De Rensis F. 2005c. The effects of GnRH treatment at the time of AI and 12 days later on reproductive performance of high producing dairy cows during the warm season in northeastern Spain. *Theriogenology*, 65:820-830.

López-Gatius F, Santolaria P, Mundet I, Yániz JL. 2005a. Walking activity at estrus and subsequent fertility in dairy cows. *Theriogenology*, 63:1419-1429.

Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQ, Hansen PJ. 2009. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced *in vitro*. *Endocrinology*, 150:5046-5054.

Mendonça LGD, Mantelo FM, Stevenson JS. 2017. Fertility of lactating dairy cows treated with gonadotropin-releasing hormone at AI, 5 days after AI, or both, during summer heat stress. *Theriogenology*, 91:9-16.

Nabenishi H, Ohta H, Nishimoto T, Morita T, Ashizawa K, Tsuzuki Y. 2012. The effects of cysteine addition during *in vitro* maturation on the developmental competence, ROS, GSH and apoptosis level of bovine oocytes exposed to heat stress. *Zygote* 20:249-259.

Nebel RL, Jobst SM, Dranfield MBG, Pandolfi SM, Bailey, TL. 1997. Use of radio frequency data communication system, HeatWatch®, to describe behavioral estrus in dairy cattle. *J Dairy Sci.* 80 (Suppl. 1):179 (Abstract).

Nguyen TTT, Bowman PJ, Haile-Mariam M, Pryce JE, Hayes BJ. 2016. Genomic selection for tolerance to heat stress in Australian dairy cattle. *J Dairy Sci*, 99:2849-2862.

Ortega MS, Rocha-Frigoni NAS, Mingoti GZ, Roth Z, Hansen PJ, 2016. Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in *HSPA1L*. *J Dairy Sci*, 99:9152-9164.

Ortiz-Colón G, Fain SJ, Parés IK, Curbelo-Rodríguez J, Jiménez-Cabán E, Pagán-Morales M, Gould WA. 2018. Assessing climate vulnerabilities and adaptive strategies for resilient beef and dairy operations in the tropics. *Climate Change*, 146:47-58.

Paula-Lopes FF, Chase CC Jr, Al-Katanani YM, Krininger CE III, Rivera RM, Tekin S, Majewski AC, Ocon OM, Olson TA, Hansen PJ. 2003b. Genetic divergence in cellular resistance to heat shock in cattle: Differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures. *Reproduction*, 125:285-294.

Paula-Lopes FF, Al-Katanani YM, Majewski AC, Mc Dowell LR, Hansen PJ. 2003a. Manipulation of antioxidant status fails to improve fertility of lactating cows or survival of heat-shocked embryos. *J Dairy Sci*, 86:2343 -2351.

Pegorer MF, Vasconcelos JL, Trinca LA, Hansen PJ, Barros CM. 2007. Influence of sire and sire breed (Gyr versus Holstein) on establishment of pregnancy and embryonic loss in lactating Holstein cows during summer heat stress. *Theriogenology*, 67:692-697.

Pereira MH, Rodrigues AD, Martins T, Oliveira WV, Silveira PS, Wiltbank MC, Vasconcelos JL. 2013. Timed artificial insemination programs during the summer in lactating dairy cows: comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol. *J Dairy Sci*, 96:6904-6914.

Pinedo PJ, De Vries A. 2017. Season of conception is associated with future survival, fertility, and milk yield of Holstein cows. *J Dairy Sci*, 100:6631-6639.

Porto-Neto LR, Bickhart DM, Landaeta-Hernandez AJ, Utsunomiya YT, Pagan M, Jimenez E, Hansen PJ, Dikmen S, Schroeder SG, Kim ES, Sun J, Crespo E, Amati N, Cole JB, Null DJ, Garcia JF, Reverter A, Barendse W, Sonstegard TS. 2018. Convergent evolution of slick coat in cattle through truncation mutations in the prolactin receptor. *Front Genet*, 9:57.

Putney DJ, Drost M, Thatcher WW. 1988a. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between Days 1 to 7 post insemination. *Theriogenology*, 30:195-209.

Putney DJ, Drost M, Thatcher WW. 1989b. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology*, 31:765-778.

Putney DJ, Mullins S, Thatcher WW, Drost M, Gross TS. 1989a. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. *Anim Reprod Sci*, 19:37-51.

Putney DJ, Thatcher WW, Drost M, Wright JM, DeLorenzo MA. 1988b. Influence of environmental temperature on reproductive performance of bovine embryo donors and recipients in the southwest region of the United States. *Theriogenology*, 30:905-922.

Richards JI. 1985. Effect of high daytime temperatures on the intake and utilization of water in lactating Friesian cows. *Trop Anim Health Prod*, 17:209-217.

Rocha de Zouza, F, Campos CC, Marques da Silva NA, dos Santos RM. 2016. Influence of seasonality, timing of insemination and rectal temperature on conception rate of crossbred dairy cows. *Semina: Ciências Agrárias*, *Londrina*, 37:155-162.

Rodrigues TA, Tuna KM, Alli AA, Tribulo P, Hansen PJ, Koh J, Paula-Lopes FF. 2019. Follicular fluid exosomes act on the bovine oocyte to improve oocyte competence to support development and survival to heat shock. *Reprod Fertil Dev*, 31:888-897.

Roth Z. 2017. Effect of heat stress on reproduction in dairy cows: insights into the cellular and molecular responses of the oocyte. *Annu Rev Anim Biosci*, 5:151-170.

Roth Z, Arav A, Bor A, Zeron Y, Braw-Tal R, Wolfenson D. 2001b. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. *Reproduction*, 122:737-744.

Roth Z, Arav A, Braw-Tai R, Bor A, Wolfenson D. 2002. Effect of treatment with follicle-stimulating hormone or bovine somatotropin on the quality of oocytes aspirated in the autumn from previously heat-stressed cows. *J Dairy Sci*, 85:1398-1405.

Roth Z, Meidan R, Braw-Tal R, Wolfenson D. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *J Reprod Fertil*, 120:83-90.

Roth Z, Meidan R, Shaham-Albalancy A, Braw-Tal R, Wolfenson D. 2001a. Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles. *Reproduction*, 121:745-751.

Sakatani M, Alvarez NV, Takahashi M, Hansen PJ. 2012. Consequences of physiological heat shock beginning at the zygote stage on embryonic development and expression of stress response genes in cattle. *J Dairy Sci*, 95:3080-3091.

Sakatani M, Kobayashi S, Takahashi M. 2004. Effects of heat shock on *in vitro* development and intracellular oxidative state of bovine preimplantation embryos. *Mol Reprod Dev*, 67:77-82.

Sakatani M, Yamanaka K, Kobayashi S, Takahashi M. 2008. Heat shock-derived reactive oxygen species induce embryonic mortality in *in vitro* early stage bovine embryos. *J Reprod Dev*, 54:496-501.

Santolaria P, López-Gatius F, García-Ispierto I, Bech-Sàbat G, Angulo E, Carretero T, Sánchez-Nadal JA, Yániz J. 2010. Effects of cumulative stressful and acute variation episodes of farm climate conditions on late embryo/early fetal loss in high producing dairy cows. *Int J Biometeorol*, 54:93-98.

Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank, MC. 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci*, 85:2803-2812.

Skibiel AL, Peñagaricano F, Amorín R, Ahmed BM, Dahl GE, Laporta J. 2018. In utero heat stress alters the offspring epigenome. *Sci Rep*, 8:14609.

Shabankareh HK, Zandi M, Ganjali M. **2010**. First service pregnancy rates following post-AI use of HCG in Ovsynch and Heatsynch programmes in lactating dairy cows. *Reprod Domest Anim*, 45:711-716.

Silva CF, Sartorelli ES, Castilho AC, Satrapa RA, Puelker RZ, Razza EM, Ticianelli JS, Eduardo HP, Loureiro B, Barros CM. 2013. Effects of heat stress on development, quality and survival of *Bos indicus* and *Bos taurus* embryos produced *in vitro*. *Theriogenology*, 79:351-357.

Stewart BM, Block J, Morelli P, Navarette AE, Amstalden M, Bonilla L, Hansen PJ, Bilby TR. 2011. Efficacy of embryo transfer in lactating dairy cows during summer using fresh or vitrified embryos produced *in vitro* with sex-sorted semen. *J Dairy Sci*, 94:3437-3445.

Stott GH, Wiersma F. 1976. Short term thermal relief for improved fertility in dairy cattle during hot weather. *Int J Biometeorol*, 20:344-350.

Thatcher WW, Collier RJ. 1986. Effects of climate on bovine reproduction. In: *Current Therapy in Theriogenology 2*. (D. A. Morrow, ed.), W. B. Saunders, Philadelphia pp. 301-309.

Torres-Júnior JR de S, Pires M de FA Pires, de Sá WF, Ferreira A de M, Viana JH, Camargo LS, Ramos AA, Folhadella IM, Polisseni J, de Freitas C, Clemente CA, de Sá Filho MF, Paula-Lopes FF, Baruselli PS. 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in *Bos indicus* cattle. *Theriogenology*, 69:155-166.

Trout JP, McDowell LR, Hansen PJ. 1998. Characteristics of the estrous cycle and antioxidant status of lactating Holstein cows exposed to heat stress. *J Dairy Sci*, 81:1244-1250.

Vasconcelos JL, Jardina DT, Sá Filho OG, Aragon FL, Veras MB. 2011. Comparison of progesterone-based protocols with gonadotropin-releasing hormone or estradiol benzoate for timed artificial insemination or embryo transfer in lactating dairy cows. *Theriogenology*, 75:1153-1160.

Voelz BE, Rocha L, Scortegagna F, Stevenson JS, Mendonça LGD. 2016. Treatment of lactating dairy cows with gonadotropin-releasing hormone before first insemination during summer heat stress. *J Dairy Sci*, 99:7612-7623.

Wilson SJ, Marion RS, Spain JN, Spiers DE, Keisler DH, Lucy MC. 1998. Effects of controlled heat stress on ovarian function of dairy cattle. 1. Lactating cows. *J Dairy Sci*, 81:2124-2131.

Wolfenson D, Flamenbaum I, Berman A. 1988. Hyperthermia and body energy store effects on estrous behavior, conception rate, and corpus luteum function in dairy cows. *J Dairy Sci*, 71:3497-3504.

Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, Braw-Tal R, Berman A. 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. *Biol Reprod*, 52:1106-1113.

Wolff LK, Monty DE, Jr. 1974. Physiologic response to intense summer heat and its effect on the estrous cycle of nonlactating and lactating Holstein-Friesian cows in Arizona. *Am J Vet Res*, 35:187-192.

Zolini AM, Ortiz WG, Estrada-Cortes E, Ortega MS, Dikmen S, Sosa F, Giordano JO, Hansen PJ. 2019. Interactions of human chorionic gonadotropin with genotype and parity on fertility responses of lactating dairy cows. *J Dairy Sci*, 102:846-856.

DOI: 10.21451/1984-3143-AR2019-0054

Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Oocytes, embryos and pluripotent stem cells from a biomedical perspective

Poul Hyttel[1,*], Laís Vicari de Figueiredo Pessôa[1], Jan Bojsen-Møller Secher[2], Katarina Stoklund Dittlau[3,4], Kristine Freude[1], Vanessa J Hall[1], Trudee Fair[5], Remmy John Assey[6], Jozef Laurincik[7,8], Henrik Callesen[9], Torben Greve[10], Lotte Björg Stroebech[1]

[1]Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark.
[2]Department of Veterinary Clinical Sciences, University of Copenhagen, Denmark.
[3]KU Leuven – University of Leuven, Department of Neurosciences, Experimental Neurology, and Leuven Brain Institute (LBI), Leuven, Belgium.
[4]VIB, Center for Brain & Disease Research, Laboratory of Neurobiology, Leuven, Belgium.
[5]School of Agriculture and Food Science, University College Dublin, Belfield , Dublin 4, Ireland.
[6]Department of Anatomy and Pathology, Sokoine University of Agriculture, Tanzania.
[7]Constantine the Philosopher University in Nitra, Nitra, Slovakia.
[8]The Czech Academy of Sciences, Institute of Animal Physiology and Genetics, Liběchov, Czech Republic.
[9]Department of Animal Science, Aarhus University, Tjele, Denmark.
[10]Nøddehaven, Værløse.

Abstract

The veterinary and animal science professions are rapidly developing and their inherent and historical connection to agriculture is challenged by more biomedical and medical directions of research. While some consider this development as a risk of losing identity, it may also be seen as an opportunity for developing further and more sophisticated competences that may ultimately feed back to veterinary and animal science in a synergistic way. The present review describes how agriculture-related studies on bovine *in vitro* embryo production through studies of putative bovine and porcine embryonic stem cells led the way to more sophisticated studies of human induced pluripotent stem cells (iPSCs) using e.g. gene editing for modeling of neurodegeneration in man. However, instead of being a blind diversion from veterinary and animal science into medicine, these advanced studies of human iPSC-derived neurons build a set of competences that allowed us, in a more competent way, to focus on novel aspects of more veterinary and agricultural relevance in the form of porcine and canine iPSCs. These types of animal stem cells are of biomedical importance for modeling of iPSC-based therapy in man, but in particular the canine iPSCs are also important for understanding and modeling canine diseases, as e.g. canine cognitive dysfunction, for the benefit and therapy of dogs.

Keywords: embryonic stem cells, induced pluripotent stem cells, *in vitro* fertilization, Alzheimer's disease, dementia.

Introduction

The veterinary and animal science professions are rapidly developing in a shifting scientific environment. Worldwide institutional reorganizations towards larger entities result in absorption of veterinary and animal science faculties into broader entities with a focus on life, biomedical and medical sciences. While this development has a range of advantages creating novel scientifically rewarding collaborative landscapes it also challenges the conventional identity of the veterinary and animal science professions and their inherent and historical connection to agriculture. Consequently, the focus of veterinary and animal sciences has been extended with a major biomedical and even medical dimension; a development which is also sparked by a shift in funding opportunities with biomedicine and medicine having higher leverage than agriculture. Some consider the gradual increase in biomedical and medical focus, at the expense of agricultural attention, a risk. On the other hand, this development gives more room for investigating the complex area of "One Health" and may also give veterinarians and animal scientists access to new sets of competences, that may, in a synergistic and constructive way, feedback to more core classical veterinary and animal science.

It is fair to say that the biomedical and medical trend in science cannot be rejected and should be contemplated as an opportunity for contemporary development of the veterinary and animal science professions. It is the focus of this review to present a scientific development where research in assisted reproductive technologies (ARTs) and embryonic stem cells (ESCs) in the large domestic species has given opportunities for establishing a stem cell center of excellence in neurology focusing on human induced pluripotent stem cell (iPSC)-models for neurodegeneration and, finally, how the competences gained through these medical activities allowed for investigations of porcine and canine iPSCs feeding positively back to veterinary and animal science (Fig.1).

*Corresponding author: poh@sund.ku.dk
orcid.org/0000-0002-6297-2863
Received: May 8, 2019
Accepted: June 18, 2019

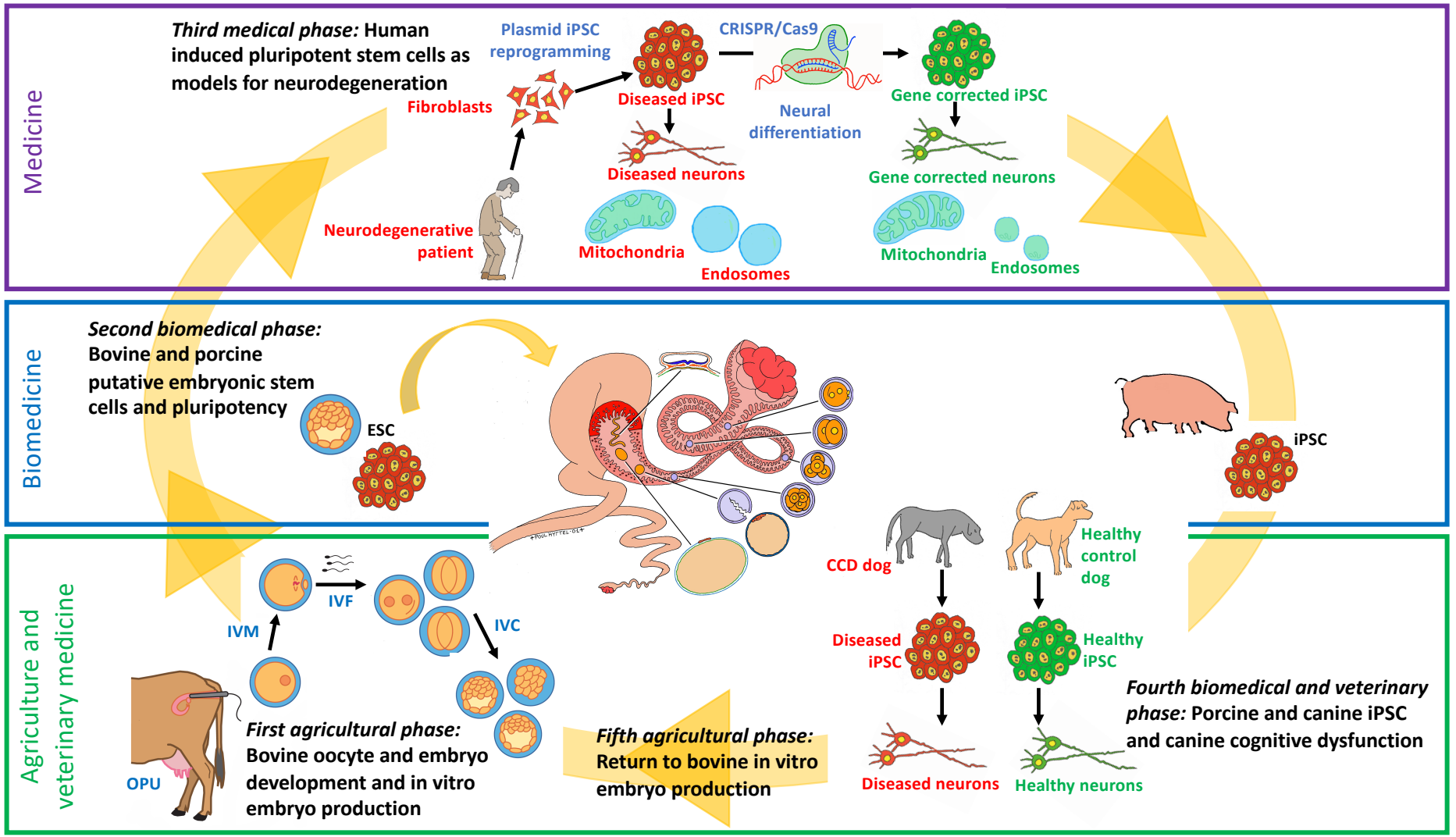


Figure. 1. The progression of research activities moving from agricultural and veterinary medicine through biomedicine and medicine and feeding back to agricultural and veterinary medicine. OPU: Ultrasound-guided ovum pickup; IVM: *In vitro* maturation; IVF: *In vitro* fertilization; IVC: *In vitro* culture.

First agricultural phase: Bovine oocyte and embryo development and *in vitro* embryo production

In vitro embryo production in cattle

Over the past 40 years there has been a gradual agricultural implementation of novel ARTs in large animal husbandry with particular focus on cattle (Greve and Callesen, 2005; Lonergan, 2007). Major components of this development have been the development and refinement of the multiple ovulation and embryo transfer (MOET), including cryopreservation of blastocysts, and of *in vitro* fertilization (IVF) culminating with the birth of the first IVF calf in 1981 (Brackett *et al*., 1982). Whereas contemporary *in vitro* production (IVP) of embryos in cattle includes *in vitro* oocyte maturation, *in vitro* fertilization and *in vitro* culture of the resultant embryos to the blastocyst stage, Brackett and colleagues flushed *in vivo* matured oocytes from the oviducts, performed IVF and transferred a 4-cell stage back to the oviduct. In 1987, the first European IVF calf, now resulting from *in vitro* oocyte maturation, was born in Copenhagen (Xu *et al*., 1987) where we invested great efforts in fundamental investigations of oocyte maturation and fertilization (Hyttel *et al*., 1986a; Hyttel *et al*., 1986b; Hyttel *et al*., 1988a; Hyttel *et al*., 1988b).

In parallel, the quest for defining optimal culture conditions allowing for the development of bovine zygotes to blastocysts took place including focus on coculture systems (Edwards *et al*., 1997), media composition (Holm *et al*., 1999) as well as the physical design of the culture platforms (Vajta *et al*., 2008; Smith *et al*., 2012). These efforts all became extremely relevant in the light of the astonishing adverse effects of improper *in vitro* culture conditions that were reported in 1997, and which coined the term Large Offspring Syndrome (LOS) (Kruip and den Daas, 1997). The risk of LOS caused severe drawbacks for the technologies, and in Denmark the practical implementation of *in vitro* embryo production in cattle breeding was abandoned mainly for this reason. Refined serum-free culture conditions, based on BSA supplementation, have now been developed allowing for improved fetal development and calving (George *et al*., 2008), and in 2013 an entire serum-free ready-to-use media suite for all the steps, maturation, fertilization and culture, was made commercially available by IVF Bioscience, UK, combining synthetic serum replacements and BSA. Finally, the combination of IVP and ultrasound-guided ovum pickup (OPU) has allowed for more sophisticated practical implementation of IVP in cattle breeding, and the year of 2018 became a turning point as the numbers of transferred bovine IVP embryos for the first time officially exceeded that of their *in vivo*-derived counterparts. According to the numbers collected by the IETS, almost 1.5 million (1,487,343) bovine embryos were produced by MOET or IVP worldwide in 2017 and two thirds (almost 1 million) were derived by IVP (Viana, 2018).

In order to pave the way for successful IVP of bovine embryos, we undertook a series of fundamental studies of oocyte development, fertilization and initial embryonic development in cattle, which are summarized in the following. Hence, we have characterized oocyte development and maturation, fertilization and initial embryonic development in cattle extensively by transmission electron microscopy (TEM).

The basic ultrastructure of the oocyte is generated during its growth phase in the primordial to the tertiary follicle. When the tertiary follicles in a cohort reach a diameter of about 3-5 mm in cattle, one dominant follicle is selected, and the structure of the oocyte in this particular follicle is modified during a process that may be referred to as capacitation or pre-maturation. The estrous cycle in cattle generally comprises 2 or 3 follicular waves, and the dominant follicle of the last wave becomes ovulatory. In the ovulatory follicle the oocyte undergoes a final maturation during an approximately 24 hour period between the peak of the LH-surge and ovulation.

Oocyte growth in cattle

During the growth of the bovine oocyte, the inside zona pellucida diameter of the gamete increases from less than 30 μm in the quiescent primordial follicle to more than 120 μm in the tertiary follicle. We have carefully characterized the ultrastructure, transcriptional activity and developmental competence of bovine oocytes in relation to the sequential stages of follicular development (Fair *et al*., 1996; Fair *et al*., 1997a; Fair *et al*., 1997b).

In the quiescent **primordial follicle** gap and intermediate junctions are present between adjacent granulosa cells, whereas exclusively intermediate junctions are seen between the granulosa cells and the oocyte. The transcriptionally quiescent nucleus of the oocyte, i.e. the germinal vesicle, occupies a central or slightly off center position and the organelles are concentrated in the perinuclear region. The **primary follicle** occasionally exhibits small portions of zona pellucida substance between the cuboidal granulosa cells and the oocyte. The continued zona-formation in the **secondary follicle** is associated with the embedding of granulosa cell processes and erect oocyte microvilli into the zona pellucida, and gap junctions are established between the granulosa cell processes and the oocyte. The oocyte nucleoli develop into a fibrillo-granular appearance and transcription is initiated. The oocyte in the **small tertiary follicle** up to about 1 mm in diameter exhibits a complete zona pellucida traversed by numerous cumulus cell projections forming gap and intermediate junctions to the oocyte. Clusters of cortical granules are numerous. The oocyte nucleoli are typical fibrillo-granular and transcription abundant. In the **larger tertiary follicles** the oocyte ultrastructure may be classified according to the inside zona pellucida diameter of the cell. In oocytes <100 μm the particular hooded mitochondria, unique to ruminants, are observed for the first time. Oocytes from 100 to 110 μm in diameter typically display formation of a perivitelline space, the process of which is associated with the release of the previously embedded microvilli from the

zona pellucida. The oocyte nucleus is displaced towards the periphery as are Golgi complexes and mitochondria, amongst which the hooded form becomes more numerous. The fibrillar centers of the nucleoli have typically migrated towards the nucleolar periphery and transcription is decreased. Oocytes from 110 to 120 µm typically present a well-developed perivitelline space and a peripherally located nucleus. The process of nucleolar inactivation has proceeded leaving the nucleolus to consist of a spherical nucleolar remnant with a fibrillar center attached. At a diameter of 120 µm, the oocyte has completed the growth phase and achieved the ultrastructure characterizing the fully developed gamete.

Interestingly, the oocyte achieves the competence to complete meiotic maturation to metaphase II *in vitro* at a diameter of about 110 µm coinciding with the de-activation of its transcriptional machinery, indicating that the necessary compartment of proteins and mRNAs has been formed at this stage of development.

Oocyte capacitation or pre-maturation in cattle

Further, we have carefully mapped the ultrastructural development of bovine oocytes in the dominant vs. the subordinate follicles (Assey *et al*., 1994a). With the growth of the dominant follicle, the ultrastructure of the fully grown oocyte is modified during its so-called capacitation or pre-maturation. During the days approaching the regression of the corpus luteum, i.e. the final period of the luteal phase, the cortical granule clusters are dislocated to more superficial locations and some granules migrate to solitary positions along the oolemma. During the period between luteolysis and the LH-surge individual cumulus cells exhibit elongation and some of the cumulus cell process endings are retracted to a more superficial location on the surface of the oolemma. Also, the oocyte nuclear envelope becomes undulating, especially in the regions facing the zona pellucida, and the nucleolar remnant displays vacuolization. Both of these phenomena are presumably related to the subsequent breakdown of the oocyte nucleus, i.e. germinal vesicle breakdown (GVBD). There are indications that the competence of the oocyte to produce blastocysts *in vitro* increases with completion of capacitation or pre-maturation in the dominant follicle (Hendriksen *et al*., 2000). Superovulation with exogenous gonadotropins may have an adverse effect on this process as indicated by a lack of at least the vacuolization of the nucleolar remnant (Assey *et al*., 1994b).

Oocyte maturation in cattle

The maturation of the oocyte, which in cattle occurs during the approximately 24 hour period from the LH-peak to ovulation, comprises the progression of meiosis from the diplotene stage of prophase I to metaphase II accompanied by a series of ultrastructural and molecular changes in the ooplasm. The ultrastructural changes have been described in detail in relation to the time of the LH-peak in unstimulated (Kruip *et al*., 1983) as well as gonadotropin stimulated cattle (Hyttel *et al*., 1986a). The breakdown of the oocyte nucleus (GVBD) occurs 9 to 12 hours after the LH-peak when the nuclear envelope becomes extremely undulating, the chromatin condenses, the nucleolar remnant is dissolved and there is a gradual decoupling of the cumulus cell endings from the oocyte (Hyttel, 1987). At about 15 and 20 hours after the LH-peak most oocytes have reached metaphase I and II, respectively, and the first polar body is abstricted. During the last hours of maturation, lipid droplets and mitochondria attain a more central location in the ooplasm and the cortical granules migrate to solitary positions along the oolemma. The peripheral migration of the cortical granules appears to be compromised to a certain degree during oocyte maturation *in vitro* (Hyttel *et al*., 1986b).

Growing and dominant follicles are capable of maintaining oocyte meiosis arrested at the diplotene stage of prophase I. However, numerous subordinate tertiary follicles undergo atresia. Interestingly, such atretic follicles may lose the ability to retain the oocyte in meiotic arrest. Hence, oocytes in atretic follicles may display different stages of meiotic maturation; even reaching metaphase II (Assey *et al*., 1994a). Through the described phases of growth, capacitation and maturation, the oocyte has now acquired the ultrastructural architecture for sustaining fertilization and initial embryonic development.

Fertilization and development of the zygote in cattle

The ultrastructure of bovine fertilization has precisely been described in relation to the estimated time of ovulation as determined by timing of the LH-peak in gonadotropin stimulated cows (Hyttel, *et al*., 1988a), and bovine *in vitro* fertilization have added to this understanding (Hyttel *et al*., 1988b; Hyttel *et al*., 1988c).

Upon acrosome reaction and penetration of the zona pellucida, the oocyte microvilli contact the equatorial segment of the sperm head where fusion between the two gametes initially occurs resulting in oocyte activation and cortical granule exocytosis establishing the block against polyspermic fertilization. With a correct Greek term, gamete fusion is termed syngamy; a term that erroneously is also widely used for the apposition of the pronuclei (see later). Within the first 2-3 hours after ovulation, the paternal chromatin is denuded from its membrane coverings and decondensed. In parallel, the maternal chromatin is advancing through anaphase and telophase II forming the second polar body. Pronucleus formation is initiated with smooth endoplasmic reticulum (SER) moving towards both the paternal and maternal chromatin to form nuclear envelope. About 4 hours after ovulation, the two sets of chromatin are completely surrounded by nuclear envelopes. The midpiece of the sperm tail remain spatially associated with the paternal pronucleus. Subsequently, the pronuclei swell to their characteristic spherical appearance accompanied by chromatin decondensation, and about 10 hours post ovulation most

zygotes exhibit spherical pronuclei (Laurincik *et al*., 1998). Along with this process, so-called nucleolus precursor bodies, very similar to the oocyte nucleolar remnant, which later act as enucleation sites for nucleolus formation, are formed in the pronuclei (Laurincik *et al*., 1996). The precursor bodies are not active in rRNA transcription and ribosome formation. The two pronuclei migrate to a close apposition, and about 14 hours after ovulation most zygotes exhibit apposed pronuclei. The S-phase of the first post-fertilization cell cycle takes place 12-19 hours after ovulation (Laurincik *et al*., 1994). Upon pronuclear apposition, pronounced undulations of the nuclear envelopes of the pronuclei are seen in the apposed regions probably preparing for breakdown of the envelopes, which is seen at about 24 hours after ovulation. This process is often referred to as synkaryosis, but it should be emphasized that the two pronuclei do not fuse, but undergo dissolution of the nuclear envelopes similar to the one seen at the breakdown of the oocyte nucleus (GVBD) at resumption of oocyte meiosis. Immediately after synkaryosis, karyokinesis and cytokinesis proceed resulting in the formation of two daughter nuclei enclosed in each their blastomere.

Pre-hatching embryonic development in cattle

Along with the initial cleavages, the embryonic genome is gradually activated during the so-called maternal-embryonic transition. Thus, a low rate of transcription of the embryonic genome has been detected as early as during the 1st, i.e. the zygote (Hay-Schmidt *et al*., 2001), and 2nd post-fertilization cell cycles (Hyttel *et al*., 1996; Viuff *et al*., 1996), and during the 4th cell cycle a major transcriptional activation occurs (Camous *et al*., 1986).

A number of other researchers have contributed to the understanding of the general embryonic ultrastructure based on either *in vivo* or *in vitro* developed embryos (Mohr and Trounson, 1981; Camous *et al*., 1986; Betteridge and Fléchon, 1988; King *et al*., 1988; Kopecný *et al*., 1989; Abe *et al*., 1999; Laurincik *et al*., 2000; Laurincik *et al*., 2003).

Early during the second cell cycle, i.e. the 2-cell stage, nucleolus precursor bodies resembling those described for the pronuclei are established in the nuclei. Hence, functional nucleoli are lacking and protein synthesis must be based on the ribosome pool inherited from the oocyte. Early during the third and fourth cell cycle, i.e. the tentative 4- and 8-cell stages, respectively, nucleolus precursor bodies resembling those from the previous cell cycles are again established. During the fourth cell cycle, however, the nucleolus precursor bodies develop into fibrillo-granular nucleoli displaying the typical components of actively ribosome-synthesizing nucleoli: Fibrillar centers, dense fibrillar component and granular component. The development of the nucleoli is a prerequisite for continued embryonic development and is a sensitive marker for the normality of this process. Abundant activation of embryonic transcription during the fourth cell cycle allows for the first cell differentiation and lineage commitments. External cells become connected by tight junctions while internal cells are only connected by focal membrane contacts. Mitochondria of the hooded form, which were established back during the development of the oocyte in the early tertiary follicle, become fewer, and elongated types with transverse cristae become more numerous.

The competences gained by our studies of oocyte maturation, fertilization and initial embryonic development in cattle allowed us to move into the stem cell area for creating novel potentials in agriculture and biomedicine.

Second biomedical phase: Bovine and porcine putative embryonic stem cells and pluripotency

Embryonic stem cells

Mouse embryonic stem cells (ESCs) were derived in 1981 (Evans and Kaufman, 1981; Martin, 1981) and paved the way for production of genetically modified mice (Thomas and Capecchi, 1987). Along with this development, an interest emerged in investigating the potentials for genomic modifications of the large domestic species for production, health, environmental and biomedical purposes.

Further studies of murine ESCs revealed that there are distinct states of pluripotency (naïve and primed) that differ both morphologically and functionally (De Los Angeles *et al*., 2012). Naïve murine pluripotent stem cells are derived from the inner cell mass (ICM) or early epiblast cells, proliferate in culture as packed dome-like colonies, are maintained in the undifferentiated state by LIF and BMP4 signaling, readily contribute to germline transmitting chimeric embryos, maintain two active X chromosomes (in female cells) and are relatively resistant to differentiation into primordial germ cells (PGCs) and extra-embryonic lineages (Kuijk *et al*., 2011). In contrast, primed pluripotent stem cells are derived from the epiblast of post-hatching murine blastocysts, are termed epiblast stem cells (EpiSCs), are molecularly and epigenetically different from murine ESCs (Brons *et al*., 2007; Tesar *et al*., 2007), have a more flattened colony morphology, depend on bFGF or TGFa/activin signaling, exhibit a limited ability to contribute to chimeras and have undergone X-chromosome inactivation (Brons *et al*., 2007). Human ESCs were first derived in 1998 (Thomson *et al*., 1998) and, surprisingly, they exhibit characteristics more like those of primed murine EpiSCs than their naïve murine ESC counterparts (Thomson *et al*., 1998).

The potentials of murine ESCs for the generation of transgenic mice sparked an interest in deriving ESCs in the large domestic species including activities in our laboratories focusing on cattle and pig. We and many others attempted to derive bovine ESCs (for review, see Ezashi *et al*., 2016) from different developmental stages from 2-cell embryos (Mitalipova *et al*., 2001) up to Day 12 hatched blastocysts (Gjørret and Maddox-Hyttel, 2005). However, even though

ESC-like cell lines were established and some of them could be cultured for extended periods of time, their characterization, especially with respect to functional contribution to chimeras, remained obscure. At present, it must be concluded that none of the derived cell lines have been capable of contributing to germline transmitting chimeras (Iwasaki *et al*., 2000) and , thus, can not be classified as *bona fide* ESCs. A very recent breakthrough indicates that a combination of FGF2 and an inhibitor of the canonical Wnt-signaling pathway may be the key to maintain bovine ESCs (Bogliotti *et al*., 2018).

Similar activities materialized in the pig where we and many others attempted to establish porcine ESCs (for review, see Telugu *et al*., 2010; Ezashi *et al*., 2016). However, even though a single report on a porcine ESC-derived chimera is found (Chen *et al*., 1999), none of the derived cell lines were capable of contributing to germline transmitting chimeras. Interestingly, cells from the inner cell mass from Day 6 to 7 porcine blastocysts are capable of contributing to such germline transmitting chimeras (Anderson *et al*., 1994; Onishi *et al*., 1994; Nagashima *et al*., 2004), and are, by this criterion, pluripotent and hereby a potential source of ESCs. Clearly, however, such is pluripotent cells loose this potential when cultured for even a short period of time. More recent data, where porcine ESCs again have been demonstrated to give rise to chimeric contribution, indicate that a novel medium including a combination of bFGF and LIF may represent a breakthrough although follow up with respect to germline transmission is warranted (Xue *et al*., 2016).

ICM and epiblast differentiation in the pig

In order to explain our lack of success in deriving bovine and porcine ESC, we undertook a set of fundamental studies of the porcine ICM and epiblast which clearly demonstrated that ungulate ICM and epiblast development and pluripotency show distinct differences as compared with its murine counterpart. A dynamic change in gene expression is the driving force for the first cell differentiation, i.e. the segregation of the compacting blastomeres into the ICM and trophectoderm. In the mouse, the ICM develops a stable regulatory circuit, in which the transcription factors Nanog (Chambers *et al*., 2003; Mitsui *et al*., 2003), OCT4 (Nichols *et al*., 1998; Schöler *et al*., 1990), SOX2 (Avilion *et al*., 2003), and SAL4 (Elling *et al*., 2006; Zhang *et al*., 2006) promote pluripotency and suppress differentiation. In contrast, in the trophectoderm-destined cells, the transcription factors CDX2 and EOMES are upregulated together with ELF5 and TEAD4, which are transcription factors acting upstream of CDX2 to mediate trophectoderm differentiation (Ng *et al*., 2008; Nishioka *et al*., 2008; Yagi *et al*., 2007). On the other hand, expression of the trophectoderm-associated transcription factors, CDX2, TEAD4, and ELF5, are repressed in the ICM by the regulatory circuit of Nanog, SOX2, and OCT4 (Ralston and Rossant, 2005). In the pig, the expression of CDX2 during preimplantation development appears conserved as compared with the mouse (Kuijk *et al*., 2007). OCT4 is, on the other hand, expressed in both the ICM and trophectoderm as opposed to the mouse (Keefer *et al*., 2007; Kuijk *et al*., 2008), and Nanog expression has not been observed in the porcine ICM (Hall *et al*., 2009). Hence, there are marked species differences with respect to the molecular background for ICM and trophectoderm specification.

The embryo hatches from the zona pellucida by Days 7 to 8, and in parallel the OCT4 expression, which was earlier present in both the ICM and the trophectoderm, becomes confined exclusively to the ICM (Vejlsted *et al*., 2006), whereas expression of Nanog is still lacking (Wolf *et al*., 2011) as opposed to the mouse. At the time of hatching, the ICM separates into two distinct cell populations. Hence, the most "ventral" cell layer towards the blastocyst cavity flattens and, finally, delaminates forming the hypoblast, whereas the "dorsal" cell population establishes the epiblast. The hypoblast subsequently extends along the inside of the trophectoderm forming a complete inner epithelial lining. The polar trophectoderm covering the epiblast (Rauber's layer) becomes very thin around Day 9 of gestation and gradually disintegrates exposing the epiblast to the uterine environment, which is very unlike the situation in the mouse, where the trophectoderm stays intact. Before the shedding of Rauber's layer, tight junctions are formed between the epiblast cells and the adjacent trophectoderm to maintain the epithelial sealing of the embryo despite the loss of the polar trophectoderm. Apparently, the porcine epiblast forms a small cavity, which finally opens dorsally followed by an "unfolding" of the complete epiblast upon the disintegration of Rauber's layer forming the embryonic disc (Hall *et al*., 2010). In parallel with the formation of the embryonic disc, the porcine epiblast starts to express not only OCT4, but also Nanog (Wolf *et al*., 2011b). At this stage of development, the first sign of anterior-posterior polarization develops in the embryonic disc: As mentioned earlier, the epiblast is underlaid by the hypoblast, and an area of increased cell density of closely apposed hypoblast cells develops. This area is approximately the same size as the embryonic disc, but it is dislocated about one third of its diameter anteriorly as compared with the epiblast of the embryonic disc (Hassoun *et al*., 2009; Wolf *et al*., 2011b). It is likely that this dense hypoblast region emits signals to the epiblast which suppress mesoderm-formation in the anterior epiblast regions. In this sense, the hypoblast may carry the blue-print for the specification of the epiblast.

During Days 11 to 12, the porcine embryonic disc develops into an oval shape, and a crescent-shaped accumulation of cells are found in the posterior region of the disc (Vejlsted *et al*., 2006). This crescent includes mesodermal progenitors which express the mesodermal markers, T (Brachyury) and Goosecoid (Blomberg *et al*., 2006; Wolf *et al*., 2011a), and apparently ingression of Brachyury-expressing extra-embryonic mesoderm is initiated from this crescent even before the "true" gastrulation starts with the appearance of the primitive streak (Wolf *et al*., 2011a), again, as opposed to the

mouse.

With the development of the embryonic disc, a very peculiar pattern of OCT4 and Nanog expression develops in the porcine epiblast: The majority of epiblast cells express OCT4, but small groups or islands of cells are OCT4 negative (Wolf *et al*., 2011b). The latter cells, on the other hand, express Nanog resulting in a mutually exclusive expression pattern. Subsequently, Nanog expression is lost in almost the entire epiblast, except for a few cell in the most posterior region of the embryonic disc, in which OCT4 is also expressed (Wolf *et al*., 2011b). The latter cells are believed to be the primordial germ cells (PGCs).

In conclusion, the efforts on establishing bovine and porcine ESCs have been plentiful but none of them resulted in *bona fide* ESC lines that were capable of giving rise to germ line transmitting chimeric embryos. Reasons for this lack of success are probably multifactorial (Ezashi *et al*., 2016). First of all, the initial embryonic development in cattle and pig differs significantly from that in the mouse: Bovine and porcine embryos have a more protracted development of the epiblast from the inner cell mass, in contrast to the mouse, the bovine and porcine epiblast penetrates the trophectoderm (Rauber's layer) and become exposed to the uterine environment and, finally, the bovine and porcine embryo adheres to the uterine epithelium instead of implanting through the epithelium as their murine counterpart. Second, the well-established markers of pluripotency are much less distinct and well-defined in bovine and porcine ESC-like cells than in their murine counterparts. Finally, the pluripotency states, i.e. naïve vs. primed, are not well recognized in bovine and porcine ESC-like cells. Hence, the culture conditions and needs for supplementation for maintenance of pluripotency are putative and in many studies both LIF and bFGF are used.

Importantly, in 1996 it was elegantly demonstrated that cloned sheep could be established by somatic cell nuclear transfer (SCNT) from a cultured cell line established from embryonic discs (Campbell *et al*., 1996). This breakthrough later led to the birth of Dolly (Wilmut *et al*., 1997), cloned from an adult mammary epithelial cell line, and to an alternative avenue for production of genetically modified large domestic species by SCNT utilizing genetically modified cell lines (Schnieke *et al*., 1997; McCreath *et al*., 2000). With this development, the practical importance of bovine and porcine ESCs became less evident as seen in an agricultural and biomedical perspective.

Third medical phase: Human induced pluripotent stem cells as models for neurodegeneration

Through our struggles towards establishing bovine and porcine ESCs, we developed a skill set that allowed us to embark on human iPSCs and the use of these fascinating cells for modelling neurodegeneration. Eminent funding opportunities prompted us to move from an agricultural focus into the medical arena.

Human iPSC reprogramming and mesenchymal-to-epithelial transition

In 2006, Takahashi and Yamanaka published their conceptual work on the establishment of murine iPSCs, where they elegantly narrowed down the need of reprogramming factors to the so-called Yamanaka factors: *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM), which were introduced by retroviral vectors (Takahashi and Yamanaka, 2006). Only one year later, two groups independently reported on the establishment of human iPSCs (Takahashi *et al*., 2007; Yu *et al*., 2007). Since these first publications a range of iPSC reprogramming technologies have been developed and refined with respect to both reprogramming factors (gene sequences, mRNA, miRNA, protein) and vectors (integrating and non-integrating viruses, minicircle vectors and episomal plasmids) in combination with different epigenetic modifiers (for review, see Malik and Rao, 2013). As the most novel approach, it has been demonstrated that the use of CRISPR transcriptional activators for prompting endogenous pluripotency gene expression can result in iPSC reprogramming (Weltner *et al*., 2018).

We have refined and characterized a non-integrative episomal plasmid-based human iPSC reprogramming strategy first published by Okita *et al*. (2011). Our reprogramming is based on the use of electroporation of fibroblasts with three plasmids encoding a short hairpin to TP53 (*shp53*) combined with human *OCT4*, *SOX2*, *KLF4*, *L-MYC* and *LIN28*. We have clearly demonstrated that this strategy, including transient p53 suppression, increases reprogramming of human fibroblasts without affecting apoptosis and DNA damage (Rasmussen *et al*., 2014). Moreover, we have performed a detailed investigation of the gene expression and ultrastructural changes associated with the mesenchymal-to-epithelial transition (MET) that is a vital component of the iPSC reprogramming process (Høffding and Hyttel, 2015). We clearly demonstrated that the sequential acquisition of an epithelial epiblast-like ultrastructure was accompanied by a reorganization of actin and beta-catenin localization from the cytoplasm to the plasma membrane region as well as appearance of plasma membrane-associated E-cadherin and Occludin and of Nanog in the nucleus. In parallel, the mesenchymal marker vimentin disappeared. At the transcriptional level, the relative expression of the epithelial markers *CDH1*, *OCLN* and *EPCAM* was, accordingly, dramatically increased through MET. On the other hand, transcription of the mesenchymal markers *VIM*, *ZEB1* and *SLUG* appeared constant or slightly downregulated. The true downregulation was probably masked by the large number of non-reprogrammed fibroblasts in the samples. These studies clearly demonstrated that a well-orchestrated MET is a major component of iPSC reprogramming.

The investigations referred to above gave us a solid platform for iPSC-based disease modelling, which was materialized in the stem cell center of excellence in neurology, BrainStem.

Human iPSCs for modelling neurodegeneration

The iPSC technology gives access to an infinite source of pluripotent cells from an individual, offering great potentials for future disease modelling and cell therapy (Condic and Rao, 2010). *In vitro* disease modelling has become a major tool in the potential identification of novel disease phenotypes and drug targets as well as in drug screening. Worldwide, iPSCs are used for modelling a variety of disorders, but they are especially useful in research focusing on late progressive disorders such as neurodegenerative diseases, like frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Alzheimer's (AD) and Parkinson's disease (PD), where early symptomatic brain samples are impossible to obtain (Hargus *et al*., 2014; Hedges *et al*., 2016; Lee and Huang, 2017). The iPSC technology allows for creation of "micro-brains" in a dish and studies of the specific pathology and disease progression in an easily assessable and manipulated environment.

Previously, research in the underlying mechanisms of neurodegeneration, as e.g. AD, has been based on data from transgenic AD mice models, which are unfaithful in mimicking AD, or post-mortem AD brain tissue, which exclusively represents the terminal disease pathology. These shortcomings are a major setback for the development of novel therapeutics, which need to combat early disease progression. The iPSC technology, on the other hand, offers the opportunity to investigate early disease mechanisms in the relevant targets: The human neurons, astrocytes and microglia. An example of modelling of frontotemporal dementia (FTD) is presented in the following as it encompasses all components of stem cell biology and gene editing required for dissecting early disease mechanisms.

Frontotemporal dementia linked to chromosome 3 (FTD3) is a rare heterozygous early-onset form of frontotemporal dementia, which is caused by a point mutation in the gene encoding the charged multivesicular protein 2B (CHMP2B) located to the human chromosome 3. FTD3 is characterized as a behavioral variant of frontotemporal dementia mainly associated with initial mild personality changes and social inabilities and as the disease commences potential development of apathy and aggressive behavior (Seelaar *et al*., 2011). FTD3 slowly progresses from the age of onset around the late 50'ties with a mean duration of approximately 10 years, and is thus defined as an early-onset form of dementia (Isaacs *et al*., 2011; Rossor *et al*., 2010; Tang *et al*., 2012). The Danish version of FTD3 has spread in a large family and is caused by a single nucleotide mutation translated into shortened and altered C-terminus of the CHMP2B protein (Skibinski *et al*., 2005; Urwin *et al*., 2010; Zhang *et al*., 2017). CHMP2B is an important part of the endosomal sorting complex required for transport-III (ESCRT-III) and for a proper function of the intracellular endolysosomal pathway (Krasniak and Ahmad, 2016; Urwin *et al*., 2010; van der Zee *et al*., 2008). The mutation results in truncated CHMP2B unable to mediate the endosomal-lysosomal fusion and processing. Patient-derived iPSCs have proven to be very useful in identification of cellular and molecular FTD3 phenotypes and future studies utilizing such models will likely reveal potential therapeutic targets. The stepwise process of FTD3 disease modelling is presented in the following (Zhang *et al*., 2017).

Skin fibroblasts were harvested from the patients and reprogrammed into iPSCs as by means of the non-integrative episomal plasmid approach described above (Rasmussen *et al*., 2014). Before using the iPSC lines for experimentation, they were carefully characterized with respect to their expression of pluripotency markers, ability to differentiate into all three germ layers in-vitro, normality of karyotype and absence of episomal plasmids in their genome.

For disease modelling of neurons, the iPSCs were submitted to neural induction via a dual SMAD inhibition using the small molecules SB431542 and LDN193189, which inhibits the TGFβ and the BMP pathway, thus promoting ectodermal and neuronal differentiation, respectively (Zhang *et al*., 2017). The maturation of the neuronal progenitor cells into glutamatergic forebrain cortical neurons was initiated and maintained with growth factor supplements of BDNF, GDNF and the γ-secretase inhibitor DAPT (Zhang *et al*., 2017).

Until recently, reference iPSCs were derived from healthy age- and gender matched control individuals and used as a comparison to the iPSCs derived from the patients. With the introduction of clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing, it is now possible to create isogenic controls from the patient's own cells to use as a reference instead, eliminating obvious bias due to genomic variance (Poon *et al*., 2017). This so called CRISPR/Cas9 technology is derived from a natural adaptive immune defence mechanism in bacteria providing protection against DNA sequences invading from bacteriophages (Rath *et al*., 2015). Today, this microbial immune mechanism has been biotechnologically transformed into a versatile tool for genome editing. Hence, a single stranded guide-RNA sequence (sgRNA), designed to recognize a specific site in the genome, is combined with a Cas9 protein, capable of cleaving double stranded DNA. Once the target DNA is cut by Cas9 by a double stranded break, the cell repairs the break by either non-homologous-end-joining (NHEJ) or homology-directed repair (HDR) (Ran *et al*., 2013). NHEJ, which is by far the most common of the two, is a random default-prone mechanism where the DNA-recombinase repairs the break by adding random nucleotides until the two DNA strands once again are connected. NHEJ is likely to result in insertions or deletions (Indels), which often results in formation of a codon shift creating a premature stop codon. NHEJ can, however, be bypassed by HDR where an alternative, often single stranded, DNA template carrying a designed sequence with overhangs matching the DNA regions beside the cut is provided. This oligo will, in successful cases, function as a template for DNA-repair (Hsu *et al*., 2014; Ran *et al*., 2013; Yumlu *et al*., 2017).

In the case of FTD3, isogenic controls were created in three different patients (Zhang *et al*., 2017).

Based on the use of patient iPSC-derived neurons and their isogenic controls, we have clearly demonstrated specific disease phenotypes in the FTD3 neurons, all of which can be rescued by correction of the disease-causing mutation. These include mis-regulated expression of genes related to endosomes, mitochondria and iron homeostasis, which was verified by immunocytochemistry, electron microscopy and cellular assays demonstrating large neuronal accumulations of endosomes, lack of mitochondrial axonal distribution and cristae formation, reduction in mitochondrial respiration capacity and intracellular iron accumulation (Zhang *et al*., 2017). Further studies in disease modelling of FTD3 using iPSCs will potentially reveal additional novel disease phenotypes and therapeutic strategies.

The central nervous system holds a glial/neuron ratio of 1.48 (Friede and Van Houten, 1962; Sica *et al*., 2016), which emphasize the glial importance and points towards potential pathological implications of glia in neurodegenerative disorders like FTD. Consequently, we applied an astrocyte differentiation protocol where growth factor supplements, mimicking *in vivo* embryonic astrogenesis, promoted the differentiation and maturation of the neuronal progenitor cells into astrocyte progenitors and further towards astrocytes expressing the astrocytic markers AQP4, S100β, SOX9 and GFAP (unpublished data). Our studies of FTD3-derived astrocytes and their isogenic controls clearly demonstrated that the FTD3 astrocytes displayed accumulation of autophagosomes and increased astrocyte reactivity with a subsequent toxic effect on neurons (unpublished data). Hence, not only neurons, but also the prominent glial compartment is affected by FTD3. Continued research on co-cultures between neurons and glial cells, including both astrocytes and microglia, will further aid unravelling the molecular mechanisms behind this autophagic imbalance and induced neurotoxicity.

Fourth biomedical and veterinary phase: Porcine and canine iPSCs and canine cognitive dysfunction

The competences gained from the human iPSC-based disease modeling allowed us to return our focus to studies of porcine and canine iPSCs of more biomedical, veterinary and, potentially, agricultural relevance.

Porcine iPSCs

Porcine iPSCs have attracted great attention due to the fact that pigs are excellent biomedical models where potentials, but also risks associated with iPSC-based therapy may be investigated. The use of this model enables long-term studies of, for example, cell or organ transplantation, and a multitude of genetically modified pigs are emerging as models for human diseases (Perleberg *et al*., 2018). In addition to being used for modeling cell-based therapy, porcine iPSCs may also facilitate the generation of genetically modified pigs for use as preclinical models and, potentially in the future, production of animals with valuable traits through the use of chimeric or nuclear transfer technologies. For these reasons we set out to derive integration-free porcine iPSCs.

As alluded to earlier, *bona fide* porcine ESCs have not been generated (Gandolfi *et al*., 2012). The derivation of iPSCs, therefore, is of great importance, and at least 25 studies have already described putative porcine iPSC production (for review, see Pessôa *et al*., 2019). The production of porcine iPSCs until now has predominantly utilized integrative viral vectors carrying human or murine *OCT4*, *SOX2*, *KLM4* and *C-MYC*, including some variations such as *NANOG* and *LIN-28*. However, persistent expression of the integrated transgenes has been widely reported, as opposed to the mouse, and failure to inactivate the exogenous factors is considered a major flaw in the generation of *bona fide* porcine iPSCs (Ezashi *et al*., 2016).

Contribution of porcine iPSCs to live chimeric offspring and germline transmission has only been achieved by one group thus far (West *et al*., 2010; West *et al*., 2011). In this study, porcine mesenchymal stem were used for the iPSC reprogramming and this approach resulted in more than 85% of the live-born piglets being chimeras. Interestingly, this approach also allowed for germline transmission where 2 out of 43 next generation piglets were of iPSC-origin. One of these piglets was, however, stillborn and the other only lived to Day 3 indicating that underlying potential epigenetic aberrancies are incurred.

As for the putative porcine ESCs, the pluripotency state, i.e. naïve vs. primed, of the porcine iPSCs has remained elusive and unclarified. Interestingly, the porcine iPSCs giving rise to germline transmission were cultured in the presence of bFGF being typical for primed murine ESCs, which are not capable of giving rise to germline transmitting chimeras (West *et al*., 2010; West *et al*., 2011). Again, this underlines the lack of clarity regarding the pluripotency states in the pig.

We have particularly focused on the derivation of integration-free porcine iPSCs according to the protocol we optimized for human iPSC reprogramming (Rasmussen *et al*., 2014). Porcine iPSCs were successfully generated by this methodology and cultured in the presence of bFGF as well as MEK/ERK (PD0325901) and GSK-3β (CHIR99021) inhibitors (Li *et al*., 2018). In order to assess the transgene status with respect to genomic integration or plasmid persistence in our iPSCs, PCR analysis on total DNA extractions, which included genomic DNA and episomal plasmid DNA, were performed. These revealed that at least two of the three episomal plasmids were still present in all lines examined at passage 10. However, at passage 20 the abundance of the two plasmids was significantly diminished in all iPSC lines with one plasmid being completely undetectable. This promoted us to select the a porcine iPSC line, which showed the weakest PCR products for the two plasmids, for single cell subcloning under the assumption that the cell line might show a

certain diversity with respect to plasmid integration or retention. Indeed, 6 out of 8 subclones were completely free of episomal vector DNA. We hereby succeeded in generating porcine iPSCs free of the reprogramming constructs. One of the most striking findings during this quest was that subcloning appears to be crucial in order to obtain integration- and episomal-free porcine iPSCs using the plasmid approach.

During our efforts in implementing the plasmid-based iPSC reprogramming in the pig, we discovered a small population of stage-specific embryonic antigen 1 positive (SSEA-1+) cells in Danish Landrace and Göttingen minipig embryonic fibroblasts, which were absent in their Yucatan counterparts (Li *et al*., 2017). Interestingly, reprogramming of the SSEA-1+ cells after cell sorting led to higher reprogramming efficiency. These SSEA-1+ cells exhibited expression of several genes that are characteristic of mesenchymal stem cells.

Canine iPSCs

Dogs are considered as very interesting models for human diseases; not only due to the over 200 hereditary canine diseases with equivalents in humans, but also due to the physiological similarities as well as equivalence in response to therapy (Starkey *et al*., 2005; Gilmore and Greer, 2015). Based on the competences gained from our human iPSC modelling of neurodegeneration, we extended our studies back to the veterinary field focusing on the dog. Recently, the neurobehavioral syndrome canine cognitive dysfunction (CCD), which shares many clinical and neuropathological similarities with human aging and early stages of AD, has been characterized in dogs, and it is increasingly evident that humans and dogs demonstrate commonalities in brain aging associated with cognitive dysfunction (Studzinski *et al*., 2005; Cotman and Head, 2008). The prevalence of CCD in dogs over 8 years of age has been estimated to 14.2-22.5 % (Azkona *et al*., 2009; Salvin *et al*., 2010). Hence, we set out to further characterize the CCD condition in iPSC-derived neurons from aged demented and control dogs, which will also allow the comparison of CCD with human AD at the cellular level. Such studies have several perspectives: The dog may in the future serve as a model for spontaneous AD in humans and from a veterinary point of view, novel treatment modalities of CCD may become available.

The first information on potential canine iPSCs was reported some years after Yamanaka's breakthrough (Takahashi and Yamanaka, 2006), and the quest for deriving fully reprogrammed and stable canine iPSCs is still ongoing (Shimada *et al*., 2010; Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Koh *et al*., 2013; Baird *et al*., 2015; Nishimura *et al*., 2017; Gonçalves *et al*., 2017; Chow *et al*., 2017; Tsukamoto *et al*., 2018). In the first studies on canine iPSCs, canine reprograming factors were utilized for reprogramming (Shimada *et al*., 2010). The presumptive iPSCs were positive for OCT4 and alkaline phosphatase and were capable of directed differentiation into representatives of all three germ layers. However, the cells were not extensively characterized. In the subsequent work, researchers used mostly human or mouse OSKM reprogramming factors, occasionally with addition of *LIN28* and *NANOG* (Whitworth *et al*., 2012), introduced using retroviral (Shimada *et al*., 2010; Koh *et al*., 2013; Baird *et al*., 2015) or lentiviral approaches (Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Nishimura *et al*., 2017; Gonçalves *et al*., 2017). Lastly, non-integrative Sendai virus have been attempted (Chow *et al*., 2017; Tsukamoto *et al*., 2018). Again, as earlier described for the pig, the silencing of the integrated transgenes in the canine iPSCs seems to represent a consistent problem and was only described in a few studies (Baird *et al*., 2015; Gonçalves *et al*., 2017). Regarding culture conditions and supplementation requirements, canine iPSCs seem to be dependent of both LIF and bFGF, with some exceptions (Whitworth *et al*., 2012; Nishimura *et al*., 2017; Gonçalves *et al*., 2017; Chow *et al*., 2017), as well as the cells are dependent on culture with feeder cells, except for a single report (Nishimura *et al*., 2017).

In general, the reports on putative canine iPSCs do not refer to the naïve vs. primed pluripotency state of the generated cells. However, based on the expression of pluripotency markers, one can speculate that most of the generated cell lines represent a primed status, characterized by expression of markers such as SSEA4, TRA-1-60 and TRA-1-80 (Lee *et al*., 2011; Luo *et al*., 2011; Whitworth *et al*., 2012; Baird *et al*., 2015; Nishimura *et al*., 2017; Chow *et al*., 2017). Nevertheless, canine iPSCs expressing naïve pluripotency markers, like SSEA1, have also been described (Koh *et al*., 2013; Tsukamoto *et al*., 2018). The expression of pluripotency markers, however, may differ between species making it difficult to draw firm conclusions on the state of pluripotency just based upon such markers. Overall, the potential canine iPSCs have been reported to show different combinations of classic pluripotency markers, such as OCT4, Nanog, SOX2, amongst others (for review, see Pessôa *et al*., in press), and some of these cells lines were also able to form teratomas (Lee *et al*., 2011; Whitworth *et al*., 2012; Koh *et al*., 2013; Gonçalves *et al*., 2017; Chow *et al*., 2017; Tsukamoto *et al*., 2018). Contribution of iPSCs to the development of chimeric embryos has, however, not been described so far.

All the previously cited reports deal with reprogramming of fibroblasts or adipose tissue cells from canine embryos, fetuses or younger adults, the oldest donors being 3-year-old beagles and a 6-year-old male standard poodle (Koh *et al*., 2013; Chow *et al*., 2017; respectively). Our studies of CCD focused on geriatric dogs, and it turned out that iPSC reprogramming of fibroblasts from such elderly dogs is a major challenge. Our efforts, however, have just started to pay off. We have attempted to reprogram adult fibroblasts to pluripotency using an excisable lentiviral vector containing human and/or murine OSKM (Sommer *et al*., 2009; Gonçalves *et al*., 2017). After a longer series of experiments, the first iPSCs colonies have now emerged around 14 days after transduction with human factors in skin fibroblasts of a 14-year and 9 month-old female west highland white terrier. So far, colonies obtained are flat, present high

nuclei to cytoplasm ratio, are tightly packed, present well defined edges, and are positively stained for alkaline phosphatase and hanog The potential iPSCs are dependent on both LIF and bFGF and are in the process of expansion for further characterization. Once these cell lines are well stablished and characterized, we hope they will provide valuable information for iPSC-based disease modeling and veterinary research.

Fifth agricultural phase: Return to bovine *in vitro* embryo production

This review began with *in vitro* production of bovine embryos. Even though great advances were made in this area in Denmark during the eighties and nineties, the practical implementation of the technologies failed due to concerns related to animal welfare and ethical considerations related to the OPU procedure and the risk of LOS. As alluded to earlier in the text, the refinement of media for bovine IVP has more or less eliminated the risk for LOS, and the OPU procedures have also become less harmful. These developments have led to a Danish reconsideration of the use of the technologies in cattle breeding and have given leverage to funding of the project EliteOva by Innovation Fund Denmark. EliteOva aims at implementing OPU and IVP combined with genomic selection of the embryos in commercial Danish Holstein dairy breeding.

It has been a great privilege to encompass a full circle of scientific progress from bovine oocytes and embryos through bovine and porcine stem cells into human stem cell-based disease modeling and back to animal stem cells and, finally, practical implementation of bovine oocyte and embryo technologies.

Conclusions

The veterinary and animal science professions are rapidly developing and their inherent and historical focus on agriculture has been extended with a major biomedical and even medical dimension. This biomedical and medical trend in science cannot be rejected and should be contemplated as an opportunity for contemporary development of the veterinary and animal science professions. With an open scientific mind it is possible to embark on such biomedical and medical adventures and gain new competences that can feed back to novel ideas and projects in the veterinary and animal science field. Hence, seek opportunistic scientific avenues and see the possibilities in gaining novel competences that will, in turn, benefit veterinary and animal science.

Author contributions

PH: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; LVFP: Conceptualization, Investigation, Writing – original draft, Writing – review & editing; JB-MS: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; KSD: Conceptualization, Investigation, Writing – original draft, Writing – review & editing; KF: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; VJH: Funding acquisition, Conceptualization, Investigation, Writing – review & editing; TF: Investigation, Writing – review & editing; RJA: Investigation, Writing – review & editing; JL: Funding acquisition, Investigation, Writing – review & editing; HC: Writing – review & editing; TG: Funding acquisition, Supervision, Writing – review & editing; LBS: Funding acquisition, Conceptualization, Investigation, Writing – review & editing.

Conflict of interest

The authors declare that they have no competing interests. LBS: Scientific Advisor for IVFBioscience.

Acknowledgments and Funding disclosure statement

PH: Innovation Fund Denmark is acknowledged for funding of the projects BrainStem, NeuroStem and EliteOva and Independent Research Fund Denmark, Technology and Production, for the project "Establishment of demented dogs as a superior model for Alzheimer's disease" (4184-00061B) and Independent Research Fund Denmark, Natural Sciences, for the project "Cell reprogramming - basic developmental studies in the pig" (8021-00048B). JL: The work was, furthermore, supported by "EXCELLENCE in molecular aspects of the early development of vertebrates", CZ.02.1.01/0.0/0.0/ 15_003/0000460 from the Czech Operational Programme Research, Development and Education and by Slovak VEGA 1/0001/19. KSD: Additional support was granted from the VIB, the KU Leuven (C1 (C14/17/107) and "Opening the Future" Fund), the Agency for Innovation by Science and Technology (IWT; SBO-iPSCAF n°150031) and the ALS Liga België.

References

Abe H, Otoi T, Tachikawa S, Yamashita S, Satoh T, Hoshi H. 1999. Fine structure of bovine morulae and blastocysts *in vivo* and *in vitro*. *Anat Embryol (Berl)*, 199:519-527.

Anderson GB, Choi SJ, BonDurant RH. 1994. Survival of porcine inner cell masses in culture and after injection into blastocysts. *Theriogenology*, 42:204-212.

Assey RJ, Hyttel P, Greve T, Purwantara B. 1994a. Oocyte morphology in dominant and subordinate follicles. *Mol Reprod Dev*, 37:335-344.

Assey RJ, Hyttel P, Roche JF, Boland M. 1994b. Oocyte structure and follicular steroid concentrations in superovulated versus unstimulated heifers. *Mol Reprod Dev*, 39:8-16.

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev*, 17:126-140.

Azkona G, García-Belenguer S, Chacón G, Rosado B,

León M, Palacio J. 2009. Prevalence and risk factors of behavioural changes associated with age-related cognitive impairment in geriatric dogs. *J Small Anim Pract*, 50:87-91.

Baird A, Barsby T, Guest DJ. 2015. Derivation of Canine Induced Pluripotent Stem Cells. *Reprod Domest Anim*, 50:669-676.

Betteridge KJ, Fléchon J-E. 1988. The anatomy and physiology of pre-attachment bovine embryos. *Theriogenology*, 29:155-187.

Blomberg LA, Garrett WM, Guillomot M, Miles JR, Sonstegard TS, Van Tassell CP, Zuelke KA. 2006. Transcriptome profiling of the tubular porcine conceptus identifies the differential regulation of growth and developmentally associated genes. *Mol Reprod Dev*, 73:1491-1502.

Bogliotti YS, Wu J, Vilarino M, Okamura D, Soto DA, Zhong C, Sakurai M, Sampaio RV, Suzuki K, Izpisua Belmonte JC, Ross PJ. 2018. Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. *Proc Natl Acad Sci U S A*, 115:2090-2095.

Brackett BG, Bousquet D, Boice ML, Donawick WJ, Evans JF, Dressel MA. 1982. Normal development following *in vitro* fertilization in the cow. *Biol Reprod*, 27:147-158.

Brons I Gabrielle M, Smithers LE, Trotter MWB, Rugg-Gunn P, Sun B, Chuva de Sousa Lopes SM, Howlett SK, Clarkson A, Ahrlund-Richter L, Pedersen RA, Vallier L. 2007. Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature*, 448:191-195.

Camous S, Kopecný V, Fléchon JE. 1986. Autoradiographic detection of the earliest stage of [3H]-uridine incorporation into the cow embryo. *Biol Cell*, 58:195-200.

Campbell KHS, McWhir J, Ritchie WA, Wilmut I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, 380:64-66.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell*, 113:643-655.

Chen LR, Shiue YL, Bertolini L, Medrano JF, BonDurant RH, Anderson GB. 1999. Establishment of pluripotent cell lines from porcine preimplantation embryos. *Theriogenology*, 52:195-212.

Chow L, Johnson V, Regan D, Wheat W, Webb S, Koch P, Dow S. 2017. Safety and immune regulatory properties of canine induced pluripotent stem cell-derived mesenchymal stem cells. *Stem Cell Res*, 25:221-232.

Condic ML, Rao M. 2010. Alternative sources of pluripotent stem cells:ethical and scientific issues revisited. *Stem Cells Dev*, 19:1121-1129.

Cotman CW, Head E. 2008. The canine (dog) model of human aging and disease:dietary, environmental and immunotherapy approaches. *J Alzheimers Dis*, 15:685-707.

Edwards LJ, Batt PA, Gandolfi F, Gardner DK. 1997. Modifications made to culture medium by bovine oviduct epithelial cells:Changes to carbohydrates stimulate bovine embryo development. *Mol Reprod Dev*, 46:146-154.

Elling U, Klasen C, Eisenberger T, Anlag K, Treier M. 2006. Murine inner cell mass-derived lineages depend on Sall4 function. *Proc Natl Acad Sci U S A*, 103:16319-16324.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature*, 292:154-156.

Ezashi T, Yuan Y, Roberts R Michael. 2016. Pluripotent Stem Cells from Domesticated Mammals. *Annu Rev Anim Biosci*, 4:223-253.

Fair T, Hyttel P, Greve T, Boland M. 1996. Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. *Mol Reprod Dev*, 43:503-512.

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. 1997a. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. *Mol Reprod Dev*, 46:208-215.

Fair T, Hulshof SC, Hyttel P, Greve T, Boland M. 1997b. Oocyte ultrastructure in bovine primordial to early tertiary follicles. *Anat Embryol (Berl)*, 195:327-336.

Friede RL, Van Houten WH. 1962. Neuronal extension and glial supply:functional significance of glia. *Proc Natl Acad Sci U S A*, 48:817-821.

Gandolfi F, Pennarossa G, Maffei S, Brevini T. 2012. Why is it so Difficult to Derive Pluripotent Stem Cells in Domestic Ungulates? *Reprod Domest Anim*, 47:11-17.

George F, Daniaux C, Genicot G, Verhaeghe B, Lambert P, Donnay I. 2008. Set up of a serum-free culture system for bovine embryos:Embryo development and quality before and after transient transfer. *Theriogenology*, 69:612-623.

Gilmore KM, Greer KA. 2015. Why is the dog an ideal model for aging research? *Exp Gerontol*, 71:14-20.

Gjørret JO, Maddox-Hyttel P. 2005. Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures. *Reprod Fertil Dev*, 17:113-124.

Gonçalves NJN, Bressan FF, Roballo KCS, Meirelles F V, Xavier PLP, Fukumasu H, Williams C, Breen M, Koh S, Sper R, Piedrahita J, Ambrósio CE. 2017. Generation of LIF-independent induced pluripotent stem cells from canine fetal fibroblasts. *Theriogenology*, 92:75-82.

Greve T, Callesen H. 2005. Embryo technology:implications for fertility in cattle. *Rev Sci Tech*, 24:405-412.

Hall VJ, Christensen J, Gao Y, Schmidt MH, Hyttel P. 2009. Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. *Dev Dyn*, 238:2014-2024.

Hall VJ, Jacobsen J V., Rasmussen MA, Hyttel P. 2010. Ultrastructural and molecular distinctions between the porcine inner cell mass and epiblast reveal unique pluripotent cell states. *Dev Dyn*, 239:2911-2920.

Hargus G, Ehrlich M, Hallmann A-L, Kuhlmann T. 2014. Human stem cell models of neurodegeneration:a novel approach to study mechanisms of disease development. *Acta Neuropathol*, 127:151-173.

Hassoun R, Schwartz P, Feistel K, Blum M, Viebahn C. 2009. Axial differentiation and early gastrulation

stages of the pig embryo. *Differentiation*, 78:301-311.

Hay-Schmidt A, Viuff D, Greve T, Hyttel P. 2001. Transcriptional activity in *in vivo* developed early cleavage stage bovine embryos. *Theriogenology*, 56:167-176.

Hedges EC, Mehler VJ, Nishimura AL. 2016. The Use of Stem Cells to Model Amyotrophic Lateral Sclerosis and Frontotemporal Dementia:From Basic Research to Regenerative Medicine. *Stem Cells Int*, 2016:9279516.

Hendriksen PJ, Vos PL, Steenweg WN, Bevers MM, Dieleman SJ. 2000. Bovine follicular development and its effect on the *in vitro* competence of oocytes. *Theriogenology*, 53:11-20.

Høffding MK, Hyttel P. 2015. Ultrastructural visualization of the Mesenchymal-to-Epithelial Transition during reprogramming of human fibroblasts to induced pluripotent stem cells. *Stem Cell Res*, 14:39-53.

Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H. 1999. High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. *Theriogenology*, 52:683-700.

Hsu PD, Lander ES, Zhang F. 2014. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157:1262-1278.

Hyttel P, Xu KP, Smith S GT. 1986a. *Ultrastructure of in-vitro oocyte maturation in cattle*. *J Reprod Fertil*, 78(2):615-625.

Hyttel P, Callesen H, Greve T. 1986b. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. *J Reprod Fertil*, 76:645-56.

Hyttel P. 1987. Bovine cumulus-oocyte disconnection *in vitro*. *Anat Embryol (Berl)*, 176:41-44.

Hyttel P, Greve T, Callesen H. 1988a. Ultrastructure of in-vivo fertilization in superovulated cattle. *J Reprod Fertil*, 82:1-13.

Hyttel P, Xu KP, Greve T. 1988b. Scanning electron microscopy of *in vitro* fertilization in cattle. *Anat Embryol (Berl)*, 178:41-46.

Hyttel P, Xu KP, Greve T. 1988c. Ultrastructural abnormalities of *in vitro* fertilization of *in vitro* matured bovine oocytes. *Anat Embryol (Berl)*, 178:47-52.

Hyttel P, Viuff D, Avery B, Laurincik J, Greve T. 1996. Transcription and cell cycle-dependent development of intranuclear bodies and granules in two-cell bovine embryos. *J Reprod Fertil*, 108:263-270.

Isaacs AM, Johannsen P, Holm I, Nielsen JE, FReJA consortium. 2011. Frontotemporal dementia caused by CHMP2B mutations. *Curr Alzheimer Res*, 8:246-251.

Iwasaki Shizue, Campbell KHS, Galli C, Akiyama K, Iwasaki Setsuo. 2000. Production of Live Calves Derived from Embryonic Stem-Like Cells Aggregated with Tetraploid Embryos1. *Biol Reprod*, 62:470-475.

Keefer CL, Pant D, Blomberg L, Talbot NC. 2007. Challenges and prospects for the establishment of embryonic stem cell lines of domesticated ungulates. *Anim Reprod Sci*, 98:147-168.

King WA, Niar A, Chartrain I, Betteridge KJ, Guay P. 1988. Nucleolus organizer regions and nucleoli in preattachment bovine embryos. *J Reprod Fertil*, 82:87-95.

Koh S, Thomas R, Tsai S, Bischoff S, Lim J-H, Breen M, Olby NJ, Piedrahita JA. 2013. Growth requirements and chromosomal instability of induced pluripotent stem cells generated from adult canine fibroblasts. *Stem Cells Dev*, 22:951-963.

Kopecný V, Fléchon JE, Camous S, Fulka J. 1989. Nucleologenesis and the onset of transcription in the eight-cell bovine embryo:fine-structural autoradiographic study. *Mol Reprod Dev*, 1:79-90.

Krasniak CS, Ahmad ST. 2016. The role of CHMP2BIntron5 in autophagy and frontotemporal dementia. *Brain Res*, 1649:151-157.

Kruip TAM, Cran DG, van Beneden TH, Dieleman SJ. 1983. Structural changes in bovine oocytes during final maturation *in vivo*. *Gamete Res*, 8:29-47.

Kruip TAM, den Daas JHG. 1997. *In vitro* produced and cloned embryos:Effects on pregnancy, parturition and offspring. *Theriogenology*, 47:43-52.

Kuijk EW, du Puy L, van Tol HT, Haagsman HP, Colenbrander B, Roelen BA. 2007. Validation of reference genes for quantitative RT-PCR studies in porcine oocytes and preimplantation embryos. *BMC Dev Biol*, 7:58.

Kuijk EW, Du Puy L, Van Tol HTA, Oei CHY, Haagsman HP, Colenbrander B, Roelen BAJ. 2008. Differences in early lineage segregation between mammals. *Dev Dyn*, 237:918-927.

Kuijk EW, Chuva de Sousa Lopes SM, Geijsen N, Macklon N, Roelen BAJ. 2011. The different shades of mammalian pluripotent stem cells. *Hum Reprod Update*, 17:254-271.

Laurincík J, Hyttel P, Baran V, Eckert J, Lucas-Hahn A, Pivko J, Niemann H, Brem G, Schellander K. 1998. A detailed analysis of pronucleus development in bovine zygotes *in vitro*:cell-cycle chronology and ultrastructure. *Mol Reprod Dev*, 50:192-199.

Laurincik J, Kopecny V, Hyttel P. 1994. Pronucleus development and DNA synthesis in bovine zygotes *in vivo*. *Theriogenology*, 42:1285-1293.

Laurincik J, Kopecny V, Hyttel P. 1996. Detailed analysis of pronucleus development in bovine zygotes *in vivo*:Ultrastructure and cell cycle chronology. *Mol Reprod Dev*, 43:62-69.

Laurincik J, Thomsen PD, Hay-Schmidt A, Avery B, Greve T, Ochs RL, Hyttel P. 2000. Nucleolar Proteins and Nuclear Ultrastructure in Preimplantation Bovine Embryos Produced *In vitro*1. *Biol Reprod*, 62:1024-1032.

Laurincik J, Schmoll F, Mahabir E, Schneider H, Stojkovic M, Zakhartchenko V, Prelle K, Hendrixen PJM, Voss PLAM, Moeszlacher GG, Avery B, Dieleman SJ, Besenfelder U, Müller M, Ochs RL, Wolf E, Schellander K, Maddox-Hyttel P. 2003. Nucleolar proteins and ultrastructure in bovine *in vivo* developed, *in vitro* produced, and parthenogenetic cleavage-stage embryos. *Mol Reprod Dev*, 65:73-85.

Lee AS, Xu D, Plews JR, Nguyen PK, Nag D, Lyons JK, Han L, Hu S, Lan F, Liu J, Huang M, Narsinh KH, Long CT, de Almeida PE, Levi B, Kooreman N, Bangs C, Pacharinsak C, Ikeno F, Yeung AC, Gambhir SS, Robbins RC, Longaker MT, Wu JC. 2011. Preclinical derivation and imaging of autologously transplanted canine induced pluripotent

stem cells. *J Biol Chem*, 286:32697-704.
Lee S, Huang EJ. 2017. Modeling ALS and FTD with iPSC-derived neurons. *Brain Res*, 1656:88-97.
Li D, Secher JO, Juhl M, Mashayekhi K, Nielsen TT, Holst B, Hyttel P, Freude KK, Hall VJ. 2017. Identification of SSEA-1 expressing enhanced reprogramming (SEER) cells in porcine embryonic fibroblasts. *Cell Cycle*, 16:1070-1084.
Li D, Secher J, Hyttel P, Ivask M, Kolko M, Hall VJ, Freude KK. 2018. Generation of transgene-free porcine intermediate type induced pluripotent stem cells. *Cell Cycle*, 17:2547-2563.
Lonergan P. 2007. State-of-the-art embryo technologies in cattle. *Soc Reprod Fertil Suppl*, 64:315-325.
De Los Angeles A, Loh Y-H, Tesar PJ, Daley GQ. 2012. Accessing naïve human pluripotency. *Curr Opin Genet Dev*, 22:272-282.
Luo J, Suhr ST, Chang EA, Wang K, Ross PJ, Nelson LL, Venta PJ, Knott JG, Cibelli JB. 2011. Generation of leukemia inhibitory factor and basic fibroblast growth factor-dependent induced pluripotent stem cells from canine adult somatic cells. *Stem Cells Dev*, 20:1669-1678.
Malik N, Rao MS. 2013. A review of the methods for human iPSC derivation. *Methods Mol Biol*, 997:23-33.
Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A*, 78:7634-7638.
McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. 2000. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature*, 405:1066-1069.
Mitalipova M, Beyhan Z, First NL. 2001. Pluripotency of bovine embryonic cell line derived from precompacting embryos. *Cloning*, 3:59-67.
Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, 113:631-642.
Mohr LR, Trounson AO. 1981. Structural Changes Associated with Freezing of Bovine Embryos. *Biol Reprod*, 25:1009-1025.
Nagashima H, Giannakis C, Ashman RJ, Nottle MB. 2004. Sex Differentiation and Germ Cell Production in Chimeric Pigs Produced by Inner Cell Mass Injection into Blastocysts. *Biol Reprod*, 70:702-707.
Ng RK, Dean W, Dawson C, Lucifero D, Madeja Z, Reik W, Hemberger M. 2008. Epigenetic restriction of embryonic cell lineage fate by methylation of Elf5. *Nat Cell Biol*, 10:1280-1290.
Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell*, 95:379-391.
Nishimura T, Hatoya S, Kanegi R, Wijesekera DPH, Sanno K, Tanaka E, Sugiura K, Hiromitsu Tamada NK, Imai H, Inaba T. 2017. Feeder-independent canine induced pluripotent stem cells maintained under serum-free conditions. *Mol Reprod Dev*, 84:329-339.
Nishioka N, Yamamoto S, Kiyonari H, Sato H, Sawada A, Ota M, Nakao K, Sasaki H. 2008. Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech Dev*, 125:270-283.
Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, Okamoto S, Hong H, Nakagawa M, Tanabe K, Tezuka K, Shibata T, Kunisada T, Takahashi M, Takahashi J, Saji H, Yamanaka S. 2011. A more efficient method to generate integration-free human iPS cells. *Nat Methods*, 8:409-412.
Onishi A, Takeda K, Komatsu M, Akita T, Kojima T. 1994. Production of Chimeric Pigs and the Analysis of Chimerism using Mitochondrial Deoxyribonucleic Acid as a Cell Marker. *Biol Reprod*, 51:1069-1075.
Perleberg C, Kind A, Schnieke A. 2018. Genetically engineered pigs as models for human disease. *Dis Model Mech*, 11:dmm030783.
Pessôa LVF, Bressan, Freude KK. 2019. Induced pluripotent stem cells throughout the animal kingdom: Availability and applications. *World J Stem Cells*. In press.
Poon A, Zhang Y, Chandrasekaran A, Phanthong P, Schmid B, Nielsen TT, Freude KK. 2017. Modeling neurodegenerative diseases with patient-derived induced pluripotent cells:Possibilities and challenges. *N Biotechnol*, 39:190-198.
Ralston A, Rossant J. 2005. Genetic regulation of stem cell origins in the mouse embryo. *Clin Genet*, 68:106-12.
Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*, 8:2281-2308.
Rasmussen MA, Holst B, Tümer Z, Johnsen MG, Zhou S, Stummann TC, Hyttel P, Clausen C. 2014. Transient p53 suppression increases reprogramming of human fibroblasts without affecting apoptosis and DNA damage. *Stem Cell Reports*, 3:404-413.
Rath D, Amlinger L, Rath A, Lundgren M. 2015. The CRISPR-Cas immune system:biology, mechanisms and applications. *Biochimie*, 117:119-128.
Rossor MN, Fox NC, Mummery CJ, Schott JM, Warren JD. 2010. The diagnosis of young-onset dementia. *Lancet Neurol*, 9:793-806.
Salvin HE, McGreevy PD, Sachdev PS, Valenzuela MJ. 2010. Under diagnosis of canine cognitive dysfunction:A cross-sectional survey of older companion dogs. *Vet J*, 184:277-281.
Schnieke AE1, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman ACK. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science*, 278:2130-2133.
Schöler HR, Ruppert S, Suzuki N, Chowdhury K, Gruss P. 1990. New type of POU domain in germ line-specific protein Oct-4. *Nature*, 344:435-439.
Seelaar H, Rohrer JD, Pijnenburg YAL, Fox NC, van Swieten JC. 2011. Clinical, genetic and pathological heterogeneity of frontotemporal dementia:a review. *J Neurol Neurosurg Psychiatry*, 82:476-486.
Shimada H, Nakada A, Hashimoto Y, Shigeno K, Shionoya Y, Nakamura T. 2010. Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. *Mol Reprod Dev*, 77:2.

Sica RE, Caccuri R, Quarracino C, Capani F. 2016. Are astrocytes executive cells within the central nervous system? *Arq Neuropsiquiatr*, 74:671-678.

Skibinski G, Parkinson NJ, Brown JM, Chakrabarti L, Lloyd SL, Hummerich H, Nielsen JE, Hodges JR, Spillantini MG, Thusgaard T, Brandner S, Brun A, Rossor MN, Gade A, Johannsen P, Sørensen SA, Gydesen S, Fisher EM, Collinge J. 2005. Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nat Genet*, 37:806-808.

Smith GD, Takayama S, Swain JE. 2012. Rethinking *in vitro* embryo culture:new developments in culture platforms and potential to improve assisted reproductive technologies. *Biol Reprod*, 86:62.

Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. 2009. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells*, 27:543-549.

Starkey MP, Scase TJ, Mellersh CS, Murphy S. 2005. Dogs really are man's best friend--canine genomics has applications in veterinary and human medicine! *Brief Funct Genomic Proteomic*, 4:112-128.

Studzinski CM, Araujo JA, Milgram NW. 2005. The canine model of human cognitive aging and dementia:pharmacological validity of the model for assessment of human cognitive-enhancing drugs. *Prog Neuropsychopharmacol Biol Psychiatry*, 29:489-498.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126:663-676.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131:861-872.

Tang D-Q, Wang Q, Burkhardt BR, Litherland SA, Atkinson MA, Yang L-J. 2012. *In vitro* generation of functional insulin-producing cells from human bone marrow-derived stem cells, but long-term culture running risk of malignant transformation. *Am J Stem Cells*, 1:114-127.

Telugu BPVL, Ezashi T, Roberts RM. 2010. The promise of stem cell research in pigs and other ungulate species. *Stem Cell Rev*, 6:31-41.

Tesar PJ, Chenoweth JG, Brook FA, Davies TJ, Evans EP, Mack DL, Gardner RL, McKay RDG. 2007. New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*, 448:196-199.

Thomas KR, Capecchi MR. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*, 51:503-512.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. *Science*, 282:1145-1147.

Tsukamoto M, Nishimura T, Yodoe K, Kanegi R, Tsujimoto Y, Alam ME, Kuramochi M, Kuwamura M, Ohtaka M, Nishimura K, Nakanishi M, Inaba T, Sugiura K, Hatoya S. 2018. Generation of Footprint-Free Canine Induced Pluripotent Stem Cells Using Auto-Erasable Sendai Virus Vector. *Stem Cells Dev*, 27:1577-1586.

Urwin H, Authier A, Nielsen JE, Metcalf D, Powell C, Froud K, Malcolm DS, Holm I, Johannsen P, Brown J, Fisher EMC, van der Zee J, Bruyland M, FReJA Consortium C, Van Broeckhoven C, Collinge J, Brandner S, Futter C, Isaacs AM. 2010. Disruption of endocytic trafficking in frontotemporal dementia with CHMP2B mutations. *Hum Mol Genet*, 19:2228-2238.

Vajta G, Korösi T, Du Y, Nakata K, Ieda S, Kuwayama M, Nagy ZP. 2008. The Well-of-the-Well system:an efficient approach to improve embryo development. *Reprod Biomed Online*, 17:73-81.

Vejlsted M, Offenberg H, Thorup F, Maddox-Hyttel P. 2006. Confinement and clearance of OCT4 in the porcine embryo at stereomicroscopically defined stages around gastrulation. *Mol Reprod Dev*, 73:709-718.

Viana J. 2018. Statistics of embryo production and transfer in domestic farm animals, International Embryo Transfer Technology Society, Newsletter Dec 2018.

Viuff D, Avery B, Greve T, King WA, Hyttel P. 1996. Transcriptional activity in *in vitro* produced bovine two- and four-cell embryos. *Mol Reprod Dev*, 43:171-179.

Weltner J, Balboa D, Katayama S, Bespalov M, Krjutškov K, Jouhilahti E-M, Trokovic R, Kere J, Otonkoski T. 2018. Human pluripotent reprogramming with CRISPR activators. *Nat Commun*, 9:2643.

West FD, Terlouw SL, Kwon DJ, Mumaw JL, Dhara SK, Hasneen K, Dobrinsky JR, Stice SL. 2010. Porcine induced pluripotent stem cells produce chimeric offspring. *Stem Cells Dev*, 19:1211-1220.

West FD, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, Gallegos-Cardenas A, Pratt SL, Stice SL. 2011. Brief report:chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. *Stem Cells*, 29:1640-1643.

Whitworth DJ, Ovchinnikov DA, Wolvetang EJ. 2012. Generation and characterization of LIF-dependent canine induced pluripotent stem cells from adult dermal fibroblasts. *Stem Cells Dev*, 21:2288-2297.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385:810-813.

Wolf Xenia Asbaek, Serup P, Hyttel P. 2011a. Three-dimensional immunohistochemical characterization of lineage commitment by localization of T and FOXA2 in porcine peri-implantation embryos. *Dev Dyn*, 240:890-897.

Wolf Xenia Asbæk, Serup P, Hyttel P. 2011b. Three-dimensional localisation of NANOG, OCT4, and E-CADHERIN in porcine pre- and peri-implantation embryos. *Dev Dyn*, 240:204-210.

Xu KP, Greve T, Callesen H, Hyttel P. 1987. Pregnancy resulting from cattle oocytes matured and fertilized *in vitro*. *J Reprod Fertil*, 81:501-514.

Xue B, Li Y, He Y, Wei R, Sun R, Yin Z, Bou G, Liu Z. 2016. Porcine Pluripotent Stem Cells Derived from IVF Embryos Contribute to Chimeric Development *In vivo* A.J. Cooney (ed.). *PLoS One*, 11:e0151737.

Yagi R, Kohn MJ, Karavanova I, Kaneko KJ, Vullhorst D, DePamphilis ML, Buonanno A. 2007. Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian

development. *Development*, 134:3827-3836.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. 2007. Induced pluripotent stem cell lines derived from human somatic cells. *Science*, 318:1917-1920.

Yumlu S, Stumm J, Bashir S, Dreyer A-K, Lisowski P, Danner E, Kühn R. 2017. Gene editing and clonal isolation of human induced pluripotent stem cells using CRISPR/Cas9. *Methods*, 121-122:29-44.

van der Zee J, Urwin H, Engelborghs S, Bruyland M, Vandenberghe R, Dermaut B, De Pooter T, Peeters K, Santens P, De Deyn PP, Fisher EM, Collinge J, Isaacs AM, Van Broeckhoven C. 2008. CHMP2B C-truncating mutations in frontotemporal lobar degeneration are associated with an aberrant endosomal phenotype *in vitro*. *Hum Mol Genet*, 17:313-322.

Zhang J, Tam W-L, Tong GQ, Wu Q, Chan H-Y, Soh B-S, Lou Y, Yang J, Ma Y, Chai L, Ng H-H, Lufkin T, Robson P, Lim B. 2006. Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. *Nat Cell Biol*, 8:1114-1123.

Zhang Y, Schmid B, Nikolaisen NK, Rasmussen MA, Aldana BI, Agger M, Calloe K, Stummann TC, Larsen HM, Nielsen TT, Huang J, Xu F, Liu X, Bolund L, Meyer M, Bak LK, Waagepetersen HS, Luo Y, Nielsen JE, FReJA Consortium B, Holst B, Clausen C, Hyttel P, Freude KK. 2017. Patient iPSC-Derived Neurons for Disease Modeling of Frontotemporal Dementia with Mutation in CHMP2B. *Stem Cell Reports*, 8:648-658.

DOI: 10.21451/1984-3143-AR2019-0055

Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Developments of reproductive management and biotechnology in the pig

Olli Peltoniemi[1,*], Stefan Björkman[1], Marianne Oropeza-Moe[2], Claudio Oliviero[1]

[1]Department Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Finland.
[2]Norwegian University of Life Sciences in Sandnes, Norway.

Abstract

This review aims to describe changes in production environment, management tools and technology to alleviate problems seen with the present hyperprolific sow model. Successful parturition in the pig includes the possibility to express adequate maternal behaviour, rapid expulsion of piglets, complete expulsion of placenta, elimination of uterine contamination and debris, neonatal activity and colostrum intake. We focus on management of large litters, including maternal behaviour, ease of parturition, colostrum production, piglet quality parameters and intermittent suckling. There are also some interesting developments in technology to assess colostrum and immune state of the piglet. These developments may be utilized to improve the success rate of reproductive management around farrowing, lactation and after weaning. We also discuss new insights in how to examine the health of the mammary gland, uterus and ovaries of hyperprolific sows. Finally, we assess the latest developments on breeding and technology of hyperprolific sows, including artificial insemination (AI), real-time ultrasound of the genital tract and embryo transfer (ET). We conclude that 1) for the sow to produce sufficient colostrum, both the behavioural and physiological needs of the sow need to be met before and after parturition. Furthermore, 2) new ultrasound and biopsy technology can be effectively applied for accurate diagnosis of inflammatory processes of the udder and uterus and timing of AI regarding ovulation to improve insemination efficiency. Finally, 3) developments in cryopreservation of germ cells and embryos appear promising but lack of valid oocyte collection techniques and nonsurgical ET techniques are a bottleneck to commercial ET. These latest developments in management of parturition and reproductive technology are necessary to cope with the increasing challenges associated with very large litter sizes.

Keywords: large litter, sow, piglet, management, biotechnology.

Introduction

The pig appears to be superior in its reproductive ability at least when compared to other domestic animal species. This ability is based on the extremely high rate of fertility. Over the past three decades, efficient breeding and management has almost doubled the litter size of the domestic European sow breeds (Oliviero, 2019). During the same period, the duration of farrowing (second stage, from the first to the last fetus expulsed) has extended remarkably and is now four to five times longer than in the early 1990s (Oliviero *et al*., 2019). This may have resulted in an increase in farrowing complications such as postpartum dysgalactia syndrome (PDS, Kaiser *et al*., 2018a, b) and retention of placenta and a decrease in subsequent fertility (Björkman *et al*., 2017c; 2018c). Along with this development, we have seen a constant downward trend in the birth weight of the piglets and a similar trend in colostrum intake, which are connected and are the most important risk factors for piglet mortality (Oliviero *et al*., 2019). In the other hand, we have seen a tremendous increase in efficiency of production, which has considerably improved farming economy and related industry in a highly positive way. However, this may have come, at least to some extent, at the expense of animal health and welfare.

A large litter may be challenging for the metabolism of the sow such that there may be difficulties in resumption of ovarian cyclicity after weaning, especially in young sows in certain European breeds (Oliviero *et al*., 2013; Peltoniemi *et al*., 2016; Björkman *et al*., 2018c; Oliviero *et al*., 2019). Therefore, there appear to be major challenges associated with increasing litter sizes that are evident at farrowing, lactation and after weaning, which are periods when the foundations of the subsequent pregnancy are laid (Algers and Uvnäs-Moberg, 2007; Martineau *et al*., 2012). This paper discusses some of the key applications of reproductive biotechnology for the modern hyperprolific sow and her numerous offspring (beyond 17 piglets in a “large litter”). The first focus is on management and technology-related innovations used to address the challenges that sows and piglets face in terms of the ambient parturition environment and development of immunity around parturition and lactation. These innovations include optimizing colostrum intake and evaluation of colostrum yield and quality produced by the sow. Among the newer management interventions, intermittent suckling is aimed at not only hastening the production cycle, but also more importantly to improve the resilience of piglets after weaning. Secondly, we review some novel approaches to examine ovarian, uterine and mammary gland function *in vivo*. These involve both sampling and diagnostic imaging

Corresponding author: olli.peltoniemi@helsinki.fi
orcid.org/0000-0002-9481-1837
Received: May 8, 2019
Accepted: June 20, 2019

techniques that have been recently either discovered or considerably developed. Finally, we provide an updateon the use of artificial insemination (AI), which has been successful regarding use of fresh semen since inception of this technique, and future prospects of embryo transfer (ET) in the pig.

Management of large litters

Developments in parturition management of hyperprolific sows

Prolonged farrowing increases the risks of piglet asphyxia during parturition and less vital piglets at birth (Herpin *et al*., 2001). Yun et al. (2014) demonstrated that providing space and abundant nest building material before farrowing tended to increase sow plasma oxytocin concentrations (25 *vs*. 18 pg / ml in sows with abundant nesting material *vs*. sows with crates, respectively). Abundant nesting material also increased piglet serum IgG and IgM concentrations during early lactation (15 *vs*. 10 mg / ml (IgG) and 0,9 *vs*. 0,7 mg/ml (IgM) of sows with abundant nesting material vs. with sows in crates, respectively; Yun *et al*., 2014). Allowing for the intrinsic nesting behaviour to occur can reduce farrowing duration and therefore allow for more vital piglets (Jensen, 1986; Islas-Fabila *et al*., 2018) and for greater colostrum intake due to a shorter time interval gap from the start of farrowing to first suckling (Manjarin *et al*., 2018). Uncomplicated farrowing also reduces pain and inflammation in the sow (Björkman *et al*., 2017c; Kaiser *et al*., 2018a). Allowing the sow to farrow free and providing a substrate (straw, sawdust, paper) 1 to 2 days before farrowing can support the physiological nest building behaviour of the sow. This can significantly reduce farrowing duration and stillbirth rate (Oliviero *et al*., 2008; Gu *et al*., 2011).

With increasing occurrence of large litters, providing the sow with a good basis to produce enough colostrum is fundamental. Loss of back fat in late gestation and consequently sows arriving at farrowing with inadequate body condition affect colostrum yield (Decaluwé *et al*., 2013). Therefore, it appears essential that sows improve their body condition gradually during the whole pregnancy, arriving to farrowing in good body condition (backfat of 17 ± 3 mm) to fulfil protein turnover and sufficient colostrum yield (Oliviero *et al*., 2010; Decaluwé *et al*., 2013). During late pregnancy, not only adequate energy intake but also feeding composition seems to be of key importance in supporting the physiology of farrowing and colostrum quality. Many studies reported that specific essential fatty acids (conjugated linolenic, pinolenic and oleic acids) supplemented in gestating and lactating diets can improve sow colostrum immunoglobulins, piglet performance, average daily gain and weaning weight (Bontempo *et al*., 2004; Corino *et al*., 2009; Yao *et al*., 2012; Hasan *et al*., 2018). The feeding timing during pregnancy and especially in relation to parturition also seems to be of relevance regarding the success of farrowing. Feyera et al. (2018) observed that if the time lapse between the last feeding occasion prior to onset of farrowing lapsed beyond 3 hours, there was a positive linear correlation for time lapse and farrowing duration (Feyera *et al*., 2018). Glucose metabolism was considered to be of highest relevance behind this finding. However, other factors such as feeding fibre (involving bacterial metabolism of the GI tract) were also suggested to support more successful, quicker process of farrowing (Feyera *et al*., 2018). In conclusion, a proper ambient environment regarding food, metabolism, enrichment and space around farrowing are of key importance for successful processes of farrowing and colostrum yield, intake and quality.

Improving colostrum intake

Increased competition for colostrum intake is a critical factor for neonate piglets. These piglets are born without the protection of maternal immunoglobulins, as the epitheliochorial nature of the porcine placenta does not permit transfer of such large molecular weight structures from maternal to foetal blood circulation. Neonate piglets must acquire maternal immunoglobulins from ingested colostrum for passive immune protection before they produce sufficient endogenous immunoglobulins at approximately 3 to 4 weeks of age (Rooke and Bland 2002; Oliviero, 2013). The concentration of IgG piglet plasma shortly after birth is positively correlated with survival. Dead piglets have lower serum IgG concentrations than their surviving fellow piglets, which indicates low colostrum intake (Vallet *et al*., 2013). At farrowing, the IgG levels in colostrum are approximately 60 to 80 mg/ml. Within 10 to 12 h later IgG levels are reduced by half (35 to 40 mg/ml) and after 24 h a 70% reduction occurs (10 to 16 mg/ml), which is no longer an adequate level (Devillers *et al*., 2011; Quesnel *et al*., 2011; Hasan *et al*., 2016). Therefore, in large litters with prolonged farrowing of more than 6 hours, the immunity and viability of piglets are compromised. Furthermore, hyperprolific sows give birth to more piglets with low birth weight and with signs of intrauterine growth restriction (IUGR). There is an inverse relationship between number of piglets born in a litter and piglet birth weight; large litters are also associated with high variation in piglet birth weight within the litter (Quesnel *et al*., 2008; Akdag *et al*., 2009; Beaulieu *et al*., 2010; Smit *et al*., 2013; Matheson *et al*., 2018). A greater number of piglets born than the available teats at the sow's udder, lower birth weight and greater birth weight variation increase piglet competition for colostrum intake (Declerck *et al*., 2017). Similarly, lower birth weight and long farrowing duration are associated with lower piglet viability at birth, which can delay the access to the udder (Hoy *et al*., 1994; Islas-Fabila *et al*., 2018). Therefore, all underprivileged piglets should be provided with additional support to acquire a sufficient amount of good quality colostrum (e.g., should be assisted in suckling). To provide the best passive immunity, the procedure of split and assisted suckling should be effectively operated within the first 6 hours from the

beginning of parturition, when the colostrum immunoglobulin content is at the maximum (Devillers *et al*., 2011; Quesnel *et al*., 2011; Hasan *et al*., 2016). As small piglets or those with IUGR have difficulties to suckle from large nipples, the smallest functioning nipples should be used when assisting suckling. In conclusion, due to decreasing birth weight and colostrum intake per piglet, colostrum management around farrowing is of key importance for survival of piglets.

Technology to assess colostrum quality and immune state of neonatal piglets

Both colostrum yield and IgG content vary greatly among sows (Foisnet *et al*., 2010). Factors that affect the total colostrum yield are attributed to environment-related factors as well as to sow and piglet characteristics (Devillers *et al*., 2007; Farmer and Quesnel, 2009; Quesnel, 2011). IgG concentration in maternal colostrum significantly affects the acquisition of passive immunity (Kielland *et al.*, 2015) and therefore knowledge on IgG content of colostrum may be essential to determine the correct action to reduce piglet pre-weaning mortality. The major practical point in assessing colostrum IgG content at the farm level may be identifying the sows with low colostrum IgG levels. Those sows are a risk for a successful acquisition of passive immunity in the piglets. This is of great importance particularly when large litters are present and cross-fostering and split suckling are common management practices employed to maximize colostrum intake. Therefore, if the estimated colostrum IgG content appears to be insufficient, a farmer with this advance knowledge can pay additional attention to the relevant management practices. Hasan et al. (2016) have proposed the use of a Brix refractometer to estimate IgG content in sow colostrum. When used in non-sucrose-containing liquids, the Brix percentage approximates the total solids (TS) percentage (Quigley *et al*., 2013; Hasan *et al*., 2016). At the start of farrowing, immunoglobulins represent a significant portion of the TS (Klobasa *et al*., 1987) and IgG represents 80% of the immunoglobulins in sow colostrum (Porter, 1969; Curtis, 1970). Colostrum samples evaluated with a Brix refractometer are positively correlated with the IgG level measured with ELISA (Hasan *et al*., 2016). Therefore, the Brix refractometer can be an inexpensive, rapid and satisfactorily accurate method for estimating IgG concentration. Differentiation between good and poor IgG content of colostrum is possible by interpreting the results with the categories proposed in Table 1. Hasan et al. (2016) proposed this classification following the nature of the IgG physiological curve during the first 24 h post-partum, when IgG levels peak in the first 3 h and decrease rapidly until values of 10 mg/ml are reached 24 h post-partum (Quesnel *et al*., 2015; Hurley, 2015). Brix values of <20% were correlated with very low IgG levels (14.5 mg/ml), which are not expected during early colostrogenesis. In conclusion, the Brix refractometer is an acceptable method to assess colostrum IgG content at the herd level during the initial hours of parturition, when IgG levels are expected to peak.

Table 1. Brix value categories to estimate sow colostrum IgG content according to Hasan et al. (2016). This interpretation scale is valid if the sample is obtained within 0-3 hours from the start of farrowing using a Brix refractometer with a scale range 0-53% (adapted from Hasan *et al*., 2016).

Brix %	IgG estimation categories
< 20	Poor
20-24	Borderline[a]
25-29	Adequate
≥ 30	Very good

[a]The category "Borderline" should not always be considered to estimate a not adequate IgG content, especially if the found Brix values are on the highest range of this category (23-24%), on the contrary levels falling at the lowest range of this category (20-21%) can be considered not optimal. Taking another sample, after 1-2 h, can allow better interpretation of the results, to see if the development of the estimated IgG content is stable, increasing or decreasing from the initial value (Hasan *et al*., 2016). In conclusion, IgG can be considered as a reliable indicator of colostrum quality. Use of Brix refractometers provide an effective tool to assess colostrum quality, which is essential in the management of a large litter.

Intermittent suckling

Management strategies to support large litters are numerous. They include at least use of nurse sows (Schmitt *et al*., 2019a, b), split suckling (Donovan and Dritz, 2000), use of substitute milk and automated milk replacers (Difilippo *et al*., 2015) and general neonatal management (Kirkden *et al*., 2013). Among the strategies, intermittent suckling (Kemp and Soede, 2012) is especially interesting, since it may provide a useful tool to postpone weaning of piglets, which becomes relevant for the industry based on the decreasing trend in colostrum intake and birth weights of piglets (Oliviero *et al*., 2019). Therefore, applying an intermittent suckling (IS) protocol, which encourages sows to become pregnant in the middle of lactation, seems like an appealing alternative.

However, IS also involves resumption of reproductive function in the middle of lactation, which may become a further metabolic challenge for the sow.

Alternative reproductive management strategies as IS have a considerable impact on grouping dynamics and reproductive functions in the pig (Peltoniemi *et al*., 2016). Sows are in anoestrus during lactation and maturation of follicles is bound to the process of weaning. It is only after weaning that follicles are provided with circumstances for growth and ovulation. This process heralding ovulation stems mainly from the continuous lack of suckling stimulus on the udder, high intake of feed rich in energy and daily application of boar stimulus.

Ovulation in the middle of lactation can be induced by essentially the same means as used after weaning, specifically temporary, transient interruption of suckling stimulus, high feed intake and proper application of boar stimulus. Recent studies (see Kemp and Soede 2012 for a review) have demonstrated that intermittent suckling can induce lactation oestrus especially when IS starts around the normal weaning and is combined with adequate boar stimulation. Oestrus may be induced in up to 90% of the older sows (Gerritsen *et al*., 2008; Soede *et al*., 2012) and over 70% in first parity sows (Chen *et al*., 2017) within 6 days during lactation; farrowing rates and litter size are comparable to controls. Thus, success is dependent on parity as primiparous sows do not appear to respond as well as older sows and there seems to be differences in the response to the IS protocol and in the breed used. The success rate of IS also seems to depend on the management issues around IS (van Nieuwamerongen *et al*., 2014). These include a proper audio-visual isolation of sow and the piglets during IS. Furthermore, group management during boar stimulation around separation time is essential for IS success (Tabe 2; Hasan *et al*., 2019; van Nieuwamerongen *et al*., 2014). In conclusion, lactation oestrus has the potential advantage that the lactation period can be extended while sows are pregnant and this allows piglets to be more developed when eventually weaned. Piglets seem to respond well in terms of growth performance and resilience to the opportunity for extended, although interrupted, suckling possibilities (van Nieuwamerongen *et al*., 2014).

Table 2. Descriptive result of individual herd data for a successful intermittent suckling program. Data presented in mean ± SD. Data adapted from Hasan *et al*., 2019.

Type of production	Herd number					
	1	2	3	4	5	6
	Traditional	Traditional	Traditional	Traditional	Traditional	Intermittent suckling
Gestation length, days	115	115.6	116.2 ± 0.1	115	114.4 ± 0.1	115.2 ± 0.2
Farrowing duration, min	211.9 ± 10.7	200.6 ± 12.9	329.2 ± 24.2	261.7 ± 22.1	306.7 ± 27.4	287.8 ± 23.9
Litter size	16.1 ± 0.5	16.7 ± 0.6	14.6 ± 0.6	17.1 ± 0.6	16.5 ± 0.5	16.1 ± 0.5
Live-born piglets	15.3 ± 0.5	15.5 ± 0.5	13.1 ± 0.5	16.5 ± 0.6	14.9 ± 0.4	15.4 ± 0.5
Stillborn piglets	0.8 ± 0.1	1.1 ± 0.2	1.4 ± 0.2	0.6 ± 0.2	2.7 ± 0.5	0.6 ± 0.2
Birth interval, min	14.4 ± 0.9	13.7 ± 0.8	26.4 ± 2.7	16.6 ± 1.8	18.6 ± 1.2	-
Birth time, min	112.1 ± 3.1	100.3 ± 2.9	180.8 ± 7.9	142.2 ± 6.7	147.5 ± 4.1	-
Litter characteristics						
Piglet BW_B (live born), g	1445.7 ± 14.1	1275.0 ± 12.4	1413.6 ± 14.5	1220.48 ± 16.5	1279.2 ± 10.4	1446.1 ± 21.7
Piglet weight (weaning: ear tagged), g	6918.8 ± 105.8	6757.4 ± 106.3	7718.4 ± 161.2	5392.0 ± 149.2	7939.5 ± 55.28	6061.0 ± 135.5
ADG* (ear tagged), g	257.8 ± 4.3	246.1 ± 4.6	212.9 ± 5.0	224.0 ± 7.5	228.2 ± 1.7	246.3 ± 7.1
Piglet age (weaning) days	21.0 ± 0.03	21.6 ± 0.02	29.6 ± 0.09	18.1 ± 0.09	28.9 ± 0.03	19.4 ± 0.2
CY**, g	4658.5 ± 221.5	4009.4 ± 145.9	4132.2 ± 223.1	4336. 1 ± 268.4	4710.6 ± 129.4	3846.5 ± 367.3
CI***, g	332.0 ± 6.6	274. 3 ± 5.8	343.5 ± 7.2	270.9 ± 8.1	331.1 ± 4.5	262.5 ± 10.0

ADG* average daily gain, CY** colostrum yield, CI*** colostrum intake.

Management of hyperprolific sows after parturition

Mammary gland function

The most important disease of the mammary gland of the postpartum sow is generally considered to be mastitis as part of PDS (Farmer *et al*., 2019), although the role of mastitis as part of the complex in modern sow lines has recently been questioned (Kaiser *et al*., 2018a,b). This disease (PDS) is suggested to be associated with large litters as a connection between farrowing duration and PDS has been established (Tummaruk *et al*., 2013; Björkman *et al*., 2018c). Diagnosis of mastitis is based mainly on clinical signs, as has been reviewed by Gerjets and Kemper (2009).

Recently, other methods such as ultrasound examination and biopsy isolation have been tested for feasibility as diagnostic tools for udder diseases (Baer and Bilkei, 2005; Spiegel *et al*., 2017; Björkman *et al*., 2017a, 2018a, 2018b). In the study by Baer and Bilkei

(2005), the sows that had PDS had more hyperechoic images in the ultrasonographic examination of their mammary glands than sows without PDS. Björkman et al. (2017a) made the same observation in sows suffering from severe udder oedema prior to parturition, which is considered a risk factor for subsequent mastitis. In this case report, ultrasound of the mammary glands showed thickened dermal and subdermal tissues, hyperechoic lobuloalveolar tissue with enlarged blood vessels and severe shadowing (Fig. 1). Sows with severe udder oedema also had lower colostrum quality (Björkman *et al*., 2018a). Therefore, PDS must be prevented to ensure the immunity of newborn piglets.

The objective of the study by Spiegel et al. (2017) was to verify by comparative bacteriological examinations of milk samples and mammary gland biopsies whether a better assessment of bacteriological status is possible using biopsies. Diagnostic investigations based on bacteriological examination are complicated, as a similar bacterial content can be detected in milk samples from both healthy and diseased sows. Contamination during sample collection may be a possible reason. Spiegel et al. (2017) obtained biopsies after local anaesthesia using a 7-cm biopsy needle and revealed that biopsy samples of the mammary gland did not provide advantages for bacteriological diagnosis compared to milk sampling. Furthermore, Spiegel et al. (2017) observed complications such as abscess formation following biopsy. The same method was also tested by Björkman et al. (2018b) using an automatic needle with a 14-gauge diameter, 10-cm length and a 22-mm penetration depth. Biopsies were obtained from the lateral-caudal part of three different mammary glands. Before biopsy, glands were disinfected three times with a povidone-iodine solution but no local anaesthesia was used. Sows were monitored until weaning and no complications (such as abscess formation) were observed. There was also no effect of the biopsy before parturition on colostrum production (Han *et al*., 2018). Biopsies can thus be collected in a rapid and humane way. This method seems to be of minor value for diagnosis of mastitis but can be used to study mammary gland function for research purposes, especially for comparison of sows with low and high colostrum or milk yield. Ultrasound imaging of the mammary glands can provide an effective tool for diagnosis of inflammatory processes of the udder, such as PDS.

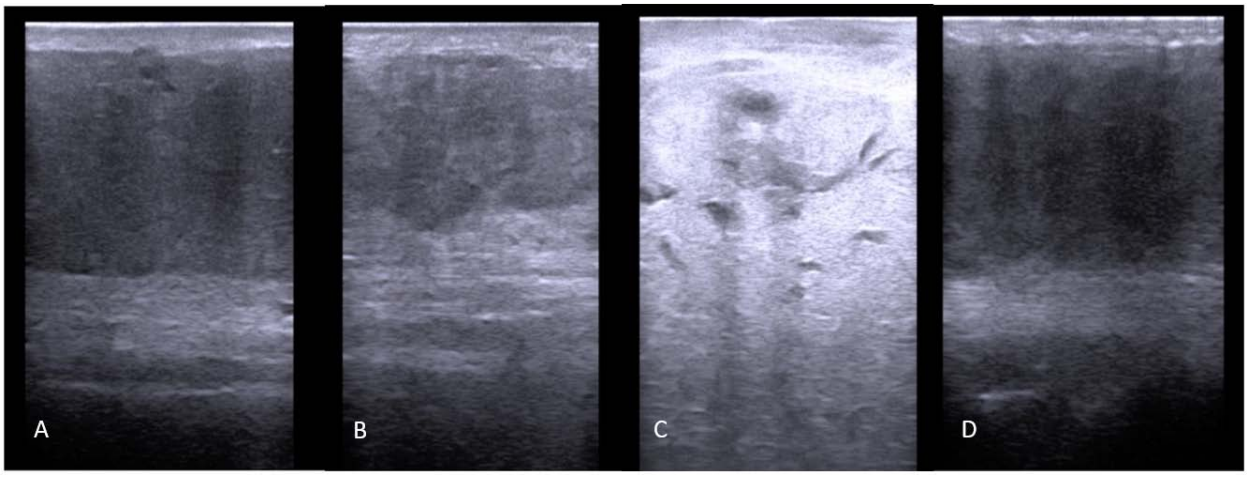


Figure 1. Ultrasound images of a mammary gland of a healthy sow (A) and from sows with from severe udder oedema (B-C). These images show thickened dermal and subdermal tissues (B, C), hyperechoic lobuloalveolar tissue (B, C) with enlarged blood vessels (C) and severe shadowing (D) (Björkman *et al*., 2017a, 2018a).

Uterine function

In recent years progress has been made in the use of ultrasonography to examine the non-gravid uterus. Timely and correct diagnosis of uterine disease, especially post-partum uterine disease, is essential to prevent subsequent subfertility (Kauffold and Wehrend, 2014). Oliviero et al. (2013) have shown that prolonged parturition can reduce subsequent fertility in the sow that may be associated with an increased incidence of post-partum uterine disease (Björkman *et al*., 2018c). In addition to prolonged parturition, obstetrical intervention, retained placenta and ≥2 stillborn piglets at birth have been shown to affect the incidence of post-partum endometritis (Björkman *et al*., 2018c). Ultrasonography is considered the best tool for diagnosis, not only for endometritis but also for cases in which placenta is retained (Björkman *et al*., 2017c). Examination of uterine structures currently utilizes the following three criteria: fluid echogenicity, echotexture and size (Kauffold and Althouse, 2007). Changes in echotexture reflect changes in endometrial oedema. Increased echotexture, unless attributed to circulating oestrogens originating from enlarged follicles, must be considered abnormal (Kauffold and Althouse, 2007). Furthermore, any fluid echogenicity, unless attributed to pregnancy, semen or oestrus, must be considered abnormal and indicative of an exudative inflammation of an acute or acute-chronic type (Kauffold and Althouse, 2007). Fluid echogenicity is often associated with uterine oedema and therefore increased echotexture and size of uterine cross-sections (Björkman *et al*., 2018c). In contrast, chronic

endometritis, representing the most common type of uterine inflammation in pigs, cannot be definitively diagnosed by ultrasonography based on any of the criteria described above (Kauffold and Althouse, 2007). Therefore, it is essential to recognize acute endometritis in time. This can be achieved based on the criteria mentioned above. However, fluid echogenicity, uterine oedema and increased uterine size during the first few days after parturition are not unusual or abnormal (Björkman *et al*., 2018c). Furthermore, when interpreting uterine size, the age and parity of the sow and the number of postpartum days must be considered. Björkman et al. (2018c) provide some reference values for the first postpartum week in Large White x Yorkshire sows.

Recently, the feasibility of transabdominal Doppler sonography (colour, power, pulse wave) to define uterine perfusion characteristics throughout the oestrous cycle in gilts (German Landrace x Pietrain) has been tested (Herlt, *et al*., 2018). These characteristics were perfused area, blood-flow velocity and intensity and resistance and pulsatility index. Colour Doppler sonography was the only feasible technique, as it was less affected by animal movements than power and pulse wave sonography. As determined by colour Doppler sonography, all five parameters determined showed specific patterns throughout the oestrous cycle. Perfused area and blood-flow velocity and intensity increased in proestrus, decreased in oestrus and remained low in midoestrus and most parts of dioestrus. The resistance and pulsatility index showed inversely paralleled patterns. Herlt et al. (2018) encourage the use of colour Doppler sonography for studying uterine capacity or uterus-related infertility, such as in cases of clinically unapparent endometritis. In conclusion, real-time ultrasound examination of the uterus is a fast, practical, efficient and accurate tool for diagnosis of acute inflammatory processes after parturition. Further developments in ultrasound technology, such as use of colour Doppler, may broaden the use of this technique beyond diagnosis of clinical disease of the uterus. In the future, it would be desirable to develop a uterine biopsy method for the sow for diagnosis of chronic uterine disease, like in the equine (Rua *et al*., 2018).

Ovarian function

Ovarian function postpartum can be monitored using ultrasonography. The use of B-mode ultrasound to determine follicular and corpus luteum size and the factors that affect the size of these structures have been reviewed (Soede *et al*., 2011; Langendijk, 2015; Soede and Kemp, 2015).

Recently, transabdominal colour Doppler sonography was used to assess ovarian blood flow characteristics during the course of the oestrus cycle in gilts (Stark, *et al*., 2019). These characteristics were perfused area, blood-flow velocity and intensity and resistance and pulsatility index. All parameters showed oestrous cycle-dependent patterns. Perfused area and blood-flow velocity were highest in diestrus, followed by proestrus, whereas the patterns of resistance and pulsatilty index were inversely proportional. Stark et al. (2019) concluded that ovarian blood flow was dependent on the stage of the oestrous cycle and was highest during the luteal phase and thus encouraged the use of colour Doppler ultrasonography to also investigate the reasons for ovary-based infertility, including corpus luteum insufficiency or seasonal effects on ovarian function.

Another technique that has recently been used is transvaginal ultrasound-guided biopsy of ovarian tissue. Björkman et al. (2017b) developed this method to obtain luteal tissue and to study corpus luteum function (Fig. 2). Biopsies were performed in four multiparous sows on days 9 and 15 of three consecutive oestrous cycles and the size and histological composition of the samples obtained were evaluated and the reproductive tract of the sows was monitored. Furthermore, biopsies were performed on 26 multiparous sows on days 10 and 13 after insemination and pregnancy rate, gestation length and subsequent litter size were evaluated. Altogether, tissue samples were obtained in 50% of the biopsy attempts. Sows from which one or more samples were obtained were older, heavier and had higher back fat compared to sows where no samples were obtained. No effects of the biopsies were observed on the cyclicity or reproductive organs of the sows or on subsequent corpus luteum diameter, pregnancy rate, gestation length and subsequent litter. The samples obtained had a diameter of 1 mm and contained heterogeneous tissue with various cell types. Björkman et al. (2017b) concluded that a transvaginal ultrasound-guided biopsy method for ovarian tissue can be used to study ovarian function. This method is relatively fast, minimally invasive and humane (Yun *et al*., 2017). Nevertheless, it should be noted that this method may not be used in young and small animals and tissue may be obtained in only half of the attempts. Furthermore, methods to select cells (e.g., laser microdissection) may be used to separate luteal from other ovarian cell types. In conclusion, advanced ultrasound techniques such as colour Doppler may be used to study ovarian dysfunction and seasonal infertility. A transvaginal ultrasound-guided biopsy of ovarian tissue has been developed for the pig and can be used for research purposes.

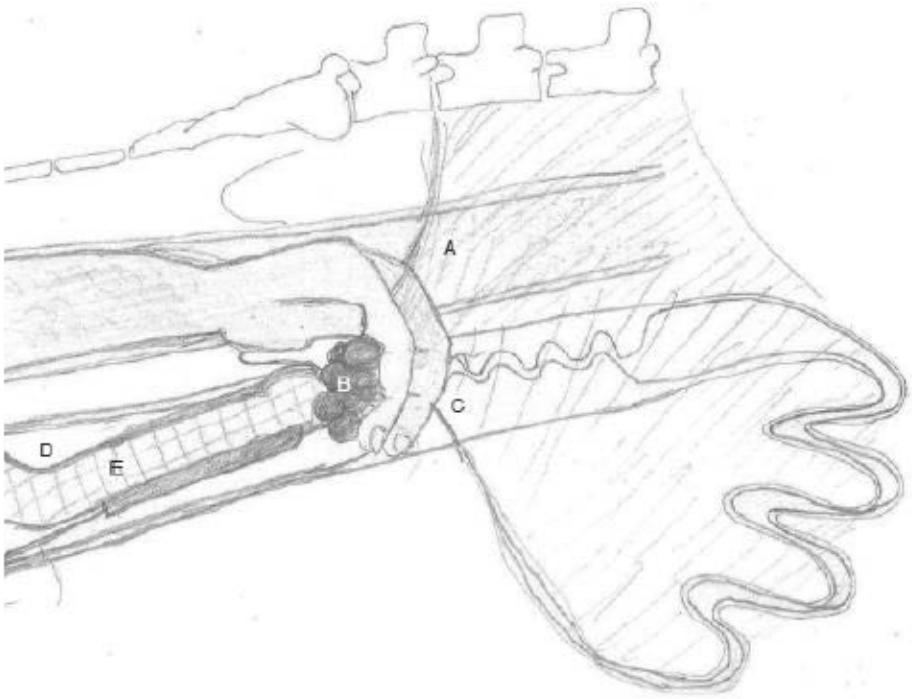


Figure 2. Illustration of the positioning of the transvaginal ultrasound-guided biopsy device. One hand is placed in the rectum (a) and the suspensory ligament of the ovary is palpated. After pulling on the ligament, the ovary is located and the proper ligament of the ovary held between the index and middle fingers, with the ovary on the palm side of the hand. The ovary (b) is pulled along the uterine cervix (c) into the peritoneal part of the pelvic cavity towards the cranial part of the vagina (d). With the other hand, the probe (e) is inserted into the vagina adjacent to the caudal part of the uterine cervix until the ovary becomes visible on the ultrasound screen (Björkman *et al*., 2017b).

Breeding hyperprolific sows

Artificial insemination

The pig is considered an intrauterine ejaculator (Senger, 2012). Therefore, deposition of semen in the uterus may be considered more of a physiological method than using the caudal portion of the cervix as the primary site of semen deposition. Generally speaking, intrauterine insemination (post-cervical, semen deposited into the uterine base) and deep intra-uterine (semen deposited into uterine horn) have been practiced to allow for a reduction of sperm number per dose, improved fertility or both (Watson and Behan, 2002; Martinez *et al*., 2002; Peltoniemi *et al*., 2009). A similar technique was developed to allow transcervical ET (Martinez *et al*., 2004). Results by Watson and Behan (2002) suggested that 2 or 3 billion spermatozoa/dose using intrauterine AI improve live-born litter size when compared with 1 billion spermatozoa/dose. However, it was subsequently shown that the number of spermatozoa may be reduced to 500 million spermatozoa/dose without detrimental effects on fertility (Martinez *et al*., 2006; Sumransap *et al*., 2007; Tummaruk *et al*., 2007; Roca *et al*., 2016; García-Vázquez *et al*., 2019). Post-cervical insemination seems to provide a number of advantages, such as a reduced sperm number requirement, less time required to perform insemination and faster genetic improvement (reviewed by García-Vázquez *et al*., 2019).

AI is used widely and globally by the industry. Despite these developments, some constraints such as cryopreservation of porcine semen prohibit efficient use of AI in international trade. Current research is focused on issues that affect AI such as freezing rates, cryoprotectants and storage (Yeste *et al*., 2016). Addition of antioxidants and the role of seminal plasma are being explored. As in other species like the horse, there seems to be large individual variation in semen freezability between boars (Yeste *et al*., 2016).

Timing of AI is another important factor in ensuring good fertility. Inseminating too early may not be successful, whilst if the sow is bred too late after ovulation, endometritis resulting in decreased litter size may be observed. Currently, two inseminations per oestrus is a commonly used practice to achieve a high pregnancy rate and large litter size. In a typical sow in oestrus, standing oestrus lasts for about 48 hours on average and ovulation occurs when two thirds of the standing oestrus has passed (Peltoniemi and Kemp, 2019). However, variation in the weaning to oestrus interval may affect the timing of AI. The later the sow enters oestrus after weaning, the sooner the optimal window for insemination (Roca *et al*., 2016). Ultrasound technology, in addition to a fixed-time AI after hormonal treatment protocol, may be used to pinpoint the optimal timing for AI in a specific herd, allowing for a good outcome after a single AI/oestrus (De Rensis and Kirkwood, 2016; Peltoniemi and Kemp, 2019). In conclusion, despite its wide use, application of AI in terms of dose deposition site within the uterus, cryopreservation of spermatozoa and timing towards a single AI are being further developed to advance the use of AI technology.

Use of ultrasound in the boar

Due to the increase in AI use in the pig breeding industry, there is interest in identifying males with suboptimal fertility to discard them or reduce their use (Pinho *et al*., 2018). This is especially important if a low number of spermatozoa per insemination dose is used and to meet the genetic potential of hyperprolific sows. In addition to proper mating management and insemination technique, high-quality semen from genetically superior sires is of high importance. Assessment of semen quality is one of the major evaluations for the selection of boars for breeding. For this reason, methods to assess the quality of semen before a boar starts reproductive life, or before using his semen for AI are required to predict their "fertility potential" (Pinho *et al*., 2018).

Therefore, studies in the past have focused on examining pre-pubertal and pubertal boars with ultrasound (Clark and Althouse, 2002; Clark *et al*., 2003; Ford and Wise, 2011; Kauffold *et al*., 2011; Pinho *et al*., 2018). The aims of these studies were to establish normal ultrasound parameters to identify subfertile boars and to establish correlations between these parameters and subsequent semen parameters. The first ultrasonographic evaluation of normal boar testes was performed more than 30 years ago. Cartee et al. (1986) compared the ultrasonographic appearance and testicle measurements with semen parameters in 14 Landrace boars but did not find any correlations. Nevertheless, they found significant differences in these parameters between 9-month-old and 15-month-old boars. Likewise, Clark et al. (2003) found an increased paired-testicular diameter in 18-month-old boars compared to 12-month-old boars. However, no correlation between paired-testicular diameter and the average total sperm number was established (Clark *et al*., 2003). Ford and Wise (2011) assessed pubertal development of boars derived from ultrasonographic determination of testicular diameter and length in 160 boars at 4, 5, 6, or 7 months of age. Boars were subsequently castrated and the weight of the testes, mean diameter of seminiferous tubules and percentage of the testis occupied by tubules were determined. At 4 and 5 months of age, although testicular diameter correlated positively with diameter of seminiferous tubules, this relationship was not significant at older ages.

Previously, Kauffold et al. (2011) conducted a study to describe the echogenicity pattern of the epididymis in boars using B-mode ultrasound together with grey-scale analysis. Ejaculate parameters were also determined for investigating the relationships between them and ultrasonographic findings. In the ultrasound images, all parts of the epididymis appeared homogeneous and regular in echotexture. However, while the echotexture of the caput and the corpus was normal, the cauda had a rather marbled echotexture (Kauffold *et al*., 2011). The echogenicity, expressed as the mean grey value, was different in comparison between the three segments of the epididymis (caput > corpus > cauda). The echotexture of the caput of the epididymis correlated slightly positively with the ejaculate volume and the total sperm count. Thus, ultrasound examination of the epididymis with analysis of caput echotexture provides information on semen parameters before semen collection.

Ultrasound examination of the accessory sex gland has also been successfully conducted and the appearance of each accessory sex gland has been described (Clark and Althouse, 2002) but no correlations with semen parameters have been made. It is unlikely that ultrasound examination of accessory sex glands can be implemented into practice. This method is quite challenging and dangerous as it requires rectalizing the boar. It is also not applicable in pubertal boars because of the anatomically small pelvic canal. This method may only be used in adult boars as a diagnostic tool in the work-up of subfertility. In conclusion, ultrasonographic determination of testicular diameter can be used to monitor testicular development during puberty but no correlations have been established with total sperm number in the ejaculate or with subsequent reproductive performance. It would be of particular interest to study whether ultrasound of the epididymis could also be used in prepubertal or pubertal boars to predict their future "fertility potential."

Embryo transfer in sows

Global need for foods and animals requires the development of strategies beyond traditional breeding to ensure offspring of high genetic quality and productivity while preserving genetic diversity. Demand for pork has been rising in recent decades due to changes in consumption patterns as incomes increase in developing countries with rapidly growing economies. Genetics from superior sows best meeting with breeding goals are sought internationally. The export of live animals is contentious due to animal welfare issues, biosecurity, economy and sustainability due to long transport times and crossing of borders. The challenges with AI regarding export of porcine genetics have been discussed earlier. Although sensitive to chilling and highly susceptible to intracellular ice formation, recent progress in oocyte and embryo cryopreservation is promising (Saragusty and Arav, 2011; Cuello *et al*., 2016; Nohalez *et al*., 2018). Porcine embryos have the potential to substantially accelerate genetic gain in pig populations and to facilitate international transport of genetics, while decreasing the carbon footprint due to reduced live animal transportation. New knowledge on ET in sows is therefore essential. ET in pigs was described for the first time in 1950 at the Pig Breeding Research Institute in Poltava, Ukraine (Kvasnitski, 1950). To our knowledge, no standardized and commercial ET service in sows exist. Today, porcine ET is carried out in private companies and institutes engaged in biomedical research (Petersen *et al*., 2008; Zheng *et al*., 2016). The main oocyte source for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is ovaries mainly from prepuberal sows collected from the local slaughterhouse. Antral follicles are punctured in the laboratory for oocyte collection. Embryo collection after slaughter has the disadvantage of using donor sows only once.

Additionally, the stage of the oestrus cycle at slaughter or the reason for slaughter is commonly unknown. Therefore, when oocytes are recovered in this manner, they are heterogenous in terms of developmental competence (Bertoldo *et al*., 2012).

To establish a viable, commercial ET concept, oocyte and embryo retrieval should be feasible for trained veterinarians under field conditions. This suggests non-surgical oocyte or embryo retrieval. Recent reports by Björkman et al. (2017b) and Yun et al. (2017) are encouraging as transvaginal ultrasound-guided biopsies of the ovaries may not cause appreciable pain or distress in non-sedated sows. However, no successful non-surgical embryo collection has been reported in pigs, except for the studies of Hazeleger et al. (1989) and Kobayashi et al. (1989) that used surgical resection of uterine horns (Brüssow *et al*., 2000). The major reason for this restriction is the anatomy of the porcine genital tract.

Non-surgical ovum pick-up (OPU) has not gained significant importance in live sows. This is probably also due to anatomical challenges and the fact that sow ovaries must be placed near the cervix for proper visualization before transvaginal follicle puncture and oocyte isolation can be conducted. Rectal palpation of pig ovaries in can be challenging due to the long uterus horns and the limited length of the rectal mesentery (Okuyama *et al*., 2017). A recent report from Japan investigated transvaginal OPU and examined the effects of different aspiration vacuum pressures and the phases of oestrous cycle on oocyte recovery, the morphology of cumulus oocyte

complexes (COCs) and blastocyst formation in Berkshire pigs. The proportion of oocytes with several compact cumulus layers in 90 mmHg (27.2%) was significantly higher ($P < 0.01$) than in 120 mmHg (5.2%). The OPU technique enables repeated oocyte collection from highly valuable live pigs (Ikoma *et al*., 2014).

IVM, IVF and IVC have been extensively investigated in pigs taking known obstacles such as polyspermy into account (Romar *et al*., 2012; Yuan and Krisher 2012; Gil *et al*., 2017). IVM influences both nuclear and cytoplasmatic maturation of porcine oocytes and therefore pronuclear formation and cleavage (Laurincik *et al*., 1994). By modifying maturation media via addition of thiols and organic osmolytes, low incidents of male pronuclear formation after IVF can been counteracted (Funahashi and Day 1993). Polyspermic penetration of porcine oocytes range between 13% and 90% (Niwa, 1993). By simulating the oviductal environment, polyspermy is reduced and the final IVF increases the final efficiency by more than 48%. This was seemingly due to reduced sperm motility and lower capacitating status (Soriano-Úbeda *et al*., 2017). For IVC, NCSU23 containing taurine and hypotaurine promote the highest success rates in development from the single cell to blastocyst stage (Brüssow *et al*., 2000).

Table 3. *In vitro* and *in vivo*-related embryo transfer (ET) technologies in sows.

	Procedure	Need for research and development	References
I.	Selection of the indicated sows with superior fertility traits	Follicular fluid composition, seasonal infertility and follicle size effects on oocyte developmental competence and embryonic survival.	Peltoniemi *et al*., 1999; Bertoldo *et al*., 2013; Da Silva *et al*., 2018.
I.	Oocyte/ embryo retrieval from donor sows	Flushing equipment for sows, skill acquisition *in vivo*	Hazeleger *et al*., 1989; Kobayashi *et al*., 1989; Brüssow and Rátky 1996; Besenfelder *et al*., 1997
	Ovum pick-up (OPU) in donor sows	OPU device for sows, OPU technique optimization, skill acquisition on live animals	Brüssow *et al*., 1997; Antosik *et al*., 2007; Ikoma *et al*., 2014
II.	Gametes: *In vitro*	*In vitro* maturation (IVM), fertilization (IVF) and culture	Funahashi and Day 1993; Laurincik *et al*.,, 1994; Brüssow *et al*., 2000; Romar *et al*., 2012; Gil *et al*., 2017; Soriano-Úbeda *et al*., 2017
	Oocytes/ embryos: *In vitro*	Cryopreservation/ vitrification of embryos/ oocytes	Berthelot *et al*., 2000; Cuello *et al*., 2016; Nohalez *et al*., 2018
III.	Recipient sow synchronization	Hormonal synchronization protocol	Wilson *et al*., 1998; Martynenko *et al*., 2004; Brüssow *et al*., 2018
	ET on recipient sows	ET into the cranial portion of the corpus uteri	Webel *et al*.,, 1970; Galvin *et al*.,, 1994; Hazeleger and Kemp 1994; Li *et al*., 1996; Yonemura *et al*., 1996; Rátky *et al*., 2001; Martinez *et al*., 2004; Martinez *et al*., 2016

The selection of recipient gilts or sows will have a major impact on ET results (Brüssow *et al*., 2018). Recipient sows must be hormonally synchronous to the donors. Both the breed of the recipients and the recipient uterine environment can influence the ET results. Meishan pigs have been suggested as recipients due to their higher placental efficiency (Wilson *et al*., 1998), and post-ovulatory AI followed by ET could increase ET efficiency (Martynenko *et al*., 2004).

Embryos have mainly been transferred surgically into recipients, either into the oviducts or the cranial tip of the uterus. The need for surgical ET has certainly hampered the progress of bringing this method closer to implementation under field conditions. Multiple research groups have attempted to develop a nonsurgical ET procedure (Galvin *et al*., 1994; Hazeleger and Kemp 1994; Li *et al*., 1996; Yonemura *et al*., 1996; Martinez *et al*., 2004; Martinez *et al*., 2016).

Considerable research effort is still necessary before ET can be offered as a commercial breeding tool (Tab. 3). In conclusion, the prospects for ET in pigs have improved with recent developments in cryopreservation of oocytes and embryos. However, despite some recent developments, repeated collection of oocytes from live animals and the need for a surgical ET remain as bottlenecks for wider commercial use of ET in genetic improvement and international trade.

Conclusions

Management of the large litters of the present hyperprolific breeds involve a sufficient appreciation of the physiological and behavioural needs of the sow prior to and around farrowing. Meeting these needs improve the capacity of the sow to produce adequate colostrum, the quantity and quality of which can be managed and monitored by modern tools such as the Brix test. Feeding sows with higher levels of fibre and decreasing the time lapse between last feeding prior to onset of parturition provide new insights for the management of parturition. Use of intermittent suckling will hasten the production cycle while extending the lactation length of small piglets. This would make the process of weaning easier for piglets, but metabolically more demanding for the sow. Recent developments in real-time ultrasonography, together with ultrasound-guided biopsy techniques provide new and novel means to study inflammatory processes of the mammary gland, dysfunction of the uterus and the ovary, timing of AI and seasonal infertility. Advancements in cryopreservation of semen, oocytes and embryos appear encouraging in terms of establishing a foundation for further development of breeding, ET and trade of germ cells and embryos across borders. While litter size in domestic European pig breeds has doubled over the past two decades, duration of farrowing has extended four to five-fold. The birth weight of piglets and colostrum intake per piglet continue to decrease. These challenges of modern breeding need to be addressed in the future. We also urge more research into this area to resolve these emerging challenges of the hyperprolific sows lines.

Author contributions

OP: Funding acquisition, conceptualization, writing - original draft, review & editing, commenting all phases; SB: Conceptualization, writing – original draft, review & editing, commenting all phases; MO-M: writing – original draft, review & editing, commenting all phases; CO: Conceptualization, writing – original draft, review & editing, commenting all phases.

Conflict of interest

There is no conflict of interest regarding any of the authors.

Acknowledgments and Funding disclosure statement

This work was funded by Ministry of agriculture, Finland, grants1788/312/2014, 1803/312/ 2014, 1487/03.01.02/2016, Agrimarket ltd., Atria ltd. and Vetcare ltd.

References

Akdag F, Arslan S, Demir H. 2009. The Effect of parity and litter size on birth weight and the effect of birth weight variations on weaning weight and pre-weaning survival in piglet. *J Anim Vet Adv*, 8:2133-2138.

Algers B, Uvnäs-Moberg K. 2007. Maternal behavior in pigs. *Horm Behav*, 52:78-85.

Antosik P, Jaśkowski JM, Jeziorkowski M, Lechnowicz J. 2007. The influence of vaccum pressure on quality and number of recovered oocytes aspirated from ovarian follicles of swine and cows. *Arch Tierz Dummerstorf*, 50:260-266.

Baer C, Bilkei G. 2005. Ultrasonographic and gross pathological findings in the mammary glands of weaned sows having suffered recidiving mastitis metritis agalactia. *Reprod Domest Anim*, 40(6):544-547.

Beaulieu AD, Aalhus JL, Williams NH, Patience JF. 2010. Impact of piglet birth weight, birth order, and litter size on subsequent growth performance, carcass quality, muscle composition, and eating quality of pork. *J Anim Sci*, 88:2767-2778.

Berthelot F, Martinat-Botté F, Locatelli A, Perreau C, Terqui M. 2000. Piglets Born after Vitrification of Embryos Using the Open Pulled Straw Method. *Cryobiology*, 41:116-124.

Bertoldo MJ, Holyoake P K, Evans G, Grupen C G. 2012. Seasonal variation in the ovarian function of sows. *Reprod Fertil Dev*, 24:822-834.

Bertoldo MJ, Nadal-Desbarats L, Ge´rard N, Dubois A, Holyoake PK, Grupen CG. 2013. Differences in the metabolomic signatures of porcine follicular fluid collected from environments associated with good and poor oocyte quality. *Reproduction*, 146:221-231.

Besenfelder U, Mödl J, Müller M, Brem G. 1997. Endoscopic embryo collection and embryo transfer into

the oviduct and the uterus of pigs. *Theriogenology*, 5:1051-1060.

Björkman S, Oliviero C , Hasan S, Peltoniemi O . 2017a. Mammary gland edema as a cause of postpartum dysgalactia in the sow-a case report. *Reprod Domest Anim*, 52:72.

Björkman S, Yun J, Niku M, Oliviero C, Soede NM, Peltoniemi O AT. 2017b. Serial transvaginal ultrasound-guided biopsy of the porcine corpus luteum *in vivo*. *Reprod Fertil Dev*, 29:931-939.

Björkman S , O liviero C , R ajala-Schultz P J, So ede NM, P eltoniemi O AT. 2017c. The effect of litter size, parity, farrowing duration on placenta expulsion and retention in sows. *Theriogenology*, 92:36-44.

Björkman S , Grahofer A , Han T , Oliviero C , Peltoniemi O. 2018a. Severe udder edema as a cause of reduced colostrum quality and milk production in sows - a case report. In European Symposium of Porcine Health Management,10th, 2018, Barcelona, Spain.

Björkman S, Han T , Oliviero C , Peltoniemi O . 2018b. Biopsy of mammary gland in sows: A tool for studying colostrum production. *Reprod Domest Anim*, 53:111-112.

Björkman S , Oliviero C , Kauffold J , Soede NM, Peltoniemi O AT. 2018c. Prolonged parturition and impaired placenta expulsion increase the risk of postpartum metritis and delay uterine involution in sows. *Theriogenology*, 106:87-92.

Bontempo V , Sciannimanico D , Pastorelli G , Rossi R, Rosi F, Corino C. 2004. Dietary conjugated linoleic acid positively affects immunologic variables in lactating sows and piglets. *J Nutr*, 134:817-824.

Brüssow KP, Rátky J. 1996. Endoscopic collection of porcine embryos. *Reprod Dom Anim*, 31:711-715.

Brüssow K P, T orner H , Rátky J , Hunter M G, Nürnberg G. 1997. Ovum pick up in swine: The influence of aspiration vacuum pressure on oocyte recovery from preovulatory follicles. *Acta Vet Hung*, 45:189-196.

Brüssow KP, Torner H, Kanitz W, Rátky J. 2000. *In vitro* technologies related to pig embryo transfer. *Reprod Nutrit Dev*, 40(5):469-480.

Brüssow K P, R átky J, A ntosik P , K empisty B , Jaśkowski JM. 2018. Embryo transfer in swine - an indispensable key for the application of reproductive techniques. *Electronic Journal of Polish Agricultural Universities*, 21(3):3. DOI:10.30825/5.EJPAU.78.2018.21.3

Cartee R E, P owe T A, G ray B W, H udson R S, Kuhlers D L. 1986. Ultrasonographic evaluation of normal boar testicles. *Am J Vet Res*, 47(12):2543-2548.

Clark S G, Althouse G C. 2002. B-mode ultrasonographic examination of the accessory sex glands of boars. *Theriogenology*, 57(8):2003-2013.

Clark SG, Schaeffer DJ, Althouse GC. 2003. B-mode ultrasonographic evaluation of paired testicular diameter of mature boars in relation to average total sperm numbers. *Theriogenology*, 60(6):1011-1023.

Chen TY, Turpin DL, K night A L, B ouwman E G, Soede N M, K irkwood R N, L angendijk P . 2017. Lactational oestrus and reproductive performance following a delayed limited nursing schedule in primiparous sows. *Theriogenology*, 96:42-48.

Corino C, Pastorelli G, Rosi F, Bontempo V, Rossi R. 2009. Effect of dietary conjugated linoleic acid supplementation in sows on performance and immunoglobulin concentration in piglets. *J Anim Sci*, 87:2299-2305.

Cuello C, Martinez C A, N ohalez A, P arrilla I, R oca J, G il M A, M artinez E A. 2016. Effective vitrification and warming of porcine embryos using a pH-stable, chemically defined medium. *Sci Rep*, 6:33915. Doi: 10.1038/srep33915.

Curtis SE . 1970. Environmental-thermoregulatory interactions and neonatal piglet survival. *J Anim Sci*, 31:576-587.

Da S ilva C LA, M ulder H A, B roekhuijse M LWJ, Kemp B , So ede N M, K nol EF. 2018. Relationship between the estimated breeding values for litter traits at birth and ovarian and embryonic traits and their additive genetic variance in gilts at 35 days of pregnancy. *Front Genet*, 9:111. Doi.org/10.3389/fgene.2018.00111.

Decaluwé R, Maes D, Declerck I, C ools A, Wuyts B, De S met S , J anssens G. 2013. Changes in back fat thickness during late gestation predict colostrum yield in sows. *Animal*, 7:1999-2007.

De Re nsis F , K irkwood RN . 2016. Control of estrus and ovulation: Fertility to timed insemination of gilts and sows. *Theriogenology*, 86:1460-1466

Declerck I, Sarrazin S, Dewulf J, Maes D. 2017. Sow and piglet factors determining variation of colostrum intake between and within litters. *Animal*, 11:1336-1343.

Devillers N, Farmer C, Le Dividich J and Prunier A. 2007. Variability of colostrum yield and colostrum intake in pigs. *Animal*, 1:1033-1041.

Devillers N, Le Dividich J, Prunier A. 2011. Influence of colostrum intake on piglet survival and immunity. *Animal*, 5:1605-1612.

Difilippo E, Bettonvil M , Wi llems RH, Braber S , Fink-Gremmels J, Jeurink PV, Schoterman MH, Gruppen H , Sc hols H A. 2015. Oligosaccharides in Urine, Blood, and Feces of Piglets Fed Milk Replacer Containing Galacto-oligosaccharides. *J Agric Food Chem*, 63(50):10862-10872. Doi: 10.1021/acs.jafc.5b04449.

Donovan TS, Dritz SS. 2000. Effect of split nursing on variation in pig growth from birth to weaning. *J Am Vet Med Assoc*,1:217(1):79-81.

Farmer C , Quesnel H . 2009. Nutritional, hormonal, and environmental effects on colostrum in sows. *J Anim Sci*, 87:56-64.

Farmer C, Maes D, Peltoniemi OAT. 2019. Mammary system. In: Zimmerman, Karriker, Ramirez, Schwartz, Stevenson and Zhang (Ed.): Diseases of swine, 11th ed. Wiley-Blackwell: Nova Jersey, EUA, pp.313-338.

Feyera T, Pedersen T, Krogh U, Foldager L, Theil P. 2018. Impact of sow energy status during farrowing on farrowing kinetics, frequency of stillborn piglets, and farrowing assistance. *J Anim Sci*, 96:2320-2331.

Foisnet A, Farmer C, David C and Quesnel H. 2010. Relationships between colostrum production by primiparous sows and sow physiology around parturition. *J Anim Sci*, 88:1672-1683.

Ford JJ, Wise TH. 2011. Assessment of pubertal development of boars derived from ultrasonographic determination of testicular diameter. *Theriogenology*, 75(2):241-247.

Gerjets I, Kemper N. 2009. Coliform mastitis in sows: a review. *J Swine Health and Prod*, 17(2):97-105.

Funahashi D, Day BN. 1993. Effect of follicular fluid at fertilization *in vitro* on sperm penetration in pig oocytes. *J Reprod Fertil*, 99:97-103.

Galvin J M, K illian D B, Stewart A NV. 1994. A procedure for successful nonsurgical embryo transfer in swine. *Theriogenology*, 41:1279-1289.

García-Vázquez F A, M ellagi AP G, Ul guim RR, Hernandez-Caravaca I , L lamas-Lopez P J, Bortolozzo F P. 2019. Post-cervical artificial insemination in porcine: The technique that came to stay. *Theriogenology*, 129:37-45.

Gerritsen G, Soede NM, Langendijk P, Hazeleger W, Kemp B. 2008. The Intermittent Suckling Regimen in Pigs: Consequences for Reproductive Performance of Sows. *Reprod Dom Anim*, 43(Suppl. 5):29-35.

Gil M A, No halez A, Martinez CA, Ak e-Villanueva JR, C enturion-Castro F , Maside C, Cu ello C, Ro ca J, P arrilla I , M artinez E A. 2017. Effects of meiotic inhibitors and gonadotropins on porcine oocytes *in vitro* maturation, fertilization and development. *Reprod Domest Anim*, 52:873-880.

Gu Z, Gao Y, Lin B, Zhong Z, Liu Z, Wang C, Li B. 2011. Impacts of freedom farrowing pen design on sow behaviours and performance. *Prev Vet Med*, 102:296-303.

Han T, B orkman S , O liviero C , P eltoniemi O. 2018. Mammary gland biopsy does not affect colostrum yield of sows: a pilot study. *Reprod Domest Anim*, 53:144.

Hasan S, J unnikkala S, V alros A, P eltoniemi O, Oliviero C. 2016. Validation of Brix refractometer to estimate colostrum immunoglobulin G content and composition in the sow. *Animal*, 10:1728-1733.

Hasan S, Saha S, Junnikkala S, Orro T, Peltoniemi O, O liviero C . 2018. Late gestation diet supplementation of resin acid-enriched composition increases sow colostrum immunoglobulin G content, piglet colostrum intake and improve sow gut microbiota. *Animal*, 13:1599-1606. Doi: 10.1017/S1751731118003518.

Hasan S, Orro T, Junnikkala S, Valros A, Peltoniemi O, O liviero C . 2019. Factors affecting sow colostrum yield and composition, and their impact on piglet growth and health. *Livest Sci*, In press.

Hazeleger W, v an d er M eulen J , v an d er Lende T . 1989. A method of transcervical embryo collection in the pig. *Theriogenology*, 32:727-734.

Hazeleger W, Kemp B. 1994. Farrowing rate and litter size after transcervical embryo transfer in sows. *Reprod Domest Anim*, 29:481-487.

Herlta C , S tarka R , S igmarsson H L , Kauffold J. 2018. Feasibility of transabdominal Doppler sonography for studying uterine blood flow characteristics in cycling gilts. *Tierärztliche Praxis* G: Großtiere/Nutztiere, 46(03):154-163.

Herpin P , H ulin J C, L e Dividich J , F illaut M. 2001. Effect of oxygen inhalation at birth on the reduction of early postnatal mortality in pigs. *J Anim Sci*, 79:5-10.

Hoy S, L utter C , Wä hner M , P uppe B . 1994. The effect of birth weight on the early postnatal vitality of piglets. *Dtsch Tierarztl Wochenschr*, 101:393-396.

Hurley W . 2015. Composition of sow colostrum and milk. Chapter 9. In: Farmer, C. (Ed.) The gestating and lactating sow. Wageningen Academic Publishers, Wageningen, The Netherlands, pp.193-229.

Ikoma E, Suzuki C, Ishihara Y, Komura K, Ookoda T, M aruno H . 2014. Transvaginal ultrasound-guided ovum pick up (OPU) in Berkshire breed during natural estrous cycle. *JPN J Swine Sci*, 51(2): 45-53.

Islas-Fabila P , M ota-Rojas D , M artínez-Burnes J , Mora-Medina P , G onzález-Lozano M , Roldan-Santiago P . 2018. Physiological and metabolic responses in newborn piglets associated with the birth order. *Anim Reprod Sci*, 197:247-256.

Jensen P. 1986. Observations of the maternal behaviour of free-ranging domestic pigs. *Appl Anim Behav Sci* 16:131-142.

Kaiser M, Jacobsen S, H aubro-Andersen P, Bækbo P, Cerón J, Dahl J, Escribano D, Jacobson M. 2018a. Inflammatory markers before and after farrowing in healthy sows and in sows affected with postpartum dysgalactia syndrome. *BMC Vet Res*, 14:83. Doi.org/10.1186/s12917-018-1382-7.

Kaiser M, Jacobsen S, H aubro-Andersen P, B ækbo P, C erón J, D ahl J, E scribano D , Th eil P, Jac obson M. 2018b. Hormonal and metabolic indicators before and after farrowing in sows affected with postpartum dysgalactia syndrome. *BMC Vet Res*, 14:334. Doi.org/10.1186/s12917-018-1649-z.

Kauffold J, Althouse GC. 2007. An update on the use of B-mode ultrasonography in female pig reproduction. *Theriogenology*, 67(5):901-911.

Kauffold J , K essler M, Richter A , B eynon N, Wehrend A. 2011. B-mode Ultrasound and Grey-Scale Analysis of the Epididymis in Boars, and the Relationship to Semen Parameters. *Reprod Domest Anim*, 46(1):108-113.

Kauffold J, Wehrend A. 2014. Fertilitätsstörungen beim weiblichen Schwein. *Tierärztliche Praxis Ausgabe G: Großtiere/Nutztiere*, 42(03):179-186.

Kemp B a nd So ede N . 2012. Reproductive Issues in Welfare-Friendly Housing Systems in Pig Husbandry: A review. *Reprod Domest Anim*, 47 (Suppl 5):51-57.

Kielland C, Ro otwelt V, Reksen O , Framstad T . 2015. The association between immunoglobulin G in sow colostrum and piglet plasma. *J Anim Sci*, 93:4453-4462.

Kirkden RD, Broom DM, Andersen IL. 2013. Invited review: piglet mortality: management solutions. *J Anim Sci*, 7:3361-89. doi: 10.2527/jas.2012-5637.

Klobasa F and Butler JE. 1987. Absolute and relative concentrations of immunoglobulins G, M, and A, and albumin in the lacteal secretion of sows of different lactation numbers. *Am J Vet Res*, 48:176-182.

Kobayashi K , Hayashi S, O htubo Y , H onda A , Mizuno J, Hirano S. 1989. *Theriogenology*, 32(1):123-129.

Kvasnitski A V. 1950. The research on interbreed ova transfer in pigs. *Social Livest Breed J*, 12-15.

Langendijk P . 2015. Early gestation feeding and management for optimal reproductive performance. In The gestating and lactating sow. Wageningen Academic Publishers, Wageningen, The Netherlands, pp.1145-1153. Doi: 10.3920/978-90-8686-803-2_2.

Laurincik J , R ath D , N iemann H . 1994. Differences in pronucleus formation and first cleavage following *in vitro* fertilization between pig oocytes matured *in vivo* and *in vitro* . *J Reprod Fertil*, 102(2):277-84.

Li J , Ri eke A, Day B N, P rather RS . 1996. Technical note: porcine non-surgical embryo transfer. *J Anim Sci*, 74(9):2263-8.

Manjarin R , M ontano Y A, K irkwood R N, B ennet DC, P etrovski K R. 2018. Effect of piglet separation from dam at birth on colostrum uptake. *Can J Vet Res*, 82: 239-242.

Martineau G P, F armer C , P eltoniemi O AT. 2012. Mammary system. In: Diseases of swine (10th edition). Zimmerman JJ, Locke A, Karriker AR, Ramirez A, Schwartz KJ, Stevenson GW (Ed). John Wiley & Sons, Inc. Wiley-Blackwell, Ames, Iowa, USA, pp.270-293.

Martinez E A, V azquez J M, R oca J , L ucas X , Gil MA, Parrilla I, Vazquez JL, Day BN. 2002. Minimum number of spermatozoa required for normal fertility after deep intra- uterine insemination in non-sedated sows. *Reproduction*, 123:163-170.

Martinez E A, Ca amano J N, G il M A, Ri eke A, McCauley T C, C antley T C, V azquez JM , R oca J, Vazquez J L, D idion B A, Murphy C N, P rather R S, Day B N. 2004. Successful nonsurgical deep uterine embryo transfer in pigs. *Theriogenology*, 61:137-146.

Martinez E A, V azquez J M, Parrilla I, C uello C, Gil MA, R odriguez-Martinez H, R oca J , V azquez J L. 2006. Incidence of unilateral fertilizations after low dose deep intrauterine insemination in spontaneously ovulating sows under field conditions. *Reprod Domest Anim*, 41:41-47.

Martinez E A, N ohalez A , Martinez C A, P arrilla I , Vila J, C olina I, D iaz M , R eixach J, Vazquez J L, Roca J , Cu ello C, Gil M A. 2016. The Recipients' Parity Does Not Influence Their Reproductive Performance Following Non-Surgical Deep Uterine Porcine Embryo Transfer. *Reprod Domest Anim*, 51(1):123-9. doi: 10.1111/rda.12654. Epub 2015 Dec 14.

Martynenko N A, T chjrkov A G, D enisjuk P V, Korennoij S N. 2004. Transcervical transplantation of porcine embryos and neutralization of superinduction using inseminated recipients. *Vistnik Poltavskoij Dershavnoij Agrarnoij Akademii*, 2:13-17.

Matheson SM , Wa lling GA, E dwards SA . 2018. Genetic selection against intrauterine growth retardation in piglets:a problem at the piglet level with a solution at the sow level. *Genetics Selection Evolution*, 50(1):46. Doi:10.1186/s12711-018-0417-7.

van Nieuwamerongen SE, Bolhuis JE, van der Peet-Schwering CMC, Soede NM. 2014. A review of sow and piglet behaviour and performance in group housing systems for lactating sows. *Animal*, 8:448-460.

Niwa K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. *J Reprod Fertil Suppl*, 48:49-59.

Nohalez A, Martinez CA, Parrilla I, Roca J, Gil MA, Rodriguez-Martinez H , Martinez E A, C uello C . 2018. Exogenous ascorbic acid enhances vitrification survival of porcine *in vitro* -developed blastocysts but fails to improve the *in vitro* embryo production outcomes. *Theriogenology*, 113:113-119.

Okuyama MW, Sugiura T, Moriyoshi M, Yamashita K, T amura J , K atagiri S . 2017. A transvaginal endoscopy-based technique for performing ovarian examinations in sows. *J Reprod Dev*, 63:617-622.

Oliviero C , H einonen M , V alros A, H alli O , Peltoniemi OAT. 2008. Effect of the environment on the physiology of the sow during late pregnancy, farrowing and early lactation. *Anim Reprod Sci*, 105:365-377.

Oliviero C, Heinonen M, Valros A, Peltoniemi OAT. 2010. Environmental and sow-related factors affecting the duration of farrowing. *Anim Reprod Sci*, 119:85-91.

Oliviero C . 2013. Management to improve neonate piglet survival. In Control of pig reproduction IX. H Rodriqez-Martinez, N Soede, W Flowers (Eds.). Leicestershire, United Kingdom: Context Products Ltd, 345p.

Oliviero C , K othe S , H einonen M , V alros A , Peltoniemi O. 2013. Prolonged duration of farrowing is associated with subsequent decreased fertility in sows. *Theriogenology*, 79(7):1095-1099.

Oliviero C, Junnikkala S and Peltoniemi OAT. 2019. The challenge of large litters on the immune system of the sow and the piglets. *Reprod Domest Anim*. In press.

Peltoniemi OAT, Love RJ, Heinonen M, TuovinenV, Saloniemi H. 1999. Seasonal and management effects on fertility of the sow:a descriptive study. *Anim Reprod Sci*, 55:47-61.

Peltoniemi OAT, Alm K, Andersson M. 2009. Uterine insemination with a standard AI dose in a sow pool system. *Reprod Domest Anim*, 44(3):414-418.

Peltoniemi O AT, Björkman S, O liviero C . 2016. Parturition effects on reproductive health in the gilt and sow. *Reprod Domest Anim*, 51:36-47.

Peltoniemi O, Kemp B. 2019. Infertility in the pig and the control of pig herd fertility. In Noakes DE, Parkinson TJ, England GCW (Eds.), Veterinary Reproduction and Obstetrics. 10 ed. Elsevier. p. 581-592. Doi.org/10.1016/B978-0-7020-7233-8.00032-X.

Petersen B , Lu cas-Hahn A , O ropeza M, Hornen N , Lemme E, Hassel P, Queisser AL, Niemann H. 2008. Development and Evaluation of a Highly Efficient Protocol of Porcine Somatic Cloning Using Preovulatory Embryo Transfer in Peripubertal Gilts. *Cloning and Stem Cells*, 10:355-362.

Pinho RO, Camilo B S, Lima DMA, Villadiego F A C, Vergara JCM, Shiomi HH, Guimarães, JD. 2018. The use of ultrasonography in the reproductive evaluation of boars. *Reprod Domest Anim*, 53(2):393-400.

Porter P. 1969. Transfer of immunoglobulins IgG, IgA and IgM to lacteal secretions in the parturient sow and their absorption by the neonatal piglet. *Biochim Biophys Acta*, 181:381-392.

Quesnel H , B rossard L, V alancogne A, Q uiniou N . 2008. Influence of some sow characteristics on within-

litter variation of piglet birth weight. *Animal*, 2:1842-1849.

Quesnel H . 2011. Colostrum production by sows:Variability of colostrum yield and immunoglobulin G concentrations. *Animal*, 5:1546-1553.

Quesnel H, Farmer C, Theil PK. 2015. Colostrum and milk production. In The Gestating and lactating sow. C Farmer (Ed), Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 173-192.

Quigley JD, Lago A, Chapman C, Erickson P, Polo J. 2013. Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. *J Dairy Sci*, 96:1148-1155.

Rátky J, Brüssow KP, Solti L, Torner H, Sarlós P. 2001. Ovarian response, embryo recovery and results of embryo transfer in a Hugarian native pig breed. *Theriogenology*, 56:969-978.

Roca J, Parrilla I, Bolarin A, Martinez EA, Rodriguez-Martinez H. 2016. Will AI in pigs become more efficient? *Theriogenology*, 86:187-193.

Romar R, Coy P, Rath D. 2012. Maturation conditions and boar affect timing of cortical reaction in porcine oocytes. *Theriogenology*, 15:1126-1139.

Rooke JA, Bland, IM. 2002. The acquisition of passive immunity in the new-born piglet. *Livest Prod Sci*, 78:13-23.

Rua MAS, Quirino CR, Ribeiro RB, Carvalho ECQ, Bernadino MLA, Bartholazzi Junior A, Cipagalta LF, Barreto MAP. 2018. Diagnostic methods to detect uterus illnesses in mares. *Theriogenology*, 114:285-292. Doi:10.1016/j.theriogenology.2018.03.042.

Saragusty J, Arav A. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, 141:1-19.

Schmitt O, Baxter EM, Boyle LA, O'Driscoll K. 2019a. Nurse sow strategies in the domestic pig: I. Consequences for selected measures of sow welfare. *Animal*,13(3):580-589. Doi:10.1017/S175173111800160X.

Schmitt O, Baxter EM, Boyle LA, O'Driscoll K. 2019b. Nurse sow strategies in the domestic pig:II. Consequences for piglet growth, suckling behaviour and sow nursing behaviour. *Animal*, 13(3):590-599. Doi:10.1017/S1751731118001702.

Senger PL. 2012. The organization & function of the female reproductive tract. In Pathways to pregnancy & parturition, 3.ed. Current Conceptions Inc., Redmond, OR, USA, pp.10-43.

Soede NM, Langendijk P, Kemp B. 2011. Reproductive cycles in pigs. *Animal Rep Sci*, 124(3-4):251-258.

Soede NM, Laurenssen B, Abrahamse-Berkeveld M, Gerritsen R, Dirx-Kuijkend N, Langendijk P, Kemp B. 2012. Timing of lactational oestrus in intermittent suckling regimes:Consequences for sow fertility. *Anim Reprod Sci*, 130:74-81.

Soede NM, Kemp B . 2015. Best practices in the lactating and weaned sow to optimize reproductive physiology and performance. In The gestating and lactating sow. Wageningen, The Netherlands: Wageningen Academic Publishers, pp.99-106.

Soriano-Úbeda C, García-Vázquez FA, Aguirregomezcorta R, Matás C . 2017. Improving porcine *in vitro* fertilization output by simulating the oviductal environment. *Nature Scientific Reports*, 7:43616.| Doi:10.1038/srep4361.

Spiegel F, Spiegel S, von Altrock A, Verspohl J, Seehusen F, Wendt M. 2017. Occurrence of bacteria in milk samples and biopsy samples of the mammary gland from health sows. In Proceedings of the ninth European Symposium of Porcine Health Management, CZECH-IN, 9th, May 3 to 5, Prague, Czech Republic.

Smit M, Spencer J, Almeida F, Patterson J, Chiarini-Garcia H, Dyck M, Foxcroft G. 2013. Consequences of a low litter birth weight phenotype for postnatal lean growth performance and neonatal testicular morphology in the pig. *Animal*, 7:1681-1689. Doi:10.1017/S1751731113001249.

Stark R, Herlt C, Sigmarsson HL, Kauffold J. 2019. Feasibility of transabdominal Doppler ultrasonography for studying ovarian blood flow characteristics in cycling gilts. *Tierarztl Prax Ausg G Grosstiere Nutztiere*, 47(02):77-85.

Sumransap P, Tummaruk P, Kunavongkrit A. 2007. Sperm distribution in the reproductive tract of sows after intrauterine insemination. *Reprod Domest Anim*, 42:113-117.

Tummaruk P, Sumransap P, Techakumphu M, Kunavongkrit A . 2007. Distribution of spermatozoa and embryos in the female reproductive tract after unilateral deep intra uterine insemination in the pig. *Reprod Domest Anim*, 42:603-609.

Tummaruk P, Sang-Gassanee K . 2013. Effect of farrowing duration, parity number and the type of anti-inflammatory drug on postparturient disorders in sows:a clinical study. T*rop Anim Health Prod*, 45(4):1071-1077.

Vallet J, Miles J, Rempel L. 2013. A simple novel measure of passive transfer of maternal immunoglobulin is predictive of preweaning mortality in piglets. *Vet J*, 195:91-97.

Watson PF, Behan JR. 2002. Intrauterine insemination of sows with reduced sperm numbers:results of a commercially based field trial. *Theriogenology*, 57:1683-1693.

Webel SK, Peters JB, Anderson LL. 1970. Synchronous and asynchronous transfer of embryos in the pig. *J Anim Sci*, 30:565-568.

Wilson ME, Biensen NJ, Youngs CR, Ford SP. 1998. Development of Meishan and Yorkshire littermate conceptuses in either a Meishan or Yorkshire uterine environment to day 90 of gestation and to term. *Biol Reprod*, 58:905-910.

Yao W, Li J, jun Wang J, Zhou W, Wang Q, Zhu R, Wang F, Thacker P. 2012. Effects of dietary ratio of n-6 to n-3 polyunsaturated fatty acids on immunoglobulins, cytokines, fatty acid composition, and performance of lactating sows and suckling piglets. *J Anim Sci Biotechn*, 3:43.

Yeste M, Rodríguez-Gil JE, Bonet S. 2016. Artificial insemination with frozen-thawed boar sperm. *Mol Reprod Dev*, 84:802-813.

Yonemura I , F ujino Y , Irie S, M iura Yl. 1996. Transcervical transfer of porcine embryos under practical conditions. *J Reprod Dev*, 42:89-94.

Yuan Y, Krisher RL. 2012. *In vitro* maturation (IVM) of porcine oocytes. *Methods Mol Biol*, 825:183-98. Doi:10.1007/978-1-61779-436-0_14.

Yun J , Sw an K M, V ienola K, K im Y Y, Oliviero C, Peltoniemi OAT, Valros A. 2014. Farrowing environment has an impact on sow metabolic status and piglet colostrum intake in early lactation. *Livest Sci*, 163:120-125.

Yun J, Björkman S, Pöytäkangas M, Peltoniemi OAT. 2017. The effects of ovarian biopsy and blood sampling methods on salivary cortisol and behaviour in sows. *Res Vet Sci*, 114:80-85.

Zheng C, Ji an-Tao Z , H ai-Feng L , H ong-Bin W . 2016. Laparoscopic Embryo Transfer in Pigs: Knowledge for Surgical Procedures. *J Northeast Agric Univ (Engl Ed)*, 23:52-58.

DOI: 10.21451/1984-3143-AR2019-0057

Proceedings of the 35th Annual Meeting of the European Embryo Transfer Association (AETE); Murcia, Spain, September 12th and 14th, 2019.

Inflammation: friend or foe of bovine reproduction?

Sylvie Chastant[1,*], Marie Saint-Dizier[2]

[1]Reproduction, UMR INRA/ENVT 1225, Toulouse National Veterinary School, Toulouse, France.
[2]Université de Tours, UMR85 Physiologie de la Reproduction et des Comportements, Centre INRA Val-de-Loire, Nouzilly, France.

Abstract

Inflammation is not only the first line of defense of the organism but is also required in many reproductive processes such as ovulation, corpus luteum development, luteolysis, uterine clearance after insemination and post partum. Nevertheless, if excessive or persistent, inflammation can switch from a positive mechanism to a deleterious process, impairing oocyte quality and embryo development. Not only uterine but also non genital inflammatory sites can depreciate reproductive performances, with a carry over effect of 2 to 4 months. Since the metabolic challenges of the peripartum transition period make difficult for the cow to control inflammation, dairy cows are frequently in a pro-inflammatory stage, suggesting that inflammation, rather than infection, is a limiting factor of fertility in modern dairy cows. Within the first week after calving, cows have to mount an intense inflammatory response to the bacterial invasion of the uterine cavity with the challenge of being able to switch it off in no more than 5-6 weeks. The absence of neutrophils on endometrial smear is associated with the highest success rate at insemination. Since a fine tuning – rather than an absence - of inflammation is required along the reproductive cycle, anti-inflammatory drugs do not allow any improvement of pregnancy rate, except in the specific case of embryo transfer. Appropriate management of the transition period (especially nutritional) and in a long term perspective, genetic selection contribute to improve the aptitude of cows to controls the intensity of inflammatory process.

Keywords: inflammation; ovulation; post partum; cytokines; neutrophils.

Introduction

(Bacterial) infection has been long considered as an essential component of reproductive disorders, whereas (sterile) inflammation is nowadays identified as a major and frequent limiting factor of reproductive performances. In the medical approach, inflammation, hallmark of "–itis" diseases, is classically considered as a deleterious process, an unwanted response leading to immune dysfunction, diversion of nutrients from productive purposes, tissue damage, sepsis, organ failure and even death. Nevertheless, from a biological perspective, inflammation, involving chemokines and cytokines release, blood vessel dilation and immune cell infiltration, is the first line immune response of an organism facing a microbial infection or a tissue injury. Since the female genital tract is physiologically exposed to a range of tissue injuries (such as ovulation) and intrauterine bacterial challenges (after calving, at insemination/mating through sperm), inflammation also belongs to the physiology of reproduction. Moreover, some other reproductive processes, such as corpus luteum development and demise, or maternal recognition of pregnancy share some similarities with inflammatory events. The objective of this paper is to review the positive and negative relationships between inflammation and cow reproduction, to finally question the rationale of the use of anti-inflammatory drugs to improve reproductive performances. This review focuses on inflammation, trying to distinguish it from the effects of bacterial infections (including Lipopolysaccharide - LPS) and on the bovine female, despite inflammation is closely associated to many physiological and pathological aspects of reproduction in many other species, if not all (e.g. Freeman *et al*., 2013 in the bitch or Katila, 2012 in the mare).

The female genital tract is physiologically able to mount an inflammatory reaction

The female genital tract is naturally equipped to recognize pathogens and damages (Sheldon *et al*., 2018): some uterine, tubal and ovarian cells of the cow express receptors (Pattern recognition receptors, PRRs, sensors of 'danger') recognizing highly conserved microbial molecular signatures (MAMPs, Microbe-associated molecular patterns) or host-derived molecules indicative of cell injury (DNA fragments, mitochondrial content, but also free fatty acids and carbohydrates), referred to as DAMPs (for Damage-associated molecular patterns). Transmembrane toll-like receptors (TLRs) are probably the most classical PRRs and are expressed by bovine granulosa cells (Price and Sheldon, 2013), bovine oviductal epithelial cells, epithelial and stromal cells of the endometrium (Herath *et al*., 2009; Turner *et al*., 2014; Dadarwal *et al*., 2017; Danesh Mesgaran *et al*., 2018).

These different compartments are able to mount an early immune response: recognition of MAMPs or DAMPs by the genital cells initiate several signaling cascades (through NFκB or MAPkinase pathways for example), resulting in the expression of pro-inflammatory mediators (e.g. Tumor Necrosis Factor α -TNFα-, interleukin- IL 1 and 8),

*Corresponding author: s.chastant@envt.fr
orcid.org/0000-0003-0790-6377
Received: May 9, 2019
Accepted: July 30, 2019

antimicrobial peptides and anti-apoptotic factors. Immune cells (mainly polymorphonuclear cells - PMN) are consequently attracted to the site of infection/injury, ensuring phagocytosis of invading microorganisms or cell fragments (Broom and Kogut 2018; Sheldon *et al*., 2019) together with the generation of *reactive oxygen species* (ROS) and the release of proteolytic enzymes. Pro-inflammatory cytokines also induce important microcirculatory events, at short (vasodilation) and long term (neoangiogenesis contributing to tissue healing).

Physiological inflammation in reproductive processes

Apart from playing a central role into innate immunity, inflammation is essential for successful cow reproduction since inflammatory (or inflammatory-like) processes are implicated in every step of fertility: in the cycle (ovulation, corpus luteum development, luteolysis), early pregnancy (maternal recognition of pregnancy) and later, in expulsion of fetal membranes and post partum uterine involution.

Ovulation

The ovulation exhibits many classical signs of local inflammation, with production of inflammatory mediators, locally increased blood flow, leukocyte infiltration, swelling, tissue digestion and ultimately tissue repair (Espey, 1980; Duffy *et al*., 2019). First responders to the LH surge are granulosa and theca cells, which produce chemokines and cytokines within hours after the LH surge. High concentrations of TNFα, IL1 and IL8 are found in follicular fluid at the preovulatory stage; consequently, not only the preovulatory follicle is invaded by high numbers of neutrophils and macrophages, but ovarian resident immune cells are activated (Jiemtaweeboon *et al*., 2011). Through proteolytic pathways, crucial within the ovulation process, these exogenous and endogenous cells regulate the reorganization of follicular stroma, the disruption of the granulosa basal lamina, and its invasion by vascular endothelial cells. LH-induced mediators also initiate cumulus expansion and cumulus-oocyte-complex detachment, together with extensive extracellular matrix remodeling and loss of the surface epithelium at the follicular apex. All these inflammatory phenomenons play a crucial role in the ovulatory process since treatment with antibodies directed against IL8 or neutrophils respectively suppress or decrease ovulation rate; administration of anti-proteases blocks ovulation; no blood flow increase is observed around large follicles that will finally fail to ovulate (Murdoch *et al*., 1997; Miyamoto *et al*., 2006).

Corpus luteum development

After ovulation, the remainder of the follicle undergoes intra-antral bleeding, colonization by a large variety of immune cells (mainly macrophages, neutrophils and eosinophils), secreting numerous cytokines (TNFα, interferon gamma, interleukins, prostaglandins) together with angiogenic factors. Follicular wall is rapidly remodeled, thanks to rapid angiogenesis and granulosa/thecal cells differentiation into luteal tissue, that finally fills the former follicular antral cavity. If ovulation can be assimilated to a specific physiological injury, corpus luteum (CL) development can be compared to a phase of tissue repair and organ healing.

Luteolysis

Not only CL formation but also lysis are inflammatory-like processes. Due to the short delay between prostaglandin F2α (PGF2α) secretion and the intraluteal immune reaction, luteolysis is even considered as an acute phenomenon (Shirasuna *et al*., 2012a). Leukocytes, especially eosinophils, macrophages and T lymphocytes, are recruited into the CL within the 5 first minutes after a PGF2α injection; as early as after 30 minutes, the expression of endothelial nitric oxide synthase is stimulated, accompanied by an increase in luteal blood flow and IL8 expression (Neuvians *et al*., 2004). Luteal blood flow increases within minutes in response to each peak of uterine PGF2α during spontaneous luteolysis in cattle (Miyamoto *et al*., 2005; Ginther and Beg, 2012). Interestingly, this “preluteolytic” blood flow increase is not observed in PGF2alpha refractory CL (Miyamoto *et al*., 2006). A little bit later, but as early as two hours, expression of pro-inflammatory cytokines (TNFα, IL1beta and interferon gamma) is increased and made responsible for apoptosis of luteal cells. CL regresses primarily through the loss of cells by apoptosis and apoptotic luteal cells are phagocytosed by macrophages. The large number of immune cells observed within the CL 6-24 hours after PGF2α are considered essential for a rapid demise of the CL tissue (Neuvians *et al*. 2004; Shirasuna *et al*., 2012b). As previously described, TNFα is also found involved into CL development: this dual effect may be due to a dose-effect, luteotropic at high doses or luteolytic at low doses, probably depending on the type of receptors activated (TNFRI or II) (Korzekwa *et al*., 2008). Four hours after PGF2α release, blood flow has felt back to the preluteolytic level and totally disappears after 24 hours (Miyamoto *et al*., 2005).

After insemination/mating: post-mating reaction

Spermatozoa, seminal plasma or extenders are recognized as “dangers” by the genital tract and can induce an inflammatory reaction though PRRs activation. Mating or artificial insemination (AI) are thus followed by a physiological influx of neutrophils into the uterine lumen which peaks between 1 and 12 hours after. This so called post mating reaction has been observed in the uterus, cervix and vagina but not into the oviduct (despite less well studied and probably more complex). Like bacteria, sperm are phagocyted by neutrophils either directly through cell-cell attachment or entrapped with neutrophil extracellular traps (NETs) which ensnare sperm and hinder their motility (Marey *et*

al., 2016). Rapid removal of sperm is thought to prevent acquired immune responses against sperm in dams since it is important for further embryo development that the female genital tract remains tolerant to paternal antigens (Katila 2012). In cattle, 60% of sperm are voided by 6 hours after AI and by 12-24 hours, only a few percent of sperm are left in the reproductive tract, the majority found within the vagina (Mitchell *et al*., 1985; Hawk, 1987). The duration of PMN infiltration is short, with a peak at less than 2 hours or at around 8-16 hours post AI or mating in cattle according to the different studies (reviewed by Katila, 2012): sperm and bacteria are rapidly eliminated, afterward the endometrium rapidly returns to a non-inflamed status, prepared to receive the embryo after its oviductal transit. If one can easily conceive than an excessive or persistent post mating response could decrease embryo survival rate, Kaufmann *et al*. (2009) suggested that the absence of post mating reaction in cows (no leukocytes intrauterine mobilization 4 hours after insemination) is associated with decreased pregnancy rates.

The situation is different in the oviduct whose epithelial cells face two opposite challenges: first, the protection against bacteria ascending from the uterus (and especially in oestrus, due to the opening of the cervical barrier and eventually insemination) and second, to favor fertilization and embryo development, whereas sperm and embryos are (semi) allogeneic to the maternal host. Interestingly, in presence of LH and estradiol, the oviduct generates a state of immunotolerance that ensures sperm survival until fertilization (Marey *et al*., 2016). Once sperm bound to oviductal epithelial cells, these cells are stimulated to secrete high levels of PGE2 that strongly suppress the PMN phagocytic activity to sperm and pro-inflammatory cytokines synthesis. Sperm binding thus favors the development of an anti-inflammatory immune environment and suppresses PMN sperm phagocytosis. More precisely, follicular fluid collected from pre-ovulatory follicle enhanced sperm phagocytosis by neutrophils in vitro whereas the oviductal fluid suppressed this activity. The oviductal environment seems thus to minimize the inflammatory effect of the follicular fluid released at the time of ovulation to allow sperm capacitation and fertilization (Yousef et al., 2019).

Placental expulsion

Placental maturation leading to fetal membranes expulsion also involves inflammatory mechanisms, mainly protease activity and leukocytes chemotaxis (Beagley *et al*., 2010). During the third trimester of pregnancy, fetal major histocompatibility complex (MHC) Class 1 molecules begin to be expressed by placental cells and initiate a maternal response (the fetus being an allograft) (Davies *et al*., 2000). Leukocytes are recruited through the placenta via several chemoattracting cytokines (TNFα, IL2 and IL 8) and phagocyte placental cells (Heuwieser and Grunert, 1987; Kimura *et al*, 2002). In addition, Matrix MetalloProteinase and collagenase activities increase in the maternal and the fetal part of the placenta (Maj and Kankhofer, 1997; Beagley *et al*., 2010). Both inflammatory components (leukocytes and enzymes) contribute to the loosening and subsequently the detachment of the villi. Importance of efficient inflammatory processes into placental expulsion in the cow is well demonstrated by the overexpression of anti-inflammatory associated genes and decreased expression of promoters of proteolytic activity in case of spontaneous placental retention (Nelli *et al*., 2019) even if not systematically reported (Walter and Boos, 2001).

Post partum uterine involution

Following the delivery of the calf, the uterine lumen, fulfilled with cellular and tissular debris, from placental and maternal origin, is physiologically colonized by bacteria (Sheldon *et al*., 2006). Both damages and bacterial invasion elicit a massive immediate cellular influx, whose intensity affects reproductive performances. Cows able to mount an early inflammatory response with more than 35-40% of neutrophils on endometrial smears 7 days after calving have shorter intervals from calving to pregnancy (Gilbert and Santos 2016; Cheong *et al*., 2017). This may be attributable to an early clearance of the uterine cavity from inflammatory stimuli. Inflammation is thus beneficial for the animal in the very early times after calving. However, it is important to distinguish local intrauterine cell mobilization - associated with a higher probability of ovulation from the first dominant follicle - and systemic inflammation, evaluated through haptoglobin concentration, conversely associated with a decreased ovulation rate (Cheong *et al*., 2017).

Excessive or persistent uterine inflammation

Once the initial danger of post-partum microbial invasion is contained, it is important that inflammation is resolved, otherwise chronic inflammation persists to the detriment of tissue function. Optimal reproductive performances thus require that the animal is able to mount a rapid, acute inflammatory response to control in a short term delay the microbial invasion. In a second step, after pathogen clearance, it is of equal importance that the animal is able to control the inflammation itself, to extinct it through a timely transition to an anti-inflammatory state, favorable to tissue repair processes. Rapid, targeted, effective and quick resolution are the hallmarks of a desired inflammatory response (Broom and Kogut 2018). Considering uterine health, after the intense PMN mobilization of the first week after calving, optimal reproductive performances are obtained if the percentage of PMN on endometrial smears falls below 5% between 21 and 35 days after calving, reaching a nadir (0-1%) around 45 days after calving and being maintained at this almost null level until the time of insemination (Deguillaume, 2010; Drillich *et al*., 2012; Bogado Pascottini *et al*., 2016; Machado Pfeifer *et al*., 2018; Fig. 1).

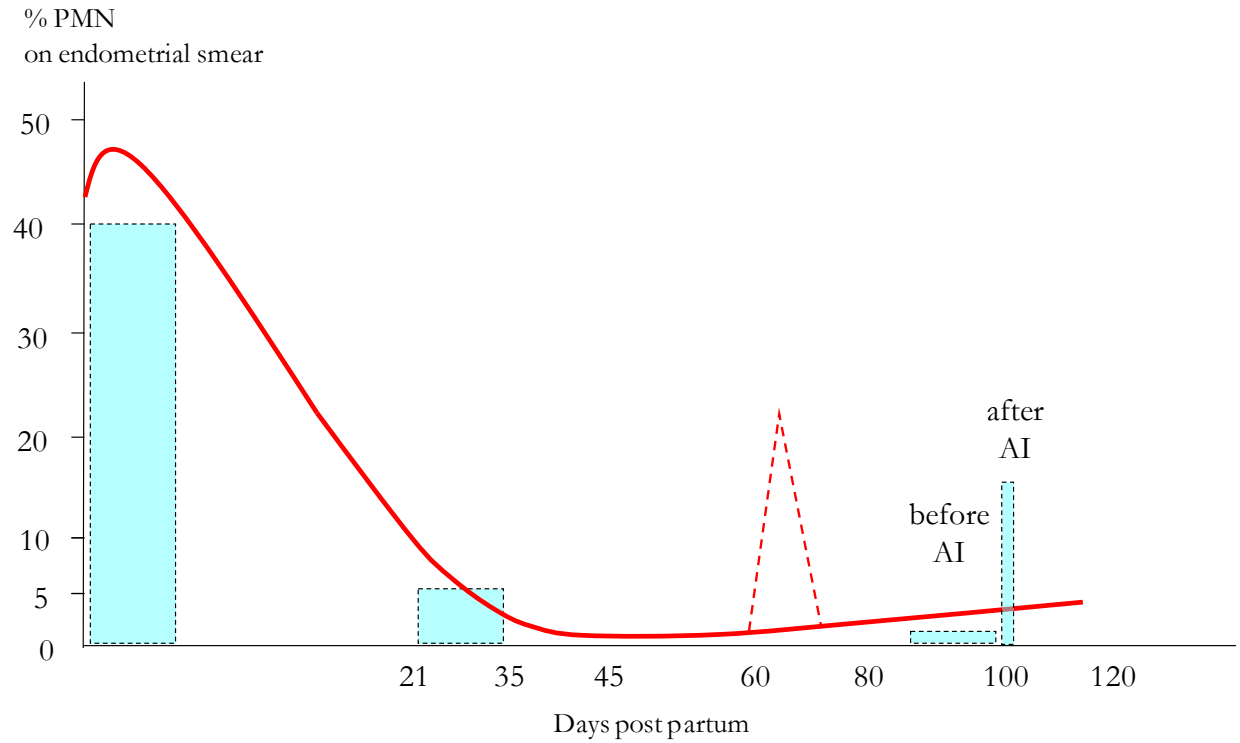


Figure 1. Intensity of endometrial inflammation from calving to insemination (% neutrophils on endometrial smear). All the thresholds indicated were determined based on a significant decrease of pregnancy rate. After an early intense mobilization of neutrophils after calving (>40%), inflammation is down regulated, becoming null around 40-45 days after calving and remaining null at the time of insemination. Between the nadir and the time of insemination, inflammation can be reactivated (interrupted lines). During a few hours after insemination, inflammation is transiently reactivated (post mating response).

But this fine tuning of uterine inflammation (massive during the first week after calving, rapidly controlled and finally extinct at the end of the first month and transiently reactivated during a few hours after insemination) is a difficult exercise for dairy cows, due to the delicate metabolic context of the post partum period (LeBlanc, 2014). Inflammation control is not just a passive extinction but rather requires the activation of anti-inflammatory pathways (including for example lipoxins and resolvins, Sheldon *et al*., 2017). Genital health relies on a fragile equilibrium between pro- and anti-inflammatory systems, difficult to maintain in dairy cows: the persistence of uterine inflammation at the time of insemination is a frequent situation (28 to 57% of Holstein cows according to the different studies). From three weeks before and until three weeks after calving (transition period), dairy cows are facing a negative energy balance (with production of non esterified fatty acids), oxidative stress (ROS production), together with digestive acidosis and social stress (Fig. 2), all situations that put the cow in a pro-inflammatory situation. Moreover, a vicious circle installs due to the huge energy expenditure associated to the inflammatory phenomenon itself. Dairy cows use more than 1 kg glucose in the first 12 hours after an LPS challenge (Kvidera *et al*., 2017), an expenditure corresponding to about 100 kcal/kg $BW^{0.75}$ (calculation from Gilbert, 2019), i.e. almost equivalent to maintenance. The depletion of the key cellular nutrients (such as glucose) reduces inflammatory responses, compromising the ability of animals to respond sufficiently to pathogens, resulting in the persistence of infections and chronic inflammation.

The tendency to an overactivity of pro-inflammatory systems and the instability of inflammation control in post partum dairy cows are pictured in endometrial smears follow-up: even when cows solved their uterine inflammation at 40-45 days post partum (0% PMN), transient episodes of reactivation of the uterine inflammation (up to 40% PMN) were observed after 60 days post partum (unpublished data). This explains why cows diagnosed as free from endometritis around 30 days post partum can be found with purulent uterine content at the time of insemination, probably due to a disruption of the equilibrium between pro- and anti-inflammatory systems.

To date, the unability of cows to down regulate inflammation is probably one important limiting factor of modern dairy cows fertility, due to the frequency of excessive uterine inflammation at the time of insemination, and its dramatic impact on insemination success rate (around 15 points decrease). As developed by Sheldon *et al*. (2019), uterine health is rather dependent on the endometrial tolerance to pathogens (ability to limit the disease severity induced by pathogens) than on its resistance (ability to limit the pathogen development).

Deleterious effects of inflammation on reproduction

Excessive or persistent inflammation has deleterious impact on fertility. But this applies not only to uterine inflammation, but also to extragenital inflammation. Due to cytokine release into the general circulation, ovaries, uterus and embryos may be somewhat "contaminated" by distant inflammatory sites, such as mastitis, podal inflammation, digestive inflammation consecutive to acidosis, all highly prevalent in dairy cows. Inflammatory diseases affect many steps of the reproductive process: GnRH and LH synthesis, folliculogenesis, follicular steroidogenesis, oocyte quality, ovulation, estrus expression, corpus luteum quality and lifespan, fertilization, embryo development and survival (Ribeiro and Carvalho, 2017; Fig. 3).

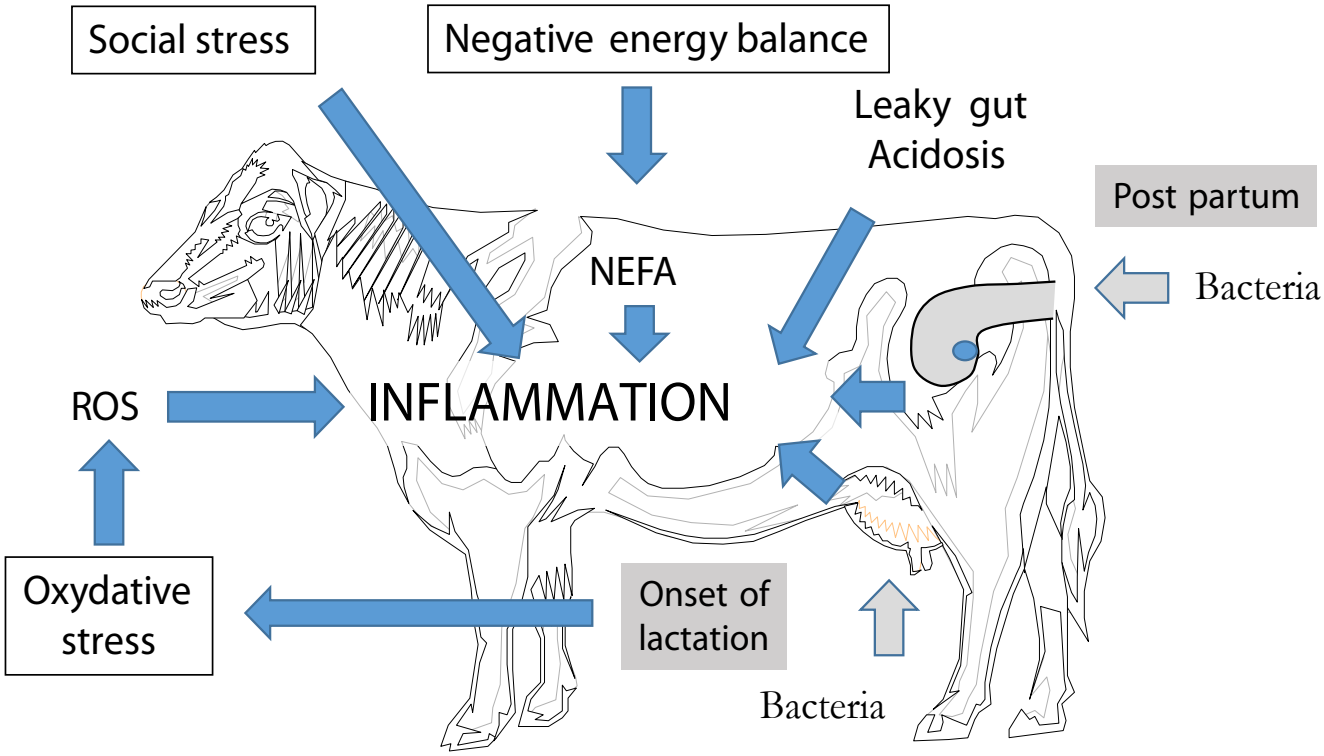


Figure 2. Determinants of the pro-inflammatory status during the post partum period of dairy cows. NEFA: Non Esterified Fatty Acids.

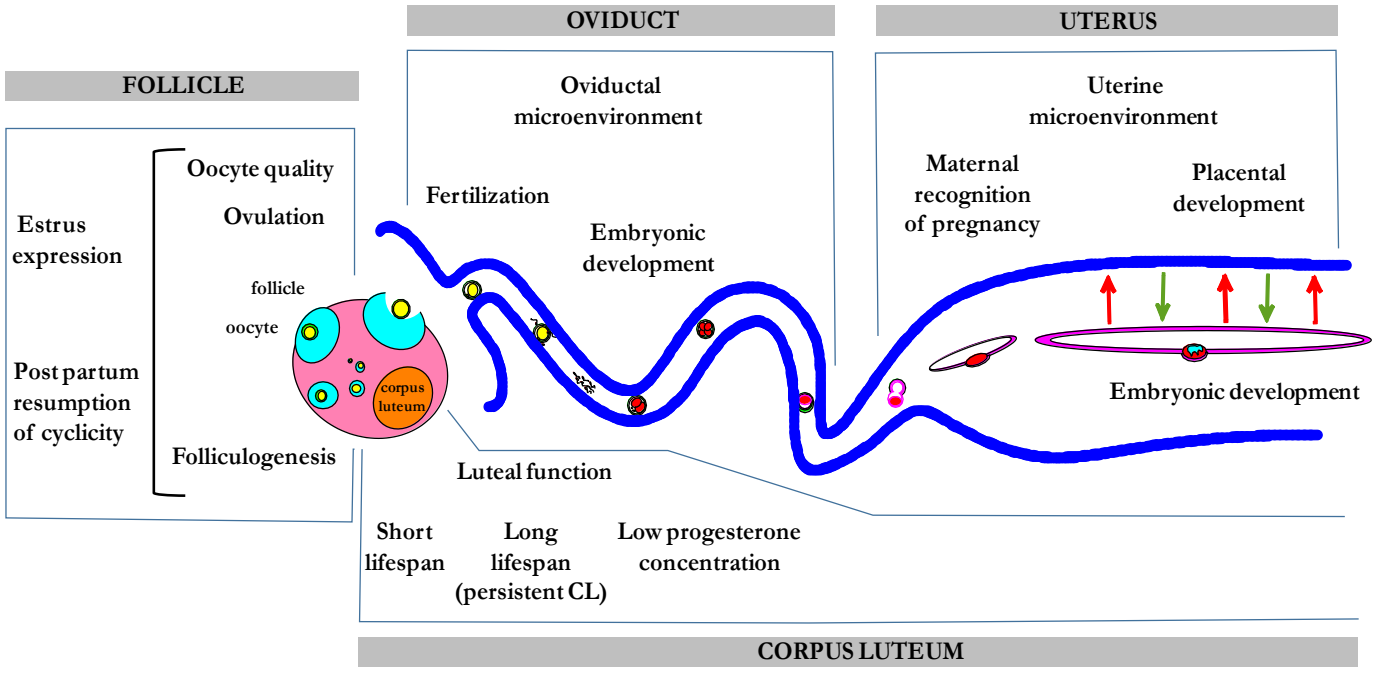


Figure 3. Steps of the reproductive process sensitive to inflammation.

Ovarian reserve

In humans and mouse, chronic inflammation is made responsible for destruction and/or premature activation of primordial follicles, leading to a decrease of the ovarian reserve, and thus Premature Ovarian Failure (phenomenon so called "inflamm-aging") (Huang *et al*., 2019). In the bovine, considering the post partum period as a prolonged period of inflammation with excessive oxidative stress and fatty acids release, Gilbert (2019) estimated plausible that inflammatory damages could be inflicted on developing oocytes and the resting oocyte pool, resulting in chronically diminished fertility (Sheldon *et al*., 2017).

Anovulation – Follicular cyst

Several anovulatory situations are associated with an increased expression of pro-inflammatory cytokines in the granulosa (IL1α, IL6 and TNF α) in humans (Luteinized unruptured follicle syndrom; Polycystic ovary syndrom) and in the cow (ovulation failure and follicular persistence, follicular cyst; Baravalle *et al*., 2015; Stassi *et al*., 2017).

Oocyte competence

Inflammation mediates changes in follicular fluid that diminish the ability of the oocyte to complete meiosis, undergo fertilization and support development of a conceptus. By the activation of granulosa PRRs, steroidogenesis and the interaction between oocyte and cumulus can be impaired (Herath *et al*., 2007). Inflammatory mediators have been also described to result into aberrant spindle formation and meiosis abnormalities (Bromfield and Sheldon, 2011; Banerjee *et al*., 2012).

Luteal insufficiency

Since inflammation affects granulosa and thecal cell function (before ovulation) and luteal cells (after ovulation), it is associated with inadequate function of the CL and insufficient circulating concentrations of progesterone, one of the major causes of infertility of modern cows (Diskin *et al*., 2011; Ribeiro *et al*., 2016).

Embryo/placental development

Inflammation may affect embryo survival both by its deleterious effect on oocyte quality and CL function but also by providing an inadequate uterine microenvironment and through direct effect of cytokines on embryonic/placental cells. The direct influence of inflammation *per se* on embryo has been elegantly demonstrated by Hill and Gilbert (2008) who induced a non infectious endometrial inflammation; after culture into the conditioned uterine medium, blastocyst cell number was decreased, affecting trophectoderm but not inner cell mass. Other authors observed consistently impaired elongation and decreased interferon tau secretion. Inflammation thus interferes with maternal recognition of pregnancy and later, if pregnancy is maintained, decreases placental weight from Day 42 of gestation (Lucy *et al*., 2016; Ribeiro *et al*., 2016). Interestingly, maternal inflammatory diseases even caused inflammation-like changes in the transcriptome of conceptus cells (Ribeiro *et al*., 2016).

Inflammation is thus involved into many reproductive diseases, namely abnormalities in ovarian resumption of cyclicity (delayed ovulation, short luteal phases, persistent corpus luteum), metritis/endometritis and repeat breeder syndrome.

Carry over effects of inflammation

The variety of targets sensitive to inflammation (oocyte, embryo, placenta) explains that inflammation affects reproductive performances at various distances from insemination. For example, mastitis negatively impacts on reproductive performances whatever it occurred before the first AI (even during the first month after calving), between first AI and conception or after conception, with a period at higher risk extending from 3 weeks before AI until 30 days after (Loeffler *et al*., 1999; Perrin *et al*., 2007; Lavon *et al*., 2011; Albaaj *et al*., 2017). Same observation was made with long lasting consequences of metritis on ovarian function, long after the resolution of the disease (Piersanti *et al*., 2019). This delayed effect of inflammation is reminiscent of what is known as the "Britt hypothesis" explaining the carry-over effect of negative energy balance on fertility (Britt, 1992). The carryover effect of inflammatory diseases on reproduction is attributable to the impact on oocyte quality together with an durably modified uterine environment. In case of uterine disease, inflammation can persist during several months as inflammatory lymphocytic foci within the endometrial wall, even during pregnancy (Lucy *et al*., 2016). The uterus may also be long-lasting impaired secondary to altered steroid synthesis. When previously diseased cows (retained fetal membranes, metritis, mastitis, lameness, and respiratory and digestive problems) are used as embryo recipients, establishment of diagnosed pregnancy is reduced and pregnancy loss rate is increased relative to that of previously healthy cows. The effect of inflammation on reproduction extends long beyond the resolution of the disease, until 4 months later (Ribeiro *et al*., 2016).

Transgenerational (epigenetic) effects of maternal inflammation are also suspected but with controversial observations. For Ribeiro and Carvalho (2017), female calves born from multidiseased cows have significantly lower incidence of mortality and morbidity before their first calving. Conversely, Ling *et al*. (2018) described that calves born to cows with a higher serum haptoglobin concentration (acute phase protein) during late gestation showed a lower TNFα plasma concentration after challenge, suggesting a compromised immune response to microbials.

Suppression of inflammation: NSAID and reproduction

Since inflammation (rather than infection) is now recognized as the limiting factor of reproductive performances and in the context of the reduction of the use of antibiotics, the interest of non steroidal anti-inflammatory drugs (NSAID) has been evaluated. When used as additional treatment, NSAID allowed to limit the reproductive impact of mastitis (MacDougall *et al*., 2016). Their administration at the time of AI did not improve pregnancy rates (Heuwieser *et al*., 2011); administration before ovulation was deleterious due to an inhibition of the ovulation process and follicular cyst formation (Pugliesi *et al*., 2012). Conversely, administration at the time of embryo transfer showed an improvement of pregnancy rates (+10 to 25 points), especially when transfer was qualified as difficult (Aguiar *et al*., 2013) or after transfer of low quality embryos (Scenna *et al*., 2005). Administration at mid luteal phase targeting maternal recognition of pregnancy did not show any significant improvement of insemination success rate.

Conclusion: Inflammation is not to be suppressed but regulated

Inflammation is a dual process, together mandatory at numerous steps of the reproduction process and deleterious for reproductive performances if excessive or persistent. Optimisation of insemination success rate depends not on the suppression of inflammation but on its fine regulation. The cow has to be able to mount intense inflammatory episodes and, more difficult, to control and shut them down rapidly, what is made complex by metabolic challenges post partum. Better regulation of the inflammation can be obtained through an appropriate dietary management during the transition period, targeting energy balance, Dietary Anions-Cations Difference, and anti oxidant reserves (LeBlanc 2012). Immunomodulators rather than anti-inflammatory drugs are an elegant strategy (such as pegbovigrastim, long acting-analog of bovine granulocyte colony-stimulating factor; Ruiz *et al*., 2017; Heiser *et al*., 2018). The genetic option is also promising, with the selection of females with high immune regulatory competences (Thompson-Crispy *et al*., 2012; Silva Silveira *et al*., 2019; König and May, 2019).

Author contributions

SCM: Conceptualization, Writing – original draft, Writing – review & editing. MSD: Conceptualization, Writing – review & editing.

Conflict of interest

The Authors declare no conflict of interest.

References

Aguiar T S, Ar aujo CV, Tirloni RR, M artins L R. 2013. Effect of meloxicam on pregnancy rate of recipient heifers following transfer of in vitro produced embryos. *Reprod Domest Anim*, 48:984-988.

Albaaj A , F oucras G , R aboisson D . 2017. High somatic cell counts and changes in milk fat and protein contents around insemination are negatively associated with conception in dairy cows. *Theriogenology*, 88:18-27.

Banerjee J, S harma R , A garwal A , Maitra D , Diamond M P, A bu-Soud H M. 2012. IL-6 and mouse oocyte spindle. *PLoS One*, 7(4):e35535.

Baravalle M E, Stassi AF, V elazquez MML, B elotti EM, R odriguez F M, Ortega HH, Salvetti N R. 2015. Altered expression of proinflammatoru cytokines in ovarian follicles of cows with cystic ovarian disease. *J Comp Patho*l, 153:116-130.

Beagley JC, Whitman KJ, Baptiste KE, Scherzer J. 2010. Physiology and Treatment of Retained Fetal Membranes in Cattle. *J Vet Intern Med*, 24:261-268.

Bogado Pascottini O, Hostens M, Sys P, Vercauteren P, O psomer G. 2016. Cytological endometritis at artificial insemination in dairy cows: Prevalence and effect on pregnancy outcome. *J Dairy Sci*, 100:588-597.

Britt J H. 1992. Impacts of early postpartum metabolism on follicular development and fertility. *The Bovine Proceedings*, 24:39-43.

Bromfield JJ, Sheldon IM. 2011. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. *Endocrinology*, 152(12):5029-40.

Broom L J, K ogut M H. 2018. Inflammation: friend or foe for animal production? *Poult Sci*, 97:510-514.

Cheong SH , S a F ilho O G, A bsalon-Medina V A, Schneider A . Butler WR, Gilbert RO. 2017. Uterine and systemic inflammation influences ovarian and follicular function in postpartum dairy cows. *PLos One*, 12(5): e0177356.

Dadarwal D , Palmer C , G riebel P . 2017. Mucosal immunity of the postpartum bovine genital tract. *Theriogenology* ,104:62-71.

Danesh M esgaran S , G artner MA, Wa gener K, Drillich M , E hling-Schulz M, E inspanier R , G abler C. 2018. Different inflammatory responses of bovine oviductal epithelial cells in vitro to bacterial species with distinct pathogenicity characteristics and passage number. *Theriogenology*, 106:237-246.

Davies C J, F isher P J, S chlafer D H. 2000. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. *Placenta*, 21:194-202.

Deguillaume L. 2010. L'inflammation génitale post partum de la vache. PhD dissertation. AgroParisTech, Paris, France.

Diskin M G, P arr MH, Morris D G. 2011. Embryo death in cattle: an update. *Reprod Fertil Dev*, 24:244-251.

Drillich M, Tesfaye D, Rings F, Schellander K, Heuwieser W, Hoelker M. 2012. Effects of polymorphonuclear neutrophil infiltration into the endometrial environment on embryonic development in superovulated cows. *Theriogenology*, 77:570-578.

Duffy DM, Ko CM, Jo M, Brannstrom M, Curry Jr TE. 2019. Ovulation: parallels with inflammatory processes. *Endocr Rev*, 40:369-416.

Espey LL. 1980. Ovulation as an inflammatory reaction - a hypothesis. *Biol Reprod*, 22:73-106

Freeman SL, Green MJ, England GCW. 2013. Uterine fluid from bitches with mating-induced endometritis reduces the attachment of spermatozoa to the uterine epithelium. *Vet J*, 198:76-80.

Gilbert RO, Santos NR. 2016. Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility in dairy cows. *Theriogenology*, 85:1367-74.

Gilbert RO. 2019. Mechanisms of disruption of fertility by infectious diseases of the reproductive tract. *J Dairy Sci*, 102:3754-3765.

Ginther OJ, Beg MA. 2012. The hour of transition into luteolysis in horses and cattle: A species comparison. *Theriogenology*, 77:1731-1740.

Hawk HW. 1987. Transport and fate of spermatozoa after insemination of cattle. *J Dairy Sci*, 70:1487-1503.

Heiser A, LeBlanc SJ, McDougall S. 2018. Pegbovigrastim treatment affects gene expression in neutrophils of pasture-fed, periparturient cows. *J Dairy Sci*, 101:1-14.

Herath S, Williams EJ, Lilly ST, Gilbert RO, Dobson H, Bryant CE, Sheldon IM. 2007. Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. *Reproduction*, 134:683-693.

Herath S, Lilly ST, Santos NR, Gilbert RO, Goetze L, Bryant CE, White JO, Cronin J, Sheldon IM. 2009. Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. *Reprod Biol Endocrinol*, 7:55.

Heuwieser W, Iwersen M, Goetze L. 2011. Efficacy of carprofen on conception rates in lactating dairy cows after subcutaneous or intrauterine administration at the time of breeding. *J Dairy Sci*, 94:146-151.

Heuwieser, W, Grunert, E. 1987. Significance of chemotactic activity for placental expulsion in cattle. *Theriogenology*, 27: 907- 912.

Hill J, Gilbert R. 2008. Reduced quality of bovine embryos cultured in media conditioned by exposure to an inflamed endometrium. *Aust Vet J*, 86:312-316.

Huang Y, Chuan H, Haifeng Y, Ruichen L, Xinxin F, Xiaoyan L, Jian H, Weiyun C, Yuehui Z. 2019. Inflamm-Aging: a new mechanism affecting premature ovarian insufficiency. *J Immunol Res*, 1-7.

Jiemtaweeboon S, Shirasuna K, Nitta A, Kobayashi A, Schuberth HJ, Shimizu T, Miyamoto A. 2011. Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. *Reprod Biol Endocrinol*, 9:79.

Katila T. 2012. Post mating inflammatory responses of the uterus. *Reprod Domest Anim*, 47(Suppl 5), 31-41.

Kaufmann TB, Drillich M, Tenhagen BA, Forderung D, Heuwieser W. 2009. Prevalence of bovine subclinical endometritis 4h after insemination and its effects on first service conception rate. *Theriogenology*, 71:385-91.

Kimura K, Goff JP, Kehrli ME, Reinhardt TA. 2002. Decreased neutrophil function as a cause of retained placenta in dairy cattle. *J Dairy Sci*, 85: 544- 550.

König S, May K. 2019. Phenotyping strategies and quantitative-genetic background of resistance, tolerance and resilience associated traits in dairy cattle. *Animal*, 13:897-908.

Korzekwa A, Murakami S, Wocławek-Potocka I, Bah MM, Okuda K, Skarzynski DJ. 2008. The influence of TNFalpha on the secretory function of bovine corpus luteum; TNF and its receptor expression during the estrous cycle. *Reprod Biol*, 8:245-262.

Kvidera SK, Horst EA, Abuajamieh M, Mayorga EJ, Fernandez MV, Baumgard LH. 2017. Glucose requirements of an activated immune system in lactating Holstein cows. *J Dairy Sci*, 100(3):2360-2374.

Lavon Y, Ezra E, Leitner G, Wolfenson D. 2011. Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. *J Dairy Sci*, 94:4538-4535.

LeBlanc SJ. 2012. Interactions of Metabolism, Inflammation, and Reproductive Tract Health in the Postpartum Period in Dairy Cattle. *Reprod Domest Anim*, 47:18-30.

LeBlanc SJ. 2014. Reproductive tract inflammatory disease in postpartum dairy cows. *Animal*, 8(s1):54-63

Ling T, Hernandez-Jover M, Sordillo LM, Abuelo A. 2018. Maternal late-gestation metabolic stress is associated with changes in immune and metabolic responses of dairy calves. *J Dairy Sci*, 101:6568-6580.

Loeffler SH, de Vries MJ, Schukken YH. 1999. The effects of time of disease occurrence, milk yield, and body condition on fertility of dairy cows. *J Dairy Sci*, 82:2589-2604.

Lucy MC, Evans TJ, Pock SE. 2016. Lymphocytic foci in the endometrium of pregnant dairy cows: characterization and association with reduced placental weight and embryonic loss. *Theriogenology*, 86:1711-1719.

Machado Pfeifer LF, de Souza Andrade J, Moreira EM, Reis da Silva R, Araújo Neves PM, Moreira da Silva G, Lemos IC, Schneider A. 2018 Uterine inflammation and fertility of beef cows subjected to timed AI at different days post partum. *Anim Reprod Sci*,197:268-277.

Maj JG, Kankofer M. 1997. Activity of 72-kDa and 92-kDa matrix metalloproteinases in placental tissues of cows with and without retained fetal membranes. *Placenta*, 18: 683- 687.

Marey MA, Yousef MS, Kowsar R, Hambruch N. 2016. Local immune system in oviduct physiology and pathophysiology: attack or tolerance? *Domest Anim Endocrinol*, 56:S205-S211.

McDougall S, Abbeloos E, Piepers S, Rao AS, Astiz S, Van Verwen T, Statham J, Perez-Villalobos N. 2016. Addition of meloxicam to the treatment of clinical mastitis improves subsequent reproductive performance. *J Dairy Sci*, 99:1-17.

Mitchell J R, S enger P L, Rosenberger J L. 1985. Distribution and retention of spermatozoa with acrosomal and nuclear abnormalities in the cow genital tract. *J Anim Sci*, 61:956-967.

Miyamoto A, Shirasuna A, Hayashi KG, Kamada D, Kawashima C, Kaneko E, Acosta T, Matsui M. 2006. Potential use of color ultrasound as a tool for reproductive management: new observations using color ultrasound scanning that were not possible with imaging only in black and white. *J Reprod Dev*, 52:153-160.

Miyamoto A, Shirasuna K, Wijayagunawardane MP, Watanabe S , H ayashi M , Yamamoto D , M atsui M , Acosta TJ . 2005. Blood flow: a key regulatory component of corpus luteum function in the cow. *Domest Anim Endocrinol*, 29:329-39.

Murdoch WJ , C olgin D C, E llis J A. 1997. Role of Tumor Necrosis Factor-alpha in the ovulatory mechanism of ewes. *J Anim Sci*, 75:1601-5.

Nelli RK, De Koster J, Roberts JN, De Souza J, Lock AL, Ra phael W, Agnew D, Co ntreras GA. 2019. Impact of uterine macrophage phenotype on placental retention in dairy cows. *Theriogenology*, 127:145e152.

Neuvians TP, S chams D, Berisha B , P faffl MW. 2004. Involvement of proinflammatory cytokines, mediators of inflammation and basic Fibroblast Growth Factor in prostaglandin F2alpha - induced luteolysis in bovine corpus luteum. *Biol Reprod*, 70:473-480.

Perrin L , B ostelmann R W, She ldon I M. 2007. Reduced conception rates associated with bovine mastitis during a «window of opportunity». *Vet Rec*,161:61-62.

Piersanti R L, H orlock A D, B lock J, S antos JE P, Sheldon IM, Bromfield JJ. 2019 Persistent effects on bovine granulosa cell transcriptome after resolution of uterine disease. *Reproduction*, pii: REP-19-0037.R1.

Price J C, Sheldon I M. 2013. Granulosa cells from emerged antral follicles of the bovine ovary initiate inflammation in response to bacterial pathogen-associated molecular patterns via Toll-like receptor pathways. *Biol Reprod*, 89(5):119.

Pugliesi G , K han F .A., H annan M .A., Beg M .A., Carvalho G .R., Ginther O J. 2012. Inhibition of prostaglandin biosynthesis during postluteolysis and effects on CL regression, prolactin, and ovulation in heifers. *Theriogenology*, 78: 443-454.

Ribeiro E S, Carvalho M R. 2017. Impact and mechanisms of inflammatory diseases on embryonic development and fertility in cattle. *Anim Reprod*, 14:589-600.

Ribeiro ES, Gomes G Greco LF, C erri RLA, Vieira-Neto A , Monteiro P LJ Jr , Lima F S, B isinotto R S, Thatcher WW, Sa ntos JEP. 2016. Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. *J Dairy Sci*, 99:2201-2220.

Ruiz R, Tedeschi LO, Sepúlveda A. 2017. Investigation of the effect of pegbovigrastim on some periparturient immune disorders and performance in Mexican dairy herds. *J Dairy Sci*, 100:1-13.

Scenna F N, H ockett M E, T owns T M, Sa xton A M, Rohrbach N R, We hrman ME, Sc hrick F N. 2005. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. *Prostaglandins Other Lipid Mediat*, 8:38-45

Sheldon IM, C ronin JG, Bromfield JJ. 2019. Tolerance and innate immunity shape the development of postpartum uterine disease and the impact of endometritis in dairy cattle. *Annu Rev Anim Biosci*, 7:361-384.

Sheldon I M, C ronin J G, P ospiech M , T urner M L. 2018. Mechansisms linking metabolic stress with innate immunity in the endometrium. *J Dairy Sci* 101:3655-3664.

Sheldon IM, Lewis GS, LeBlanc S. Gilbert RO. 2006. Defining post-partum uterine disease in cattle. *Theriogenology*, 65:1516-1530.

Sheldon IM, O wens SE , Turner M L. 2017. Innate immunity and the sensing of infection, damage and danger in the female genital tract. *J Reprod Immunol* 119:67-73.

Shirasuna K, J iemtaweeboon S, R addatz S, N itta A, Schuberth HJ, B ollwein H, Shimizu T, M iyamoto A. 2012a. Rapid accumulation of polymorphonuclear neutrophils in the corpus luteum during prostaglandin F2alpha-induced luteolysis in the cow. *PlosOne*, 7: e29054.

Shirasuna K , N itta A , Si neenard J , Sh imizu T , Bollwein H , Miyamoto A . 2012b. Vascular and immune regulation of corpus luteum development, maintenance and regression in the cow. *Domest Anim Endocrinol*, 43:198-211.

Silva Silveira PA, B utler WR, LaCount SE, O verton TR, C astilho B arros C , S chneider A . 2019. Polymorphisms in the anti-oxidant paraoxonase-1 (PON1) gene associated with fertility of post partum dairy cows. *Theriogenology*, 125:302-309.

Stassi AF, B aravalle ME, B elotti EM, R ey F, G areis NC, Díaz PU, R odríguez FM, Leiva CJ, O rtega HH, Salvetti NR. 2017. Altered expression of cytokines IL-1alpha, IL-6, IL-8 and TNFalpha in bovine follicular persistence. *Theriogenology*, 97:104-112.

Thompson-Crispi K A, H ine B , Q uinton M , M iglior F, M allard BA. 2012. Short communication: association of disease incidence and adaptive immune response in Holstein dairy cows. *J Dairy Sci*, 95:3888-3893.

Turner ML, C ronin J G, H ealey G D, Sheldon I M. 2014. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. *Endocrinology*, 155(4):1453-65.

Walter I , B oos A . 2001. Matrix metalloproteinases (MMP-2 and MMP-9) and tissue inhibitor-2 of matrix metalloproteinases (TIMP-2) in the placenta and interplacental uterine wall in normal cows and in cattle with retention of fetal membranes. *Placenta*, 22(5):473-83.

Yousef M S, A bd-Elhafeez H H, Talukder A K, Miyamoto A . 2019. Ovulatory follicular fluid induces sperm phagocytosis by neutrophils, but oviductal fluid around oestrus suppresses its inflammatory effect in the buffalo oviduct in vitro. *Mol Reprod Dev*, 86(7):835-846.

001 TAI/FTET/AI

Time of ovulation after the induction and reproductive outcomes in mares inseminated with frozen semen

Maria Fernanda Zamai[1], Isabele Picada Emanuelli[1], Antonio Hugo Bezerra Colombo[1], Josmar Mazucheli[2], Márcia Aparecida Andreazzi[1], Fábio Luiz Bim Cavalieri[1]

[1]UniCesumar - UniCesumar, Maringá, PR, Brasil; [2]UEM - Universidade Estadual de Maringá, Maringá, PR, Brasil.

The use of cryopreserved equine semen has some limiting factors inherent to gametes: the reduction of sperm viability inside the female reproductive tract compared to fresh semen; and the viability of the oocyte in the physiologically reduced genital tract (8 to 12 h), varying according to the stage of development in which it is at the time of ovulation. In this way, controlling follicular growth, inducing ovulation and controlling the time of ovulation could optimize pregnancy rate. This was a retrospective study aimed at investigating whether the moment of ovulation after artificial induction of ovulation interferes with the fertility of mares inseminated with frozen semen. A total of 107 estrus cycles from the reproductive season for the years 2017 and 2018 were analyzed from 23 Quarter horse mares, all submitted to the same protocol of induction of ovulation and inseminated with frozen semen. The ovulation induction protocol started on day -2 with administration of hCG 1633 IU IV (only in the first two cycles of each animal) + 1.0 mg / animal GnRH (deslorelin acetate) via IM. After 32h four ultrasound evaluations were started to identify ovulation every 4h, in order to divide the animals into 3 groups according to the time of ovulation after induction of ovulation: G36; G40; G44 (ovulation 36, 40 and 44 hours after the inducer protocol). Animals that had ovulated at the first evaluation in 32 hours were discarded from the experiment. Identified at ovulation, artificial insemination was performed using the same stallion. Embryo collection was performed in D9 (D0 = ovulation time) and after transfer in recipient mares, the diagnosis of gestation was performed in D14. The dependent variables analyzed were embryo recovery rate (TER) and pregnancy rate (TP). The data were analyzed in the program R by the chi-square test ($P < 0.05$). The embryo recovery rate (p-value = 0.8116) and the pregnancy rate (p-value = 0.177) were similar in all groups of ovulation times, respectively: G1- 60% (29/48) and 86% (25/29); G2-65% (31/47) and 70% (22/31); G3 - 58% (7/12) and 57% (4/7). Instead of few number animal in G3, this study indicated that the time of ovulation after the artificial induction of ovulation does not interfere on the rate of embryo recovery and the pregnancy rate in mares inseminated with frozen semen.

002 TAI/FTET/AI

Fertility of lactating dairy cows submitted to a progesterone-based FTAI protocol initiated with GnRH or GnRH plus estradiol benzoate

Natália Picoli Folchini[1], Carlos Eduardo Cardoso Consentini[1], Jéssica Cristina Lemos Motta[1], Leonardo de França e Melo[2], Rodrigo Lemos Olivieri Rodrigues Alves[1], Lucas Oliveira e Silva[1], Matheus Cruz Silva[1], Mayara Silvestri[1], Danielle Nunes Gurgeira[1], Milo Charles Wiltbank[3], Roberto Sartori[1]

[1]ESALQ/USP - Department of Animal Science - Luiz de Queiroz College of Agriculture of University of São Paulo, Piracicaba-SP, Brazil; [2]Geneal - Geneal - Genetics and Animal Biotechnology, Uberaba-MG, Brazil; [3]UW, Madison, Department of Dairy Science - University of Wisconsin-Madison, Madison, WI, USA.

The aim was to compare two strategies to initiate resynchronization FTAI protocols in lactating dairy cows. A total of 543 lactating Holstein cows from two farms, producing 41.7 ± 0.71 kg/d of milk and with BCS of 3.1 ± 0.02 (1 to 5 scale) were used. Weekly, at time of pregnancy diagnosis, which was performed 31 ± 3 d after previous insemination, cows were randomly assigned to 1 of 2 groups. On D0, cows from Group EB/G received 2 mg estradiol benzoate (EB) and 16.8 μg buserelin acetate (GnRH), whereas in Group G, cows only received 16.8 μg GnRH. All cows received a new 2 g intravaginal progesterone (P4) device on D0. Seven d later (D7) every cow received 0.530 mg sodium cloprostenol (PGF), and on D8 another PGF was administered concomitant with 1 mg estradiol cypionate (EC) and P4 withdrawal. Cows received FTAI on D10. All hormones were from GlobalGen Vet Science, Jaboticabal, Brazil. Cows received a tail-head device for estrus detection (BOViFLAG) on D8 and were considered in estrus when the paint of the device had been removed by D10. Statistical analyses were done by PROC GLIMMIX of SAS 9.4 ($P \leq 0.05$). Ovulation rate after D0 was 54.6% (283/518) and did not differ between groups. Cows initiating the protocol with CL had lower ovulation rate than cows without CL on D0 [45.8 (173/378) vs. 78.6% (110/140)]. Luteolysis between D0 and D7 was greater in EB/G group on D0 compared to G group [41.4 (75/181) vs. 29.4% (58/197)]. Consequently, the proportion of cows with CL on D7 was higher in G group compared to EB/G group [87.0 (234/269) vs. 81.0% (209/258)], as well as number of CL on D7 [1.24 (269) vs. 1.08 (259)]. Cows receiving only GnRH on D0 expressed more estrus than cows from EB/G group [84.3 (231/274) vs. 77.8% (203/261)]. Pregnancy per AI (P/AI) of primiparous and multiparous were similar [38.1 (77/202) vs. 36.2% (118/326), respectively]. Cows with BCS>2.75 had greater P/AI compared to cows with BCS ≤ 2.75 [42.4 (139/328) vs. 29.3% (60/205)]. Cows with or without CL on D0 had similar P/AI [38.4 (147/383) vs. 34.9% (52/149), respectively], as well as cows with or without CL on D7 [38.4 (170/443) vs. 34.5% (29/84), respectively]. Luteolysis prior to the PGF treatment did not affect P/AI [36.8 (49/133) vs. 39.2% (96/245), for cows with or without CL regression after D0]. The P/AI was greater in cows expressing estrus compared to cows not showing estrus at the end of the protocol [44.0 (191/434) vs. 9.9% (10/101)] Finally, cows initiating the FTAI protocol with EB plus GnRH had similar fertility to cows initiating only with GnRH [37.8 (101/267) vs. 37.3% (103/276)]. In summary, even with higher incidence of luteolysis in cows receiving EB at the beginning of the protocol, P/AI was similar between experimental groups, maybe due to the high ovulation rate achieved in the study and the lack of effect of CL presence on D7 on fertility.
Acknowledgements: FAPESP, CNPq, GlobalGen Vet Science.

003 TAI/FTET/AI

The effect of subclinical endometritis on reproductive performance in postpartum *Bos indicus* beef cattle

Ramiro Vander Oliveira Filho[1], Gustavo Azevedo de Mello[2], Abigayle Pollock[3], Jose Luiz Moraes Vasconcelos[4], Ronaldo Cerri[5], Ky Pohler[1]

[1]TAMU - Texas A&M University, College Station, Texas, USA; [2]PUC - Pontifical Catholic University of Minas Gerais, Poços de Caldas, MG, Brazil; [3]UTK - University of Tennessee, Knoxville, Tennessee, USA; [4]UNESP - São Paulo State University, Botucatu, São Paulo, Brazil; [5]UBC - University of British Columbia, Vancouver, British Columbia, Canada.

Reproductive inefficiency can be caused by several factors including management issues, cow and bull infertility, heat stress, embryonic mortality and uterine disease, which leads to major losses for both the beef and dairy industries. Postpartum uterine disease has been well documented in dairy cows as well as the resulting decrease in fertility and increased reproductive culling. However, there is very little known about the incidence or its effect in beef cattle. The objective of the present study was to diagnose subclinical endometritis postpartum during a timed AI protocol and assess the presence of polymorphonuclear neutrophils (PMN) in the uterine lumen and its effect on pregnancy outcomes of Bos indicus beef cattle. A total of 320 multiparous Nellore cows (days postpartum 28 - 61) were assigned to receive an estradiol-based estrus synchronization + timed-AI protocol (TAI, d0). Concurrently with AI, estrus expression was evaluated using Estrotect Heat Detector patches. Endometrial cytology samples were taken by Cytobrush right before the beginning of the protocol (d-11). For each slide, 300 cells (neutrophils and/or endometrial epithelial cells) were counted in three different locations by two operators and the polymorphonuclear neutrophils ratio (% PMN) was assessed. Cows with 5% or higher PMN were considered as having subclinical endometritis. Pregnancy diagnosis was performed by transrectal ultrasonography 30 days after TAI. Data were analyzed with PROC GLIMMIX model in SAS 9.4. Cows that expressed estrus at or before d0 had increased ($P < 0.01$) pregnancy per AI compared to no estrus expression (58.9% ± 6.8 vs. 23.7% ± 5.2; respectively); however, no interaction between PMN and estrus expression was observed ($P = 0.84$). The prevalence of cows diagnosed as positive for subclinical endometritis was 7.5% (n = 24). Cows presenting PMN higher than 5.0 % had less pregnancies per AI ($P = 0.02$) compared to cows presenting PMN lower than 5% (29.6% ± 9.7 vs. 53.1% ± 3.1; respectively). In conclusion, subclinical endometritis does happen in Bos indicus postpartum cows, which led to a lower probability of these cows becoming pregnant at the first TAI.

004 TAI/FTET/AI

Effect of follicle diameter at the moment of pregnancy diagnosis by Doppler (22 days after TAI) on pregnancy rate of Nellore (*Bos indicus*) cows

Gabriel Cunha Cruz[1], Laísa Garcia da Silva[2], Odair Antonio Alves de Melo Neto[1], David Bueno Lourenço Filho[1], Matheus Furtado Pereira[1], Emiliana Oliveira Santana Batista[2], Alexandre Henryli Souza[2], Everton Luiz Reis[3], Guilherme Machado Zanatta[2], Romulo Germano de Rezende[2], Bruna Lima Chechin Catussi[2], Pietro Sampaio Baruselli[2]

[1]CRIA Fértil - CRIA Fértil, Goiânia, GO, Brasil; [2]USP - Universidade de São Paulo, São Paulo, SP, Brasil; [3]Agener - Agener União Química, São Paulo, SP, Brasil.

The objective of this study was evaluate the relationship of follicle diameter at the moment of pregnancy diagnosis by Doppler (22 days after TAI) with pregnancy rate of the first and second TAI. For this experiment, 844 multiparous Nellore (Bos indicus) cows were early-resynchronized 14 days after the first TAI. Considering day 0 the day of first TAI, on day 14, cows received an intravaginal device containing 0.75g of P4 (Prociclar®, Ceva, Paulínea) and were randomly distributed to receive a injectable P4 (short action-140mg of Progecio®, Agener União, São Paulo, or short action-100mg of Sincrogest® injetável, Ourofino, Cravinhos). On day 22, P4 device was removed and pregnancy diagnosis was performed by US Color Doppler (M5, Mindray, China). Cows with CL vascularization greater than 25% were considered pregnant. In addition, the largest follicle of all cows was measured. Cows considered non-pregnant received 300IU of eCG (Novormon®, Zoetis, Brasil), 1mg of EC (ECP®, Zoetis, Brasil) and 12.5mg of dinoprost (Lutalyse®, Zoetis, Brasil) and received second TAI on D24. Pregnant cows were submitted to pregnancy confirmation 10 days later (D32) when the false positive rate was evaluated. Follicle diameters were classified by tercile for first TAI [small (4 to 7.8 mm; mean: 5.4±1.5 mm), medium (7.9 to 10.4 mm; mean: 9.1±0.7mm) and large (10.5 to 28.4 mm; mean 13,2±2,7mm); n= 844], and for second TAI [small (4 to 11.1 mm; mean: 9.0±2.0mm), medium (11.2 to 13.2 mm; mean 12.2±0.5mm) and large (13.3 to 27.7 mm; mean 15.7±2.7 de mm); n=289] to analyze the effect of the follicle diameter on pregnancy rate of the first and second TAI. Statistical analyses were performed by GLIMMIX procedure of SAS®. There was an effect of the dominant follicle diameter on D22 on pregnancy rate of the first TAI [small: 92.5%(266/287), medium: 83.1%(231/279) and large: 20.5%(57/278); P<0.0001] and on pregnancy confirmation [small: 83.8%(231/266), medium: 79.9%(198/231), large: 80.7%(46/57); P<0.0001]. There was no difference for false positive rate of first TAI between terciles [small: 13.1% (35/266), medium: 14.2% (33/231), large: 19.3% (11/57); P=0.4326. There was no effect of follicle diameter on the second TAI [small= 57.7% (56/96), medium: 64.8% (64/96) and large: 50.9% (55/97); P=0.2005]. There was no effect of injectable P4 source on pregnancy rate of first TAI [Progecio®: 63.7%(226/355), Sincrogest®: 67.1%(328/489); P=0.3019], on second TAI [Progecio®: 56.75%(82/129), Sincrogest®: 52.03%(93/160); P= 0.4520] and on false positive rate [Progecio®: 11.8%(28/226), Sincrogest®: 15.0%(51/328); P=0.2763]. It is concluded that cows positive for pregnancy diagnosis by Doppler that present large follicles (>10.5mm) on day 22 have lower pregnancy rate on first TAI. It was not verified effect of follicle diameter on false positive rate (pregnant on D22 then non-pregnant on D30). There was no effect of follicle diameter of non-pregnant cows on pregnancy rate of the second TAI.

005 TAI/FTET/AI

Effect of nutritional supplementation with blocks on pre and/or post partum in reproductive efficiency of Nellore (*Bos indicus*) primiparous cows submitted to TAI

Bruna Lima Chechin Catussi[1], Laísa Garcia da Silva[1], Guilherme Machado Zanatta[1], Rafaela Maria Sutiro Angelieri[4], Emiliana Santana Batista[1], Juan Fernando Morales Gómez[2], Fernando José Schalch Júnior[3], Pietro Sampaio Baruselli[1]

[1]VRA-FMVZ/ USP - Departamento de Reprodução Animal da Universidade de São Paulo, São Paulo, SP; [2]FZEA/USP - Departamento de Qualidade e Produtividade Animal da Universidade de São Paulo, Pirassununga, SP; [3]Minerthal - Minerthal Produtos Agropecuários Ltda, São Paulo, SP; [4]Mater - Fazenda Mater, Santa Rita do Pardo, MS, Brasil.

The objective of this study was evaluate the reproductive efficiency and calves performance of Nellore primiparous cows that received block supplementation (SB) or control supplementation (SC). The study was conducted in Farm Mater, with 417 Nellore (Bos indicus) pregnant heifers, aging 31±2.3 months. 90 days before calving date (D-90), heifers were randomly distributed in 4 groups: Group B-B: received SB 90 days before and 120 after calving (n=89); Group B-C: received SB 90 days before and SC 120 days after calving (n=103), Group C-B: received SC 90 days before and SB 120 days after calving (n=117) and Group C-C: received SC 90 days before and SC 120 days after calving (n=108). Supplementation: SB=350g/kg of crude protein; 210g/kg NNP e 300mg/kg of sodium monensin (40g/100kg of live weight/day/animal; offered weekly); SC= 400g/kg of crude protein e 320g/kg NNP (40g/100kg of live weight/day/animal; offered every two days). Body weight (BW) and body condition score (BCS) of cows and body weight of calves (WC) over time were evaluated. The subcutaneous rump fat thickness (RUFAT) and rib fat thickness (RFAT) were measured by US (D40). Blood samples were also taken in subgroup to measure the insulin concentration (INS) and IGF-1 (D-90, D40 and D80). Cows were synchronized to receive two TAI (8 days P4/E2-based protocol,PGF/eCG/EC at P4 removal and AI 48 hours later) followed by natural breeding for 50days. Pregnancy diagnosis was done by US 30 days after TAI and 30 days after bull exposure. Statistical analyses were performed by orthogonal contrast using SAS®[Contrast 1(C1): SC vs. SB (C-Cvs.B-B+B-C+C-B); Contrast 2(C2): SB in 2 periods (pre and post-partum) vs. SB in 1 period (pre or post-partum; B-B vs. B-C+C-B); Contrast 3(C3): SB on prepartum vs. SB on pos-partum (B-Cvs.C-B). On C1, there was a difference for pregnancy rate (PR) of first TAI (SC=41.7% vs. SB=51.5%; P=0.04), on final PR (SC=74.1%vs. SB=80.9%; P=0.07), on RUFAT (SC=2.9mm vs. SB=3.4mm;P=0.003) and on RFAT (SC=1.2mm vs. SB=1.6mm; P=0.004]. SB increased BCS (P=0.02), IGF-1 (P=0.05), INS (P=0.05) and WC over time (P=0.01). On C2, SB in 2 periods increased BCS (P=0.05) and WC over time (P=0.0004). There was an interaction BCS*time on C3 (P<0.0001). Supplemented cows on pre-partum presented higher BCS on D40 and animals supplemented on post-partum presented higher BCS on D80 and D170. There was also an interaction insulin*time (P=0.06). Animals supplemented on pre-partum had higher INS on D40 and cows supplemented on post-partum presented higher INS on D80. In conclusion, cows supplemented with blocks presented an increase on RFAT, RUFAT, BCS, INS, IGF-1, PR for first TAI, final PR and on CW. Still, cows supplemented on pre and post partum had higher BCS and heavier calves. Also, cows only supplemented on pre partum presented higher BCS on D80 and D170. On D40, Insulin increases for supplemented cows on pre-partum. However, on D80 insulin is higher in animals supplemented on post partum.

006 TAI/FTET/AI

Estrus synchronization and reproductive performance of ewes treated with dinoprost or intravaginal progesterone device outside the breeding season

Guilherme Henrique Freitas Seugling[1], Maria Paula Marinho de Negreiros[1], Marcela Louvaes Rodrigues[1], Ana Clara Bertolini Pereira[1], Rian Lolico Chamorro[1], Gabriella Carolina Silva[1], Luiz Aguinaldo Ricetto Pegorari Júnior[1], José Gabriel Rigo Kairuz[2], Petrônio Pinheiro Porto[1], Thales Ricardo Rigo Barreiros[1], Wanessa Blaschi[1]

[1]UENP - Universidade Estadual do Norte do Paraná; [2]UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

A trial was conducted to investigate the estrus manifestation, conception and pregnancy rates using different hormonal treatments outside the natural breeding season. A total of 83 multiparous crossbreed Santa Inês x Dorper ewes, 3–5 years of age, a body weight of between 40 and 45 kg and body condition score (BCS) 2.9±0.4 (scale 1: emaciated to 5: obese) were used. The experiment was conducted during spring at the commercial ranch in Bandeirantes city, Paraná State, Brazil (latitude 23.1054, longitude 50.3715, altitude 419m). The seasonality for crossbred ewes at this latitude is low. The animals were divided in a similar way to BCS in two experimental groups. Group PGF (G-PGF, n=43), the animals received 2.5 mg of dinoprost (Lutalyse, Zoetis, Brazil) by intramuscular route (IM); (D-8). Eight days later (D0), ewes received again 2.5 mg of dinoprost by IM route. Group CIDR (G-CIDR, n=40), ewes received (D8) a 0.33 g of progesterone intravaginal device (CIDR, Zoetis, Brazil). Eight days later (D0), the devices were removed plus and 2.5 mg of dinoprost by IM route. Approximately 12 hours after the dinoprost application, ewes were submitted to natural mating in the proportion of one ram to 10 ewes, at night (6 PM to 6 AM), for six consecutive days. The rams were diagnosed able to reproduction after an andrological examination. The rams were marked daily with paint in the sternal region and manifestation of estrus was measured by marking with paint on the pelvic region of the females at the end of each natural mating period. The pregnancy diagnosis was made by transrectal ultrasonography (Mindray, China, 7.5 MHz) 30 days after the end of natural mating. The results were analyzed by logistic regression ($P < 0.10$). There was no interaction of BCS with estrus manifestation rate ($p = 0.34$), conception rate ($p=0.53$) and pregnancy rate ($p = 0.45$). The rate of estrus manifestation was higher ($p= 0.06$) in G-CIDR [82.5%, (33/40)] than G-PGF [44.1% (19/43)]. Conception rate was similar ($p = 0.24$) in G-PGF [73.6% (14/19)] and G-CIDR [54.4% (18/33)]. The pregnancy rate was similar ($p=0.15$) in G-CIDR: 45.0 %(18/40) and G-PGF: 32.5 %(14/43). In conclusion, progesterone treatment possibly induced cyclicity in sheep since the study was performed outside the breeding season, although the genetic group is considered of low reproductive seasonality. The conception and pregnancy data presented promising results for the development of new studies to understand the use of estrus synchronization. Acknowledgments: Fundação Aralcária do Estado do Parana, Cabanha Sal no Cocho e Laboratórios Zoetis

007 TAI/FTET/AI

Effect of treatment with GnRH at the moment of embryos transfer on the follicular dynamics and conception rate of buffalo recipients

Damiana Chello[1], Julio Cesar Barboza da Silva[2], Guilherme Pugliesi[1], Nelcio Antonio Tonizza de Carvalho[4], Julia Gleyci Soares[1], Igor Garcia Motta[1], Carlos Alberto Souto Godoy Filho[1], Rodolfo Daniel Mingoti[3], Pietro Sampaio Baruselli[1]

[1]FMVZ/USP - Faculdade de Medicina Veterinária e Zootecnia/Univeridade de São Paulo; [2]UniFAJ - Centro Universitário de Jaguariúna, Jaguariúna, SP; [3]MSD - MSD - Saúde Animal, São Paulo - SP; [4]UPD/APTA - Unidade de Pesquisa e Desenvolvimento de Registro, Vale do Ribeira, SP, Brasil.

The objective of the present study is to evaluate the effect of GnRH treatment at the time of TET on the conception rate of buffalo embryo recipients. The hypothesis of the present study is that treatment with GnRH on the TET day induces the formation of an accessory corpus luteum (CL), increases the plasma concentrations of progesterone (P4), and, consequently, increasing conception rate. This study involved 265 Murrah and crossbreeding Murrah x Mediterranea buffaloes, aged from 2 to 15 years, without any fertility problems and with a good body condition score (BCS> 2.5). The experiment was carried out in 4 different farms: F1 (n=70), F2 (n=97), F3 (n=63) and F4 (n=35). The recipients were synchronized with the following protocol: on day 0 (D0) the buffaloes received an intravaginal P4 device (Prociclar® 750 mg; Ceva) associated with i.m. administration of 2 mg of estradiol benzoate (Sincrodiol®, Ourofino) and 0.53 mg of sodium cloprostenol (PGF2α; Sincrocio®, Ourofino). On day 9 (D9), the P4 device was removed and 0.53 mg PGF, 400 IU equine chorionic gonadotrophin (eCG; Sincro eCG®, Ourofino) and 1.0 mg estradiol cypionate (SincroCP®, Ourofino) were administrated (i.m.). On day 18 (D18), an ultrasound evaluation (DP 2200®, Mindray) was performed. Only the buffaloes that had a CL> 10 mm received a in vitro produced embryo. At TET (D18), recipients were divided into two groups: Group GnRH (G-GnRH; n=134) which received 25 μg of lecirelin i.m. (Gestran Plus, Tecnopec) and the Control Group (G-CONT; n=131), which did not receive any treatment. Additionally, a subset of 22 recipients was subjected to Doppler ultrasound (MyLabDelta, Esaote) examination in the following days: D18, D22, D25, D28 to evaluate the ovulation rate, the formation of an accessory CL and the vascularization of the CLs. Blood was taken from 35 animals in the same days for plasma P4 concentration analysis. Thirty days following TET, a new ultrasound examination was performed to evaluate conception rate. Statistical analyzes were performed using the SAS® GLIMMIX procedure. Conception rate differed across farms (P <.0005) [F1 = 34.2% (24/70); F2 = 6.1% (6/97); F3 = 31.7% (20/63); F4= 28.3% (10/35)]. Animal category also influenced conception outcomes and lactating buffaloes had lower fertility (12.8%) than non-lactating buffaloes (30%) or heifers (33.3%; P = 0.004). No significant difference in terms of conception rate was observed between the two experimental groups (G-GnRH = 23.8%, G-CONT = 21.4%, P = 0.6). Out of the 22 buffaloes examined with Doppler ultrasound, 36% (n = 4/11) of them showed an ovulation following the treatment with GnRH with a subsequent creation of an accessory CL. The GnRH treatment didn't have any effect on the plasma P4 concentration (P = 0.4). However, the recipients that ovulated after GnRH treatment presented higher P4 concentration (P=0.02). In conclusion, treatment with GnRH at the time of ET did not increase conception rate in buffalo recipients.

008 TAI/FTET/AI

Efficiency of the protocol of cyclicity induction with injectable progesterone in prepubertal 2-year-old Nelore (*Bos indicus*) Heifers

João Abdon Santos[1], Bruno González Freitas[2], Bruna Martins Guerreiro[2], Evandro Davanço Ferreira de Souza[2], Fabio Rocha[2], Fainer Lincoln[1], Ygor Ernandes Vaz da Silva Braga[1], Bruna Lima Chechin Catussi[3], Laísa Garcia da Silva[3], Augusto Rodrigues Felisbino Neto[2,3], Pietro Sampaio Baruselli[3]

[1]JA Reprogen - JA Reprogen, Eunápolis/BA; [2]Ourofino Saúde Animal - Ourofino Saúde Animal, Cravinhos, SP, Brasil; [3]VRA - FMVZ/USP - Departamento de Reprodução Animal - FMVZ/USP, São Paulo, SP, Brasil.

The present study evaluated the efficacy of a single-dose management protocol using long-acting injectable progesterone (P4) in 2-year-old Nellore (Bos indicus) heifers to induce cyclicity. The experiment was carried out in 3 farms and 259 Nellore (Bos indicus) heifers of 23.6 ± 1.5 months of age, weighing 335.4 ± 27.7 kg were used. All animals were evaluated by ultrasonography (M5 Vet, Mindray®, China) at day -10 to detect the presence of corpus luteum (CL). Only prepubertal heifers (without CL) were randomly distributed in three experimental groups. In the group Control Device (GCD; n = 88), the animals received a 1g progesterone P4 intravaginal device previously used (fourth use, Sincrogest®, Ourofino, Cravinhos, SP), associated with application of 1 mg i.m. of estradiol cipionate (EC) (SincroCP®, Ourofino, Cravinhos, SP) on the day of P4 device removal. In the Group P4 Injectable + EC (GP4I + EC; n = 84) heifers received 150mg i.m. of long-acting P4 (Sincrogest®, Ourofino, Cravinhos, SP) on day 0 and 1mg i.m. of EC (estradiol cipionate; SincroCP®, Ourofino, Cravinhos, SP) on day 12. Finally, in the Group P4 Injection Only (GP4I; n = 87) the animals received only 150mg im of long-acting P4 Injection (Sincrogest®, Ourofino, Cravinhos, SP) on day 0. In all groups, TAI protocol was started 24 days after the induction protocol. After 30 days an ultrasound examination was performed to evaluate the pregnancy rate. Variables were analyzed by the GLIMMIX procedure of SAS® version 9.4. There was no interaction between treatment and farm (P = 0.34). The rate of cyclicity at the start of the IATF protocol was higher for the Group Device (GCD: 93.7% a; GP4I + EC: 80.1% b; GP4I: 80.3% b; P = 0.047). Heifers above 320 kg body weight had an increase in the rate of cycling in response to the induction protocol in relation to heifers below 320 kg (88.9% vs. 71.5%, P = 0.003). The pregnancy rate for the TAI protocol did not differ between the experimental groups (GCD = 42.8%, GP4I + EC = 60.8%, GP4 = 44.8%, P = 0.061). It can be concluded that the single-dose cycling induction protocol provides similar pregnancy rates to TAI compared to the protocols with intravaginal P4 and EC by the day of P4 device removal and to treatment with injectable P4 associated with EC.

Acknowledgments: Ouro Fino Saúde Animal, JA Reprogen

009 TAI/FTET/AI

Evaluation of early resynchronization protocol with the onset by 14 or 15 days after first TAI in Nellore (*Bos indicus*) heifers

Stella Mara Aparecida Morais[1], Laísa Garcia Da Silva[2], Bruna Lima Chechin Catussi[2], José Eduardo Pereira Lima Filho[1], Pietro Sampaio Baruselli[2]

[1]FAZENDA SEGREDO - Fazenda Segredo, MS, Brasil; [2]VRA-FMVZ/USP - Departamento de Reprodução Animal da Universidade de São Paulo VRA-FMVZ/USP, São Paulo, SP, Brasil.

The objective of this study was to evaluate the efficiency of early resynchronization protocol in Nellore heifers with the onset by 14 or 15 days after the first TAI and the efficiency of two different sources of injectable P4 on the beginning of resynchronization protocol. The study was performed on Fazenda Segredo (Bataguassu, MS, Brasil), where 747 Nellore (Bos indicus) heifers, with mean age of 13.5±1.3 months, weighing 279.3±40.9 kg, were randomly distributed in a 2 X 2 factorial arrangement of treatments after first TAI (Day 0). All heifers were resynchronized with a 1g intravaginal P4 device used thrice (Sincrogest®, Ourofino, Cravinhos, SP), than 4 experimental groups were formed: Group 14A [on day 14 received 50mg i.m. of injectable P4 (Afisterone®); n= 198], Group 14S [on day 14 received 50mg i.m. of injectable P4 (Sincrogest injetável®); n= 169], Group 15A [on day 15 received 50mg i.m. of injectable P4 (Afisterone®); n= 170] and Group 15S [on day 15 received 50mg i.m. of injectable P4 (Sincrogest injetável®); n= 210]. P4 devices were removed eight days later by the day of pregnancy diagnosis (D22 for Group 14 and D23 for Group 15) using Color Doppler ultrasonography (Mindray® M5Vet, China) (Ginther, 2007). Heifers with a CL vascularization greater than 25% were considered pregnant. Heifers diagnosed as non-pregnant (with low or no CL vascularization) received 0,530 mg i.m. of cloprostenol (Sincrocio®, Ourofino, Brasil), 200 IU of eCG (SincroeCG®, Ourofino, Brasil) and 0,5 mg of CE (SincroCp®, Ourofino, Brasil), and they were inseminated 48 hours later. Heifers diagnosed as pregnant by Doppler were submitted to another US 15 days later, for pregnancy confirmation and false positive evaluation. Heifers that received the second TAI were checked for pregnancy 22 days after bred by US Color Doppler again. Statistical analyses were performed by GLIMMIX procedure of SAS® 9.4 version. There was no interaction between injectable P4 and day of the beginning of the protocol (P= 0.64). There was no effect on pregnancy rate of the first TAI [D14= 52.3% (192/367) vs. D15= 51.6% (196/380); P=0.90] and second TAI [D14= 40.6% (71/175) vs. D15= 42.6% (78/183); P= 0.78] according to the day of the beginning of resynchronization protocol. False positive rate was lower for the group resynchronized on Day 15 [D14= 23.4% (45/192) vs. D15=13.3% (26/196); P=0.002]. It is concluded that the type of injectable P4 and the day of the beginning of resynchronization protocol have no effect on the pregnancy rate. However, the resynchronization started on day 15 contributed to a lower false positive rate and may be an alternative for the early resynchronization protocol. Acknowledgment: Fazenda Segredo

010 TAI/FTET/AI

Strategies for induction of ovulation for fixed-time ai in lactating dairy cows submitted to a novel presynchronization protocol

Carlos Eduardo Cardoso Consentini[1], Leonardo de França e Melo[2], Jessica Cristina Lemos Motta[1], Rodrigo Lemos Olivieri Rodrigues Alves[1], Lucas Oliveira e Silva[1], Mariana Contini[1], Felipe Belchior Sargaço[1], Natália Picoli Folchini[1], Guilherme Madureira[1], Milo Charles Wiltbank[3], Roberto Sartori[1]

[1]ESALQ/USP - Department of Animal Science, Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, Piracicaba, SP; [2] GENEAL - Geneal – Genetics and Animal Biotechnology, Uberaba, MG, Brazil; [3]UW - Madison - Department of Dairy Science, University of Wisconsin - Madison, Madison, WI 53706, USA.

The study evaluated strategies for induction of ovulation for first fixed-time AI (FTAI) postpartum in dairy cows submitted to Ovsynch-type protocols initiated after a presynchronization. A total of 909 lactating dairy cows (36.7±0.3 DIM and 38.9±0.6 kg/d of milk) from 6 herds were used. On D-15, cows received a used intravaginal progesterone (P4) device, and 7 d later (D-8), 1 mg estradiol cypionate (EC) and 0.530 mg cloprostenol (PGF), concomitant with P4 withdrawal. On D0 (onset of FTAI protocol), cows were randomly assigned to 1 of 3 groups, that differed only on the strategy to induce ovulation at the end of the protocol. On D0, cows received 16.8 μg buserelin acetate (GnRH) and insertion of a 2 g P4 device. On D6, cows received 0.530 mg PGF followed by a second PGF on D7, concomitant with P4 withdrawal. In Group EC, cows received 1 mg EC on D7 as inducer of ovulation. In Group EC/G, cows received EC on D7 and 8.4 μg GnRH administered 16 h before AI (56 h after the first PGF). Finally, in Group G, cows only received 8.4 μg GnRH 16 h before AI. The AI was performed on D9 (48 h after P4 removal) in all treatments and pregnancy diagnosis was performed 31 and 60 d after AI. Statistical analyses were performed with PROC GLIMMIX of SAS 9.4 ($P \leq 0.05$). The proportion of cows with CL at the beginning of presynchronization was 67.7% (427/631), and on D0 of FTAI protocols, it increased to 80.9% (433/535). On D6 (time of first PGF) 91.0% (342/376) of the cows had a CL. Ovulation rate after D0 was 63.3% (229/362). Pregnancy per AI (P/AI) was not different between cows with or without CL on D-15 [44.7 (191/427) vs. 38.7% (79/204)] and on D0 [44.3 (192/433) vs. 37.3% (38/102)], however, cows with CL on D6 had higher P/AI than cows without CL [45.9 (157/342) vs. 17.7% (6/34)]. Estrus expression was greater in cows receiving EC than cows only receiving GnRH [80.2 (203/253) vs. 46.1% (53/115)]. There was a tendency ($P = 0.07$) for interaction between treatment and estrus expression on P/AI on D31, in which estrus increased P/AI in EC group [53.7 (58/108) vs. 15.0% (3/20)], but not in cows with estrus in G group [41.5 (22/53) vs. 40.3% (25/62)]. The P/AI of primiparous was 1.5 times higher than multiparous in all groups, and overall, it was 55.3% (130/235) vs. 35.7% (101/283). There was no interaction between BCS and treatment, however, cows with BCS greater than 2.75 had higher P/AI than thinner cows [44.0 (243/552) vs. 30.7% (61/199)]. There were no differences between treatments on P/AI on D31, with overall P/AI of 40.4% (367/909). There was a tendency ($P=0.09$) for higher pregnancy loss in cows receiving only GnRH as ovulation inducer [19.5 vs. 10.1 vs. 12.2% for G, EC/G, and EC group, respectively]. In summary, although all ovulation treatments produced similar P/AI, further research is needed to optimize the presynchronization strategy and definitively confirm if lack of EC does not increase pregnancy loss. Acknowledgments: FAPESP, CAPES, CNPq and GlobalGen

011 TAI/FTET/AI

Effect of eCG on conception rate in 15 months old beef heifers, submitted to a FTAI program using a Progesterone device

A. Vater[1], J. Rodriguez[1], A.A Vater[1], J. Cabodevila[2], S. Callejas[2]

[1]Grupo IA total, Veterinarians Private Activity; [2]Área de Reproducción, Centro de Investigación Veterinaria de Tandil, (CIVETAN, CONICET-CICPBA), Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina.

The use of eCG is widely employed in Bos indicus breeds, however the use in Bos taurus is still controverse. The objective of this study was to evaluate if the use of eCG can influence conception rate of heifers submitted to FTAI. A total of 318 Hereford (Boss Taurus) heifers with 13 to 15 month old, were used from a commercial farms located in the state of Benito Juarez, Buenos Aires Province, Argentine. All heifers were evaluated in BSC (1-9), average (± S.D) 5.3±0.6; as well as ovarian structure (OS) by ultrasonography (Wed-3000 V), 1= corpus luteum; 2= Follicle ≥10 mm diam; 3= follicle <10 mm diameter). All heifers received on day 0 an intravaginal device with 0.6 g of P4 (Fertilcare 600®, PASRL, Argentine) and 2 mg of estradiol benzoate via i.m (Fertilcare Synchronization®, PASRL, Argentine). In D7, the device was removed and 0.265mg Cloprostenol Sodium (DProst®, PASRL, Argentine) and 0.5mg estradiol cypionate (Cipionato, PASRL, Argentine) were administered, both via i.m. The heifers where homogeneously subdivided into two groups: G0-control, n=159; and G250 [250 IU of eCG (Novormon®, Syntex; Argentine); n= 159. The FTAI was performed 52 to 54 hours after the device was removed and the pregnancy diagnosis was performed 32 days after FTAI by ultrasonography (Wed-3000V). The obtained data were analyzed by logistic regression through PROC Logistic of SAS® (α=0.05). There was no difference in the pregnancy rate (PR) between G0= 50.9% (81/159) and G-250 = 54.7% (87/159); however, the number of animals used does not allow to detect the differences observed as significant. The PR were not different between OS on day 0 [OS1 = 55.4% (31/56); OS2= 53.0% (44/83); OS3= 52.0% (93/179)]. Also, no differences were verified between PR and BSC group interaction: BSC5 = 52.4% (121/231); BSC6 = 53.7% (36/67); BSC7 = 55.0 % (11/20); P> 0.05). It is concluded that the use of 250 IU of eCG does not increase conception rate in Boss Taurus 13-15 months old heifers. More research will be necessary to conclude if the difference in the conception rate could be increased.

012 TAI/FTET/AI

Evaluation of a modification of the j-synch synchronization protocol on follicular dynamics and pregnancy rates in *Bos indicus* embryo recipients

Andrés Cedeño[1,2,4], Luis Morales[4], Luis Pinargote[1,3], Carlos Rivera[4], Ignacio Macías[4], Camila Guadalupe[4], Vanessa Figueroa[4], Gabriel Amilcar Bó[1,2]

[1]IRAC - Instituto de Reproducción Animal Córdoba, Córdoba, Argentina; [2]UNVM - Doctorado en Ciencias, Universidad Nacional de Villa María, Instituto A.P. de Ciencias Básicas y Aplicadas, Córdoba, Argentina; [3]INIAP - Instituto Nacional de Investigaciones Agropecuarias, Quevedo, Ecuador; [4]ESPAM - Laboratorio de Biotecnología de la Reproducción Animal, Escuela Superior Politécnica Agropecuaria de Manabí, Manabí, Ecuador.

The objective of this study was to compare the effect of the period of insertion of the progesterone (P4) device in the J-Synch protocol on follicular dynamics and pregnancy per embryo transfer (P/ET) in recipients. Sixty Bos indicus crossbreed beef cows, with a body condition between 2.5 and 3 (scale 1 to 5) were used. All the recipients were allocated to one of three groups [7-day J-Synch (n = 20), 6-day J-Synch (n = 20) and Conventional (n = 20)]. On Day 0, all recipients received 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Brazil) and the insertion of a P4 device (1g P4, Sincrogest®, Ourofino). All the recipients received at the time of P4 device removal (Day 6 in the 6-day J-Synch and Day 7 in the other two treatment groups), 500 μg Cloprostenol sodium (Sincrocio®, Ourofino), 400 IU of equine Chorionic Gonadotropin (SincroeCG® 6000UI, Ourofino). Cows in the Conventional group also received 0.5 mg ECP (SincroCP®, Ourofino) at the same time, whereas the recipients in the two J-Synch groups did not receive any further treatment. All recipients were also tail-painted for estrus detection (CeloTest, Biotay, Argentina) and all cows that had >50% of the paint removed at 72 h (J-Synch groups) or 54 h (Conventional group) from P4 device removal were considered in estrus. All cows were examined twice-daily by transrectal ultrasonography (Mindray DP50 Vet®, Shenzhen, China) from the time of P4 device removal until ovulation or 120 h after P4 device removal. In vitro produced fresh embryos were transferred on Day 17 in the 7-day J-Synch or Day 16 in the other two groups. Results were analyzed using the mixed GLM procedure (InfoStat, 2019). The diameter of the dominant follicle on the day of P4 device removal was larger (P=0.04) in cows in 7-day J-Synch group (9.94 ± 0.60 mm) than in those in the 6-day J-Synch (9.28 ± 0.71 mm) and Conventional (7.82 ± 0.59 mm) groups. The diameter of the preovulatory follicle was larger (P=0.03) in cows in the 7-day J-Synch (16.4 ± 0.2 mm) than in in the 6-day J-Synch (15.5 ± 0.3 mm) and Conventional (13.8 ± 0.2 mm) groups. The diameter of the CL on the day of the ET was also larger (P=0.01) in the 7-day J-Synch (21.89 ± 0.81 mm) than in the 6-day J-Synch (18.66 ± 0.78 mm) and Conventional (16.81 ± 0.95 mm) groups. The interval from P4 device removal to ovulation was longer (P=0.03) in cows in the J-Synch groups (7-day J-Synch: 86.0 ± 3.0 h, 6-day J-Synch: 83.0 ± 4.0 h) than in the Conventional group (64.0 ± 3.0 h). Although more cows were transferred in the Conventional group (P=0.05, 18/20, 90.0%) than in the 7-day J-Synch (85.0%, 17/20) and 6-day J-Synch groups (80.0%, 16/20), P/ET tended (P=0.09) to be higher in cows of 7-day J-Synch (59.0%) than in the 6-day J-Synch (50.0%) and Conventional (39.0%) groups. In conclusion, extending the period of P4 device insertion one more day in the J-Synch protocol is an alternative to synchronize estrus and ovulation in Bos indicus embryo recipients.

013 TAI/FTET/AI

A low dose of hCG (150IU) associated to inducing ovulation with estradiol benzoate can successfully replace eCG in timed AI protocols in Nelore cows

Alexandre Souza[1,3], Alessio Valenza[1], Tiago Carneiro[2], Guilherme Zanatta[3], Rodolfo Mingoti[3], Laísa Silva[3], Emiliana Batista[3], Pietro Baruselli[3]

[1]CEVA - Ceva Animal Health, Brazil & France, Libourne, France; [2]DVM - Independent Bovine Vet practitioner, São Paulo, Brazil; [3]VRA-USP - VRA-USP University of São Paulo, São Paulo, Brazil.

The objective of this study was to compare follicle growth and conception results in postpartum Nelore cows receiving either a low dose of hCG (150IU) vs eCG 300 IU during the final stages of the timed AI protocol. A total of 561 anestrous (absence of CL) Nelore cows, with average BCS of 2.6 (scale 0-5) and at 35 to 70 days postpartum were treated on D0 with 2mg of estradiol benzoate (Benzoato-HC, Ceva) and received an intravaginal progesterone device (Prociclar, Ceva). Eight and a half days later, the intravaginal device was removed and all cows were treated with a PGF2a (Luteglan, Ceva), 1 mg of estradiol benzoate, and randomized to receive either saline (Control, n = 145), 150 IU of hCG (Fertigon, Ceva, n = 204), or 300 IU of eCG (Folligon, MSD, n = 212). Timed AI was performed at 36 to 40h after device was removed. A subset of cows were evaluated for follicular dynamics and CL development (Control n = 13, hCG n = 13, eCG n = 12). Ultrasound exams for follicular growth evaluation were performed at device removal, and at 24h intervals until 96h, and 7 days post ovulation. Pregnancy diagnosis was performed at 30 days post AI. Data were analyzed by logistic regression using the GLIMMIX procedure of SAS (version 9.4). There were no differences in time to ovulation and distribution of ovulations across time among groups ($P>0.10$). Dominant follicle growth within the 24h following the device removal was similar between cows treated with hCG (2.04 mm) and eCG (1.98 mm) and both were greater than cows in the Control (1.60 mm) group ($P<0.05$). In addition, Corpus Luteum diameter was greater for hCG and eCG compared to Control cows ($P<0.01$). More importantly, conception results were similar for cows receiving hCG (53.2%) compared to cows in the eCG group (53.8%), and both yielded greater conception than Control cows (43.3%). In conclusion, hCG did not alter time to ovulation and produced similar follicular growth rates and CL development after device removal as compared to cows receiving eCG, both treatments resulting then in improved fertility compared to cows that did not receive neither stimulatory gonadotrophic treatment.

014 TAI/FTET/AI

Short-term alternative protocols for synchronized induction of estrus in dairy goats: preliminary results

Jenniffer Hauschildt Dias[1], Cleber Jonas Carvalho de Paula[2], Joanna Maria Gonçalves Souza-Fabjan[2], Aline Matos Arrais[3], Gilmar Pereira Alvim[4], Jeferson Ferreira da Fonseca[5]

[1]UFV - Universidade Federal de Viçosa, Viçosa, MG, Brasil; [2]UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil; [3]UFRRJ - Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brasil; [4]EMBRAPA - EMPRAPA Gado de Leite, Coronel Pacheco, MG, Brasil; [5]EMBRAPA - EMPRAPA Caprinos e Ovinos, Coronel Pacheco, MG, Brasil.

In order to increase productivity and allow genetic improvement of Brazilian dairy goats, the use of estrus induction protocols associated with fixed time artificial insemination (FTAI) has great importance. However, for FTAI success, a great estrus synchrony is necessary. This study aimed to assess the efficiency of a classic protocol of estrus induction of six days of duration (Fonseca et al. Reprod. Biol. 17: 268-73, 2017) and an alternative protocol increasing the duration of the protocol by 12 h. A total of 19 pluriparous Saanen goats with mean body condition score of 3.3 ± 0.12 were submitted to one of two treatments. All goats received 60 mg of medroxyprogesterone acetate sponges (MAP, Progespon®, Zoetis, Brazil) for 6 d (G6; n = 9) or 6.5 d (G6.5; n = 10). At 24 (G6) and 36 h (G6.5) before sponge removal, 30 µg of d-cloprostenol i.m. (Prolise®, Agener União, Brazil) and 200 IU of eCG i.m. (Novormon®, Zoetis, Brazil) were administered. Estrus detection was performed twice daily aided by teaser males and goats were artificially inseminated with frozen-thawed semen by transcervical via 24 h after the onset of estrus. Pregnancy diagnosis was performed 45 days after sponge removal. The data are presented in a descriptive way. The estrus response rate was 100% in both groups. The interval from sponge removal to estrus onset was 30.6 ± 6.3 h and 36 ± 0 h for the G6 and G6.5 group, respectively. After sponge removal, four and six goats started estrus at 24 and 36 h, respectively, for G6 group, while all animals from G6.5 group started estrus at 36 h. The pregnancy rate was 88.9% (8/9) in G6 and 70.0% (7/10) in the G6.5 group, with a total pregnancy rate of 78.9% (15/19). These preliminary results pointed to two efficient synchronous estrus induction protocols resulting in elevated pregnancy rates in goats. Both protocols are also associated to less time consuming related to estrus detection. The fact that 100% of animals started estrus at the same time in G6.5 animals appeared to be very interesting for FTAI, but this must be confirmed in larger trial study. Financial support: Embrapa (02.13.06.026.00.04) and Fapemig (CVZ-PPM 00201-17).

015 TAI/FTET/AI

Reproductive efficiency in dairy heifers and its relationship with dam fertility, net merit and herd size

Emiliana de Oliveira Santana Batista[1], Alexandre Henrily Souza[2,1], Pietro Sampaio Baruselli[1]

[1]USP - Universidade do Estado de São Paulo, Butantã; [2]Ceva - Ceva Saúde Animal, Paulínia, SP, Brasil.

The objective of this retrospective study was to assess factors that can influence reproductive efficiency in Holstein heifers raised in confined systems. The database was criated in collaboration with the Dairy Herd Improvement Association (DHI – Agritech Analytics, USA). Records were from 45.951 Dam-daughter pairs, housed at 346 dairy herds located across 12 states in the US territory. Only records from nulliparous Dam and daughter pairs and with a minimum of 305 milk equivalent of 8 thousand kg were included in the analysis. Also, age at calving for Dam and daughter was limited at 22 to 35 months and breeding records were collected throughout 2012. Variables considered in the statistical analysis included net merit, age at first calving for the Dam and her respective daughter, conception at first AI following first calving (daughters only), as well as herd location and lactating herd size. Data was analyzed with the PROC HPMIXED and PROC CORR of SAS (Version 9.4). As expected, larger herds were more efficient, and in general heifers calved at earlier ages for both Dam and her respective daughter (PIn conclusion, larger herds clearly had better reproductive efficency in heifers. In addition, although there seem to be a slight carry over effect from Dams with poor fertility to their offspring, this negative effect on fertility appeared to be minimized through better management and genetic improvement, particularly in larger herds.

Agradecimentos: CAPES processo: 88882.315553/2019-01

016 TAI/FTET/AI

Interrelation between the Follicular and Luteal Characteristics of Nellore Females submitted to a Fixed-Time Artificial Insemination Program

Artur Azevedo Menezes[1], Aldo Barbosa Sousa[1], Lucas André Silva Batista[1], Marcus Vinícius Galvão Loiola[2], Antonio de Lisboa Ribeiro Filho[2], Marcos Chalhoub[2], Rodrigo Freitas Bittencourt[2], Alexandra Soares Rodrigues[1]

[1]UFOB - Universidade Federal do Oeste da Bahia, Barra, Bahia, Brasil; [2]UFBA - Universidade Federal da Bahia, Ondina, BA, Brasil.

The objective of this study was to correlate the structural characteristics of the preovulatory follicle and the corpus luteum in Nellore females submitted to a protocol for Fixed-Time Artificial Insemination (FTAI). For this experiment, 39 Nellore lacting female, multiparous category, 5 to 7 years old, body condition score of 3.03±0.03 (1 to 5 scale) were used and maintained in pasture with mineral supplementation and water ad libitum. In a randomized day denominated day zero (D0) the synchronization protocol was initiated through the insertion of intravaginal progesterone (P4) device (DIB®, Zoetis, São Paulo, Brazil) and administration of 2.0 mg of estradiol benzoate (Gonadiol®, Zoetis, São Paulo, Brazil) intramuscularly (im). On day nine (D9) the P4 devices were removed and then12.5 mg of dinoprost tromethamine (Lutalyse®, Zoetis, Sao Paulo, Brazil), 0.6 mg estradiol cypionate (ECP®, Zoetis, St. Paulo, Brazil) and 300UI Equine Chorionic Gonodotrophin (eCG) (Novormon®, Zoetis, São Paulo, Brazil) im were administered. On day 11 (D11), to determine the structural characteristics of the preovulatory follicle, the animals were examined by ultrasonography (US) in B mode and color doppler by measuring the follicular diameter (DFOL), the area of the follicular wall (AFOL) and the area of vascularization of the follicular wall (VFOL). In this experiment all 39 animals responded to the protocol. On day 24 (D24), we performed US in B mode and color doppler of the corpus luteum (CL), analyzing the luteal diameter (DCL), luteal area (ACL) and the area of vascularization of the CL (VCL). The statistical analysis was performed using SPSS, version 19, considering $P<0.05$. The correlation between the follicular (DFOL, AFOL and VFOL) and luteal characteristics (DCL, ACL, VCL and P4) were performed using the pearson correlation test. The overall means for DFOL, AFOL and VFOL were, respectively, 1.12±0.20cm; 0.42±0.13 cm² e 0.22±0.10 cm². For the luteal parameters, were observed an average, of DCL, ACL and VCL of, 1.92±0.29 cm; 3.18±0.78 cm²; 1.25±0.45 cm² respectively. There was positive and significant association between DFOL and ACL (r=0.41 and P=0.02). The AFOL has showed a positive and significant correlation with DCL (r=0.41 and P= 0.02), ACL (r=0.41 and P=0.02) and VCL (r=0.35 and P=0.04). Concerning the VFOL, a positive and significant correlation with VCL was observed (r=0.36 e P=0.04). It was concluded that there was a positive correlation between the follicular and luteal structural characteristics, making possible the use of follicular measurement as a tool to associate to CL morphology and thus orientate mating in FTAI programs.

017 TAI/FTET/AI

Live weight and age affects pregnancy rate in precocious Nelore heifers submitted to timed artificial insemination

Kaerton Soares Campelo[1], Mateus Anastacio da Silva[1], Antônio Carlos Damasceno Tavares[1], Francisco Augusto Souza Ferreira[1], Samuel Santos do Nascimento[1], Ana Clara Canto Souza[2], Rafael Luiz Stolf[2], Gabriela Sanches Scuisato[2], Denis Vinícius Bonato[2], Fábio Morotti[2]

[1]P - Produzir Agropecuária, Santa Inês, MA, Brasil; [2]UEL - Universidade Estadual de Londrina, Campus Universitário, Londrina, PR, Brasil.

The aim of this study was to evaluate the influence of age and live weight on the pregnancy rate in precocious heifers submitted to TAI protocol, followed by two resynchronizations. For this study, 385 Bos taurus indicus Nelore heifers with ages ranging from 10 to 15 months (mean 13.48±1.2 months) and live weight from 216 to 380 Kg (mean 285.7±33.3 Kg) were kept in a pasture-grazing system based on Panicum maximum. Before the experiment, all heifers passed by a gynecological examination and only the healthy were kept in the study. On a random day of the estrous cycle, the heifers received an intravaginal progesterone device previously used (18 days, CIDR®, Zoetis) and 2mg estradiol benzoate intramuscularly (IM, Sincrodiol®, Ourofino). Eight days later, the device was removed and 500µg of sodium cloprostenol (IM, Sincrocio®, Ourofino), 0,5mg estradiol cypionate (IM, SincroCP, Ourofino), and 200IU eCG (IM, SincroeCG, Ourofino) were administrated. The TAI was performed 48 hours after the device removal. The pregnancy diagnosis was performed 30 days after the TAI by transrectal ultrasonography (Ultramedic®, China; linear transducer 5 MHz). Non-pregnant heifers were submitted up to two resynchronizations, repeating the pregnancy diagnosis after 30 days. For data analysis, the animals were classified based on the mean of the two variables: according to age in<14 months old (N=170) and ≥ 14 months old (N=215) and according to live weight in low weight (< 285 Kg, N=191) and high weight (≥ 285 Kg, N=194). The pregnancy rate was analyzed by logistic regression including age and live weight categories as a fixed effect, as the bull and inseminator were included as covariates. Interactions were considerate significant when P≤0.05. Pregnancy rate at 1st, 2nd, and 3th TAI were 31.69% (122/385), 34.22% (90/263) and 26.59% (46/173), respectively, and the overall pregnancy rate (3 protocols sum) was 67.01%. The category of heifers with high weight showed higher overall pregnancy rate compared to the low weight (73.20% vs. 60.21%; P=0.025). The overall pregnancy rate was higher too among the heifers with age ≥ 14 months than the category<14 months old (72.56 vs. 59.41%; P=0.037) at the end of the breeding season. Both age and live weight influenced the overall pregnancy rate, and an interaction was observed (P=0.05) between these two factors. Considering both extremes, the high weighted and older heifers (≥14 months) presented 72,85% of overall pregnancy rate (125/173) while the low weight <14 months heifers presented only 56,88% (84/149). It was concluded that older and heavier precocious heifers present better results when submitted to TAI protocols, considering the range of 10 to 15 months and live weight from 216 to 380 Kg.

018 TAI/FTET/AI

Adjustment of dose of long-acting progesterone during super precocious resynchronization in Nelore heifers

Mateus Anastacio da Silva, Kaerton Soares Campelo, Francisco Augusto Souza Ferreira, Antônio Carlos Damasceno Tavares, Samuel Santos do Nascimento

Produzir - Produzir Assessoria Agropecuária, Santa Inês, MA, Brasil.

The objective of this study was to evaluate the dose of long-acting progesterone (P4) during super precocious resynchronization protocols in Nelore heifers. A total of 92 Nelore heifers (Bos indicus), aging between 12 and 14 months old, body condition score between 2 and 3 (1-5 scale) and with free access to pasture and mineralized salt were used. On a random day of the estrous cycle (D0), heifers received an intravaginal device with 1 g P4 (Sincrogest, Ourofino, Cravinhos, Brazil) and 2 mg estradiol benzoate i.m. (EB, Sincrodiol, Ourofino). On D8, the P4 device was removed and 500 μg cloprostenol sodium (PGF, Sincrocio, Ourofino), 0.5 mg estradiol cypionate (EC, SincroCP, Ourofino) and 300 IU eCG (Sincro eCG, Ourofino) were administered i.m., and heifers were submitted to timed artificial insemination (TAI) 48 h later (D10). On D24, heifers received a new intravaginal device with 1 g P4 associated to the administration of 100 (n = 46) or 50 (n = 46) mg long-acting injectable P4 (Sincrogest Injectable, Ourofino). On D33 the P4 device was removed and pregnancy diagnosis was performed by color Doppler ultrasonography (Mindray, Z5 Vet, China, 7.5 MHz linear transducer), evaluating the presence of the corpus luteum (CL) and its blood flow (vascularization score using 1-3 scale, being 1 = low and 3 = high vascularization), adapted from Pugliesi et al. (Brazilian Journal of Animal Reproduction, 41:140-50, 2017). Non-pregnant heifers received the same hormonal treatments performed on D8 at first AI, and were submitted to TAI 2 d later (D35). Heifers considered pregnant at the Doppler evaluation, were confirmed by ultrasonography in B mode 47 d after the second TAI, by looking at fetal presence and viability. Statistical analyses were performed using chi-square ($P \leq 0.05$). Pregnancy per AI (P/AI) based on ovarian color Doppler evaluation was 48.9% (45/92) and by fetal evaluation was 42.4% (39/92; P = 0.5), demonstrating low diagnostic failure that was 13.3% (6/45). The P/AI was 45.6% (21/46) and 39.1% (18/46) of heifers receiving 100 mg and 50 mg of long-acting injectable P4, respectively (P = 0.6). The vascularization score was 30.8% (12/39), 41% (16/39) and 28.2% (11/39) in grades 1, 2 and 3, respectively (P = 0.3). Thus, the doses of 50 and 100 mg of long-acting injectable P4 had a similar effect for super-precocious resynchronization protocols in Nelore heifers, requiring more studies with larger numbers of animals to confirm the results. Acknowledgements: Ourofino.

019 TAI/FTET/AI

Effect of different estradiol benzoate doses for synchronization of follicle wave in a timed-AI protocol in *Bos indicus* beef cows

Amanda Guimarães Silva[1], Leonardo Marin Ferreira Pinto[1], Nadark de Amorim Silva[1], Ana Clara Degan Mattos[2], Pablo Henrique Ambrósio[4], Keila Maria Roncato Duarte[3], Rafael Herrera Alvarez[3], Guilherme Pugliesi[1]

[1]USP - Universidade de São Paulo, Jardim Elite; [2]UNESP (FCAV) - Universidade Estadual Paulista, Via de Acesso Professor Paulo Donato Castelane Castellane S/N - Vila Industrial; [3]APTA Tietê - Agência Paulista de Tecnologia do Agronegócio, Tietê - SP; [4]UFSC - Universidade Federal de Santa Catarina, Curitibanos, SC, Brasil.

We aimed with this study to evaluate the effect of three different doses of estradiol benzoate (EB) on the ovarian follicular dynamics of Zebu beef cows submitted to timed-AI (TAI). Primiparous and pluriparous Nelore cows, lactating (n=54) or not (n=19), and with a body condition score between 2.5 and 4 (1 to 5 scale) were used. On a random day of estrous cycle (D0), cows received an 8 days-used intravaginal progesterone (P4) device (Sincrogest®, Ourofino Animal Health) and was randomly assigned according to cow's category to three groups according to the EB dose. Cows in the EB-1 (n=26), EB-1.5 (n=24) and EB-2 (n=33) groups and received, respectively, an im treatment with 1, 1.5 or 2 mg EB (Sincrodiol®, Ourofino Animal Health). A subgroup (n=15/group) were subject to daily ultrasonography from D0 to D11, to evaluate ovarian follicular dynamics. On D8, P4 devices were removed and cows received via im 1 mg estradiol cypionate (SincroCP®, Ourofino Animal Health), 530 µg sodium cloprostenol (Cioprostinn®, Boehringer-Ingelheim Animal Health Brazil), and 300 IU eCG (SincroeCG®, Ourofino Animal Health). All cows were painted with chalk marker in the sacrocaudal region to identify cows that displayed estrus between D8 and D10. Thawed semen from two bulls was used for TAI on D10 and equally distributed among the treatment groups. Pregnancy diagnosis was done on D47 by transrectal B mode ultrasonography to detect the presence of a viable embryo with heartbeat. The data were evaluated by ANOVA (PROC MIXED), Fisher's exact test or logistic regression (PROC GLIMMIX) of SAS. The time of follicle emergence (days) did not differ (P>0.1) among groups (EB-1, 4.0 ± 0.3; EB-1.5, 3.9 ± 0.4; and EB-2, 4.1 ± 0.4). Similarly, no difference (P>0.1) was observed for the follicle growth rate (mm/day) from emergence to TAI (EB-1, 1.13 ± 0.11; EB-1.5, 1.25 ± 0.08; and EB-2, 1.03 ± 0.08), diameter (mm) of the largest follicle at TAI (EB-1, 11.4 ± 0.6; EB-1.5, 12.0 ± 0.6; EB-2, and 10.4 ± 0.6), and proportion of cows detected in estrus (EB-1, 77% [20/26]; EB-1.5, 75% [18/24]; EB-2, 88% [29/33]). However, an interaction of treatment and category (P=0.05) was observed for the ovulation rate within 36 h after TAI, indicating a reduction in ovulation rate for the EB-2 group in multiparous cows (53% [8/15]A, 54% [7/13]A and 27% [6/22]B, which was not observed in the primiparous category. The pregnancy rate did not differ (P>0.1) between EB-1, EB-1.5 and EB-2 groups (42.3% [11/26], 41.7% [10/24], and 39.4% [13/33], respectively). However, for the EB-2 group, primiparous had a higher pregnancy rate than multiparous (64% [7/11]A vs. 27% [6/22]B). In conclusion, the reduction of EB dose at the beginning of TAI protocol does not impact negatively on follicle dynamics, but further studies are needed to mitigate the effects on ovulation and pregnancy rates of Bos indicus cows submitted to EB/P4 based TAI protocols. Acknowledgments: FAPESP (2015/10606-9, 2019/07805-0).

020 TAI/FTET/AI

PGF2α at the moment of AI on pregnancy rate of cows displaying different scores of estrus expression

Juliana Wilke Diniz Horta[1], Isabella Marconato Noronha[2], Cícero Fleury Guedes Martins[3], Ana Carolina Bahia Teixeira[1], Leticia Zoccolaro Oliveira[1]

[1]Escola de Veterinária - UFMG - Universidade Federal de Minas Gerais, São Luiz, Belo Horizonte, MG; [2]FMVZ UNESP Botucatu - Faculdade de Medicina Veterinária e Zootecnia, UNESP Botucatu, Botucatu, SP, Brasil; [3]AFB - Agropecuária Fazenda Brasil, Nova Xavantina, MT, Brasil.

Animals expressing estrus presents higher ovulation rate, greater probability of pregnancy and better fertility at timed-AI (TAI; Pereira et al., 2016). On the other hand, PGF2α may enhance the mechanisms involved in the ovulatory process (Pfeifer et al., 2014). Thus, our study was based on the hypothesis that PGF2α could improve the ovulatory capacity of animals with lower evidency of heat expression at TAI. The objective was to evaluate the influence of PGF2α application at the moment of AI on conception rate (CR) of animals expressing higher or lower estrus behavior. Data from first service of 182 multiparous Nelore cows with 50 days postpartum (CEUA UFMG 348/2018) were collected. On Day 0 (D0), the animals received a intravaginal progesterone implant (CIDR®) and 2mg i.m. of estradiol benzoate (Gonadiol®). On D7, 12.5mg i.m. of Dinoprost (Lutalyse®). On D9, 12.5mg i.m. of Dinoprost (Lutalyse®), 1mg i.m. of ECP®, 300UI of eCG (Novormon®) and CIDR was removed. In addition, estrus detection device (Estrotec) was applied on the base of tail of the cows. The TAI was performed 48 hours after (at D11), by two experienced technitians, using semen from two bulls. Randomly, at the moment of AI, some animals received 12.5mg i.m. of Lutalyse (PGF group; n=101) and part of the animals received 2.5mL of saline solution (Saline group). Data regarding to the expression of estrus were also recorded, with a scale (1 to 4) referring to the activation of the device (estrus expression grade 1 = ≤25% removal of gray protective paint; grade 2 = ≤50% removal of gray paint ; grade 3 = ≤75% removal of gray paint; grade 4 = >75% removal of gray paint) (Pohler et al., 2016). CR was analyzed by logistic regression and means were compared by Tukey's test, considering $P>0.05$ and <0.10. Total CR was 45.6% (83/182), being higher (51.8%; n=56/108) for cows expressing evident estrus (grades 3 and 4) than for cows that expressed lower evidency of estrus behavior (grades 1 and 2; CR:36.5%, n=27/74, P=0.051). No difference (P=0.81) was observed for CR on the total number of cows receiving PGF2α at D11 (PGF group=46.5%; n=47/101) or not (Saline group=44.4%; n=36/81). For animals expressing evident estrus (grades 3 and 4), no influence of PGF2α at the moment AI on CR was observed (Estrus+PGF=52.6%, n=57; Estrus+Saline=51.0%, n=51; P=0.88), as expected. However, there was also no effect of PGF2α at AI on cows expressing estrus 1 and 2 (No estrus+PGF=38.6%, n=44; No estrus+Saline=33.3%, n=30; P=0.70). Hence, the present hypothesis was not confirmed in this preliminary study of our research group. It was concluded that animals that express more evident estrus behavior present higher conception rate, independent of the application of PGF2α at the time of AI. Acknowledgments: Zoetis and Dr. Ky Pohler.

021 TAI/FTET/AI

High fertility bulls have higher efficiency on TAI and SOV, but not on IVEP

Guilherme Machado Zanatta[1], Flávia Morag Elliff[1], Marcos Henrique Alcantara Colli[1], Rodolfo Daniel Mingoti[1], Bruna Lima Chechin Catussi[1], Laísa Garcia da Silva[1], Alessandra Bridi[2], Gabriel Armond Crepaldi[3,1], Mayra Elena Ortiz D'Avlia Assumpção[1], Felipe Perecin[2], Pietro Sampaio Baruselli[1]

[1]VRA - FMVZ/USP - Departamento de Reprodução Animal - FMVZ/USP, São Paulo, SP; [2]ZMV - FZEA/USP - Departamento de Medicina Veterinária - FZEA/USP, Pirassununga, SP; [3]ST Repro - ST Repro, Indaiatuba, SP.

The aim of this study was to evaluate pregnancy rate/TAI, and embryo quality on TAI, SOV and IVEP of high and low fertility bulls. For this, we used three high and three low fertility bulls (STRepro's evaluation). The same semen batch for each bull was used on all biotechnologies. On Exp. 1, 562 cows were synchronized with P4/E2 based protocol, (8 days of device permanence) and PGF, eCG and EC on the day of device removal. TAI was performed 48h after device removal. After 7 days, cows were randomized in two groups: embryo collection (GC; n=301) and pregnancy rate (GP; n=261). The GC group was submitted to flushing (embryos were classified according to the IETS guidelines). The GP was submitted to pregnancy diagnosis 30 days after TAI. On Exp. 2, 60 superovulations were performed with a P4/E2 based protocol (D0), with 8 decreasing doses of FSH (D4 until D8), on D6 cows received 2 doses of PGF2α with 12 hours interval. On D7, the P4 device was removed and on D8 ovulation was induced with GnRH. Two TAI were performed, 12h and 24h after GnRH application. The embryo collections were done on D15 (same methods/classifications described above). For Exp. 3, we used ovaries from slaughterhouse were used for IVEP. The follicles were aspirated and grade 1 and 2 oocytes were matured in vitro. For the bulls' distribution on the IVF, the oocytes were randomized according to quality. The semen was thawed and submitted to a percoll gradient of 45/90% by centrifugation at 6600g/5min. The zygotes were cultivated in a KSOMaa medium at 5% CO2 and 5% O2 until the 8th day. The cleavage and blastocyst rates on total oocytes and blastocyst rate on cleaved oocytes were considered. Data were analyzed by Fischer's exact test and SAS® GLIMIX procedure. On Exp. 1, (GC), there was no difference on embryos recovery rate according to the bull's fertility: high=39.7% (50/126)/low=35.9% (46/128), (P=0.5). However, there was difference on fertilization rate [high=98.0% (49/50); low=78.2% (36/46); P=0.01] and quality rate [high=88.0% (44/50); low=60.8% (28/46); P<0.01]. On GP, there was an effect on pregnancy rate [high=54.6% (71/130); low=41.2% (54/131); P=0.03]. On Exp. 2 there was a difference on the fertilization rate [high=59.0% (154/261); low=42.7% (102/239); P=0.04], on the viability of the total structures [high=54.0% (141/261); low=38.9% (93/239); P=0.05], but there was no difference on the viability over fertilized structures [high=91.6% (141/154); low=91.2% (93/102); P=0.9]. On Exp. 3, there was no effect on cleavage rate [high=82.1% (619/754); low=81.9% (584/713); P=0.9], blastocyst rate on total oocytes [high=21.5% (160/754); low=21.9% (152/693); P=0.9] and blastocyst rate on cleaved [high=25.8% (160/619); low=26.0% (152/584); P=0.9]. In conclusion, bulls with high fertility showed higher efficiency on TAI (fertilization rate, embryo quality and pregnancy rate) and SOV (fertilization rate and embryo quality). However, these differences were not observed for IVEP.

022 TAI/FTET/AI

Induction of the puberty in *Bos indicus* heifers in the western amazon region

Suellen Miguez Gonzalez[3], Geraldo Francisco dos Santos Junior[1], Igor Emanuel Gomes Assunção[2], James Duarte[2], Tales André Guedes[2], Carlos Henrique de Andrade Oliveira[2], Fernando Castro Parizi[1], Fábio Morotti[3], Marcelo Marcondes Seneda[3], Evelyn Rabelo Andrade[2]

[1]UFAC - Universidade Federal do Acre, Distrito Industrial, Rio Branco, AC; [2]UNIR - Universidade Federal de Rondônia, Porto Velho, RO; [3]UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

The western Amazon region, with more than one million head of cattle, represents a very specific location for cattle, due to unique environmental conditions. Studies on adaptation, behavior and reproductive physiology of bovine females in the Amazon are scarce. Therefore, the objective of this study was to evaluate two distinct hormonal protocols for puberty induction of Bos indicus heifers in the western Amazon region. Bos indicus prepubertal heifers (n = 127) aged 21 months (SD+/- 0.3 months) and mean weight of 318 kg (SD +/- 7 kg) were submitted to two distinct protocols for induction of cyclicity. All females were submitted to ultrasound examination of the ovaries to verify the absence of the corpus luteum (CL). Two evaluations were performed with a 12 day interval between them. Afterward, they were divided into two experimental groups, G-CIDR/ECP (n=54) and G-P4/ECP (n=73). The G-CIDR/ECP remained for 12 days with CIDR® (Pfizer Saúde Animal, São Paulo, SP, Brazil) of 4th use and on the day of its withdrawal, 0.6 mg of estradiol cypionate (E.C.P, Pfizer Saúde Animal, São Paulo, SP, Brazil; IM) was administered. The G-P4/ECP was given 150 mg of injectable progesterone (Sincrogest, Ouro Fino, Cravinhos, SP; IM) and one injection of 0.6 mg of ECP 12 after days the progesterone injection. Twelve days after the end of the hormonal protocols, the females were submitted to another ultrasound evaluation (SonoScape, Domed-Dominium Medical, EUA) for identification of cyclicity (ovulatory follicle or CL) and then the females were submitted to synchronization of ovulation for FTAI. Among the females, only the ones that were responsive to puberty induction, G-CIDR/ECP (n=30) and G-P4/ECP (n=51) were included in the FTAI protocol. After seven days of insemination, both groups were exposed to clean-up bulls. Data were analyzed by the logistic regression model, adopting a significance level of 5%. G-CIDR/ECP resulted in 81.48% (44/54) of females responsive to induction of cyclicity (presence of CL) while G-P4/ECP obtained 86.3% (63/73; p=0.463). Regarding the conception rate from FTAI and bull, the group G-CIDR/ECP and G-P4/ECP had 43.33% (13/30) and 33.33% (10/30), respectively. The G-P4/ECP received 54.9% (28/51) and 39.22% (20/51) for the evaluations above. Regarding the final pregnancy rate (FTAI+Bull), G-CIDR/ECP resulted in 76.67% (23/30) and G-P4/ECP 94.12% (48/51; p=0.023). We concluded that both cycling induction protocols were efficient in Bos indicus heifers from western Amazonia. However, injectable P4 provided a higher conception rate at the end of the reproductive season.

023 TAI/FTET/AI

Presynchronization by induction of a largest follicle using a progesterone device in GnRH-based-ovulation synchronization protocol in dairy cows

José Nélio Sousa Sales[1], Eduardo Alves Lima[1], Luiz Manoel Souza Simões[1], Luiz Augusto Capellari Leite Silva[1], Miguel Pizzolante Bottino[1], Ana Paula Castro Santos[1], Raphael Evangelista Orlandi[1], Bruna Martins Guerreiro[2], Bruno Gonzalez de Freitas[2], Michele Ricieri Bastos[2]

[1]UFLA - Universidade Federal de Lavras, Lavras, MG, Brasil; [2]Ouro Fino - Ouro Fino Saúde Animal, Cravinhos, SP, Brazil.

The objective of study was to evaluate the pre-synchronization by dominant follicle induction using a progesterone device prior to the Ovsynch protocol (P4synch) compared to estradiol/progesterone-based protocol in lactating Holstein dairy cows. Holstein dairy cows (n=349) were randomly assigned to one of two groups: P4E2 Group (n=179), cows received a progesterone intravaginal device (Sincrogest, Ouro Fino, Brazil) plus the administration of 2mg of estradiol benzoate (Sincrodiol, Ouro Fino, Brazil) and 10µg of Busereline (GnRH; Sincroforte, Ouro Fino, Brazil) on day 0 (D0). Eight days later (D8), the progesterone device was removed and 500µg of Cloprostenol (PGF; Sincrocio, Ouro Fino, Brazil) and 1mg estradiol cypionate (SincroCP, Ouro Fino, Brazil) were administered. The TAI was performed 48 hours later (D10). P4Synch Group (n=170), cows received a progesterone intravaginal device on day -10 (D-10). Ten days later (D0) starting the Ovsynch protocol (10 µg of GnRH on Day 0, 500 µg of PGF on Day 7 and 10µg of GnRH on Day 9), with withdrawal of the device on Day 7. The TAI was performed 15 to 20 hours after the second GnRH of the Ovsynch protocol. In subgroups of cows (n=92), ultrasound exams were performed on days 0, at the time of ovulation inducer and TAI. The pregnancy diagnoses were performed 30 days after TAI. Statistical analyses were performed by GLIMMIX procedure of SAS. The pre-synchronization rate (presence the follicle with more than 12 mm on D0) for P4synch group was 97.8% (45/46). There was difference among groups for presence of CL on D0 [P4E2 - 80.4% (37/46) and P4synch - 37.0% (17/46); P=0.001], follicular diameter on D0 (P4E2 - 15.0±0.8mm and P4synch 21.0±0.8mm; P=0.001), at the time ovulation induction (P4E2 - 13.9±0.9mm and P4synch 17.6±0.6mm; P=0.001) and TAI (P4E2 15.2±0.7mm and P4synch 17.2±0.8mm; P=0.05). Furthermore, there was no difference between groups for synchronization rate [presence de follicle with more than 12 mm on TAI; P4E2 – 76.1% (35/46) and P4synch - 80.4% (37/46); P=0.61], follicular persistence after ovulation induction [P4E2 - 8.7% (4/46) and P4synch - 15.2% (7/46); P=0.34] and pregnancy rate at 30 days after TAI [P4E2 - 37.4% (67/179) and P4synch - 42.4% (72/170); P=0.35]. In conclusion, that despite differences in follicular dynamics among groups, the pre-synchronization by large dominant follicle induction using progesterone intravaginal device prior to Ovsynch protocol (P4synch) presents similar results as the estradiol/progesterone-based protocol in the fertility of lactating Holstein dairy cows. Support: Ouro Fino Saúde Animal, CAPES, FAPEMIG

024 TAI/FTET/AI

Relationship of antral follicle count, animal reactivity and productive parameters with pregnancy rate to TAI in Brahman cows

Maria Eduarda Scheel Bomtempo[1], Marcela Bortoletto Cerezetti[1], Fábio Lucas Zito de Morais[1], Elis Lorenzetti[1,2], Fábio Morotti[1]

[1]UEL - Universidade Estadual de Londrina, Campus Universitário, Londrina-Paraná; [2]UNOPAR - Universidade Norte do Paraná, Jardim Aeroporto, Arapongas, Paraná, Brasil.

This study evaluated the relationship of antral follicle count (AFC), animal reactivity and variations of live weight and body condition score (BCS) with pregnancy rate in Brahman cows submitted to timed artificial insemination (TAI). Brahman cows (N=122; Bos taurus indicus) with 24-96 months old, 45-60 postpartum (50±2), BCS range 2.5-3.5(2.9±0.1; Scale 1-5) and weight between 300-560kg(414±6) were kept on the pasture of Brachiaria brizantha for this study. On a random day of the estrous cycle (D0), cows received an intravaginal progesterone device (FertilCare®1200, Vallée) and 2mg estradiol benzoate (EB; Ric-Be®, Tecnopec) intramuscularly (IM). After 8 days, the device was removed and cloprostenol (50mg, Estron®, Tecnopec), equine chorionic gonadotrophin (300IU, Folligon®, MSD) and estradiol cypionate (1mg, Fertilcare® Ovulação, Vallée) were IM applied. On the same day, the base of the tail was painted to assess estrus demonstration, and TAI was performed 48h later. The AFC (follicles ≥3mm) of each female was determined at D0. The BCS and reactivity score (in the trunk, according to Grandin, 1993) were assessed every day of the TAI protocol, and weight was measured at the D0 and at the pregnancy diagnosis (D40). For analysis the data were grouped according AFC groups (low≤15, intermediate≥16 and ≤29, and high ≥30 follicles), reactivity classification (calm -mean score=1, restlessness -mean score >1 and ≤ 2, and agitated -mean score ≥ 2.1), weight variation (gaining -positive variation +10 to +40kg, maintaining -variation of -9 to +9kg, and losing -negative variation -10 to -40kg) and BCS variation (gaining - positive variation +0.25 to +0.75points, maintaining - variation of -0.25 to +0.25points, and losing - negative variation -0.25 to -0.75points). Pregnancy rates were analyzed by logistic regression model (P<0.05) in the MINITAB18® statistical software. The overall pregnancy rate of the study was 50%(61/122), and it was not influenced (P>0.05) by the AFC groups, reactivity classification and variations weight, and BCS. Cows with low, intermediate and high AFC showed similar (P>0.1) pregnancy rate [52.6%(20/38), 50.9%(26/51) and 45.4%(15/33), respectively]. However, estrus of high intensity to TAI was present in 94.7% of cows with low AFC in relation 70.6% to intermediate and 84.8% to high AFC (P=0.05). Calm, restlessness and agitated cows exhibit pregnancy rate of 43.9%(18/41), 54.7%(35/64) and 47.1%(8/17, P>0.1), respectively. Similar (P>0.1) pregnancy rate was found in cows gaining, maintained and losing weight [44.7%(21/47), 54.6%(30/55) and 50.0%(10/20), respectively] or BCS [44.0%(11/25), 54.3%(44/81) and 37.5%(6/16), respectively]. It was concluded that the pregnancy rate of Brahman cows submitted to TAI is not related by AFC, reactivity classification and variations of BCS or live weight. However, low AFC resulted in a high proportion of cows with estrus of high intensity.

025 TAI/FTET/AI

Follicular dynamics and evaluation of ovulation rates on lactating dairy cows subjected to j-sync protocol

Ana Paula Depiere[1], Gustavo Henrique Lenz[1], Jankiel Primon[2], Adiel Cristiano Nino[3], Patrícia Diniz Ebling[1], Sabrina Parise[1], Ana Paula da Silva[1], Fernanda de Souza Rosa[1]

[1]UCEFF - Centro Universitário FAI, Itapiranga, SC; [2]Med. Vet - Médico Veterinário autônomo, Caibi, SC; [3]Med. Vet - Médico Veterinário autônomo, Palmitinho, RS, Brasil.

The J-sync protocol has been demonstrating good results in beef and dairy heifers and weaned beef cows in terms of ovulatory follicle size and consequently corpus luteum formation, as well in the endometrial preparation to receive the embryo, resulting in pregnancy high rates when compared to conventional protocols. There are no studies demonstrating its efficiency in lactating dairy cows yet, since their high hepatic metabolism of steroids hormones. Therefore the main objective of this study is measuring the follicular dynamics and ovulation rates of lactating dairy cows subjected to the J-sync protocol. 12 Holstein cows were randomly selected, averaging 3,5BSC, 4years, yielding 31Kg/Day of milk. Those were divided into 2 groups: J-Sync and PEPE (control group). On D0 all cows received an DIB containing 1g of Progesterone (Primer®, Agener União, Sâo Paulo, Brazil) and an IM administration of 2mg of Estradiol benzoate (Sincrodiol®, Ourofino, Cravinhos, Brazil). On D6 the DIB was removed from the cows of the J-synch group (n=6) and there was an IM administration of 150µg of D-cloprostenol (Prolise®, Agener União, SP, Brazil) and the oestrus was observed. On D9 (72h after the DIB removal), 0,025mg IM of gonadolreline acetate (Gestran Plus®, Tecnopec, SP, Brazil) were injected in those that didn't manifest oestrus, so they were AI 12h after the oestrus detection or on D9. However in cows from the PEPE group the DIB removal and the IM D-cloprostenol administration were on D9, and in the same day 0,5mg of IM estradiol cypionate (SincroCP®, Ourofino, Cravinhos, Brazil) was administrated. The FTAI happened on D11 54h after the DIB removal. The ultrassonography evaluations were performed with the Mindray DP3300 equipment with a retal linear transducer of 7,5MHz. In these evaluations it was observed the maximum diameter of the dominant and the subordinated follicles, the daily growth rate was also monitored from the DIB removal day until the AI scheduled moment (each 12h) and after 7 days, the ovulations of all the managed cows (n=12) were assessed. There wasn't a significant difference between the maximum diameters of the dominant follicles and the subordinated ones in the groups J-synch and PEPE (17,32vs17,8mm e 10,56vs11,1mm; $P>0,05$), however the follicular growth rate in the J-synch group trended to be higher when compared to PEPE (2vs1,64mm/d $P<0,1$). The cows subjected to the J-synch protocol obtained 100% of ovulation rate and 1 double ovulation, while 83% of ovulation rate was observed in the cows from PEPE group ($P>0,05$). We have concluded that the J-synch protocol can be efficient to lactating dairy cows, although we didn't observe difference in the size of the dominant and the subordinate follicle, the same was not observed in the ovulation rates between the protocols, just a tendency to have a higher follicular growth in J-synch, the necessity to increase the number of animals has been showed, as the necessity to evaluate the pregnancy rates.

026 TAI/FTET/AI

Progesterone pre-exposition to ovulation synchronization protocol increases follicular diameter and pregnancy rate in *Bos taurus* and *Bos indicus* suckled beef cows

Luiz Manoel Souza Simões[1], Eduardo Alves Lima[1], Mar Betjhai Perez Martinez[5], Guilherme Machado Zanatta[7], João Paulo Martinelli Massoneto[2], Mateus Felipe Osório dos Santos[6], Amanda Bilha Machado[4], Marcelo Maronna Dias[4], Bruna Martins Guerreiro[3], Bruno Gonzalez de Freitas[3], Michele Ricieri Bastos[3], José Nélio Sousa Sales[1]

[1]UFLA - Universidade Federal de Lavras, Lavras, MG, Brasil; [2]AAP - Agropecuária Água Preta, Cocalinho, MT, Brasil; [3]Ouro Fino - Ouro Fino Saúde Animal, Cravinhos, SP, Brasil; [4]MV - Médico Veterinário Autônomo, Capivari do Sul, RS, Brasil; [5]UNAM - Universidade Nacional Autônoma do México, Cidade do México, México; [6]UniRitter - UniRitter, Porto Alegre, RS, Brasil; [7]USP - Universidade de São Paulo, São Paulo, SP, Brasil.

Three studies were carried out to evaluate the effects of pre-exposition of injectable progesterone (P4i) to a TAI protocol in Bos taurus and Bos indicus suckled beef cows. In experiment 1, the effect of P4i prior to the TAI on the pregnancy rate (P/AI) was evaluated in 568 suckled Nelore cows, at 30-60 days postpartum (DPP) and BCS of 2.68±0.01. In experiment 2, the effect of P4i prior to the TAI on follicular dynamics and P/AI was evaluated in 518 suckled Bos taurus cows, at 30-90 DPP and BCS of 2.69±0.01. On D-10 from both experiments, cows were divided into two experimental groups (Control and P4i). Control cows received 2mg of estradiol benzoate and a progesterone intravaginal device on day 0 (D0). On D8, the progesterone device was removed and cows received 500µg of Cloprostenol, 300IU of eCG and 1mg of estradiol cypionate. In the P4i Group, cows received 150mg of P4i (Sincrogest Injetável®, Ouro Fino, Brazil) on D-10 and were submitted to the same synchronization protocol as the Control Group. On a subset of cows of experiment 2 (n=401), ultrasound exams were performed on days 0, 8, 10 and 12 to evaluate the diameter of largest follicle (LF), follicular growth between D8 and D10 (FG) and ovulation rate. In experiment 3, the effect of the replacing eCG with P4i prior to the TAI protocol on follicular dynamics and P/AI was evaluated in 446 suckled Nelore cows, at 30-60 DPP and BCS of 2.63±0.01. Ten days before the TAI protocol (D-10), cows were allocated in a 2x2 factorial design to either receive or not 150mg of P4i on D-10 and receive or not 300UI of eCG on D8 of the TAI protocol (Control, eCG, P4i and P4ieCG Groups). Ultrasound exams were performed on D0, D8 and D10 to evaluate the LF and FG. The pregnancy diagnoses (3 experiments) were performed 30 days after TAI. Statistical analyses were performed by the PROC GLIMMIX of SAS. In experiment 1, the P/AI was greater in cows that received P4i previous to the TAI [Control 48.1% vs P4i 57.2%; P=0.03]. In experiment 2, the LF on D0 (Control 11.6±0.2 vs P4i 13.3±0.3mm; P=0.01), LF on D8 (Control 11.3±0.2 vs P4i 11.8±0.2mm; P=0.08) and P/AI [Control 45.6% vs P4i 54.8%; P=0.03] were greater in cows that received P4i previous to the TAI. In experiment 3, there were interaction effects between P4i and eCG for the LF on D10 (Control 10.2±0.3c; P4i 10.3±0.2c; eCG 11.2±0.3b; P4ieCG 12.3±0.2amm; P=0.04). The LF on D0 (P<0.01) and D8 (P<0.01) was larger in cows receiving P4i prior to the TAI protocol. In addition, there was no effect of P4i on all other variables studied. The FG (P<0.01) and P/AI [No-eCG 25.5% vs eCG 52.7%; P<0.01] were higher in cows receiving eCG. In conclusion, pre-exposition to P4i on TAI protocol increased diameter of the LF (D0 and D8) and pregnancy rate of Bos taurus and Bos indicus suckled beef cows. However, in suckled Bos indicus cows, pre-exposition to P4i is not suitable to replace eCG in TAI protocols. Support: Ouro Fino Saúde Animal and FAPEMIG.

027 TAI/FTET/AI

Evaluation of the luteolytic efficiency of different doses of sodium cloprostenol and dinoprost tromethamine administered on days 4 and 11 of luteal phase in beef cows

Lindsay Unno Gimenes[1], Gabriel Artur Marciano do Nascimento[1,2], Rafael Rodrigues Corrêa[3], Rodrigo da Costa Maia[4], Débora Ferreira Lopes[5], Larissa Nicolau Fortunato[6], Vitor Barbosa Fialho Martins[2,1], Bruno de Souza Mesquita[2], José Ricardo Garla de Maio[2], Bruno Gonzalez de Freitas[2], Gabrielle Nellis Bragaglia[2], Vanessa Garcia Rizzi Mussi[2]

[1]UNESP - Jaboticabal - Universidade Estadual Paulista - Câmpus de Jaboticabal, Jaboticabal, SP; [2]Ourofino Saúde Animal - Ourofino Saúde Animal, Cravinhos, SP; [3]Médico Veterinário - Médico Veterinário, Jaboticabal, SP; [4]UFOP - Universidade Federal de Ouro Preto, Ouro Preto, MG; [5]FZEA/USP - Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga, SP; [6]Médica Veterinária - Médica Veterinária, Franca, SP, Brasil.

In this study the effect of different doses (0, 50 or 100% of recommended dose [RD]) of sodium cloprostenol (SC, Sincrocio®, Ourofino Animal Health, Cravinhos, Brazil) and dinoprost trometamine (DT; Lutalyse®, Zoetis, São Paulo, Brazil) administered I.M. in beef cows on days 4 (D4) and 11 (D11) of luteal phase was evaluated. The hypothesis is that the use of 50% RD of SC and DT is so effective as 100% RD to induce complete luteolysis in beef cows when administered on D11, while on D4 it is expected no effectiveness of any treatment. Non-lactating and cyclic cows (n = 92) had ovulation synchronized to receive the experimental treatments (D0 = ovulation). Animals which ovulated on D0 (n = 54) were randomized according to BCS, breed and luteal diameter to receive SC 0 µg (0%; untreated, nD4=3; nD11=3), SC 250 µg (50%; nD4=5; nD11=7), SC 500 µg (100%; nD4=5; nD11=5), DT 0 mg (0%; untreated, nD4=2; nD11=2), DT 12.5 mg (50%; nD4=5; nD11=6) or DT 25 mg (100%; nD4=5; nD11=6). Ultrasonographic examinations of the corpus luteum (CL) were made pre-treatment (0), and 24, 48, 72, 96 h post-treatment, in mode B to estimate CL diameter (cm) and Color Doppler to estimate vascularization area (VA, percentual of colored pixels). Serum progesterone concentration (P4 - ng/ml) was evaluated 0, 8, 24 and 48 h post-treatment, by radioimmunoassay. Data were analyzed by ANOVA, in a factorial arrangement 2 (SC and DT) x 2 (D4 and D11) x 3 (0, 50 and 100% RD) with time repeated measures, and Tukey test, with significance at 5% level (SAS). There was no difference in luteolytic response after administration of SC and DT. An interaction day of luteal phase (day) x time post-treatment (time) was observed: on D4, CL diameter (0h: 1.5±0.1; 96h: 1.4±0.1) and serum P4 (0h: 1.1±0.2; 48h: 1.8±0.2) remained constant ($p \geq 0.05$); whereas, on D11 a reduction ($p < 0.05$) was observed for CL diameter (0h: 1.7±0.1; 96h: 1.2±0.1) and serum P4 (0h: 4.7±0.2; 48h: 1.2±0.2). The interaction of dose x time reflected an increase in CL diameter (0h: 1.6±0.1; 96h: 1.9±0.1; $p < 0.05$) and constant serum P4 (0h: 2.1±0.4; 48h: 3.5±0.4;$p \geq 0.05$) in untreated animals, while treated animals had a reduction ($p < 0.05$) in CL diameter (50% - 0h: 1.6±0.1; 96h: 1.1±0.1; 100% - 0h: 1.7±0.1; 96h: 1.0±0.1) and serum P4 (50% - 0h: 3.1±0.2; 48h: 0.8±0.2; 100% - 0h: 3.4±0.2; 48h: 0.2±0.2). The three-way interaction in VA reflected similar ($p > 0.05$) luteal blood perfusion for untreated animals (D4 - 0h: 18.5±3.3; 96h: 20.3±3.3; D11 - 0h: 14.2±3.3; 96h: 17.6±3.3), and for those treated on D4 (50% - 0h: 17.4±2.3; 96h: 18.0±2.3; 100% - 0h: 15.7±2.3; 96h: 7.2±2.3); whereas, animals treated with 50% (0h: 21.2±2.0; 96h: 0.3±2.0) or 100% (0h: 18.7±2.2; 96h: 0.7±2.2) on D11 presented reduction in VA ($p < 0.05$). In conclusion, we accepted our hypothesis, demonstrating that the use of 50% RD of SC and DT may be an effective alternative to induce complete luteolysis when administered on day 11 of luteal phase in beef cows.

028 TAI/FTET/AI

Use of GnRH to increase the pregnancy rate from different categories in Nelore females submitted to TAI

Fábio Morotti[1], Luigi Carrer Filho[2], Eriko da Silva Santos[2], Murilo Rezende Figueira[2], Marcela Bortoletto Cerezetti[1], Marcelo Marcondes Seneda[1]

[1]UEL - Univesidade Estadual de Londrina, Londrina, PR, Brazil; [2]Neopecuária - Genética e Reprodução Bovina, Londrina, PR, Brazil.

The use of GnRH at the time of TAI has increased the pregnancy rate in cows that do not show estrus. Therefore, the objective of this study was to compare the increment in pregnancy rate among nulliparous, primiparous and multiparous treated with GnRH at the time of TAI. Nelore females (146 nulliparous heifers, 139 primiparous and 200 multiparous cows) with body condition score (BCS) ranged from 2.50 to 3.50 (range 1-5) and 35 to 60 days postpartum were submitted TAI protocol. On a random day of the estrus cycle (D0) the animals received an intravaginal progesterone device (heifers - 0.5 g, Repro one®, GlobalGen; cows - 1.0 g Repro neo®, GlobalGen) associated with intramuscular (IM) application of estradiol benzoate (2 mg, Syncrogen® GlobalGen). At the day of removal of the device (D8), the females receive (I.M) cloprostenol (50 mg, Induscio®, GlobalGen), equine chorionic gonadotropin (300 IU, eCGen®, GlobalGen) and estradiol cypionate (heifer - 0.6 mg and cow - 1 mg, Cipion®, GlobalGen). All the females receive painting at the base of the tail to evaluate the estrus demonstration. The TAI was performed 48 hours after withdrawal of the device using semen from two Nelore bulls with known fertility. Cows showing estrus (paint removed) were conventionally inseminated and those who maintained the paint on the tail (total presence or up to 50% of the paint) received 10 μ buserelin acetate (IM, Prorelinn®, Boehringer Ingelheim) concomitant to insemination. The pregnancy diagnosis was performed by transrectal ultrasonography 30 days later and non-pregnant females received a resynchronization following the same procedure already described. The data were analyzed by the logistic regression model including effects of category, bull, progesterone source and TAI order. BCS and postpartum days were included as covariables ($P \leq 0.05$). All results refer to the combined analysis of the two TAI protocols. The overall pregnancy rate was 74.43% (361/485), being similar ($P = 0.85$) among nulliparous (74.66%, 109/146), primiparous (73.38%, 102/139) and multiparous (75.00%, 150/200). In overall pregnancy analyses, there was no influence of animal category, source of P4, order of TAI or interactions ($P>0.1$). Considering females with estrus manifestation, the pregnancy rate was 58.97% (286/485), being higher ($P = 0.06$) for nulliparous (63.70%, 93/146) and multiparous categories (61.50%, 123/200) compared to primiparous (50.36%, 70/139). Considering females that did not show estrus and received GnRH, the highest pregnancy rate ($P = 0.01$) was for the primiparous category (23.02%, 32/139), compared to nulliparous (10.96%, 16/146) and multiparous (13.50%, 27/200). It is concluded that GnRH can be used strategically in category of females to increase the pregnancy rate in TAI programs.

029 TAI/FTET/AI

Relationship between antral follicle count and age at puberty and fertility of beef heifers subjected to timed AI

George Moreira da Silva[3,1], Jair Sábio de Oliveira Junior[4,3], Elizângela Mirian Moreira[5,1], Jéssica de Souza Andrade[3,4,3], Vanessa Rachele Ribeiro Nunes[1], Marcelo Marcondes Seneda[2], Luiz Francisco Machado Pfeifer[1]

[1]Embrapa - Empresa Brasileira de Pesquisa Agropecuária, Porto Velho; [2] UEL - Universidade Estadual de Londrina, Londrina; [3]UNIR - Universidade Federal de Rondonia, Porto Velho; [4]Facimed/Bionorte - Facimed/Bionorte, Porto Velho; [5]FAPERO - FAPERO, Porto Velho, RO, Brasil.

In Experiment 1, Nelore prepubertal heifers (n=30), 16 months of age, 272.3 ± 23 kg were examined with ultrasound. Videos from the ovaries were recorded for further AFC (≥ 3 mm). Heifers were divided into two experimental groups according to the number of AFC: 1) Low AFC (<22 follicles), and 2) High AFC (≥22 follicles). Further ovarian ultrasonic evaluations were performed every 15 days until the detection of a corpus luteum (CL) in the ovary. Once puberty was determined, all heifers were subjected to a estradiol progesterone-based TAI protocol [2 mg BE (Gonadiol®, Zoetis)+ CIDR®(Zoetis) on D0 / 2 mL PGF (Lutalyse®, Zoetis) + 0,6 mg ECP (E.C.P.®, Zoetis) – CIDR®on D8 / TAI 48 h] and a estrus detector device (Estrotect®) were placed in the sacrocaudal region. After CIDR removal, the dominant follicle was monitored by ultrasonography every 12 h until the ovulation. Area of the ovary, interval to estrus and ovulation were analyzed by the general linear model (GLM) procedure and Tukey's test was used to determine differences between groups. The estrus, ovulation and pregnancy rates were analyzed by Chi-square test. No differences (P>0.05) on the age at puberty, ovulation and estrus expression rates, and preovulatory follicle diameter between groups were detected. The ovary of the Low AFC heifers was smaller in the prepuberty period (4.4±1.1 vs. 5.6±0.7 cm2). At TAI, Low AFC heifers displayed estrus (48±0.0 vs. 58.5±12.3 h), and ovulated (60±6.3 vs. 72.6±15.5 h) earlier, and had higher pregnancy per AI (76.9%, 10/13 vs. 29.4%, 5/17) than High AFC heifers (P<0.05). In Experiment 2, 147 Nelore pubertal heifers, 16 months of age, 330 ± 20 kg, were subjected to a TAI protocol [2 mg BE (Syncrogen®, GlobalGen) + 0.5 g P4 (Repro one®, GlobalGen) on D0 / 2 mL PGF(Induscio®, GlobalGen) on D7 + 300 UI eCG (eCGen®, GlobalGen) + 0,6 mg ECP (Cipion®, GlobalGen) – P4 on D9 / TAI 48 h]. On Day 9, heifers were painted with chalk marker in the sacrocaudal region to identify cows that displayed estrus. On Day 0, ultrasound examinations were performed in all heifers to AFC and heifers were divided in two groups: Low AFC (<22 follicles, n=31), and High AFC (≥27 follicles, n=78). In this experiment, AFC were performed on Day 0 of the protocol, therefore, videos from the ovaries were not recorded. Thus, heifers that presented AFC between 22 - 26 were considered as intermediate AFC, and thus, were discarded from this experiment. No differences (P>0.05) on the estrus expression (77.4%, 24/31 vs. 73.1%, 57/78), and on the P/AI (41.9%, 13/31 vs. 50%, 39/78) were detected between Low and High AFC heifers, respectively. The results from these experiments demonstrate that AFC did not affect age at puberty and Low AFC heifers presented better ovarian responses when subjected to TAI protocols. However, more studies are necessary to elucidate the herd and genetic effects, since differences on fertility between Experiment 1 and 2 were detected. Acknowledgements: This study received funding support from Embrapa (MP1/PC3 Project n. 01.03.14.011.00.00) and from CNPq (Universal Project n: 407307/2016-8).

030 TAI/FTET/AI

Effect of cyclicity, body condition score, antral follicle count and body weight gain on the pregnancy rate of Nelore cows submitted to fixed-time artificial insemination

Maria Paula Marinho de Negreiros[1,2], Guilherme Henrique Freitas Seugling[1,2], Luiz Aguinaldo Ricetto Pegorari Junior[1,2], Rian Lolico Chamorro[1,2], José Gabriel Rigo Kairuz[3], Gabriella Carolina Silva[1,2], Ana Clara Bertolino Pereira[1,2], Wanessa Blaschi[1], Thales Ricardo Rigo Barreiros[1]

[1]UENP - Universidade Estadual do Norte do Paraná, Jacarezinho, PR; [2]PIBIC,FAP - PR - Fundação Araucária do Estado do Paraná, Curitiba, PR; [3] UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

The present study aimed to evaluate the effect of cyclicity, antral follicles count (AFC), body condition score (BCS) and body weight gain (BWG) on the pregnancy rate of Nelore cows submitted to timed artificial insemination (TAI). A total of 194 Nelore cows, 30 to 45 days post-partum with BCS of 2.7±0.5 (range 1 - 5); in the region of Congonhinhas, State of Parana was used. The animals were submitted to two ultrasound examinations (Mindray 2200, 7.5 MHz, China) with an interval of 14 days for evaluation of cyclicity by the presence of CL, and antral follicles>3 mm were counted by ultrasonography, using the transrectal linear probe. At the time of the second ultrasound evaluation, the animals had body weight measured and received an intravaginal progesterone device (CIDR®, Zoetis, Brazil) and 2mg of BE (Gonadiol®, Zoetis, Brazil). Nine days later, the implants were removed and the animals received 12.5 mg of dinoprost (Lutalyse®, Zoetis, Brazil), 300IU eCG (Novormon®, Zoetis, Brazil) and 1.0 mg EC (ECP®, Zoetis, Brazil). Forty-eight hours later the cows were inseminated with semen a single bull. New body weight measure and pregnancy diagnoses were performed 30 days later by ultrasonography examination. Data were analyzed by logistic regression ($p < 0.10$). The pregnancy rate had no interaction with BCS [BCS ≤ 2.5: 40.5% (30/74); BCS =2.75: 52.7%(19/36); BCS ≥ 3.0: 44.0(37/84); (p=0.50)], presence [57,5 (19/33)] or absence of CL [41,6 (67/161) p=0,23] and AFC [AFC ≤ 16: 49.1% (30/61); AFC 26 to 36: 44.1%(49/111); AFC>36: 31.8% (7/22), p=0,54]. There was an effect (p=0.05) of BWG during the experimental period (minimum: - 73.0kg and maximum: 73kg). Cows that maintenance or BWG up to 73 kg had a higher (p=0.09) pregnancy rate [48.8% (62/127)] than cows with loss of up to 73 kg of body weight [35.8% (24/67)]. In conclusion, the BWG between the TAI protocol and the pregnancy diagnosis affected the pregnancy rate. Therefore, BWG deserves to be highlighted as important aspects to increase the efficiency of biotechnology in beef cows. Acknowledgements: Fundação Araucária do Estado do Paraná.

031 TAI/FTET/AI

Hormonal associations aiming to optimize fertility outcomes of Nelore cows submitted to 7-d fixed-time AI protocols

Rodrigo Lemos Olivieri Rodrigues Alves[1], Carlos Eduardo Cardoso Consentini[1], Abraham López Oliva[5], Guilherme Madureira[1], Lucas Oliveira e Silva[1], Alexandre Barbieri Prata[2], José Renato Gonçalves[4], Milo Charles Wiltbank[3], Roberto Sartori[1]

[1]ESALQ/USP - Department of Animal Science, Luiz de Queiroz College of Agriculture of University of São Paulo, Piracicaba, SP; [2]Globalgen - Globalgen Vet Sience, Jaboticabal, SP; [3]UW - University of Wisconsin, Madison, WI, USA, Madison, WI 53706, EUA; [4]Figueira Farm - "Hildegard Georgina Von Pritzelwiltz" Experimental Station, Londrina, PR, Brazil; [5]UNAM - Cuautitlán Higher Education Faculty, Campo Uno, 54740 Cuautitlán Izcalli, Mexico.

The study evaluated fertility outcomes of Nelore cows submitted to 7-d fixed-time AI (FTAI) protocols. A total of 1461 lactating Nelore cows (911 multiparous and 550 primiparous) at 61.4±21.8 DIM and with BCS 3.1±0.01, during first (993) and second postpartum AI (468) were used. On D0, cows were randomly assigned to experimental groups and received an intravaginal progesterone (P4) device (1g) and 2mg estradiol benzoate (EB). On D7, every cow received 0.530mg cloprostenol sodium (PGF) and 300 IU eCG, concomitant with P4 withdrawal. FTAI was performed 48h later (D9). Experimental treatments were: administration (P1) or not (P0) of PGF on D0, 1 (C1) or 0.5 (C0.5) mg estradiol cypionate (EC) on D7, and 8.4μg (G1) buserelin acetate (GnRH) or no GnRH (G0) on D9, resulting in 8 treatments: P1C1G1 (189), P1C1G0 (190), P0C1G1 (176), P0C1G0 (181), P1C0.5G1 (169), P1C0.5G0 (176), P0C0.5G1 (187), and P0C0.5G0 (193). Hormones were from GlobalGen Vet Science. Statistical analyses were done by PROC GLIMMIX of SAS 9.4 (P≤0.05). Presence of CL on D0 did not differ between first AI and resynch cows [20% (279/1391)]. There was no interaction between the 3 factors of the study (PGF on D0, EC dose on D7, and GnRH at AI), and P/AI were similar between first and second AI [57% (835/1461)]. Multiparous had greater P/AI than primiparous (64 vs 46%) and cows with BCS >3 achieved higher conception rate than thinner cows [63 (413/660) vs 53% (422/801)]. There was an interaction between EC dose and GnRH treatment, in which cows receiving 0.5mg were helped by GnRH [61 (218/356) vs 49% (182/369)]. Presence of CL on D0 resulted in higher P/AI [(67 (174/260) vs 56% (594/1062)]. The GnRH treatment on D9 only improved fertility of cows that did not show estrus [47 (88/288) vs 36% (82/231)]. In addition, there was an interaction between EC dose and BCS, in which cows with low BCS (≤3) receiving 0.5mg EC had lower P/AI than cows treated with 1mg EC [49 (200/407) vs 56% (222/394)]. Treatment with 1mg EC induced more estrus than 0.5mg [72 (496/693) vs 67% (442/664)], and cows with CL on D0 also had more estrus than those without CL [81 (191/236) vs 67% (655/982)]. However, estrus was not influenced by PGF on D0 [70% (846/1218)]. Multiparous had greater dominant (DF) and ovulatory (OF) follicle than primiparous [9.9±0.1 (n=187) vs 8.8±0.1mm (n=93); 12.5±0.1 (n=208) vs 11.3±0.2mm (n=86), respectively]. Cows that expressed estrus had larger DF [9.6±0.1 (n=202) vs 9.0±0.2mm (n=63)] and OF [12.3±0.1 (n=241) vs 11.6±0.2mm (n=53)]. In conclusion, if cows are treated with 0.5 mg of EC on D7, GnRH treatment at AI is necessary, especially for cows not showing estrus. Cows with lower BCS may have fertility improved by using 1mg EC on D7. Moreover, the CL presence at the beginning of the FTAI protocol indicates a better nutritional and reproductive status, resulting in greater estrus expression and better fertility.

Acknowledgments: FAPESP, CNPq, CAPES, GlobalGen, Figueira Farm, STGenetics.

032 TAI/FTET/AI

Use of estradiol at day 14 post-TAI does not impair the pregnancy maintenance and increases the pregnancy rate in resynchronized beef heifers

Igor Garcia Motta[1], Danilo Zago Bissinoto[2], João Abdon Santos[3], Fainer Lincoln Savazzi Bertoncini[3], Vitor Hugo Guilger Gonzaga[1], Gilmar Arantes Ataide Júnior[1], João Vitor Vasconcellos[2], Bruno Gonzalez de Freitas[4], Kleber Menegon Lemes[5], Ed Hoffmann Madureira[1], Guilherme Pugliesi[1]

[1]VRA-FMVZ-USP - Departamento de Reprodução Animal, Pirassununga, SP, Brasil; [2]FZEA-USP - Faculdade de Engenharia de Alimentos e Zootecnia, Pirassununga, SP, Brasil; [3]JA Reprogen, Eunápolis, BA, Brasil; [4]Ourofino - Ourofino Saúde Animal, Cravinhos, SP, Brasil; [5]Boehringer-Ingelheim - Boehringer-Ingelheim Animal Health do Brasil Ltda, Campinas, SP, Brasil.

We aimed to evaluate the effects on pregnancy rate of using estradiol benzoate (EB) or 17β-estradiol (E2) associated to progesterone (E2+P4) in a resynchronization protocol at 14 days post-TAI in heifers. Thus, 1178 Nelore and 117 cross heifers (NelorexAngus) had the ovulation synchronized and were submitted to TAI (D0). On D14, heifers received an intravaginal P4 device (1g, Sincrogest, Ourofino Saúde Animal) and were randomly split in 3 groups: control (no treatment; n=433); EB (1mg EB, Sincrodiol, Ourofino; n=431); and E2+P4 (1mg E2 + 9mg P4, Betaproginn, Boehringer-Ingelheim; n=431). On D22, color Doppler ultrasonography was performed to detect non-pregnant (NP) heifers based on luteolysis detection (Pugliesi et al., Biol Reprod, 4: 1-12, 2014). The NP heifers received 1mg E2 cypionate (SincroCP, Ourofino), 500µg sodium cloprostenol (Sincrocio, Ourofino) and 200IU eCG (SincroeCG, Ourofino), and the largest dominant follicle (DF) was measured. A second TAI was performed on D24. In a subgroup of NP heifers (n=337), an estrous detection patch (Boviflag, ABS Pecplan) was used between D22 and D24, and DF was measured at the second TAI. Confirmatory diagnoses based on detection of embryo/fetus with heartbeat were performed between D37-67 after first TAI, and 43-47 days after second TAI. Data were evaluated by ANOVA (PROC MIXED), LSD test or logistic regression (PROC GLIMMIX) of SAS. No interaction with breed and lot was observed. Pregnancy rates (PR) after first TAI did not differ ($P>0.1$) between the control, EB and E2+P4 groups on D22 (53% [230/433], 53% [229/431] and 50% [217/431]) and confirmatory diagnoses (43% [149/344], 44% [154/349] and 46% [156/342]), respectively. Pregnancy loss between D22 and D37-67 was similar ($P>0.1$) in the control (19% [36/185]), EB (15% [28/182]), and E2+P4 (15% 28/184]) groups. On D22, the DF diameter (mm) was greater ($P<0.05$) in the control group (11.9 ± 0.14), than in the EB (11.3 ± 0.1) and E2+P4 (11.5 ± 0.1) groups. Proportion of heifers detected in estrus, and DF diameter on D24 did not differ ($P>0.1$) among the groups (overall mean: 63 ± 4.5% and 13.0 ± 0.2 mm, respectively). However, DF growth rate (mm/day) from D22 to D24 was greater ($P<0.05$) in the EB group (0.89 ± 0.08) than in the control (0.59 ± 0.07) and E2+P4 (0.66 ± 0.09) groups. The PR for the second TAI was greater ($P<0.05$) in the EB group (47% [94/200]) than in the control group (37% [76/203]), but did not differ ($P>0.1$) in the E2+P4 group (43% [93/214]) compared to the others. Cumulative PR (first and second TAIs) did not differ ($P>0.1$) between control, EB and E2+P4 groups (59% [204/344], 65% [227/349], 64% [220/342], respectively). In conclusion, administration of 1mg EB or 1mg E2 + 9mg P4 at 14 days post-TAI does not impair the pregnancy, and the 1mg EB treatment increases the pregnancy rate in resynchronized beef heifers for a second TAI within 24 days. Acknowledgments: FAPESP (2015/10606-9; 2017/18613-0); Geneplan; JA Reprogen; Faz Querência.

033 TAI/FTET/AI

Associative and isolated effect of vaccination against impacting reproductive diseases and mineral supplementation in inseminated Nelore cows

Jessica Souza Lima[1], Reiller Moraes Silva[5], Aline Gomes da Silva[1], Bruno Sivieri de Lima[2], Gustave Decuadro-Hansen[3], Luc M. Durel[4], Eliane Vianna da Costa e Silva[1], Gustavo Guerino Macedo[1]

[1]UFMS - Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brasil; [2]Virbac - Virbac Latin America, Santiago de Chile, Chile; [3]Virbac - Virbac do Brasil, São Paulo, SP, Brazil; [4]Virbac - Virbac SA, Carros, France; [5]Reprogene - Reprogene, Iporá, Goiás, Brazil.

Considering the seasonality of pasture production, cattle farms generally experience deprivation of food, decreasing mainly the body condition score (BCS) and immunological status resulting in open females after breeding season, increasing the generation interval. In tropical countries like Brazil, almost 2/3 of soil needs nutrients correction. To overcome this problem, the present study aimed to study the administration of mineral supplementation and vaccination against impacting reproductive diseases on synchronized Nelore females for TAI, considering pregnancy and pregnancy loss. Nelore females (n = 1361) had their BCS registered on D-21 with a random blood collection of 150 to measure serum biochemical status and antibodies title for Leptospira sp., IBR and BVD. Considering the D0 as the beggining of the synchronization of ovulation protocol and D10 the TAI, females were randomly assigned to four groups: Vaccine (n=314) at D-21 and D0, 5 ml i.m. of Bovigen®Repro(Virbac, Brazil; cont inactivated BoHV-1 and 5 Bov Herpesvirus, BVD-1 and 2 Bov Viral Diarrhea, Leptospira interrogans: pomona, wolffi, hardjoprajitno, icterohaemorrhagiae, canicola, copenhageni, bratislava; borgpetersenii hardjo bovis, Campylobacter fetusFetus and venerealis, venerealisintermedius, and 10 mg/dose of sodium selenite in 10% aluminum hydroxide); Supplement(n = 314) at D0 15 ml i.m. of Fosfosal® [(Virbac, Brazil; 100 ml contain sodium glycerophosphate (14g), monosodium phosphate (20.1g), copper chloride (0.4g), potassium chloride (0.6g), magnesium chloride (2.5g), sodium selenite (0.24g)]; Vacc+Supp (n = 363) 5 ml i.m. of the Vaccine at D-21 and D0 and 15 ml i.m. of supplement at D0; and Control(no treatment; n = 365). US was performed at D40 (P/IA30) and D100 (P/IA90) to evaluate pregnancy. BCS was registered on D40 (BCS30) and D100 (BCS90). Data were tested by normality using guided analysis of SAS 9.4, and the model by proc GLIMMIX. When significant, Tukey and lsmenas test were used to compair means. Serum analysis showed more than 80% of animals had at least 2 diseases. Phosphorus blood concentration was 7.2 mg/dl. Pregnancy was affected by Ca (preg= 2,55 e non-preg = 2.33 mg/dl; $p<0.05$) and creatinine (preg=5.85 e non-preg= 5.76; $p<0.05$). The BCS30 was 3.2 (1 – skinny; 5 – obese) and there was no effect of time (D-21 to DG40). Data showed P/AI30 for Vaccine, Supplement, Vacc+Supp and Control were 54 %, 52%, 57% and 49% ($p>0.05$), respectively. There was a tendency of higher pregnancy for Vacc+Supp (55%a) than Control (45%b; $p<0.1$). There was no effect of pregnancy loss Vaccine (5%), Supplement (4%), Vacc+Supp (3%) and Control (6%). Considering only pluriparous cows, there was no increase in P/AI using Vacc+Supp (59%) than Control (51%; $P<0.05$). Thus, the animals presented a very good BCS and effect of the supplement was only as immunostimulant; also starting a breeding season with supplementation and vaccination can increase the pregnancy rate and profitability of farmers.

034 TAI/FTET/AI

The Efficacy of a New Pedometer Tool For Heat Detection in Holstein Cows Raised in a Compost Barn System

Julian Scariot, Ricardo Zanella

UPF - Universidade de Passo Fundo, Passo Fundo, RS, Brasil.

Heat detection is considered the major problem related with the reproductive success in farm animals. In cows, the beginning of estrus is associated with specific behavioral changes as increase in the animal movement, reduction of feed intake and animals stand to be mounted by others. Therefore measurements of those signs could be used as an aid for heat detection in cattle. For this we have tested the efficiency of a new pedometer system developed by Gimenez® with a low total cost of less than R$500,00 per animal. This system is connected to a cell phone antenna which releases a signal every 2 hours indicating the cattle movement. Animals were submitted to a system calibration period of 10 days to evaluate the cattle regular movement. When an increase in movement is detected, the system releases a signal indicating the beginning of heat. This could predict the most accurate time for the success of the AI. In this study we have evaluated the use of a pedometer system in 25 Holstein cows raised in a compost barn system. Twenty three (n=23) open cows without any reproductive problem, and 2 pregnant cows serving as negative controls were evaluated. Cows were followed for 60 days with the use of Ultra Sound in a weekly base, for the identification of ovarian structures. The sensitivity and specificity of the pedometer system and visual observation was calculated to detect heat in cattle. The presence of a new CL seven days after the heat detection was used to confirm the ovulation. Sixteen cows (70%) out of twenty tree, were identified in heat using the pedometer system, and had a CL seven days after the detection. Six cows had the presence of a CL, however didn't show changes in their movement. Ten cows (n=10) showed heat using the pedometer and visually. Six (n=6) were identified in heat only with the use the pedometer system. Only one cow showed visual signs of heat and were not identified in heat with the pedometer and had a CL seven days after the detection. None of the pregnant cows showed heat. The sensitivity of the pedometer system was 70%, and the specificity was 100%, in contrast with the sensitivity of the visual heat detection of 47% and 100% of the specificity. Forty one percent (41%) of the cows, presented heat between 8:00am and 10:00am, 25% between 04:00am and 06:00am, and 17% between 00:00 and 12:00. The average daily milk production of cows identified in heat with the use of the pedometer was 37.66 L with an average days in lactation of 202.2 days, for the cows that did not show heat was 34.66 L and DEL of 208.5 (P=0.32 and P=0.8). No correlation was identified between milk production and efficiency of the pedometer. The pedometer system has the possibility to reduce the human labor and therefore it will reduce the error associated with the heat identification. Our data supports the use of the pedometer system, as an efficient tool for heat identification in cattle raised in a compost barn system.

035 TAI/FTET/AI

Effect of levamisole at beginning of FTAI protocol on the reproductive performance of Nellore cows

Luiz Carlos Louzada Ferreira[1], Henrique Jorge Fernandes[3,2], Luana Gomes da Silva[1], Tatiane Carvalho da Cunha[1], Ellen Caroline Soligo[1], Fernando de Almeida Borges[2]

[1]Cia Assessoria - Cia Assessoria, Campo Grande, MS; [2]UFMS - Universidade Federal de Mato Grosso do Sul, Campo Grande, MS; [3]UEMS - Universidade Estadual de Mato Grosso do Sul, Aquidauana, MS, Brasil.

The aim with this work was to evaluate the impact of the use of Levamisole at the beginning of the fixed time artificial insemination (FTAI) protocol on the fertility of cows. The study was conducted in a farm in Miranda city, Mato Grosso do Sul, Brazil. During three breeding seasons, 2016-2017, 2017-2018 and 2018-2019, we evaluated 2368 Nellore cows the first FTAI and 946 Nellore cows the second FTAI, in a program with two FTAI by season. Cows were divided in two groups: one treated with a single dose of levamisole phosphate at 4.7 mg / kg (LEV) (1315 and 404 at first and second FTAI, respectively) and the control (CON), which received a sterile saline solution of 1 ml / 40 kg, both on the day zero of the FTAI protocol. All animals were last treated for gastrointestinal nematodes five months before the beginning of the study. Pregnancy diagnosis was carried out 30 days after artificial insemination (AI). Cows that were not pregnant in this first diagnosis, received a second FTAI protocol, and a new pregnancy diagnosis was carried out 30 days after the second AI. To evaluate the anthelmintic efficacy, in the two first breeding seasons, feces were collected in D0 and D9 of the FTAI protocol. Fecal egg counts were evaluated using the MiniFlotac method, sensitivity 1:5. The efficacy of Levamisole in the first year was 86.6% and in the second year 92.48%, with a high frequency of *Haemonchus* spp. and *Cooperia* spp. in pre and post treatment. Data were evaluated in a completely randomized design using the PROC GLIMMIX of SAS University. Interactions between breeding season and treatments were evaluated and removed of the model as not significant. In the first FTAI, the pregnancy rate of the LEV cows (56.5 ± 11.7%) did not differ statistically from the CON ones (54.0 ± 11.8%). In the second FTAI, pregnancy rate of the LEV cows (45.0 ± 4.62%) also was not different (P >0.05) of the CON cows (43.7 ± 4.36%). Therefore, Levamisole used on zero day of the first FTAI protocol did not affect the fertility index in FTAI.

036 TAI/FTET/AI

Use of injectable progesterone associated to an intravaginal device (CIDR) for early resynchronization of Nelore cows and heifers submitted to three TAIs in 48 days

Gilmar Arantes Ataide Junior[1], Anderson Kloster[2], Danilo Zago Bisinotto[1], Émerson Moraes[4], Igor Garcia Motta[1], Izaias Claro Junior[3], José Henrique Tanner[5], José Luís Moraes Vasconcelos[2], Leonardo Souza[4], Guilherme Pugliesi[1]

[1]USP - Universidade de São Paulo, Pirassununga, SP; [2]UNESP - Universidade Estadual Paulista, Botucatu, SP; [3]Zoetis - Saúde Animal, São Paulo, SP; [4]Qualitas - Melhoramento Genético, Aparecida de Goiânia, GO; [5]Profissional Autônomo, Pontes e Lacerda, MT, Brasil.

We aimed to evaluate the pregnancy rate (P/AI) of beef cattle submitted to super-early resynchronization protocol using a progesterone (P4) device (CIDR, Zoetis) alone or associated to 100mg injectable P4 (iP4; Afisterone, Ceva). Nelore heifers (n=498) and cows (n=760) were underwent TAI (D0). On D13, animals were divided in two experimental groups: CIDR (insertion of a CIDR), and CIDR+iP4 (CIDR plus im 100mg iP4). On D22, an early pregnancy diagnosis (PD) was performed based on detection of luteolysis by color Doppler ultrasonography (DopplerUS; Z5 Vet, Mindray). When luteolysis was detected, non-pregnant animals (NPA) received im 12.5 mg dinoprost trometamine (Lutalyse; Zoetis), 0.6 mg estradiol cypionate (ECP; Zoetis) and 200 (heifers) or 300 (cows) IU eCG (Novormon, Zoetis). A 2nd TAI was performed on D24 in NPA (214 heifers and 302 cows). On D37, 1st AI pregnant females (absence of luteolysis) went through a PD based on detection of an embryo with heartbeat and those with pregnancy loss were resynchronized by insertion of a CIDR plus 2 mg estradiol benzoate (CIDR+EB). On D37, animals submitted to the 2nd TAI were resynchronized using the reverse experimental group of the 1st resynchronization. Another early PD by DopplerUS was done on D46 and ovulation was induced in NPA as on D22. A 3rd TAI was done on D48 in NPA (172 heifers and 211 cows). On D61 and D85, a PD was done to confirm pregnancies from the 2nd and 3rd TAI, respectively. The P/AI was evaluated by logistic regression using PROC GLIMMIX of SAS, considering the effects of group, sire, body condition score (BCS), farm, category and the possible interactions. The P/AI at the 1st TAI were 57% (284/498) for heifers and 60% (458/760) for cows. The overall P/AI for both categories tended to differ (P=0.08) between animals resynchronized with CIDR (38%, 148/387) and CIDR+iP4 (43%, 178/411). However, an interaction of group by BCS was observed (P=0.04), reflecting a greater (P=0.01) P/AI in the CIDR+iP4 group only when BCS was ≤ 2 (53% [32/60] vs. 28% [13/46]). When evaluated separately for each category, the P/AI in resynchronized heifers did not differ (P>0.1) between the CIDR (38%, 61/160) and CIDR+iP4 (44%, 73/167) groups, but an interaction of group by BCS was again observed (P=0.01). For cows, P/AI did not differ between the CIDR (38%, 87/227) and CIDR+iP4 (43%, 105/244) groups, but an effect of BCS was observed (P=0.04). The P/AI in animals receiving the CIDR+EB were 44% (26/59) and 58.5% (24/41) for heifers and cows, respectively. It was concluded that the supplementary dose of 100mg iP4 improved the P/AI in Nelore cattle submitted the super-early resynchronization protocol only when they have a BCS ≤ 2, regardless of the category (heifer or cow). Acknowledgments: Bela Vista and Longavira Farms, FAPESP (2015/10606-9; 2018/20058-7), Nelore GOU and Zoetis.

037 TAI/FTET/AI

Correlation of antral follicle count and scrotal circumference

Fábio Lucas Zito de Moraes, Ana Clara Canto Souza, Maysa Lopes Orsi, Anne Yaguinuma de Lima, Denis Vinícios Bonato, Andressa Guidugli Lindquist, Fabiana De Dio Sarapião, Fábio Morotti, Marcelo Marcondes Seneda

UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

The scrotal circumference is an important criterion of the andrology exam because it is related to the reproductive performance of the bull. The antral follicle count (AFC) has been on the spotlight of recent studies on fertility, but there is a lack of studies about the AFC of the mother and the reproductive potential of their progeny. We analyzed the correlation between the AFC of cows and the scrotal circumference of respective offspring. Nelore (Bos taurus indicus) cows (n = 63) were used. All the females were multiparous, age 36 to 96 months old and body condition between 2.5 and 3.5 (1 to 5 scale), kept on a pasture-grazing system (Brachiaria brizantha) with mineral supplementation. The antral follicle count was evaluated by transrectal ultrasonography with a 5 Hz linear transducer (Aloka SSD-500, Aloka Co. Ltda., Tokio, Japan) on the first day of the timed artificial insemination protocol. The cows were included in one of three groups: high AFC (≥25 follicles, n=20), intermediate AFC (15 to 20 follicles, n=25) and low AFC (≤12 follicles, n=18). The male calves from those cows were kept on the same farm, and they were examined at the age of 18 months. These 63 young bulls (one son of each cow) had their scrotal circumference (SC) measured around the largest point using the specific device for that purpose. The SC mean of each group (according to mother's AFC) were analyzed by the Kruskal-Wallis and Mann-Whitney-Wilcoxon tests and the correlation between the AFC and the SC were obtained by the Spearman test. All the tests were performed by the software R at 5% significance level. The AFC ranged from 5 to 80 follicles, with a mean of 22.48 ±15.65 cm (SD). The scrotal circumference of the offspring ranged from 31 to 44 cm, with a mean of 36.48 ±2.46 cm (SD). The bulls from mothers with high AFC group had the largest scrotal circumference (n= 20, 37.75 ±2.07cm), comparing to the SC from mothers with intermediate (n=25, 36.44 ±2.52 cm) and low (n= 18, 35.22 ±2.11cm) AFC (p = 0.002). We found a positive linear correlation (r = 0.447; p = 0.0002) between the AFC of the mother and the scrotal circumference of her son.

038 TAI/FTET/AI

Influence of use of protected fat in the transition period on endometrial vascular perfusion and conception rate in Holstein cows

Sebastião Inocêncio Guido[1], Fabiani Coutinho Lordão Guido[2], Leonardo Fernandes de Alencar[1], Paulo Roberto Lins de Azevedo[1], Júlio César Vieira de Oliveira[1], Joaquim Evêncio Neto[2]

[1]IPA - Instituto Agronômico de Pernambuco, Estação Experimental de São Bento do Una, PE, Brasil; [2]UFRPE - Universidade Federal Rural de Pernambuco, Recife, PE, Brasil.

The aims of this study were to evaluate the influence of the supplement of a fatty acid compound (FA) in the transition period associated the diet with spineless cactus on the uterine vascular perfusion, body condition score, beta-hydroxy-butyrate concentration (BHB) and postpartum conception rate in Holstein cows. Sixty-three cows were evaluated in the transition period and maintained in a semi-intensive system. Cows at 220 days of gestation were submitted to the pre-calving period of 60 days. They were fed with 45% of spineless cactus, 11% of sorghum silage and 44% of protein concentrate with 14% of CP added mineral supplement in the former as dry matter (DM) base diet. At 21 days before calving, protein supplementation was altered to 22% of CP. The cows were distributed in two experimental groups (G1 and G2). In the G1 (n = 30) cows received in the transition period the base diet added of 100g of the compound of FA (protected fat) consisting of linoleic acid, linolenic acid and conjugated linoleic acid (Megalac®-E, Vaccinar). G2 (n = 33) consisted of cows that received only the base diet. All cows were submitted to the assessment of the body condition score (BCS) on the scale of 1 to 5. Being evaluated at the beginning of the transition period and at the end of the voluntary waiting period (VWP) at 40 days postpartum. The blood concentrations of BHB were measured in all cows, and the dosages were performed at the beginning of the transition period and at the end of the VWP using commercial kit Ketovet®. After the VWP, all cows from both groups underwent ultrasound examination, using linear endorectal transducer with 6.0 MHz in color Doppler mode to evaluate endometrial vascular perfusion (EVP), with scores ranging from 1 to 4 for diagnosis of subclinical endometritis according to Guido (2019). As well as evaluating the appearance of vaginal mucus using Metricheck® and assigning scores from 0 to 3. Immediately after the evaluations, the cows (G1 and G2) were submitted to TAI protocol, and the diagnosis of pregnancy was performed 30 days later. The data were submitted to analysis of variance ANOVA and to the Z test at 5%. Regarding the BSC results, there was no difference ($p=0.41$) between the groups, nor in relation to the pre and postpartum periods. In relation to BHB, mean postpartum concentrations were higher ($p=0.00016$) in G2 (1.7 mmol/mL). In the evaluation of EVP, higher scores ($p = 0.00231$) was found to G2; 2 (70.8%) and 3 (39.4%). Metricheck in G2, a superior result ($p<0.0251$) was observed for the score 2 in 45.5% of the cows. The conception rate was higher ($p=0.00214$) in G1 cows (62.5%) than G2 (42.4%). Therefore, it was concluded that cows supplemented with protected fat during the transitional period presented satisfactory results regarding BHB concentrations and higher postpartum conception rate. As well, proportionally cows not supplemented had elevated endometrial vascular perfusion scores, suggestive of subclinical endometritis.

A043 OPU-IVF and ET

In vitro production and transfer of embryos from 12 and 24 months old Nellore heifers (*B. indicus*) treated or not with FSH

Laísa Garcia da Silva[1], Bernardo Marcozzi Bayeux[2], Flávia Morag Elliff[1], Naiara Nantes Rodrigues[4], Rodolfo Daniel Mingoti[1], Bruna Lima Chechin[1], Fábio Morato Monteiro[3], Pietro Sampaio Baruselli[1]

[1]USP - Universidade de São Paulo, São Paulo, SP; [2]BOVIGÊNESE, Cravinhos, SP; [3]IZ - Instituto de Zootecnia de Sertãozinho, Sertãozinho, SP; [4] UNESP, Jaboticabal, SP, Brasil.

The present study evaluated the *in vitro* production and transfer of embryos produced by 12 and 24-month-old Nellore (*Bos indicus*) heifers that were treated or not with FSH. For that, 126 heifers from Instituto de Zootecnia de Sertãozinho – SP were submitted to a 2x2 factorial arrangement [12-month-old heifers not treated with FSH (n=31); 12-month-old heifers treated with FSH (n=31); 24-month-old heifers without FSH (n=32) and 24-month-old heifers with FSH (n=32)] and 2 OPU (with 40 days interval; cross over). All heifers were synchronized with 2mg of estradiol benzoate (RIC-BE®, Agener, São Paulo), 0.530mg of sodium cloprostenol (Estron®, Agener, São Paulo) and received an intravaginal P4 device (360mg, Primer PR®, Agener, São Paulo). FSH groups received two injections of 30mg of FSH (Folltropin®, Agener, São Paulo) on day 4 (AM and PM) and two injections of 20mg of FSH on day 5 (AM and PM). On day 7 (after 44 hours of coasting period), the device was removed and all heifers were submitted to OPU (guia EC9-5 Novilha, WTA, Cravinhos, SP; US S8®, SonoScape, China) of all follicles that were counted, classified in small (<5mm), medium (5-8mm) and big (>8mm). Collected oocytes were selected and forwarded to IVP. Semen from 3 sires used for IVF were equally distributed within groups. Produced embryos (n=200) were frozen and transferred in synchronized recipients. Statistical analyses were done by GLIMMIX procedure of SAS®. There was no interaction between age category (12 *vs.* 24) and treatment (Control *vs.* FSH) for the studied variables (P>0.05). Treatment with FSH increased the number of small, medium and big follicles (P<0.001), and the total follicles (GC= 27.8±1.8, GFSH= 31.8±1.6; P=0.04), decreased the recovered oocytes rate (GC= 78.9%, GFSH: 52.5%; P<0.0001), increased viable oocytes rate (GC= 70.2%, GFSH: 78.6%; P=0.0002) and increased blastocyst rate over the total of recovered oocytes (GC: 18.6%, GFSH: 22.0%; P=0.0002), however there was no effect on the number of blastocysts per OPU (GC= 3.9±0.5, GFSH= 3.94±0.5; P=0.96). Still, 12-month-old heifers presented lower total of follicles (G12= 27.7±1.8, G24= 31.9±1.6; P=0.0485), same recovered oocytes rate (G12= 64.8%, G24= 66.6%; P= 0.4632), same viable oocytes rate (G12= 73.5%, G24= 75.4%; P=0.8089), lower blastocyst rate (G12= 16.5%, G24= 24.0%; P<0.0001), and lower number of blastocysts per OPU (G12= 2.8±0.4, G24= 5.9±0.6; P=0.0026). Concerning the ET, there was no effect of age (P= 0.5131) or treatment (P= 0.9623) on pregnancy rate within groups (G12C: 24.1% (7/29); G12FSH: 21.0% (8/39); G24C: 24.3% (17/70); G24FSH: 29.1% (18/62). It is concluded that the treatment with FSH in 12 and 24-month-old Nellore (*Bos indicus*) heifers decreased the recovery rate probably because the size of follicles increased. The treatment improved blastocyst rate, but there was no effect on ET pregnancy rate. Besides that, 12-month-old heifers presented lower efficiency on IVP when compared to 24-month-old heifers.

A044 OPU-IVF and ET

Effect of the anticipation of intrafollicular transfer of immature oocytes (IFIOT) in the nuclear maturation of bovine oocyte

Otávio Augusto Costa Faria[1], Felippe Manoel Caixeta[1], Luzia Renata Oliveira Dias[1], José Felipe Warming Sprícigo[2], Margot Alves Nunes Dode[3]

[1]UnB - Universidade de Brasília, Brasília, DF, Brasil; [2]University of Guelph, Canadá;[3]Embrapa Cenargen - Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brasil.

Previous results in our laboratory demonstrated that when intrafollicular transfer of immature oocytes (IFIOT) is performed 52 to 54 hours after the removal of the progesterone implant, the time that CCO´s remain in the follicle is insufficient for them to complete nuclear maturation. Therefore, the objective of this study was to evaluate nuclear maturation of bovine oocytes submitted to IFIOT 30 hours after the removal of the progesterone implant. Twenty-six Nelore ovulators (*Bos taurus indicus*) were synchronized on day 0 (D0) with the insertion of an intravaginal progesterone implant (1g) and 2mg benzoate estradiol. On day 8 (D8), the implant was removed and 500 µg Cloprostenol sodium (PGF) was administered (i.m.). Thirty hours after implant removal ($D9^{1/2}$), grades 1 and 2 COC´s, were injected into the dominant follicle (diameter> 10mm). The CCOs were obtained from slaughterhouse ovaries and in each replicate part of the oocytes were used for IFIOT and part for IVM. All manipulation of the oocytes was performed in follicular fluid. After the IFIOT, the animals were distributed into two groups: Group LH, animals (n = 5) received a dose of an analogue of LH (1.25 mg) after IFIOT or Group GnRH (n = 7), that received a dose of GnRH (50 µg). After 22 hours, oocytes from both groups were retrieved by ovum pick up (OPU). For the IVM group, immature oocytes (CT 0) and oocytes matured *in vitro* for 22 hours (CT 22) were used. Oocytes from all groups were denuded, fixed and stained with Lacmoid for the evaluation of the meiosis stage. The oocytes were classified as: germinal vesicle (VG), germinal vesicle breakdown (VGBD), metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII) and abnormal. Data were analyzed by chi-square test ($P < 0.05$). The mean size of the dominant follicle at the time of IFIOT was 11.93 (± 0.98) mm. The mean recovery rate (OPU) after 22 hours of IFIOT was 67.25%, being 76% for the LH group and 62% for the GnRH group. A total of 379 oocytes (CT 0, n = 81, CT 22, n = 56, LH 22, n = 106 and GnRH 22, n = 136) were evaluated. At 0 hour, 98.76% of the oocytes were in VG. At 22 hours of maturation, the percentage of oocytes that reached the MII was similar ($P > 0.05$) between the groups (CT 22 = 75%, GnRH = 72.05%, and LH = 67.92). The results demonstrated that the 22 hours intrafollicular maturation period is adequate for oocyte maturation within the follicle. Further studies need to be performed to evaluate the competence of these oocytes. Support: CAPES

A045 OPU-IVF and ET

Pregnancy rate after fixed-time transfer of frozen-thawed Lacaune sheep embryos recovered by transcervical route

Lucas Machado Figueira[1,8], Nadja Gomes Alves[1], Fernanda Alves Lucas[1], Ribrio Ivan Tavares Pereira Batista[2], Aline Matos Arrais[3], Gabriel Brun Vergani[4], Jader Forquim Prates[5], Lucas Corrêa de Souza[6], Jeferson Ferreira da Fonseca[7], Joanna Maria Gonçalves Souza-Fabjan[8]

[1]UFLA - Universidade Federal de Lavras, Lavras, MG, Brasil; [2]UFVJM - Universidade Federal dos Vales do Jequitinhonha e Mucuri (Diamantina-MG, Brasil); [3]UFRRJ - Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil; [4]UNESP - Universidade Estadual Paulista "Júlio de Mesquita Fillho" Jaboticabal, SP, Brasil; [5]IFSudesteMG - Instituto Federal do Sudeste de Minas Gerais, Rio Pomba, MG, Brasil; [6]CVDF - Cabanha Val di Fiemme, Soledade de Minas-MG, Brasil; [7]EMBRAPA - Embrapa Caprinos e Ovinos, Coronel Pacheco-MG, Brasil; [8]UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil.

The cervical dilation treatment with d-cloprostenol, estradiol benzoate and oxytocin is an indispensable step for efficient non-surgical embryo recovery (NSER). However, the effects of these hormones on the viability of embryos after cryopreservation and transfer are still questioned. The aim of this study was to compare the viability after fixed-time embryo transfer (FTET) of embryos obtained by NSER and cryopreserved by two techniques. Embryos were recovery by transcervical method after hormonal treatment to induce cervical dilation (Fonseca et al., Reprod. Domest. Anim. 54(1):118-125, 2019) in donors of Lacaune breed (68.3 ± 6.7 kg of body weight - BW and 3.5 ± 0.2 of body condition score) and cryopreserved by either: slow freezing (SF - Fonseca et al., Arq. Bras. Med. Vet. Zootec., 70(5):1489-1496, 2018) or vitrification(VT - Gibbons et al., Theriogenology, 52:1005-1020, 2011). Sixty-three nulliparous ewes (46.7 ± 8.3 kg of BW) received sponges with 60 mg of MAP (Progespon®, Syntex, Buenos Aires, Argentina) for six days, besides 37.5 µg d-cloprostenol (Prolise®, Tecnopec, São Paulo, Brazil) and 200 IU eCG (Folligon 5000 IU®, Intervet, São Paulo, Brazil) intramuscularly on day before sponge removal (Day 0). Ovarian transrectal ultrasonography (Mindray M5VET®, Shenzen, China - 8.0 MHz) was conducted on Day 7 for detecting the corpora lutea (CL) count and side (right or left ovary). The recipients that presented CL (92%, 58/63) were subjected to embryo transfer on Day 8.5 after sponge removal by the semi-laparoscopic technique and received embryos on the uterine horn ipsilateral to the ovary with CL. Straws containing one or two embryos (morulae and/or blastocyst) subjected to SF (*n*=33) or VT (*n*=25) were randomly used. Pregnancy diagnosis was performed on Day 31. Data were analyzed using SAS® software. The PROC GLIMMIX was used with: (1) Poisson distribution for number of embryos/recipient and (2) binomial distribution for pregnancy rate. Recipient BW was used as covariate, and models included fixed effect of cryopreservation technique. The number of embryos/recipient did not differ (P>0.05) between SF (1.9±0.1) and VT (1.8±0.1). The pregnancy rate tended to be higher (P=0.08) in SF (39%, 13/33) than VT (16%, 4/25). The pregnancy rate in SF was similar to the rates observed in commercial FTET programs in cattle, demonstrating the viability of these embryos obtained by NSER. In sheep, the FTET is employed mainly by transfer of embryos produced *in vitro*, but to our knowledge, the present study is the first worldwide reference of FTET with embryos produced *in vivo* and recovered by NSER. In conclusion, embryos recovered by NSER after cervical dilation treatment with d-cloprostenol, estradiol benzoate and oxytocin, and later cryopreserved by either slow freezing or vitrification established pregnancy after FTET, and better rates were observed with the slow freezing technique.
Financial support: Embrapa (02.13.06.026.00.05) and Fapemig (CVZ-PPM 00201-17).

A046 OPU-IVF and ET

Superovulation and nonsurgical embryo recovery in dairy goats previously affected by hydrometra

Joanna Maria Gonçalves de Souza-Fabjan[1], Ana Lucia Rosa e Silva Maia[1], Aline Matos Arrais[2], Lucia Prellwitz[1], Mariza Amanda de Rebello Costa[3], Glaucia Mota Bragança[1], Ribrio Ivan Tavares Pereira Batista[1], Jeferson Ferreira da Fonseca[4]

[1]UFF - Universidade Federal Fluminense, Niteroi, RJ, Brasil; [2]UFRRJ - Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil; [3]UNIGRANRIO - Universidade do Grande Rio, Duque de Caxias, RJ, Brasil; [4]EMBRAPA Caprinos e Ovinos - Empresa Brasileira de Pesquisa em Agropecuária, Coronel Pacheco, MG, Brasil.

Hydrometra is responsible for sub or infertility reaching over 10% of prevalence in dairy goats. Although treatment exists, its recurrence and the loss of reproductive efficiency caused usually leads the goat to be culled. This study assessed for the first time the efficiency of superovulation (SOV) treatment in dairy goats previously affected by hydrometra (HD). The study was conducted in the anestrus season, in Minas Gerais state (21º 21' S), Brazil. Pluriparous dairy goats diagnosed by transrectal ultrasound (US) with no reproductive disorder (n=11, CONT) or with HD (n=10) were used. All goats with HD were treated before used as donors. The goats aged 1-7 years old, weighted 67.4 ± 7.8 kg and had body condition score between 2.5 and 4.0 (scale 1–5). Intravaginal devices containing 0.33 g progesterone (CIDR-G®, Pfizer do Brazil, SP, Brazil) were inserted for 6 d. For SOV, 133 mg pFSH (Folltropin-V®, Bioniche, Belleville, Canada) i.m. were applied in six decreasing doses, every 12 h, starting 48 h prior to device removal. Three doses of 37.5 µg d-cloprostenol (Prolise®, Tecnopec, SP, Brazil) i.m. were administered, at the fourth and fifth doses of FSH, and 12 h before the nonsurgical embryo recovery (NSER). At 24 h after device removal, 25 µg GnRH (Gestran®, Tecnopec, SP, Brazil) i.m. were given. Moreover, three doses of 1.5 mL flunixin meglumine (Flumax®, J.A. Saúde Animal, SP, Brazil) i.m. were administered (36, 60 and 84 h) after GnRH. After device removal, estrus was checked twice a day and goats were naturally mated. At 6-7 d after estrus onset, the number of corpora lutea (CL) was counted by US (Mindray M5VET®, Shenzen, China - 8.0 MHz) and NSER was performed. Normally distributed data were submitted to ANOVA, whilst non-normally distributed data were analyzed by Mann-Whitney test ($P<0.05$ considered as significant). All goats showed estrus and were subjected to NSER. The interval from device removal to estrus, estrus duration and SOV response rate (positive when >3 CL were observed) were similar ($P>0.05$), respectively, between CONT (29.1 ± 3.9 h; 21.8 ± 2.0 h and 82%) and HD (31.0 ± 2.3 h; 18.2 ± 2.8 h and 90%). The recovery rate, number of retrieved structures and viable embryos per goat were similar ($P>0.05$) between CONT [74% (56/76), 6.9 ± 1.7 and 5.1 ± 1.5] and HD [62% (28/45), 4.5 ± 1.2 and 2.8 ± 0.9), respectively. However, there was a difference ($P<0.05$) in the number of structures in delayed stage (8-16 cells) between CONT [1% (1/76)] and HD [29% (13/45)]. This can be a result of a poor oviduct/uterine environment to promote embryo development, suggesting a possible mechanism for the reproductive failure after HD-treatment. In conclusion, although the SOV response and NSER technique were not affected in HD-goats, the quality of retrieved embryos is questionable, and caution should be taken before indicating SOV in these animals. Financial support: CNPq (479826/2013-7), FAPEMIG (CVZ-PPM 00201-17) and FAPERJ (E-26/202.268/2018).

A047 OPU-IVF and ET

Effect of FSH treatment on the IVEP of Gyr (*Bos indicus*) calves, pubertal heifers and adult cows and pregnancy rate of the ET

Flávia Morag Elliff[1], Bernardo Marcozzi Bayeux[3], Luiz Fernando Rodrigues Féres[2], Evandro do Carmo Guimarães[2], Marcos Henrique Alcântara Colli[1], Pietro Sampaio Baruselli[1]

[1]VRA/USP - Departamento de Reprodução Animal da Universidade de São Paulo, São Paulo, SP, Brazil; [2]Fazendas do Basa - Fazendas do Basa, Leopoldina, MG, Brazil; [3]Bovigênese, Cravinhos, SP, Brasil.

For this study, 90 Gyr donors were used: 30 calves (3-10 months), 30 pubertal heifers (16-22 months) and 30 cows (44-88 months), distributed into: Calves - control (CC, n=15), calves with FSH (FC, n=15); Heifers - control (CH, n=15), heifers with FSH (FH, n=15); Adult animals – control (CA; n=15), adult animals with FSH (FA; n=15). All animals received an intravaginal P4 device (calves – Primer PR, Agener União – Saúde Animal, Brazil; heifers and cows – Prociclar, Ceva Saúde Animal, Brazil) and estradiol benzoate (calves and heifers - 1 mg, cows - 2 mg; Fertilcare Sincronização, MSD Saúde Animal, Brazil) on D0. The treated groups received 80mg (FC), 100mg (FH) or 140mg (FA) of FSH (Folltropin, Vetoquinol, Brazil), split into 4 injections given twice a day in decreasing doses (coasting period: Calves - 24h; Heifers and Cows - 48h). The control animals of each category received no additional treatment. On D7 the P4 devices were removed and all animals underwent transvaginal ultrasound-guided OPU (EC9-5 Heifer, WTA, Brazil; ultrasound S8®, SonoScape, China). The recovered oocytes were sent to a commercial lab for the IVEP. The produced embryos (280 embryos) were transferred to crossbred heifer recipients. The obtained data were analyzed by the GLIMIX procedure of SAS®. Treatment with FSH increased (P=0.03) the number of medium sized follicles on D7 of all animal categories when compared to the same animal category without treatment (CC: 0.9±0.5; CH: 1.1±0.9; CA: 1.6±1.2 vs. FC: 8.3±7.3; FH: 11.8±7.0; FA: 7.4±5.3). Heifers yielded more oocytes (P=0.02) when compared to calves and cows (heifers: 19.9±0.8; calves: 12.3±0.6; cows: 11.2±0.6). The effect of FSH on the number of viable oocytes varied (P<0.001) according to animal category (CH: 15.2±10.0^{a}; FH: 12.9±10.4^{ab}; FA: 9.7±7.6^{bc}; CC: 8.1±6.9^{cd}; FC: 8.7±6.9^{cd}; CA: 6.5±4.2^{d}). The number of cleaved oocytes was greater (P<0.001) for heifers (11.3±0.6) than for calves and cows (5.8±0.4 and 7.1±0.4), the cleavage rate was greater (P=0.01) for heifers (56.7%; 11.3/19.9) and cows (63.3%; 7.1/11.2) when compared to calves (47.1%; 5.8/12.3). The number of blastocysts per OPU showed a tendency to increase (P=0.06) when calves (FC: 2.0±1.7; CC: 1.1±1.3) and cows (FA: 4.9±4.6; CA: 3.1±2.2) were treated with FSH. Regarding the ET, no difference was observed for pregnancy rate at 30 [CC: 52.9% (9/17); FC: 30.7% (8/26); CH: 47.6% (31/65); FH: 42.3% (24/58); CA: 55.5% (25/45); FA: 57.9% (40/69); P=0.38] and 60 days [CC: 41.1% (7/17); FC: 30.7% (8/26); CH: 40.0% (26/65); FH: 36.2% (21/58); CA: 51.1% (23/45); FA: 49.2% (34/69); P=0.88]. No difference was observed for pregnancy loss [CC: 22.2% (2/9); FC: 0.0% (0/8); CH: 16.1% (5/31); FH: 12.5% (3/24); CA: 8.0% (2/25); FA: 10.0% (4/40); P=0.69]. These results demonstrate that treatment with FSH increases de IVEP of Gyr calves and cows and has no effect on pregnancy rates.

A048 OPU-IVF and ET

Oocyte quality evaluation of 14 months old Nellore heifers that became pregnant or not after breeding season

Claudiney Melo Martins[1], Laísa Garcia da Silva, Romulo Germano de Rezende[2], Emiliana Oliveira Santana Batista[2], Alexandre Henryli Souza[2], Pietro Sampaio Baruselli[2]

[1]Fertiliza, Cuiabá, MT; [2]USP - Universidade de São Paulo, São Paulo, SP, Brasil.

It was aimed to investigate the influence of oocyte quality on 14 months old Nellore heifers submitted to TAI. For this study, 75 Nellore (*Bos indicus*) heifers of Fazenda Campina – Nelore CV (Caiuá, SP) were aspirated then synchronized for TAI. On day 0 of the experiment, heifers were submitted to transvaginal US guided OPU (DP2200Vet, Mindray, China) on random day of the estrous cycle. The oocytes obtained were selected and forwarded to IVF/IVP in the laboratory (Bovitran, Cuiabá, Brasil). It was used semen from 9 sires for oocytes fertilization, and the same mates were maintained for TAI. On the day of OPU (D0), all heifers were synchronized receiving an ear norgestomet device (Crestar®, MSD, São Paulo). On day 9, the ear device was removed and were administered 0.530mg of sodium cloprostenol (Ciosin®, MSD, São Paulo), 0.5mg of EC (Fertilcare Ovulação®, MSD, São Paulo) and 200IU of eCG (Folligon®, MSD, São Paulo). On day 11, heifers were artificially inseminated. Pregnancy diagnosis was done 22 days later by US Color Doppler (M5®, Mindray, China). Statistical analyses were performed by GLIMMIX procedure of SAS®. Pregnancy rate of heifers submitted to TAI was 40.5% (there was no effect of BCS, weight, womb diameter, FD diameter and ciclicity previously to experiment; P>0.05). Total oocytes (23.9±2.3 *vs*. 21.3±2.2; P=0.51), viable oocytes (15.3±2.1 *vs*. 13.0±1.1; P=0.45), cleaved number (10.3±0.9 *vs*. 11.0±1.1; P=0.39) and embryo produced by OPU (4.6±0.5 *vs*. 4.7±0.6; P=0.73) did not differ between heifers that became pregnant or non-pregnant after TAI. Pregnancy rate of embryos produced by pregnant and non-pregnant heifers did not differ as well [36.8% (28/76) *vs.* 33.0% (36/109), P=0.6155). It is concluded that heifers that became pregnant to its first TAI presented similar efficiency in OPU/IVP when compared with heifers that became non-pregnant. Acknowledgements: Nelore CV, Fertiliza, Bovitran.

A049 OPU-IVF and ET

Use of phenazine etosulfate (PES) on the *in vitro* production of bovine embryos

Pâmella Alves Correia[1], Marcelo Siqueira El Azzi[1], Thais Alves Rodrigues[4], Raphael Nunes dos Santos[4], J Jasmin[2], Marcos Brandão Dias Ferreira[3], João Pedro Araújo Campos[1], Nadja Gomes Alves[1], José Camisão de Souza[1]

[1]UFLA - Universidade Federal de Lavras, Lavras - MG; [2]UFRJ - Universidade Federal do Rio de Janeiro, Duque de Caxias, RJ; [3]EPAMIG - Empresa de Pesquisa Agropecuária de Minas Gerais, Belo Horizonte, MG; [4]Cenatte - Cenatte embriões, Pedro Leopoldo, MG, Brasil.

The aim of this study was to evaluate the effect of different doses of the drug phenazine etosulfate (PES) during *in vitro* maturation of bovine oocytes from slaughterhouse. Consequences for embryo production and survival after vitrification were evaluated. The effects of supplementation of IVM medium with PES at 0; 0.16; 0.4; 1.0 and 2.5 μM and of replicate were evaluated on the proportion of bovine embryos produced, survival, hatching and expansion rates after thawing from vitrification. Data were submitted to analysis of variance (PROCGLM) and, when binomially distributed, to the generalized linear model procedure (GENMOD), following normality tests under the SAS® UNIVARIATE procedure. The dose of PES 2.5 μM was toxic - no blastocyst production after 7 days of culture, and only one grade I embryo at day 8 (2 replicates, n= 70 oocytes). The other groups had a minimum of 550 cumulus-oocyte complexes matured for each dose tested on 12 replicates, resulting in 400 vitrified embryos. The proportions of embryos produced between the replicates ($P < 0.0001$) ranged from 23.5% to 51.0%. The proportion of embryos produced after addition of PES during *in vitro* maturation was higher ($P < 0.0035$) for the control group (C = 41.5 ± 1.8%, n = 237) than for 0.16 (32.5 ± 1.7%, n = 182) and 1.00 (32.6 ± 1.7%, n = 186) but did not differ from the 0.4 group (35.6 ± 1.7%, n = 210). All PES-treated groups did not differ. The survival rate after 48 hours post thawing ($P = 0.123$) did not differ (C = 52.3 ± 7.4%, n = 52, 0.16 = 49.2 ± 7.6%, n = 36; 0.4 = 48.8 ± 7.3%, n = 49; 1.0 = 52 ± 7.3%, n = 44). The hatching rate ($P = 0.104$) in this period tended to be better for groups 0.4 and 1 (0.4 = 32.4 ± 5.6%, n = 38, 1 = 34.3 ± 5.6%, n = 32) than control and 0.16 (C = 27.2 ± 5.7%, n = 13; 0.16 = 22.4 ± 5.8%, n = 17). The rate of expansion ($P = 0.029$) at 48 hours after thawing was the same for the control group (27.1 ± 5.2, n = 20), 0.16 (24.3 ± 5.6%, n = 20) and 1 (17.7 ± 3.5%, n = 10). The 0.4 (10.3 ± 2.0%, n = 12) and 1.0 groups did not differ from each other. Even with the careful selection of grade I and II oocytes only, because they were from slaughterhouse, possible differences between groups of animals for each replicate (subspecies, nutritional status, parity, reproductive status) may have led to this replicate effect in the embryo production results. The low rate of expansion in the embryos at the highest doses was possibly due to the high hatching rates of these treatments, which did not mean that the doses 0.4 and 1 were harmful or toxic to the expansion of the thawed embryos. The PES treatment of 0.4 μM had no deleterious effect on embryo production, 77.5% of the blastocysts that survived after 48 hours of thawing hatched whereas for the control group 25% hatched. Therefore, more studies are needed to evaluate the use of PES in maturation for vitrification of bovine embryos.

A050 OPU-IVF and ET

In vitro production of embryos from Holstein females treated with propylene glycol

Romulo Germano de Rezende[1], Rodolfo Daniel Mingotti[1], Bernardo Marcozzi Bayeux[2], Flavia Morag Elliff[1], Tiago Carneiro[1], Guilherme Machado Zanatta[1], Ana Elisa Barreto[1], Larissa Zamparone Bergamo[3], Marcos Henrique Alcantara Colli[1], Yeda Fumie Watanabe[4], Aline Oliveira[4], Pietro Sampaio Baruselli[1]

[1]USP - Universidade de São Paulo, São Paulo, SP; [2]Bovigênese, Cravinhos, SP; [3]UEL - Universidade Estadual de Londrina, Londrina, PR; [4]Vitrogen FIV, Cravinhos, SP, Brasil.

The objective of the present study was to evaluate the effects of 500 mL propylene glycol (PPG) supplementation every 12 hours for 5 days in vitro embryo production (PIVE) of Holsteins (Bos taurus). We used 323 females belonging to different animal categories randomized, into control group (CTL) and PPG group: cows at the beginning of lactation (CTL: n = 41; PPG: n = 37), repeat breeder cows (CTL: n = 38; PPG: n = 36), dry cows (CTL: n = 45; PPG: n = 49) and prepubertal heifers (CTL: n = 39; PPG: n = 38). The OPU was performed on day 0 for follicular ablation and for synchronization of the emergence of the follicular growth wave, and then the OPU was performed for PIVE on day 5. The aspirated oocytes were matured for 24 hours, fertilized with the same set of sexed semen and cultured in vitro. Statistical analyzes were performed using Statistical Analysis System (SAS, Version 9.4) software. The results suggest that there was an increase (P = 0.0583) in the rate of embryonic development in prepubertal heifers supplemented with PPG when compared to the control group (16% vs. 26%). In dry cows, a trend (P = 0.0580) of increase in the cleavage rate was observed in the PPG treated animals when compared to the control group (44% vs. 47%). For the category of lactating cows, there was a significant increase (p = 0.0189) in the rate of blastocysts in cows treated with PPG compared to the control group (18% vs. 37%). In this way, an improvement in the oocyte quality was demonstrated by the increase in the rate of blastocysts in cows at the beginning of lactation. Also, unexpectedly, there was a reduction in the number of oocytes recovered by OPU in PPG treated animals when compared to untreated animals (5.7 ± 0.69 vs. 3.7 ± 0.53, P = 0.0042). At the end, there was no difference in the number of embryos produced by OPU between the groups (0.98 ± 0.21 vs. 1.14 ± 0.19, P = 0.8349). For cows at the end of lactation (repeat breeder cows) no effects of treatment with PPG on embryo production efficiency were observed. This animal category has high levels of circulating glucose, which may explain the lack of response. In addition, repeat breeder cows may exhibit peripheral insulin resistance. Thus, it is concluded that supplementation with 500 mL of PPG every 12 hours for 5 days increases the rates of PIVE in heifers and cows at the beginning of lactation, and may improve oocyte competence, resulting in higher rates of embryo development and quantity of blastocysts produced by OPU.

A051 OPU-IVF and ET

Association between antral follicle count and *in vitro* embryo production in Holstein calves

Amanda Fonseca Zangirolamo[1,2,3], Bruno Valente Sanches [3], Anne Kemmer Souza[1,2], Fabio Morotti[2], Larissa Zamparone Bergamo[2], Marcelo Marcondes Seneda[1,2]

[1]INCT-Leite - Instituto Nacional de Ciência e Tecnologia para a Cadeia Produtiva do Leite (Universidade Estadual de Londrina, Londrina, PR; [2]UEL - Universidade Estadual de Londrina (Laboratório de Reprodução Animal (ReproA), DCV-CCA, Londrina, PR; [3]Vytelle IVF (LLC, Hermiston, OR 97838, USA.

There is an increasing interest in anticipating reproducing of high genetic value cattle. Antral follicle count (AFC) is an important tool for the selection of potential oocyte or embryo donors in adult cattle. However, little is known about AFC and in vitro embryo production from prepubertal calves. Our objective was to compare the association between antral follicle count and in vitro embryo production (IVEP) in *Bos taurus taurus* calves. Holstein donors (n = 135) between 7 and 9 months of age were retrospectively classified as Low (n = 67) or High (n = 68) AFC, according to the number of oocytes recovered by OPU (Low = 1-10 and High = 20-78). The oocytes recovered were matured for 24 hours, fertilized and cultured in vitro. The statistical analyses were performed using statistical software Minitab, version 18.1, adopting $p<0.05$, and analysis of variance was performed using the generalized linear model. After 264 procedures of OPU, the mean number of oocytes recovered by High and Low AFC was 34.60±1.78 and 6.10±0.23, respectively; $p<0.001$. Corroborating previous data from adult Holstein cattle, embryo production between the High and Low AFC differed (3.66±0.38 vs. 0.70±0.09, respectively; $p<0,00$). In conclusion, AFC is a useful criterion for selecting Holstein calves for in vitro embryo production.

A052 OPU-IVF and ET

Productive and reproductive performance of female Holstein calves born from different reproductive biotechnologies (AI, ET and IVF)

Mariana Pallú Viziack[1], Carlos Alberto Rodrigues[2], Luana Factor[1], Rodolfo Daniel Mingoti[1], Heloise Duarte[3], Pietro Sampaio Baruselli[1]

[1]FMVZ-USP - Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo, SP; [2]SAMVET - Clinica Veterinária SAMVET, São Carlos, SP; [3]Ideagri - Ideagri, Belo Horizonte, MG, Brasil.

The objective of this study was to evaluate the effect of different reproductive biotechnologies (AI, ET and IVF) on reproductive and productive performance of females Holstein calves born from lactating Holstein cows. The study was conducted at Santa Rita farm/Agrindus S.A. in Descalvado, São Paulo, Brazil. The reproductive data of the lactating cows that received contemporaneously (during the years of 2013 to 2018) these three biotechnologies (AI=3439, ET=722 and IVF=333) were analyzed. The animals were submitted to the same farm management conditions. In the female calves, the weight at birth (WB), the mortality from birth to weaning (MBW) and the weight at weaning (WW) were analysed. Also, age at first calving (AFC) and milk production on the first lactation (MP1L) were partially analyzed in the calves born by these different biotechnologies. The data were analyzed by the PROC GENMOD and GLIMMIX of SAS. The WB [AI=39.2^{B} (n=1546), ET=39.8^{AB} (n=587) and IVF=39.4^{A} (n=306) kg; P<0.001] and the WW [AI=101.6^{B} (n=1292), ET=99.4^{A} (n=583) and IVF=101.4^{B} (n=204) kg; P=0.013] differed according to the biotechnology. However, the MBW [AI=24.1% (256/1062); ET=9.8% (33/336) and IVF=25.8% (16/62), P=0.248] and the AFC [AI=445.5 (n=2207), ET=466.1 (n=496) and IVF=444.1 (n=83) days; P=0.981] did not differ according to the biotechnology. The MP1L did differ according to the biotechnology [AI=$9,860.7^{AB}$ (n=1350), ET=$10,296.5^{A}$ (n=290) and IVF=$10,856.13^{A}$ (n=46) kg; P=0.0031]. As a conclusion, even though there was difference according to the biotechnology used on the WB and WW, it could be equalized during the calves' development. The AFC did not differ between the biotechnologies. However, the MP1L was higher in the calves born by ET and IVF comparing to AI, probably due to the improve in the genetic merit. More data is still needed to conclude the influence on the productive and reproductive performance of Holstein females born from different biotechnologies.

A053 OPU-IVF and ET

Lipid profile of embryos produced *in vitro* of Nelore cows with low and high numbers of antral follicles

Camila Oliveira Rosa[1,2], Camila Bortolieiro Costa[5], Camila Bruna Lima[3], Christina Ramires Ferreira[4], Marcelo Marcondes Seneda[1]

[1]UEL - Universidade Estadual de Londrina, Londrina, PR; [2]FG - Faculdade Guarapuava, Guarapuava, PR; [3]USP - Universidade de São Paulo, São Paulo, SP; [4]UNICAMP - Universidade Estadual de Campinas, Campinas, SP; [5]UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho - Campus Assis, Assis, SP, Brasil.

The quantity of lipids available for the initial development processes of in vitro embryos produced can reflect directly on the embryonic quality and consequently on pregnancy rates. However, the objective of the present study was to investigate the lipid profile of in vitro embryos produced (IVP) from females *Bos taurus indicus* with different antral follicle counts (AFC). For in vitro culture, ovaries (n = 498) of 249 Nelore females were collected from local slaughterhouse and transported in saline solution at 30-35 °C to the laboratory. AFC number was determined by visual counting of the surface of both ovaries of each animal. The animals were classified as low (≤31; mean less SD) and high number of AFC (≥92; mean plus SD). Oocytes were matured, fertilized and cultured in vitro. The cleavage and blastocyst rates were evaluated in D3 and D7, respectively. On day 7 (D7) blastocysts (n = 18 per group) were collected and submitted to lipid profile analysis by desorption electrospray ionization-mass spectrometry (DESI-MS). The cleavage and blastocysts rates were evaluated by the logistic regression test using the Car statistical package "R (R Development Core Team – 2008), and the differences were considered significant if P <0.05. For the lipid profile analysis, the principal component analysis (PCA) was used, followed by Fisher's test. There were no differences between groups (78.9% and 41.7% in High AFC; n = 419 oocytes; 79.5% and 40.3% in Low AFC; n = 357 oocytes). The lipid profile of embryos derived from cows with higher AFC presented a higher concentration of lipids in the category of Triacylglycerols (TAGs) when compared to the embryos of cows with a low AFC. In contrast, embryos with cow low AFC had higher concentrations of cholesterol and its derivatives and Diacylglycerol (DAG). In conclusion, our results confirm differences in lipid profile when analyzing different amounts of AFC, nevertheless it was not possible to evaluate its significant interference in the cleavage and blastocysts rates. Our results may support studies related to the influence of lipid content on embryonic development.

A054 OPU-IVF and ET

OPU and IVEP from non-stimulated 2-4 and 8-10 months old Nelore (*Bos taurus indicus*) donors

Taynan Stonoga Kawamoto[2,1], Thais Preisser Pontelo[4,1], Otávio Augusto Costa de Faria[3,1], Andrei Antonioni Guedes Fidelis[3,1], Margot Alves Nunes Dode[1], João Henrique Moreira Viana[1], Ricardo Alamino Figueiredo[1]

[1]EMBRAPA Recursos Genéticos e Biotecnologia - Empresa Brasileira de Pesquisa Agropecuária Recursos Genéticos e Biotecnologia, Asa Norte, Brasília, DF; [2]UFU - Universidade Federal de Uberlândia, Uberlândia, MG; [3]UnB - Universidade de Brasília (UnB), Brasília, DF); [4]UFLA - Universidade Federal de Lavras, Lavras, MG.

In vitro embryo production (IVEP) is a powerful tool for cattle herds' genetic improvement. The Nelore breed and its crossbreeds are predominant in Brazilian beef herds, but are lesser precocious than most taurine breeds. Thus, the inclusion of prepubertal Nelore calves as oocyte donors in IVEP programs could shorten the generations interval and accelerate herds' genetic gains. However, it is controversial whether oocytes obtained from prepubertal cattle are less competent, generate fewer embryos or are less able to establish pregnancies than those obtained from adult ones. Thus, the aim of the present study was to evaluate the capacity of Nelore calves of 2-4 and of 8-10 months of age in IVEP compared to pubertal females. On that context, 8 Nelore calves had their follicles aspirated every other 15 days, from 2 to 4 months old by laparoscopic ovum pick-up (LOPU) and from 8 to10 months age by ovum pick-up (OPU). Nelore cows were used as a control group, and underwent OPU at the same times. The calves were raised on pasture (*B. decumbens*) and had *ad libitum* access to suckling and to water. The LOPU was performed by laparoscopy (Storz®, Xenon 300W) and under sedation and anesthesia induced with Xylazine 2% (IM, Anasedan, Ceva, Brazil), Atropine Sulfate 1% (UCB Brazil), Ketamine Hydrochloride 10% (IM, Dopalen, Ceva, Brazil), and Lidocaine Hydrochloride 2% (SC, Bravet, Brazil). OPU was performed with a portable ultrasound equipped with a transvaginal, 8MHz probe (MyLab 30 VetGold, Esaote®). The recovered oocytes were morphologically evaluated and their diameters were measured with the aid of a Motic camera. Viable cumulus-oocyte complexes (COC) were used for IVEP. The oocyte diameter and blastocyst rates were evaluated by ANOVA, while the oocyte morphological quality was evaluated by Kruskal-Wallis. As expected, adult cattle produced more grade I and less grade III COC than had prepubertal calves (12.9% and 30.1% in cows vs. 1.7 and 49.0% in calves [2-4 months] and 4.1% and 44.5% [8-10 months], respectively, $P<0.05$). Oocyte diameter of 8 to 10 months old calves were similar to those in cows but greater than in 2 to 4 months old calves (124.8±8.5μm and 126.0±7.5 μm vs. 121.3±7.5 μm, respectively, $P<0.05$). Coherently, blastocyst rates on day 7 of oocytes recovered from non-stimulated 8 to 10 months old calves were similar to those recovered from cows (42.0% [50/119] vs. 48.1% [130/270], respectively, $P>0.05$), while blastocyst rates of 2 to 4 months old calves were lower than those in cows, in simultaneous IVEP batches (31.0% [53/171] vs. 71.6% [177/247], respectively, $P>0.05$). In conclusion, oocyte diameter is a potential marker of acquisition of development potential throughout puberty. Moreover, oocytes recovered from non-stimulated 8 to 10-months old Nelore calves had similar competence of those from cows, suggesting that calves of that age can be used as donors in IVEP programs. Support: FAPDF (193.001.393/2016), EMBRAPA (SEG MP3 03.17.00.066.00.00)

A055 OPU-IVF and ET

The use of synthetic oviduct fluid in the *in vitro* maturation of bovine oocytes alters the expression of genes related to lipid metabolism and bovine oocyte maturation

Adriano Felipe Mendes[2], Gabriela Azenha Milani Soriano[2], Priscila Helena dos Santos[3], Ines Cristina Giometti[2], João Ricardo Scaliante Junior[4], Sheila Merlo Firetti[2], Anthony César de Souza Castilho[2], Claudia Maria Bertan Membrive[1], Caliê Castilho[2]

[1]UNESP - Universidade Estadual Paulista, Dracena, SP, Brasil; [2]UNOESTE - Universidade do Oeste Paulista, Presidente Prudente, SP, Brasil;[3]UNESP - Universidade Estadual Paulista, Botucatu, SP, Brasil; [4]UNESP - Universidade Estadual Paulista, Araçatuba, SP, Brasil.

The objective of this work was to investigate whether the use of synthetic oviduct fluid (SOF) with or without conjugated linoleic acid (CLA) during the *in vitro* maturation of bovine oocytes modulates the expression of genes linked to lipid metabolism and oocyte maturation. The *cumulus*-oocyte complexes were aspirated from ovaries obtained at slaughterhouse and distributed into four groups: standard for *in vitro* maturation (IVM), IVM with 100 μM CLA (IVM+CLA), SOF and SOF with 100 μM CLA (SOF+CLA). After maturation, the gene expression of oocytes and *cumulus* cells was evaluated. Total RNA samples were treated with DNAse before being submitted to the reverse transcription protocol. We performed qPCR analyzes of the FADS2 (Fatty Acid Desaturase 2), SCD (Stearoyl-CoA Desaturase), SREBP1 (Sterol Regulatory Element Binding Transcription Factor 1), GREM1 (Gremlin 1), AREG (Anphiregulin) e PTGS2/COX2 (Prostaglandin-Endoperoxide Synthase 2/Cyclooxygenase 2). The PPIA Gene (Peptidylprolyl Isomerase) was used as a reference, and the efficiency correction ΔΔCt method was used to calculate the relative expression values (target genes / PPIA) for each target gene using a control sample as a calibrator. The data were submitted to analysis of variance (ANOVA) and Tukey test, being transformed into logarithm when they did not present normal distribution. Analyzes were performed using JMP software (SAS Institute Cary, NC) and data are presented as mean ± standard error of the mean. Differences were considered significant at the 5% level of significance ($p<0.05$). FADS2 (1.03 ± 0.17^{a}) and SCD1 (1.04 ± 0.15^{a}) presented higher mRNA expression in the oocytes of the IVM group. GREM1 was more expressed in groups SOF (2.26 ± 0.08^{a}) and SOF+CLA (2.68 ± 0.31^{a}). PTGS2/COX2 was more expressed in groups SOF (2.19 ± 0.14^{a}) and SOF+CLA (1.96 ± 0.41^{a}). SCD1 expression was higher in the *cumulus* cells of the IVM (0.76 ± 0.16^{a}) and IVM+CLA (0.55 ± 0.06^{a}) groups. The use of SOF or SOF+CLA in the maturation of bovine *cumulus*-oocyte complexes decreases the expression of the FADS2 and SCD1 genes related to lipid metabolism and, in addition, induces greater expression of PTGS2 / COX2 and GREM1 related to the process of oocyte maturation, specifically linked to the expansion of *cumulus* cells.

A056 OPU-IVF and ET

Effect of FSH and/ or rBST treatment on the *in vitro* embryo production of Holstein (*Bos taurus*) calves

Bernardo Marcozzi Bayeux[3], Flávia Morag Elliff[1], Francisco Palma Rennó[5], Guilherme Gomes[2], Carlos Alberto Souto Godoy Filho[1], Bruna Lima Chechin Catussi[1], Yeda Fumie Watanabe[4], Pietro Sampaio Baruselli[1]

[1]VRA/USP - Departamento de Reprodução Animal da Universidade de São Paulo, São Paulo, SP, Brazil; [2]VNP/USP - Departamento de Nutrição e Produção Animal da Universidade de São Paulo, Pirassununga, SP, Brazil; [3]Bovigenese - Bovigenese, Cravinhos, SP, Brasil; [4]VITROGEN, Cravinhos, SP, Brasil.

A total of 62 Holstein females were used in this study: 49 prepubertal (3 to 9 months of age) and 13 pubertal. The prepubertal animals were distributed in four groups: Control (CTLG, n=13); Treated with rBST (BSTG, n=11); Treated with FSH (FSHG, n=13); and Treated with rBST+FSH (BFG, n=12). The pubertal heifers (PHG) were included as a positive control. All the animals received a P4 device (Primer PR, Agener União – Saúde Animal, SP, Brazil) and 1 mg IM of estradiol benzoate (Fertilcare Sincronização®, MSD Saúde Animal, SP, Brazil) on D0. The animals of CTLG received no additional treatment. The animals of BSTG received 500 mg IM of rBST (Boostin®, MSD Saúde Animal) on day -2. The animals of FSHG received 140 mg IM of FSH (Folltropin®, Vetoquinol - SP, Brazil), performed in four injection twice a day on decreasing doses (40 mg [day 4 PM], 40 mg [day 5, AM], 30 mg [day 5, PM], and 30 mg [day 6, AM]; coasting period of 24 hours). The animals of the BFG received 500 mg IM of rBST on D-2 and the same FSH protocol mentioned above. The PHG received no additional treatment. On day 7 the P4 devices were removed and the animals of all groups were submitted to ovum pick-up guided by transvaginal ultrasound (guide EC9-5 Heifer, WTA, SP; ultrasound S8®, SonoScape, China). The recovered oocytes were sent to an IVEP commercial lab. The oocytes were fertilized with sexed semen from three Holstein bulls (*Bos Taurus*), balanced between the experimental groups. Data were analyzed by the GLIMIX procedure of SAS®. No statistical differences were found between groups regarding total number of oocytes on D7 (CTLG: 9.8±1.1; BSTG: 14.7±5.5; FSHG: 13.5±3.8; BFG: 13.0±2.7; PHG: 9.8±2.4; P=0.7), number of viable oocytes (CTLG: 6.1±1.0; BSTG: 9.9±4.9; FSHG: 9.1±2.6; BFG: 9.0±1.7; PHG: 5.0±1.1; P=0.2), cleaved oocytes (CTLG: 4.5±0.9; BSTG: 7.45±3.0; FSHG: 9.1±3.1; BFG: 6.7±1.8; PHG: 5.6±1.4; P=0.7) and blastocyst rate [on total oocytes; CTLG: 2.3% (0.23/9.85); BSTG: 4.9% (0.73/14.73); FSHG: 4.5% (0.62/13.54); BFG: 8.3% (1.08/13); PHG: 14.0% (1.38/9.85); P=0.2]. Regarding the number of blastocysts produced/OPU, no statistical difference was found (P=0.9) between the experimental groups (CTLG: 0.23±0.1; BSTG: 0.73±0.3; FSHG: 0.62±0.2; BFG: 1.08±0.4; PHG: 1.38±0.4). The number of medium follicles (5-8mm) on D7 was higher (P=0.024) for the FSHG and BFG (9.0±2.38 and 7.83±2.42, respectively) when compared to the other groups (CTLG: 1.23±0.46; BSTG: 0.91±0.25; PHG: 2.08±0.52). The cleavage rate (on total oocytes) was higher (P=0.0002) for animals treated only with FSH [68.1%; (9.2/13.5)] when compared to the other groups [BSTG: 51.0% (7.5/14.7); BFG: 52.3% (6.8/13); CTLG: 45.9% (4.5/9.8) and PHG: 58.1% (5.7/9.8)]. These data show that treatment with FSH improved the cleavage rate of young Holstein donors, however, treatment with BST or the association of both treatments had no influence on the IVEP of prepubertal heifers.

A057 OPU-IVF and ET

Effect of application moment of FSH-LH during superovulation protocols on oocyte quality in Blanco orejinegro cows (BON)

Diego Andres Velasco Acosta[1], Leonardo Perez Sandoval[1], Diego Fernando Dubeibe Marin[2], Maria de los Angeles Cortes Escobar[1], Aldemar Chavez Rodriguez[1], Augusto Schneider[3]

[1]AGROSAVIA - Corporación Colombiana de Investigación Agropecuaria Cundinamarca, Colômbia; [2]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil; [3]UFPA - Universidade Federal do Pará, Belém, PA, Brasil.

The objective of this study was to evaluate the effect of the application moment of FSH-LH hormones in a superovulation (SOV) protocolo on oocyte quality in Blanco orejinegro cows (BON). Twenty non-lactating cows were used, in a cross design, randomly distributed in 5 experimental groups. For groups 1 to 4, day 0 was considered the moment of removal of all follicles ≥5mm [follicular ablation (FA)], which was performed by ultrasound-guided transvaginal aspiration (UGTA). Groups were assigned as Group 1 (G1) cows received a single dose of 60 IU im of FSH-LH (Pluset ®) 27 hours (h) after follicular FA, and 27 h later the cumulus-oocyte complexes (CCOs) were collected by UGTA; Group 2 (G2) 27 h after FA cows received a single dose of 60 IU im of FSH-LH, and 48h later the UGTA was performed; Groups 3 and 4 (G3 and G4) received 60 IU im of FSH-LH divided into two doses, at 24 and 48 h after FA, and UGAT was performed 27 and 48 h later, respectively. Control cows (G5) were submitted to UGAT in a random phase of the estrous cycle. The COCs were collected, counted and classified into four categories, according to the compaction and transparency of cumulus cells, homogeneity and transparency of the ooplasm, as follows: Grade 1, >4 layers of cumulus cells; Grade 2, three or four layers of cumulus cells; Grade 3, one or two layers of cumulus cells; Grade 4, denuded oocytes or oocytes with expanded cumulus. CCOs from grades 1 to 3 were considered viable and grade 4 were considered not viable. The data was analyzed using the MIXED MODELS procedure from SAS 9.1 software. The means were compared using Tukey test. For the total number of collected oocytes, number of viable oocytes and oocytes of grade 2 and 4 no significant differences were found among groups (P> 0.05). A lower (P0.05). In addition, a greater proportion (P = 0.02) of grade 3 oocytes was observed in the control group cows (G5: 53.98%, 122/226) when compared to G1 (38.98%, 69/177). The use of FSH-LH in SOV protocols in BON donor cows did not increase the number of total oocytes or number of viable oocytes obtained; however, the proportion of grade 1 oocytes recovered was increased, and this can be reflected in a greater embryos production in *in-vitro* programs.

A058 OPU-IVF and ET

Embryo production and future fertility of heifers after superovulation

Carlos Antônio de Carvalho Fernandes[1,2], Gustavo Henrique de Souza Pereira[1,2], Humberto Luis Del Hoyo Neri[1], Jéssica Ruiz Pereira[1], Ana Cristina Silva de Figueiredo[1,2], Vinícius Oliveira Souza[2], João Henrique Moreira Viana[2,3]

[1]Biotran - Biotran LTDA, Alfenas, MG, Brazil; [2]Unifenas - Universidade José do Rosário Valano, Alfenas, MG, Brazil; [3]Embrapa Cenargen - Embrapa - Cenargen, Brasilia, DF, Brazil.

The Multiple Ovulation and Embryo Transfer (MOET) technique for bovine genetic multiplication has been used commercially since the 1960s. In the last years, this technique has gained a great differential, with the possibility of selection of donors by genomic evaluation. However, the use of these technologies is far below the possibilities, since some farmers and technicians believe in information, usually without proper scientific evidence, that the MOET technique can affect the fertility of heifers. Thus, the objective of this study was to evaluate if the MOET is harmful to the future fertility of heifers. It was used Holstein heifers (n=1783) between 312 and 387 days old and weighing between 273 and 307kg from two commercial farms. These animals were divided among donors (446) or non-donors (G1=1327) according to the result of genomic evaluation. The donors were always super ovulated with 180mg of Folltropin (Vetoquinol-Brazil). Of these, 337 were submitted to MOET once (G2) and 109 twice (G3). The second MOET was made only in heifers who produced more than eight viable embryos in the 1st MOET. In those super ovulated twice, the interval between MOET was 45 days. Non-donor females (G1) were in seminated as of the date that reached 320kg of body weight. The donors (G2 and G3) were inseminated from 15 days after the last embryo harvest and when they reached the same weight. The age at 1st insemination, age at conception and age at birth were evaluated by anova and compared by Tukey's test. The number of services per conception was compared by chi square, considering significance at 5% probability. The first MOET in 446 donors provided 6.8±4.6 total embryos and 3.9±2.8 viable embryos. The second MOET, selecting the best donors, provided 12.6±5.3 total embryos and 8.5±3.8 viable embryos. The age at 1st insemination was 381.5±3.6b; 387.4±15.8ab and 412±19.7a days, age at conception 438.3±29.8^{b}; 449.1+32.3^{ab} and 470±31.8^{a} days and age at partum 720.3±33.8b; 730.1±35.8ab and 749±34.3a ($P<0.05$) for G1, G2 and G3, respectively. The number of services per conception was 2.3±0.8; 2.4±0.6 and 2.3±0.7-($P>0.05$) for G1, G2 and G3 respectively. It is concluded that one or two MOET processes, prior to the beginning of the insemination phase, does not interfere with the heifer fertility. A MOET before the start of the breeding phase does not interfere with age at the 1st insemination, conception and 1st calving. In addition, two MOETs delays conception by 32 days. On the other hand, heifers submitted to two MOET produced 16.4±5.3 viable embryos before the 1st conception. Acknowledgment: Biotran, Unifenas, CNPq and CAPES

A059 OPU-IVF and ET

Superovulation protocols for *in vivo* embryo production using sexed semen

Gustavo Henrique Souza Pereira[2,1], Carlos Antônio Carvalho Fernandes[1,2], Humberto Luiz Del Hoyo Neri[1,2], Ana Cristina Silve de Figueiredo[1,2], João Paulo Guimarães[2], Jéssica Ruiz Pereira[1], Guilherme Moura[3]

[1]Biotran - Biotran LTDA, Alfenas, MG, Brazil; [2]Unifenas - Universidade José do Rosário Velano, Alfenas, MG,-Brazil; [3]Vetoquinol - Vetoquinol Saúde Animal, Vila Maria, SP, Brazil.

The objective of this study was to compare the efficiency of two using two or three doses of sexed semen for donor insemination at fixed time (SOVFT) to produce bovine embryos in vivo with sexed semen. Twenty girolando heifers donors, aged between 16 and 52 months, were used in a crossover scheme, where all the animals participated in both treatments. Each donor was superovulated twice. Females were superovulated using 180 mg Folltropin™ (Vetoquinol-Brazil) in a decreasing dose schedule. The superovulation protocol was as follows: D0 - progesterone device insert (Primer™ – Brazil); D1 - 2 mg of estradiol benzoate (Ric Be™ – Brazil) IM, D5 to D8 - Folltropin every 12 hours. In D7 the afternoon 0.5 mg of cloprostenol (Estron™ – Brasil) was applied and in D8 in the morning the device was removed. In D9, donors were divided into two groups: G2AI (N = 23), received 0.05 mg of Gonadorelin (TecRelin™ – Brazil) at 2pm and was inseminated in D10 at 7am and 7pm. G3AI (N = 23) received 0.05 mg of Gonadorelin at 7am and was inseminated at D9 at 7pm and at 10 at 7am and 7pm. All inseminations were made using sexed semen from Holstein bull (ABS Pecplan-Brazil). For the same donor, the same semen was used in both treatments. Uterine flushings were made using DMPBS™ (Reprodux-Brazil) on D16. The ovaries were evaluated by ultrasonography (Mindray - M5™-China) on the day of flushing to measure the corpora lutea (CL). The number of CLs and embryonic production was compared using Anova at 5% probability. The number of CLs on the day of collection was 14.2±6.4 and 12.4±6.2 ($P > 0.05$) for G2IA and G3IA. The mean total embryos were 8.7±4.6 and 9.9±7.9 ($P<0.05$) and viable of 5.6±3.2 and 6.9±4.0 ($P<0.05$) for G2IA and G3IA. These results show that there is no difference in SOV, indicating that the design study used was correct, since this response to SOV has great individual variation. However, even with a similar ovarian response, G3IA donors produced more total and viable embryos, indicating that an insemination protocol with an additional insemination has better results, justifying the use of an additional dose of semen. In conclusion, the protocol of SOV using three doses of sexed semen to produce bovine embryos in vivo is superior to that using only two doses. Acknowledgments: Vetoquinol, ABS Pecplan, IMV, Reprodux, Biotran, Unifenas, CNPq and CAPES.

A060 OPU-IVF and ET

Embryo production and recovery in naturalized Brazilian ewes

Jeferson Ferreira da Fonseca[1], Jenniffer Hauschildt Dias[2], Gabriel Brun Vergani[3], Monalisa Sousa Dias Lima[4], Dárcio Ítalo Alves Teixeira[4], Kleibe Moraes Silva[1], Alexandre Weick Uchoa Monteiro[1], Maria Emília Franco Oliveir[3], Joanna Maria Gonçalves Souza-Fabjan[5], Ribrio Ivan Tavares Pereira Batista[6]

[1]CNPC - Embrapa Caprinos e Ovinos, Coronel Pacheco, Minas Gerais; [2]UFV - Universidade Federal de Viçosa, Viçosa, Minas Gerais; [3]UNESP - Universidade Estadual Paulista, Jaboticabal, SP, Brasil; [4]UECE - Universidade do Estado do Ceará, Fortaleza, CE; [5]UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil; [6]UFVJM - Universidade Federal do Vale do Jequitinhonha e Mucuri, Diamantina, Minas Gerais, Brasil.

This study hypothesized and tested if the same superovulation protocol and non-surgical embryo recovery (NSER) technique would be efficient to produce and recovery embryos naturalized Brazilian sheep breeds managed in different production systems. A superovulation protocol were used in Morada Nova (MN; n=20); Santa Inês (SI; n=20) and Somalis donors (SO; n=20) consisting in progesterone-based intravaginal device (0.33g; Eazi Breed CIDR®, Zoetis, São Paulo, Brazil) maintained for nine days and administration of six decreasing doses (25-25-15-15-10-10%) of p-FSH (133 mg; Folltropin V®, Vetoquinol, Brazil) injected i.m. every 12 h starting -60 h before device removal. Concomitant to the 5th and 6th p-FSH dose, were administrated i.m. d-cloprostenol (37.5 µg; Prolise®, Agener União, Brazil) and females were submitted monitored for estrous response and natural mating with fertile rams. To prevent corpus luteum (CL) regression, ewes also received three administrations i.m. of flunixin-meglumine (24.9 mg; Banamine®, MSD, São Paulo, Brazil) on Days 3, 4 and 5 after first mating. NSER (Fonseca et al., Reproduction in Domestic Animals, 54:118-125, 2019) was performed seven days after device removal only in ewes mated and embryos collected were frozen. CL was counted by transretal Doppler mode ultrasonography one before NSER. Data were presented in a descriptive form. Estrous responses were 80 (16/20), 95 (19/20) and 90 (18/20) % respectively to MN, SI and SO ewes. Successful NSER was done in 94 (15/16), 95 (18/19) and 94 % (17/18) in MN, SI and SO ewes respectively. The average number of corpora lutea found (considering only ewes collected) by breed was 8.7±0.1, 13.9±1.4 and 9.9±0.7 for Morada Nova, Santa Inês and Somalis ewes, respectively. Embryo recovery (structures/ CL counted x100) were 95.4% (125/131), 89.9% (195/217) and 77.4% (130/168) and viability rates (viable embryos/total structures x 100) were 96.0% (120/125; 104 morulae, 16 blastocyst and five unfertilized eggs), 87.2% (170/195; 140 morulae, 30 blastocyst, one 4-7 cell embryos and 24 unfertilized eggs) and 90.0% (117/130; 59 morulae, 58 blastocyst, one eight-cell embryo and 12 unfertilized eggs) for MN, SI and SO ewes, respectively. It is concluded that the *in vivo* embryo production is feasible in naturalized Brazilian ewes with the same superovulatory protocol. The results showed (1) the repeatability and efficiency of the superovulatory protocol for ovarian stimulation irrespective to breed, (2) high success of NSER related to both successful transcervical uterine flushing and total and viable structures recovered in Morada Nova, Santa Inês and Somalis ewes and (3) elevated embryo viability for embryos recovered after estradiol-cloprostenol-oxytocin based protocol for cervical relaxation and transposing in sheep.
Financial support: Embrapa (**02.13.06.026.00.04)** and Fapemig (CVZ-PPM 00201-17).

A061 OPU-IVF and ET

Bull individual effect is determinant to *in vitro* embryo production regardless of seminal profile

Mayra Elena Ortiz D Avila Assumpcao, Adriano Felipe Peres Siqueira, Camilla Mota Mendes, Thais Rose dos Santos Hamilton, Leticia Signori de Castro, Luana de Cássia Bicudo, Tamie Giubu de Almeida, Vivian Cardoso Castiglioni, Marcilio Nichi, Rodolfo Mingoti, João Diego Agostino Losano

FMVZ-USP - Faculdade de Medicina Veterinária e Zootecnia da USP, São Paulo, SP Brasil.

Bull fertility is an intriguing issue of animal reproduction and so far, different groups have tried to characterize which sperm traits contributes to fertilize and sustain embryo development. In addition, the difference between *in vivo* and *in vitro* system turns difficult to extrapolate the results from the field to the laboratory. Then, the aim of this study was to create a model with flow cytometry and computer assisted sperm analysis (CASA) to identify which sperm traits contribute and how much they contribute to create a predictive model of *in vitro* fertility. For that, we produced a database of in vitro embryo production (IVP) of 51 semen batches from 23 Nelore bulls. For each batch, at least 3 IVP manipulations were performed, in total of 184 IVP manipulations, collecting sperm traits analysis by the *in vitro* fertilization step, after Percoll® gradient selection and embryo production rates (cleavage and blastocyst rates). Sperm traits evaluated by flow cytometry were acrosome and membrane integrity (FITC-PSA/PI), mitochondrial membrane potential (JC-1) and chromatin integrity (modified SCSA) and CASA parameters related to total and progressive motility, movement kinetics and velocities were recorded. Twenty-two sperm traits, cleavage rate and bull individual effect were included to build a mathematical model, considering the blastocyst rate as predictor of the in vitro bull fertility. Statistical analysis was performed using the SAS 9.4 Software with the GLM model, and the selection of the variables was performed through the Forward Selection. The adjusted coefficient of determination (ADJRSQ; R2) and p-value $\leq$ 0.5 were used as criteria for model acceptance. Between all variables included to predict blastocyst rate, bull and cleavage rates were those, which presented higher F values and then better indicators of embryo production. The best predicted model achieved for blastocyst rate included the percentage of total motility, the percentage of static sperm, cleavage rate and the bull individual effect ($p<0.0001$ and Adj R-Sq = 0.6319): Blastocyst rate (%) = - 1.2382(Intercept) + Bull + 0.1229*(total motility) + 0.0667*(Static) + 0.4494*(cleavage rate). Our results indicated that the bull individual effect is one of the most determining factors for IVP outcome. This effect, carried by the sperm produced by these animals will directly affect blastocyst rate. The assessment of sperm traits is an attempt to explore such bull individual effect. However, none of the 22 sperm attributes analyzed alone or in combination can explain a significant part of this bull effect on blastocyst rate. Then, our results indicate that bull individual effect, which is probably independent from the different sperm traits analyzed, is the strongest effect to define the IVP rates. Further studies focusing on sperm nuclear proteins, micro RNAs and metabolism may enlighten the knowledge on such bull individual effect. Financial Support: FAPESP (n° 2016/15147-5)

A062 OPU-IVF and ET

The oviduct fluid supplementation in the *in vitro* fertilization medium does not reduce polyspermy incidence during the non-breeding season in goat species

Glaucia M. Bragança[1,2], Agostinho Alcântara Neto[2], Ribrio Ivan T. P. Batista[1], Felipe Z. Brandão[1], Vicente José F. Freitas[3], Pascal Mermillod[2], Joanna Maria G. Souza-Fabjan[1]

[1]UFF - Universidade Federal Fluminense, Niterói, Brasil; [2]INRA - Institut National de la Recherche Agronomique, Nouzilly, France; [3]UECE - Universidade Estadual do Ceará, Itaperi, Fortaleza, CE, Brasil.

Polyspermy is one limiting factor of IVP in several species. In goats, this pathologic condition affects up to 35% of *in vitro* embryos. The action of oviduct fluid (OF) on monospermic modulation has been already demonstrated in swine and bovine species. This study assessed the OF effect on polyspermy occurrence using different inseminating doses for IVF in goats. The study was performed during the non-breading season at Nouzilly, France. Goat genital tracts in late follicular phase of estrous cycle were obtained at a local slaughterhouse. At the laboratory, tracts were dissected, and the oviducts lumen were flushed with 500 µL of IVF medium. The flushing containing OF was centrifuged (10,000□*g*) two times, aliquoted and stored at -80 °C for later use. After follicular aspiration, *cumulus* oocyte-complexes (COCs) were submitted to IVM (TCM199, 10 ng/mL EGF, 100 µM cysteamine, 10% FCS) for 22 h. Then, COCs were randomly distributed in six groups, co-cultured with three different sperm concentration (1.0, 2.0 and 4.0 x 10^6 cells/mL) in SOF medium supplemented with 5 µg/mL heparin, 4 µg/mL gentamycin and 10% estrus sheep serum with 10% OF (OF groups; OF1, OF2 and OF4) or without (control groups; CTRL1, CTRL2 and CTRL4). After 18 h of IVF, presumptive zygotes (n=628) were denuded, fixed, stained with Hoechst and evaluated under fluorescence microscopy. The normality of variables was verified by Kolgomorov-Smirnov test and data were compared by Mann-Whitney test. The results are shown in mean±SE. In six runs, 1.576 COCs were used for IVP: there was no difference (P>0.05) for OF1 *vs*. CTRL1, OF2 *vs.* CTRL2 and OF4 *vs.* CTRL4, respectively, for: cleavage rate 65±7(96/148) *vs.* 70±7(109/154), 77±2(119/155) *vs.* 72±5(114/156), 75±7(114/152) *vs.* 74±4%(112/149); blastocyst rate at day 8, 41±5(61/148) *vs.* 38±7(59/154), 39±6(59/155) *vs.* 32±6(50/152), 38±4(60/156) *vs.* 33±6%(50/149); penetration rate (74±5 *vs.* 67±4; 71±6 *vs.* 69±7 and 67±7 *vs.* 72±6%) and monospermy rate (60±5 *vs.* 46±7; 52±4 *vs.* 46±5 and 38±2 *vs.* 31±4%), although OF has shown a propensity to increase monospermy at the three sperm concentrations. The production efficiency of monospermic zygotes (monospermy/penetration) tended to be higher (P=0.06) for OF2 than CTRL2. The absence of an OF positive effect could be a consequence of the strong positive photoperiod blocking the hypothalamic-pituitary-gonadal axis and inhibiting the ovulation. Ovarian steroids (progesterone and estradiol) play roles on the modulation of oviduct activity, including secretory function. The OF composition is influenced by estrous cycle phase; the oviduct-specific glycoprotein (OVGP1) reaches its peak when estradiol concentration increases at preovulatory period; and it is well known that OVGP1 has a role on zona pellucida hardening after fertilization and thus modulation of penetration. In conclusion, the OF supplementation on IVF medium was not capable to modulate the incidence of polyspermy at the non-breeding season in goats.

A063 OPU-IVF and ET

Effect of age at first calving epd on bovine *in vitro* embryo production

Guilherme Augusto Lemos[1], Tauane Antonia Xavier de Abreu[2,1], Nilson Antonio Alves Barbalho[2,1], Wagner Oliveira Pesca[2,1], Jéssica Souza Andrade[4,3], Luiz Francisco Machado Pfeifer[3], Daniela Cristina Lemos de Carvalho[1,2]

[1]Múltipla Embriões - Múltipla Embriões Ltda., Ji-Paraná, RO; [2]São Lucas Educacional - São Lucas Educacional Ji-Paraná, Ji-Paraná, RO; [3]Embrapa Rondônia - Embrapa Rondônia, Porto Velho, RO; [4]Rede Bionorte - Rede Bionorte - Pós-Graduação em Biotecnologia e Biodiversidade, Porto Velho, RO, Brasil.

Individual bulls differ in their ability to fertilize oocytes and/or to develop to blastocyst stages following *in vitro* fertilization (IVF) procedures. The objective of this experiment was to evaluate the effect of Age at First Calving - Expected Progeny Difference (AFC EPD) of the bull in the *in vitro* embryo production (IVP). The data of this study were obtained from the commercial laboratory of IVP - MÚLTIPLA EMBRIÕES LTDA (Ji-Paraná - Rondônia - Brazil), between November 2017 and November 2018. The ovum pick-up procedures were performed in six farms from Rondônia and Roraima states. A total of 1.579 Cumulus Oocyte Complexes (COCs) were collected and submitted to *in vitro* maturation (IVM), followed by *in vitro* fertilization (IVF) and culture (IVC), at 38.5ºC, 5,5% CO2 and high humidity. Seventeen bulls were used in this study. Only bulls used in more than 2 IVP procedures were included in this study. Therefore, 17 bulls were used. Considering the EPD AFC, bulls were categorized into two groups: TOP+ (n=14; DECA 1 bulls for AFC), and, TOP- (n=3, DECA 2, 3 and 4 for AFC). Chi-square test was used to compare the cleavage and blastocyst rates between bull groups. From 1.579 COCs subjected to IVP, 1.309 were inseminated with semen of TOP+ bulls and 270 with semen of TOP- bulls. The TOP+ bulls had higher cleavage (76.3%, 999/1309 x 69.3%, 188/270; P = 0.01) and blastocyst rates (53.7%, 703/1309 x 47.03%, 127/270; P = 0.04) than TOP- bulls. The results of this study may provide evidences that AFC, as a genetic parameter, is useful to select more fertile bulls to IVP. However, more detailed studies are necessary to better understand the association between specific EPDs and *in vitro* embryo production.

A064 OPU-IVF and ET

Evaluation of the pregnancy rate of poor embryos produced *in vivo* submitted to culture prior to the-transfer

Ana Paula da Silva[1], Ana Lúcia Martins Bonotto[2], Adiel Cristiano Nino[3], Jankiel Primon[4], Fernanda de Souza Rosa[1]

[1]UCEFF - Centro Universitário de Itapiranga, Itapiranga, SC; [2]ABN - ABN Agropecuária, Santiago, RS; [3]VET - Veterinário Autônomo, Palmitinho, RS; [4]VET - Veterinário Autônomo, Caibi, SC, Brasil.

One of the most important factors associated with the success of the embryo transfer (ET) techniques is the morphological embryos evaluation before cryopreservation and / or transfer to recipients. The relation between the morphological evaluation and the developmental capacity of the embryos is highly associated with pregnancies rates. The objective of this study was to evaluate the prior transfer culture of poor quality embryos produced in vivo, developmental rate on the culture, embryo transfer rates with single or doubly embryo transfer. Date were analyzed from ET of Angus beef cattle of a central of ET, located in the city of Santiago, Rio Grande do Sul, Brasil. The embryos used in this study were those that showed low quality, classified as poor (IETS, 1998) these embryos were submitted to a culture in an enriched medium (Holding Plus ®, Vitrocell, Campinas, São Paulo, Brasil) on the heating table at a temperature of 36ºC and the developmental stage was observed during some hours (2 to 12 hours). The embryos that were developed were transferred to recipients and according to availability, and in some, two embryos were transferred, so the inovulation were divided into two groups, group I: single transfer and group II: double transfer. From the 127 embryos submitted to culture, 71 (55.9%) were transferred, obtaining a developmental rate greater than 50%. Of total transfers (71), 36 pregnancies were diagnosed (pregnancy rate 50.70%). On group I, 48 transfers were performed, with 20 pregnancies (pregnancy rate 41.66%); On group II, 23 transfers were performed, confirming 16 pregnancies (pregnancy rate 69.57%), a higher pregnancy rate when two embryos were inovulated together. It was concluded that the use of lower quality embryos kept in culture before the transfer was shown to be a viable alternative, since it obtained a good developmental rate as well as a good pregnancy rate. Double inovulation can also be a viable alternative, since they significantly increase the pregnancy rate. However, should be evaluated the percentage of twin births. In conclusion, the use of prior transfer culture for poor embryos is an alternative for their use as well as a decrease in costs with recipients.

A065 OPU-IVF and ET

Addition of melatonin to the maturation medium of bovine oocytes subjected to heat shock: effects on nuclear maturation, apoptosis, reactive oxygen species, mitochondrial activity, and gene expression

Nadja Gomes Alves[1], Fabiane Angélica de Paiva Paula[2], João Pedro Araújo Campos[1], Débora Regina da Silva[2], Renato Ribeiro de Lima[3], Eliza Diniz de Souza[4], Carolina Capobiango Romano Quintao[4], Jéssica Fernanda Souza[4], Ivan Junior Ascari[1], Luiz Sérgio Almeida Camargo[4]

[1]UFLA - Universidade Federal de Lavras (Departamento de Zootecnia, Campus Universitário, Lavras, MG; [2]UFLA - Universidade Federal de Lavras (Departamento de Medicina Veterinária), Lavras, MG; [3]UFLA - Universidade Federal de Lavras (Departamento de Estatística, Lavras, MG; [4]EMBRAPA - Gado de Leite - Empresa Brasileira de Pesquisa Agropecuária, Juiz de Fora, MG, Brasil.

The effects of melatonin addition to IVM medium of bovine oocytes under heat shock (HS) on nuclear maturation, apoptosis, mitochondrial activity, reactive oxygen species (ROS) and *GDF9* gene expression were evaluated. Cumulus-oocytes complexes (COCs) were recovered from 3-8 mm follicles of crossbreed *Bos indicus ovaries* collected at a slaughterhouse. COCs were matured under HS (12h at 41.5ºC and 7% CO_2 followed by 12h at 38.5ºC and 5% CO_2) in medium with 0, 10^{-12}, 10^{-9}, 10^{-6} and 10^{-3} M melatonin (Sigma-Aldrich, St. Louis, USA). In the non-stress (NS) group oocytes were matured for 24h at 38.5ºC and 5% CO_2 without melatonin. Oocytes were processed for TUNEL assay (Promega, Madison, USA) and stained with DAPI (Vector Lab., Burlingame, USA) to evaluate apoptosis and maturation rates (six replicates, 140±36 CCOs/replicate). For mitochondrial activity (three replicates, 133±18 CCOs/replicate) and ROS (four replicates, 130±20 CCOs/replicate) oocytes were stained in MitoTrackerRed CMX-Ros (Thermo Fisher Scientific, Waltham, USA) and DCFDA (Sigma-Aldrich) and analyzed under a fluorescence microscope. Images were analyzed by the software Image J 1.49. The *GDF9* gene expression was evaluated by RT qPCR (Applied Biosystems 7300 Real-Time PCR System, Thermo Fisher Scientific, Waltham, USA; three replicates, 10 CCOs/replicate). It was considered a randomized block design. Data were analyzed by the GLIMMIX procedure (SAS® 9.3), using binomial (maturation and apoptosis rates) or gamma (mitochondrial activity and ROS) distribution. The *GDF9* gene expression was analyzed by the software REST® and the results expressed regarding the calibrator NS. Melatonin did not improve (P>0.05) the maturation rate under HS (67.8±0.6; 75.2±0.2; 59.5±0.3; 67.6±0.2 and 55.8±0.5% in the 0, 10^{-12}, 10^{-9}, 10^{-6} and 10^{-3} M, respectively). The maturation rate did not differ (P>0.05) between 0, 10^{-12}, 10^{-6} M and NS (76.6±0.14%). Apoptosis rate in the NS group (0.6±0.6%) was lower (P<0.05) than in the groups 0, 10^{-12}, 10^{-9} and 10^{-6} M (4.4±1.0; 3.9±0.9; 4.0±1.2; 3.2±0.9%, respectively) and did not differ from 10^{-3} M (2.1±0.4%). Mitochondrial activity was lower (P<0.05) in the 10^{-3} M (42.9±0.1 arbitrary units - AU) than in the other groups (0 M: 63.9±0.1; 10^{-12} M: 62.4±0.1; 10^{-9} M: 59.8±0.1; 10^{-6} M: 58.0±0.1 AU) and it was greater in 0 M than in NS (57.1±0.1 AU). ROS production was lower (P<0.05) in the 10^{-3}, 10^{-6} and 10^{-9} M (13.5±0.2; 16.2±0.2 and 16.1±0.2 AU, respectively) than in 10^{-12} M (32.5±0.2 AU) and 0 M (31.2±0.2 AU). ROS was greater in 10^{-12} M and 0 M than in NS (25.0±0.2 AU). *GDF9* gene expression was greater in the 10^{-3}, 10^{-6} and 10^{-9} M (5.8±1.6; 2.5±0.8; 1.7±0.4 folds) compared to NS. Melatonin at 10^{-6} M in the IVM protects oocytes from the damage caused by HS, as demonstrated by maturation rate similar to that observed on oocytes from NS, lower ROS production, and greater *GDF9* gene expression. Financial support: CNPq (427476/2016-0), FAPEMIG and CAPES (Financial code 001).

A066 OPU-IVF and ET

Cytoplasmic granules in oocytes do not influence embryonic and early fetal development in bovine

Paola Rosa[1,2], Marina Ragagnin[1], Joaquim Mansano Garcia[1], Clara Slade[2]

[1]UNESP/FCAV - Universidade Estadual Paulista Campus Jaboticabal, Jaboticabal, SP; [2]EMBRAPA Gado De Leite - Empresa Brasileira De Pesquisa Agropecuária, Juiz de Fora, MG, Brasil.

Morphological oocyte evaluation is imprecise and subjective. The aim of this study was to test the hypothesis that oocyte with cytoplasmic granules can be included in IVP routine. Two experiments were performed using control cytoplasm (CC- homogeneous cytoplasm) and granulated cytoplasm (GC- cytoplasmic dimorphism). Both presented intact cumulus cells (more than three layers of cells, nonatretic, with no signs of expansion). The first experiment evaluated cleavage (%Cleav), blastocyst (%Bl), pregnancy (%Pre) rates and early fetal development (Crown-rump lenght-CRL) of oocytes obtained from cow donors (CEUA / EGL-3956180316). The second experiment aimed characterization of oocyte quality by Active caspase 3 levels-C3, Gap junction activity-GJA (Calcein-AM), percentage of lipid content-LC (Oil red) and transcription profile of oocyte (P1A, IGF2R, ZAR1), cumulus cells (BMP15, IGF2R) and blastocyst (P1A, IGF2R) obtained from slaughterhouse ovaries (results were analyzed as expression of the target genes relative to the GAPDH endogenous gene using the standard curve method). Results when necessary are presented by number/mean ± SE. The %Cleav and %Bl were compared by T-Test and pregnancy rate by Fisher's Exact Test. The C3, GJA, LC and percentage of transcripts evaluated were compared by the Mann-Whitney Test. All analyses were performed using the GraphPad Instat Software. The CRL was submitted to ANOVA using the Minitab Software ($P<0.05$). In Experiment 1, none of the analyses performed differed statistically (%Cleav, CC: 813/68.8±4.8; GC: 469/74.4±5.8; %Bl, CC: 368/12.1±2.9; GC: 209/11.3±4.1; %Pre, CC: 60/24.2±10.8; GC: 30/26.3±8.0; CRL: CC/GC, n.40/25: 31d- 9.2/9.8; 37d- 16/17; 43d- 23.3/24; 49d- 31.4/33.9; 55d- 46.3/47.2). In Experiment 2, C3 of the GC group (39/172.1±16.9) was higher when compared to CC group (21/66.2±11.6), other structural analyses did not differ between groups (GJA: CC: 38/5.6±0.4, GC: 57/6.2±0.2, LC: CC: 9/21.9±7, GC: 9/19.9±6.6). In the transcription profile, only ZAR1 was higher in CC group (178.2±151.6) when compared to the GC group (0.8±0.8). All other transcripts analysis did not show significant difference in oocytes (P1A, CC: 1.140±1, GC: 0.09 ±0.04, IGF2R, CC: 0.09±0.09, GC: 0.55±0.47), embryos (P1A, CC:0.07±0.05, GC: 0.016 ± 0.02 IGF2R, CC: 0.16±0.16, GC: 0±0) and cumulus cells (BMP15, CC: 0.03±0.01, GC: 0.02±0.04, IGF2R, CC:0.14±0.03, GC: 0.31±0.1). Despite granulated oocytes presented particularities in C3 levels and expression of the ZAR1 transcript, the results demonstrate that they have the same potential for development of control oocytes, suggesting that cytoplasm heterogeneity does not reflect oocyte competence in bovine. Acknowledgments: EMBRAPA, CAPES, CNPq.

A067 OPU-IVF and ET

Does the continuous ovum pick up (OPU) routine in donors affect the characteristics of *in vitro* embryos?

Nátali Zanenga Chacha[1], Carlos Alberto Zanenga[1], Wilder Hernando Ortiz Vega[2], Katarine Rezende Coelho[1], Daniely Coelho de Menezes[1], Emivaldo de Siqueira Filho[3], Maria Clara Caldas Bussiere [4]

[1]EMBRIZA - Embriza Biotecnologia LTDA, Campo Grande, MS; [2]UVA - Universidade Estadual Vale Do Acaraú, Jerônimo de Medeiros Prado, Sobral, CE; [3]EMBRIOTEC - EMBRIOTEC REPRODUÇÃO ANIMAL, Anápolis, GO; [4]UENF - Universidade Estadual Do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil.

In the commercial practice of in vitro embryo production (IVP) in cattle, losses in the characteristics of IVP after successive OPU in donor cows for prolonged periods are reported. The aim of this study was to evaluate the effect of the number of consecutive OPU during several years on the characteristics of IVP in Gyr dairy cows. The experiment has evaluated 19 Gir donors and they were classified according to their embryo rate (ratio of number of viable oocytes to embryos produced) in classes: 1(> 50.1% n = 7, OPU = 239, average age = 9 years old), 2 (44.1 to 50% n = 5, OPU = 144, average age = 10 years old) and 3 (<44% n = 7, OPU = 197, average age = 11 years old). A total of 580 OPU sessions were held from 2013 to 2018. The donors were kept at Embriza Farm (Campo Grande, Mato Grosso do Sul, Brazil) and were maintained in Brachiaria decumbens pastures, with mineral supplementation and water at libitum. The standard reproductive management immediately after OPU consisted of a first-use progesterone device (Cidr®-Zoetis) insert in vagina on day zero (D0). On day eight (D8) the implant was taken out and 25 mg of prostaglandin (Lutalyse®-Zoetis) was injected. The blastocyst rates were evaluated 168 hours post-insemination (performed only with sexed semen of Holstein breed), and the IVP procedures were performed in the Embriza laboratory (Campo Grande, Mato Grosso do Sul, Brazil) with media produced by Embriotec laboratory (Anápolis, Goiás, Brazil). The performance over the years was evaluated through the following characteristics: total oocytes (TotOv), viable oocytes rate (TxVia - relation between TotOv and total viable oocytes) and embryo rate (TxEm). Differences between OPU along the years and within the classes were evaluated using Kruskal Wallis test with significance level of 5%. The averages were compared in pairs through post hoc analysis of the same statistical test using SPSS software v.22. In the 1 and 3 classes, there was a decrease in the total number of oocytes in year 6 ($p < 0.05$). The average category 2 did not show different performance for this variable due of collection years (4 only). TxVia showed a positive effect from the year of collection in the three categories, with increase in the last two years of collection. The TxEm presented a negative effect in category 3 only in year 5 ($p = 0.037$), but in year 6 the result was similar to years 1 to 4. In classes 1 and 2 there was no effect from the year for this characteristic. The aspects related to the expertise of those involved in the field and laboratory routines, as well as in the general and reproductive management of oocyte donors, should be considered in the evaluation of this experiment. Finally, the main characteristics of in vitro embryo production were not negatively affected by the continued use (4-6 years) of Gyr donors cows.

A085 Folliculogenesis, oogenesis and superovulation

Differences in oocyte and cumulus cells gene expression in Nelore heifers with low and high antral follicle count

Marina Amaro de Lima[1,5], Bernardo Marcozzi Bayeux[2], Rômulo Germano de Rezende[2], Ramon Cesar Botigelli[3], Tiago Henrique Camara de Bem[1], Patrícia Kubo Fontes[3], Marcelo Fábio Gouveia Nogueira[4], Flávio Vieira Meirelles[1], Pietro Sampaio Baruselli[2], Juliano Coelho da Silveira[1], Felipe Perecin[1]

[1]FZEA/USP - Department of Veterinary Medicine, Pirassununga, SP, Brazil; [2]FMVZ/USP - Department of Animal Reproduction, São Paulo, SP, Brazil; [3]IBB/UNESP - Institute of Biosciences, Botucatu, SP, Brazil; [4]FCL/UNESP - Department of Biological Sciences, Assis, SP, Brazil; [5]FMVZ/USP - Graduate Program in Anatomy of Domestic and Wild Animals, São Paulo, SP, Brazil.

Antral Follicle Count (AFC) has been associated with reproductive performance in cattle. In taurine females, higher AFC is positively associated with pregnancy rates, and is linked to greater *in vivo* and *in vitro* embryo production, both in number and efficiency. Contrarily, in indicine females, AFC is negatively correlated with pregnancy rates after IVP-ET or TFAI. How the transcriptional patters in ovarian follicular cells correlate with the AFC remains unknown. This study aimed to investigate the expression of 95 genes in oocytes and cumulus cells in females of low AFC (LAFC) and high AFC (HAFC) using Nelore heifers (*Bos taurus indicus*) as model. Nellore heifers (n=48) had the ovarian follicular wave synchronized. On Day 5, AFC was determined by ultrasound examination. The bottom 10 and top 10 females were assigned to experimental groups LAFC (number of follicles<30, AFC = 24 ± 4.73) and HAFC (number of follicles >60, AFC = 72.3 ± 15.67), respectively. On Day 5 cumulus-oocyte complexes (COCs) were retrieved by ovum pick-up. Cumulus cells and oocytes were separated by pipetting. Twenty samples (pools) of 10 oocytes (one per animal) and 20 samples (pools) of cumulus cells from 10 COCs (one per animal) were used for RNA extraction, cDNA synthesis and gene expression assessment of 94 genes by RT-qPCR. Means of normalized gene expression, using *PPIA* as the reference gene, were compared by Student's t-test and were considered significant when P<0.05. In oocytes, a total of 11 genes were differentially expressed, including eight upregulated in LAFC group (*GAPDH*, *HSF1 BMP15*, *HAS2*, *EGFR, NPR3, H1FOO* and *IGFBP2)* and three downregulated in LAFC (*TFAM*, *XBP1* and *PRDX3*) compared with HAFC. Collectively, these genes are related to COC differentiation, meiotic control, epigenetic modulation, follicular recruitment and cellular responses to stress. In cumulus cells a total of 27 genes were differentially expressed between the groups. Cumulus cells from LAFC animals had higher expression of genes associated with meiotic control (*EGFR*, *RGS2*, *NPR3* and *NPR2*) and epigenetic modulation (*DNMT3A*, *HDAC2* and *PAF1*), as well as genes associated with energetic metabolism, cellular responses to stress and others (*CDK6*, *PA2G4*, *CASP9*, *STAT3*, *XBP1*, *HSP90AA1*, *HSPA5*, *HSPD1*, *SOD1*, *IGF1R*, *GSK3A*, *ATPL5*, *TFAM* and *PFKP)*. Contrarily, *FSHR*, *GAPDH*, *SREBF1*, *NFKB2*, *ARO* and *PTGS2* were downregulated in LAFC compared with HAFC group. These results indicate that distinct cellular compartments within ovarian follicles have dissimilar transcriptional patterns comparing LAFC and HAFC females. Such variations are potentially linked to reproductive performance and are suggestive of differential modulation in terms of cumulus-oocyte complex differentiation, meiotic control and oocyte competence acquisition. Financial support: Sao Paulo Research Foundation (FAPESP; grants 2012/50533-2 and 2018/13155-6) and CAPES - Brazil - Finance Code 001.

A086 Folliculogenesis, oogenesis and superovulation

Amburana cearensis leaf extract attenuates cisplatin-induced ovarian damage in mice

Bruna Bortoloni Gouveia[1], Ricássio de Sousa Barberino[1], Vanúzia Gonçalves Menezes[1], Thae Lanne Barbosa Gama Lins[1], Raimundo Campos Palheta Jr[2], Jackson Roberto Guedes da Silva Almeida[3], Maria Helena Tavares Matos[1]

[1]UNIVASF - Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of São Francisco Valley, Petrolina, PE, Brazil; [2]UNIVASF - Laboratory of Veterinary Pharmacology, Department of Veterinary Medicine, Federal University of São Francisco Valley, Petrolina, PE, Brazil; [3]UNIVASF - Center for Studies and Research of Medicinal Plants, Federal University of São Francisco Valley, Petrolina, PE, Brazil.

Cisplatin is one of the most effective chemotherapeutic drugs, but it has a proven ovarian toxicity by increasing oxidative stress (Barberino et al., Biology of Reproduction, 96:1244–55, 2017). Thus, the pretreatment with natural compounds with antioxidant properties may reduce this toxicity. Recently, *Amburana cearensis,* a medicinal plant, has attracted attention because of its antioxidant actions (Gouveia et al., Theriogenology, 86:1275-84, 2016).The aim of this study was to evaluate the effects of pretreatment with the extract from the leaves of *A. cearensis* before cisplatin chemotherapy on ovarian follicle morphology, oxidative stress markers (reactive oxygen species [ROS] and glutathione [GSH] levels) and metabolically active mitochondria in mice. The adult female mice (n=25) were divided into five groups: the first group acting as a control, received orally by gavage (p.o.) saline solution (0.15 M, 0.3 mL/mouse) and after 1 h, intraperitoneal injection (i.p.) of saline solution (0.15 M, 0.15 mL/mouse). The second group received cisplatin (5 mg/kg body weight, i.p.) at 1 h after saline solution treatment (0.15 M, 0.3 mL/mouse; p.o.). The third group was a positive control, which received *N*-acetylcysteine (150 mg/kg body weight, p.o.) 1 h before cisplatin treatment (5 mg/kg body weight, i.p.). For the fourth and fifth groups, mice received leaf extract of *A. cearensis* at 50 or 200 mg/kg body weight (p.o.), respectively, and after 1 h, mice received cisplatin (5 mg/kg body weight, i.p.). All groups were treated once daily for 3 days. The ovaries were harvested from the mice 24 h after the last pharmacological administration and destined to histological (follicular morphology) and fluorescence (ROS, GSH, and active mitochondria levels) analyses. The percentages of normal follicles (total or in the different developmental stages) were submitted to the Chi-square test. Data from ROS, GSH, and mitochondrial activity were compared by Kruskal-Wallis nonparametric and Student Newmans Keuls tests ($P<0.05$). The results showed that cisplatin treatment decreased ($P<0.05$) the percentage of normal follicles (30%) in comparison to the control (72.12%) and *N*-acetylcysteine (67.33%) groups. Nevertheless, *A. cearensis* extract pretreatment (50 mg/kg: 58.1%; 200 mg/kg: 54.82%) prevented ($P<0.05$) the reduction in the percentage of normal follicles induced by cisplatin, maintaining it similar to *N*-acetylcysteine group. Cisplatin treatment increased ($P<0.05$) ROS levels and decreased ($P<0.05$) both levels of GSH and active mitochondria in comparison to the control and *N*-acetylcysteine groups. Pretreatment with *A. cearensis* (50 or 200 mg/kg) prevented ($P<0.05$) the oxidative stress induced by cisplatin. In addition, *A. cearensis* extract (50 or 200 mg/kg) increased ($P>0.05$) the mitochondrial activity compared to control and *N*-acetylcysteine groups. In conclusion, the leaf extract of *A. cearensis* may attenuate the ovarian damage caused by the cisplatin treatment.

A087 Folliculogenesis, oogenesis and superovulation

Autophagy is a pro-survival adaptive response to heat shock in bovine oocytes

Laís B. Latorraca[1], Marcelo T. Moura[1], Weber B. Feitosa[1], Camila Mariano[1], Patrícia K. Fontes[2], Marcelo F.G. Nogueira[2], Fabíola F. Paula Lopes[1]

[1]UNIFESP - Universidade Federal de São Paulo, Diadema, SP; [2]UNESP - Universidade Estadual de São Paulo, Botucatu, SP, Brasil.

Autophagy is a physiological mechanism that can be activated under stress conditions to recycle protein aggregates and damaged organelles for cellular metabolism. The cascade of events triggered during fertilization activates the autophagic pathway essential for early preimplantation development. However, the role of autophagy during oocyte maturation has been poorly investigated. Therefore, the objective of this study was to characterize the impact of autophagy inhibition on developmental competence and gene expression of bovine oocytes exposed to heat shock (HS). COCs were matured at Control (38.5ºC for 22 h) and Heat Shock (41ºC for 16 h followed by 38.5ºC for 6 h) temperatures in the presence of 0 and 10 mM 3-methyladenine (3MA; autophagy inhibitor). This model was used for all experiments described below. Parametric data were analyzed by ANOVA procedure of SAS and non-parametric data were analyzed by the Kruskal-Wallis test. The first study determined whether HS induces autophagy on bovine COCs. After 22 h IVM, a total of 180 oocytes and cumulus cells of 150-210 COCs were processed for microtubule-associated protein 1A/1B-light chain 3 (LC3: autophagy marker) protein abundance by 3 replicates of western blotting. High LC3-II/LC3-I ratio is indicative of autophagy induction. Heat shock increased ($P<0.05$) LC3-II/LC3-I ratio in bovine oocytes as compared to control. However, for oocytes matured with 3MA, there was no difference in LC3-II/LC3-I ratio between control and HS. On the other hand, there was no effect of temperature on LC3-II/LC3-I ratio in cumulus cells. Although, the addition of 3MA reduced ($P<0.05$) LC3-II/LC3-I ratio under control and HS. The second study determined the role of autophagy on developmental competence of heat-shocked oocytes. After IVM, a total of 150 COCs were subjected to 5 replicates of IVF and IVC. Inhibition of autophagy during IVM reduced ($P<0.01$) cleavage rate of heat-shocked oocytes as compared to all the other groups. Exposure to HS reduced ($P<0.05$) the proportion of oocytes that reached the blastocyst stage. However, this deleterious effect of HS was increased ($P<0.01$) for oocytes matured with 3MA. The third study determined the effect of autophagy inhibition on mRNA expression in heat-shocked oocytes. Gene expression analysis of 150 bovine oocytes total was performed 5 times using Applied Biosystems™ TaqMan® Assays. The qPCR was performed in the Biomark HD System. Inhibition of autophagy in heat-shocked oocytes reduced ($P<0.06$) mRNA abundance for genes related to different biological processes such as oocyte maturation (*BMP15*, *HAS2*, and *GREM1*), lipid and energy metabolism (*SREBF2* and *MTIF3*), cellular growth (*IGF2*), and heat shock response (*HSF1* and *HSPA1A*). In conclusion, autophagy is a stress response induced on oocytes exposed to HS during IVM and its inhibition modulated important functional processes in the bovine oocyte rendering the oocyte more susceptible to the deleterious effects of HS.

A088 Folliculogenesis, oogenesis and superovulation

The effect of a non-steroidal anti-inflammatory drug on PGF and PGFM concentrations in bovine follicular fluid after GnRH treatment

Bernardo Garziera Gasperin[1], Rafael Gianella Mondadori[1], Monique Rovani[4], Camila Amaral D'Avila[1], Fabiane Pereira de Moraes[1], Fernando Caetano Oliveira[1], Sergio Vargas Jr[1], Ligia Margareth Cantarelli Pegoraro[2], Thomaz Lucia Jr[1], Paulo Bayard Dias Gonçalves[3]

[1]UFPel - Universidade Federal de Pelotas, Pelotas, RS; [2]Embrapa - Embrapa Clima Temperado Pelotas, RS; [3]UFSM - Universidade Federal de Santa Maria, Santa Maria, RS; [4]IFF - Instituto Federal Farroupilha Frederico Westphalen, RS, Brasil.

Although it is known that prostaglandins are essential for ovulation, some studies suggest that PGF could also be used as an ovulation inducer in timed artificial insemination protocols. The present study tested whether an i.m. injection of PGF reverts the effect of a non-steroidal anti-inflammatory drug (NSAID) administered 17h after GnRH-induced ovulation in cattle. The procedures were approved by UFPel and UFSM Animal Ethics Committee. Nineteen non-lactating cyclic Jersey cows had their follicular growth synchronized by a hormonal protocol consisting of insertion of an intravaginal device (IVD) containing progesterone (P4; 1g) on D0 along with i.m. injections of estradiol benzoate (2mg) on D0 and of cloprostenol (150μg) on both D0 and D8. The IVDs were removed on D9 and GnRH injections (gonadorelin acetate; 100μg i.m.) were performed in all the cows 20h after IVD withdrawal (hour 0). Then, 17h after GnRH, cows were allocated to three treatments: control (saline i.m.; n=6); NSAID: 2.2mg/kg flunixin meglumine (FM) i.m. (n=6); and NSAID+PGF: 2.2mg/kg FM (n=6) and 25mg dinoprost tromethamine (i.m.), 6h later. The groups were normalized according to follicular diameter and follicular aspirations were performed 24h after hour 0. The concentrations of PGF and of PGF metabolite (PGFM) in the follicular fluid (FF) were evaluated through ELISA Prostaglandin F2α and 13,14-dihydro-15-keto Prostaglandin F2α kits (Cayman Chemical, Ann Arbor, USA), respectively. Follicular vascular flow was evaluated at 0 and 24h after GnRH administration using Color Doppler Ultrasonography (Mindray M5; 6,5 MHz) and subjectively evaluated by three trained technicians using a 4-point score. The effects of treatments on PGF and PGFM concentrations were analyzed by ANOVA, with comparisons of means using the Tukey test. The median score of follicular vascular flow was compared among treatments by the Kruskal-Wallis ANOVA for non-parametric data. The concentration of PGF in the FF was greater (P=0.0032) in the control (49563.2±7727.7 pg/ml) than in NSAID (5664.3±3473.1 pg/ml) and NSAID+PGF (3198.4±770.5 pg/ml). The PGFM concentrations in the FF were greater (P<0.0001) in NSAID+PGF (3450.4±269.6 pg/ml) than in the control and NSAID groups (1228.2±45.7 and 204.1±568.5 pg/ml, respectively). Decreased vascular flow was observed in NSAID+PGF group (P<0.05). In conclusion, systemic NSAID treatment inhibits GnRH/LH-induced PGF secretion in preovulatory follicles which, in the conditions of the present study, was not reverted by systemic PGF administration. The use of NSAID after GnRH treatment provides an adequate model to investigate the effect of PGF on ovulation in cattle. The authors are thankful to FAPERGS and CNPq (Edital PRONEX-16/2551-0000494-3) and CAPES for financial support (financial code 001).

A089 Folliculogenesis, oogenesis and superovulation

Effects of in vitro prematuration of bovine COCs on the levels of maturation-related transcripts and meiosis resumption

José Roberto Viana Silva, Francisco Taiã G. Bezerra, Laís Rayani F. M. Paulino, Bianca R. Silva, Anderson Weiny B. Silva, Ana Liza P. Souza

UFC - Universidade Federal do Ceará, Sobral, CE, Brasil.

During growth, oocytes synthesize large quantities of mRNA that accumulates to form the stock of maternal mRNA, which will have a crucial role during oocyte maturation and early embryo development. However, it is still not known if in vitro prematuration of oocytes increase the levels of these maternal mRNAs and have a positive impact on oocyte maturation. The present study evaluated the effects of prematuration and maturation of COCs on the levels of mRNA for growth and differentiation factor 9 (*GDF9)*, cyclin B1, oocyte-specific linker histone *(H1FOO*), quinase *cMOS*, poly(A) ribonuclease *(PARN)* and eukaryotic initiation factor 4E (*eIF4E)*. Cow ovaries (n= 40) were obtained from a local abattoir and the cumulus-oocyte complexes (COCs) of medium antral follicles (3.0 to 6.0 mm in diameter) were aspirated and classified according to the morphology of oocyte and cumulus cells. Fresh COCs were collected and used as uncultured control. For prematuration and maturation protocols, grade 1 and 2 COCs with a visible compact and intact cumulus cells and a dark cytoplasm were selected. The prematuration medium (pre-IVM medium) consisted of TCM-199* supplemented with 10 µM cilostamide (Sigma, St. Louis, MO, USA) and follicular hemi-sections (n=8). The COCs were pre-matured individually for 20 h. The *in vitro* maturation medium (IVM) was designated as TCM-199* supplemented with L-glutamine (Sigma) 0.2 mM pyruvic acid (Sigma), 5.0 mg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 mg/mL FSH (Bioniche), 0.4% BSA (Sigma), 10 µM of cilostamide (Sigma) and 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate (Sigma). The COCs were cultured individually for 22 h at 39°C in a saturated humidity atmosphere containing 5% CO2 and 95% air. To evaluate the levels of mRNAs, oocytes from uncultured COCs (group 1), as well as from prematured COCs (group 2) or both prematured and matured COCs (group 3) were collected and stored at -80 °C, until RNA extraction. Quantification of mRNA for *GDF9*, *cyclin B1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* was performed by real-time PCR. The results were analyzed by the Kruskal-Wallis test, followed of the post hoc nonparametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$. After prematuration of COCs, only 35.0% of the oocytes had meiosis resumption, whereas after IVM a meiosis resumption rate of 90% was observed. Prematuration and IVM of COCs increased the levels of mRNA for *cMOS* and *H1FOO* in oocytes when compared to oocytes before the IVM period. The levels of mRNA for *GDF9*, *PARN* and *eIF4E* did not show significant differences, but the levels of *cyclin B1* presented a significant increase after prematuration period, when compared to oocytes before prematuration. In conclusion, prematuration and maturation of oocytes from medium antral follicles increase expression of *eIF4E*, *PARN*, *H1FOO*, *cMOS*, *GDF9* and *cyclin B1*.

A090 Folliculogenesis, oogenesis and superovulation

Effects of in vitro culture of secondary follicles on the levels of mRNAs for *GDF9*, *cyclin B1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* in bovine oocytes

Bianca R. Silva, Francisco Taiã G. Bezerra, Francisco Edilcarlos O. Lima, Laryssa G. Barrozo, Laís Rayani F. M. Paulino, Anderson Weiny B. Silva, Ana Liza P. Souza, José Roberto V. Silva

UFC - Universidade Federal do Ceará, Sobra, CE, Brasil.

During oocyte development a prolonged phase of intensive RNA synthesis is required for the production of transcripts essential for oocyte growth and pre-implantation embryo development. In this way, the investigation of genes that are involved with the translation process, as well as transcripts that are stored in the oocytes are quite important for understand oocyte development *in vitro*. Thus, the aim of the this study was to evaluate the effect of *in vitro* culture of secondary follicles from cattle on the levels of mRNAs expression of *GDF9*, cyclin B1, *H1FOO* (oocyte-specific linker histone), quinase *cMOS*, *PARN* (poly(A) ribonuclease) and *eIF4E (*cap binding protein) in oocytes. Thus, secondary follicles (~ 0.2 mm) were isolated from the ovarian cortex and part of these follicles were ruptured and oocytes were collected and stored at -80 ºC (control group). The remaining follicles were individually cultured at 38.5°C, 5% CO2, for 18 days only in TCM-199 supplemented with 10 µg/mL insulin, 5.5µg/mL transferrin and 5 ng/mL selenium (ITS), 3.0 mg/mL bovine serum albumin (BSA), 2mM glutamine, 2 mM hypoxanthine, 50 µg/mL of ascorbic acid and 100 ng/mL FSH. After 18 days of culture, as in the control group the follicles were ruptured and the oocytes were collected and stored at -80 °C, until RNA extraction. In both groups, the levels of mRNAs were quantified by the RT-qPCR technique. The primers used specifically amplified the mRNAs for *GDF9*, cyclin B1, H1FOO, *cMOS*, *PARN* and *eIF4E* while glyceraldehyde3-phosphatedehydrogenase (*GAPDH*) was used as an endogenous control for normalization of mRNAs expression. The delta-delta-CT method was used to demonstrate the relative expression of the mRNAs studied. The data of expression for the genes evaluated in oocytes from uncultured and cultured secondary follicles were analyzed by the Kruskal-Wallis test, followed by the post hoc non-parametric Dunn's Multiple Comparison Test. Differences were considered significant when $P<0.05$. The results showed that in vitro culture of bovine secondary follicles significantly increased their diameter (232.29 $\pm$ 16.36µm) when compared with those uncultured follicles (183.19 $\pm$ 15.44). In vitro grown follicles had an increase in the levels of mRNA for *GDF9*, *cMOS*, *PARN*, *eIF4E*, *cyclin B1* and *H1FOO* in their oocytes when compared to those before culture. In conclusion, the *in vitro* culture of bovine secondary follicles for 18 days can increase the expression of mRNA for *GDF9*, *cyclin B1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* in their oocytes.

A091 Folliculogenesis, oogenesis and superovulation

The effects of culture media and FSH on PI3K-Akt pathway during bovine IVM

Gabriella Mamede Andrade, Maite Del Collado, Alessandra Bridi, Flávio Vieira Meirelles, Juliano Coelho da Silveira, Felipe Perecin

FZEA-USP - Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, Pirassununga, SP, Brasil.

Oocyte quality is a key factor determining reproductive success. Culture conditions during *in vitro* oocyte maturation may influence the oocyte's developmental potential. Some signaling pathways important for oocyte competence as Phosphatidylinositol-3-kinase/Protein kinase B (PI3K-Akt) can be altered by supplements routinely used in *in vitro* culture medium as IGF-1, EGF and FSH. Our hypothesis is that FSH supplementation and different maturation media during IVM modifies the expression of PI3K-Akt-related genes in bovine cumulus cells. In order to test this hypothesis, we performed IVM with two different maturation media supplemented with high (0.01 UI/mL; HIGH group) or low (0.000875 UI/mL; LOW group) human recombinant FSH (hrFSH) concentrations. High or low hrFSH concentrations were tested in maturation medium 1 (MM1 – TCM199 supplemented with pyruvate, gentamicin, LH and bovine fetal serum) or maturation medium 2 (MM2 – TCM 199 supplemented with pyruvate, gentamicin, BSA, AREG, IGF-1, progesterone and estradiol). Cumulus cells from immature COCs, 12 h of IVM and cumulus cells from matured COCs (at metaphase II stage) 24 h of IVM were used to evaluate gene expression of PI3K-Akt pathway related genes. Pools (n=5) of cumulus cells from 10 or 20 COCs per group were submitted to total RNA extraction using TRizol, followed by DNase treatment and cDNA synthesis using High Capacity cDNA Reverse Transcription Kit. The relative gene expression of *PI3K, AKT1, AKT3, PTEN, FOXO3, BAX, BCL2, BRCA, CDK6, EIF4B, EIF4E* and *MAPK* were determined using three genes (*PPIA, RPL15* and *YWHAZ)* as reference. Expression levels were calculated using the $2^{-\Delta Ct}$ method and data were tested by ANOVA in a 2x2x2 factorial design (time x hrFHS x MM) with additional control group (immature). Means were compared by Tukey's test. A level of 5% significance was used. After 24 h of IVM maturation rates were similar between groups. High hrFSH at 12h of maturation decreased *PI3K* and *CDK6* gene expression and increased *MAPK1* relative expression and at 24h high hrFSH treatment reduced *PTEN* and increased *BCL2* and *CDK6* gene expression, independent of maturation medium. Regarding the *PI3K* gene, lower expression was observed in MM1 group at 12h and 24h of IVM. Additionally, when COCs were maturated with MM1 the cumulus cells expression of *FOXO3*, *BRCA*, *CDK6* were higher and the expression of *PTEN* lower at 12h. No differences were observed between MM1 and MM2 after 24h of IVM. In summary, the hrFSH possibly stimulate the PI3K-Akt pathway in both maturation media studied and the maturation medium (MM1) has an inhibitory effect on PI3K-Akt based on *PTEN* and *PI3K* gene relative expression. These results demonstrated the influence of culture conditions on cumulus cells gene expression at 12 and 24h and the possibility to modulate important pathways involved in oocyte competence acquisition during IVM. Financial support: FAPESP grants 2014/22887-0, 2018/01431-9, 2018/13155-6.

A092 Folliculogenesis, oogenesis and superovulation

Repeatability of the antral follicle count and its relationship with productive and reproductive parameters in prepubertal heifers with early induction of puberty

Sofia Botsaris Delchiaro[1], Denis Vinicius Bonato[1], Pedro Victor de Luna Freire Oliveira[2], Marcelo Marcondes Seneda[1], Fábio Morotti[1]

[1]UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil; [2]UniFil - Instituto Filadélfia de Londrina, Londrina, PR, Brasil.

This study evaluated the repeatability of antral follicle count (AFC) and its relationship with productive and reproductive parameters in prepubertal heifers submitted to early puberty induction. Nelore heifers (G-N=15, *Bos indicus*) and Nelore x Angus heifers (G-NA=15, *Bos indicus-taurus*) with 12 to 14 months old and without corpus luteum (CL) were kept under the same management conditions and nutrition for this study. The AFC, body weight, body condition score (BCS), ovary and dominant follicle diameter were determined in each animal on Days -15, 0, 12 and 21. The puberty induction was performed on Day0, by the insertion of a progesterone device (previously used 3 times, CIDR®, Zoetis, Madison, USA) which remained until the 12th. On the removal of the device 1mg of estradiol benzoate was administered IM (Gonadiol®, Zoetis, Madison, USA) and an adhesive was fixed in the sacrococcygeal region (Estrotect™ Breeding Indicator, Spring Valley, USA) for estrus intensity classification (0= absent, 1=low and 2=high intensity). At Day21, the presence and diameter of the CL were registered. In all evaluations, a 7.5 MHz ultrasound with a rectal linear transducer was used. Data were analyzed by the generalized linear model (GLM) followed by the Tukey test. The Fisher exact test was used to evaluate ovulation rate and the repeatability was calculated as $\delta 2$ animal / $\delta 2$ animal + $\delta 2$ error ($P<0.05$). Regardless of hormonal induction, the AFC was highly repeatable during the study in G-N (r=0.79) and G-NA (r=0.90). The AFC average throughout the study was higher ($P<0.05$) in G-N than G-NA (24.2±8.5 vs. 17.7±9.0 follicles). BCS was similar ($P>0.1$) between G-N (2.6±0.2) and G-NA (2.7±0.3) on D-15, but on Days 0, 12 and 21 the G-NA showed higher BCS (2.6±0.2 vs. 3.0±0.2, 2.6±0.2 vs. 3.1±0.1 and 2.6±0.2 vs. 3.2±0.2, $P<0.001$). A variation of BCS throughout the study occurred in G-NA ($P<0.0001$), but not in G-N ($P>0.1$). The weight average gain was higher ($P=0.014$) in G-NA (0.69±0.33 kg/day) than in G-N (0.40±0.29 kg/day). The G-NA exhibited higher ($P<0.01$) weight than G-N in the initial (Day-15 - G-NA: 238.4±28.9 vs. G-N: 220.0±25.6 kg) and final period (Day21 - G-NA: 262.9±27.2 vs. G-N: 233.9±30.8 kg). The dominant follicle diameter was similar ($P>0.1$) between G-N and G-NA in Days -15, 0 and 21, but in Day12 G-NA had a larger diameter (9.3±1.5 vs. 11.6±2.7 mm, $P=0.007$). The estrus intensity (1.5±0.5 vs. 1.7±0.5), ovulation rate [41.67% (5/12) vs. 50.00% (7/14)] and CL diameter (11.2±2.4 vs. 11.2±5.5 mm) were similar ($P>0.1$) between G-N and G-NA, respectively. Early puberty induction had a low response and was similar between Nelore and crossbred Nelore x Angus heifers. The AFC was higher in Nelore heifers, although in both breeds AFC exhibited highly repeatability throughout puberty induction. Crossbred heifers had higher BCS, average weight gain and diameter of the dominant follicle.

A093 Folliculogenesis, oogenesis and superovulation

Follicular development of fetal bovine ovarian cortex fragments submitted to xenotransplantation

Giovanna Faria de Moraes[1], Juliano Bergamo Ronda[1], Kele Amaral Alves[1], Benner Geraldo Alves[2], Bruna de Souza Campos[1], Elso Donizete Souza Junior[1], Jéssica Cristina dos Santos Marques[1], Ana Beatriz Almeida de Moraes[1], Ricarda Maria dos Santos[1]

[1]UFU - Laboratory of Animal Reproduction, College of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, MG, Brazil; [2]UFU - Laboratory of Reproductive Biology of the Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Xenotransplantation of ovarian tissue in immunosuppressed animals may be an option to promote follicular development and maturation, allowing the preservation of preantral follicles. The conservation of bovine fetal ovarian tissue contributes to the maintenance of evolutionary intention, even if this fetus is not compatible with life. Conservation of genetic material of fetuses, by xenotransplantation and their subsequent *in vitro* maturation, decreases the generation interval, increasing genetic gain. Ovarian tissue from bovine fetuses is a good candidate for preservation of preantral follicles because it has suffered less environmental interference, besides having a greater reserve of primordial (quiescent) follicles, which is more tolerant to cryopreservation, due to its small size, relatively unspecific morphology, and the low metabolism. The aim was evaluate the follicular development and numbers of blood vessels in fetal bovine ovarian tissue fragments submitted to xenotransplantation for 7 or 14 days (X7 and X14, respectively), and *in vitro* culture for 1 or 7 days (C1 and C7, respectively), compared to freshly analyzed fragments (FC). Ten pairs of fetal bovine ovaries collected in local slaughterhouse, fragmented (3 x 3 x 1 mm) and fresh xenotransplanted, were used in 20 female mice of the Balb C line, so that each ovarian pair provided 5 fragments for X7 and 5 fragments for X14, therefore each mice received 5 fragments. *In vitro* culture and the histological analysis were performed in the Animal Reproduction Laboratory of the Federal University of Uberlândia. The number of blood vessels per section was analyzed and the follicles were classified in normal or degenerate and primordial, transitional, primary, secondary or antral follicles, according to morphological characteristics. The data was compiled and analyzed with Sigma Plot software. Follicular viability was higher in the FC (88.2 ± 3.2), with similar results to C1 and C7. X14 presented better follicular viability (67.6 ± 4.3) than X7 (62.2 ± 4.0), with similar response to the culture groups ($p<0.05$), and there was a positive correlation between the number of blood vessels and the follicular viability ($r = 0.23$, $p<0.1$). FC presented the highest number of vessels (16.7 ± 3.1) in comparison with the other groups, which presented similar results. The follicular activation in X7 and X14 presented lower results (37.0 ± 3.7 and 34.4 ± 5.8, respectively) than FC (55.5 ± 4.9), being that X7 presented a similar result to C1 ($P <0.05$). These findings suggest that xenotransplanted fetal bovine ovarian tissue fragments for 14 days had satisfactory follicular viability and preservation of the follicular reserve (CEUA/UFU/Protocol 006/17). Financial support: FAPEMIG and CNPq.

A094 Folliculogenesis, oogenesis and superovulation

Effect of adding ascorbic acid in cultured ovarian preantral follicles for twelve days in cattle

Denis Vinicius Bonato, Camila Bizarro da Silva, Francieli Gesleine Capote Bonato, Ana Clara Canto Souza, Suellen Miguez González, Sofia Botsaris Delchiaro, Fábio Morotti, Marcelo Marcondes Seneda

UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

This study aimed to evaluate the effect of antioxidant ascorbic acid addition to the media for *in vitro* culture of preantral follicles of *Bos taurus indicus* females. Five ovaries of five cyclic adult Nelore females were collected at a local slaughterhouse, with a body score ranging from 3 to 3.5 (range 0 to 5). Ovaries were washed in 70% ethanol, and ovarian cortex was divided into five fragments about 3x3x1mm. One fragment per animal was immediately fixed in Bouin (non-cultured control, D0). The others fragments (n=4) were individually cultured in 24-well culture dishes containing 1ml of minimum essential medium (MEM, Gibco BRL, Rockville, MD, USA; osmolarity 300mOsm/l, pH 7.2) supplemented (MEM+) with ITS (6.25mg/ml insulin, 6.25mg/ml transferrin, and 6.25ng/ml selenium; (Sigma, St. Louis, MO, USA)), 0.23mM pyruvate (Sigma, St. Louis, MO, USA), 2mM glutamine (Gibco BRL, Rockville, MD, USA), 2mM hypoxanthine (Sigma, St. Louis, MO, USA), 1.25mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20IU/ml penicillin (Sigma, St. Louis, MO, USA) and 200mg/ml streptomycin (Gibco BRL, Rockville, MD, USA). The culture was tested only with MEM+ (cultured control), as well as different concentrations (50, 100 and 200ng/mL) of ascorbic acid (Sigma, St. Louis, MO, USA). Ovarian fragments were cultured in the media for twelve days. The culture media were replaced by fresh aliquots every two days. To the analysis of the integrity and degree of development of the follicles, the classical histology with Periodic Acid-Schiff (PAS) and Hematoxylin staining was used. The classification of follicles was based on the evaluation of the morphological integrity (normal or degenerated) and the development stage (primordial, primary and secondary). Data were submitted to ANOVA tests ($p \leq 0.05$). We evaluated 750 preantral follicles (normal or degenerated), of which 142 were primordial follicles and 608 developing follicles. After 12 days of culture, fragment treated with 200ng/ml of ascorbic acid had 55.33% (83/150) of intact follicles, did not differ from D0 ($p=0.94$; 64% (96/150)). Fragments treated with 50ng/ml, 100ng/ml of ascorbic acid and MEM+ had bottom number of intact follicles (23.33% (35/150), 30.66% (46/150) and 24% (36/150), respectively) compared to D0 and 200ng/ml ($p < 0.05$). The concentration of 200ng/mL ascorbic acid resulted in a higher percentage of developing follicles (52%, 78/150) when compared to D0, MEM+ and 50ng/mL ($p<0,05$), which had 17.33% (26/150), 18% (27/150) and 21.33% (32/150) of developing follicles, respectively. On the other hand, the concentration of 100ng/mL of ascorbic acid did not change the number of developing follicles (30.66%, 46/150) compared to all other treatments. Thus, MEM+ supplemented with 200ng/mL of ascorbic acid for twelve days of culture, was capable of preserving morphological integrity and promote the development of bovine preantral follicles.

A095 Folliculogenesis, oogenesis and superovulation

Effects of epidermal growth factor (EGF) and progesterone (P4) on the levels of maturation-related transcripts in oocytes of bovine secondary follicles cultured in vitro

Venância Antonia Nunes Azevedo[1], Lais Raiane Feitosa Melo Paulino[1], Francisco Taia Gomes Bezerra[1], Bianca Regia Silva[1], Pedro Alves Barroso[2], Anderson Weiny Barbalho Silva[3], Ana Liza Paz Souza[3], Jose Roberto Viana Silva[3]

[1]UFC - Universidade Federal do Ceará, Campus Sobral, CE; [2]Labirep - Laboratório de Biotecnologia Reprodutiva, Sobral, CE; [3]PPGB - Programa de Pós Graduação em Biotecnologia, Sobral, CE, Brasil.

The objective of this study was to investigate the effects of EGF and P4 on growth and on the levels of mRNA of of *GDF9*, cyclin B1, *H1FOO* (oocyte-specific linker histone), quinase *cMOS*, *PARN* (poly(A) ribonuclease) and *eIF4E (*cap binding protein) in oocytes from cultured bovine secondary follicles. Therefore, secondary follicles (~ 0.2 mm) were isolated from the ovarian cortex and individually cultured at 38.5°C, 5%CO2, for 18 days in TCM-199 alone (cultured control) or supplemented with 10 ng/ml progesterone (P4), 10ng/ml EGF or both EGF and progesterone (10ng/ml each). Follicular diameters were evaluated and the levels of mRNA were quantified at the end of 18 days of cultures by real-time PCR technique. The primers used specifically amplified the RNAs for *cMOS*, *Cyclin B1*, *H1Foo*, *eIF4*, *GDF-9* and *PARN*. The housekeeping gene *β-tubulin* was used as an endogenous control. The delta-delta-CT method was used to demonstrate the relative expression of the mRNAs studied. Follicular diameter and the levels mRNAs evaluated in oocytes from secondary follicles were compared by the Kruskal-Wallis test, followed by the Dunn's test. The differences were considered significant when $P < 0.05$. The results showed that there was a progressive increase in follicular diameter in all treatments, but only the follicles cultured in EGF-supplemented medium showed significantly larger diameters than those grown in the control at the end of culture period. The presence of EGF in the culture medium also promoted a significant increase in mRNA levels of *cMOS*, *eIF4*, *GDF-9* and *PARN* compared to follicles cultured with both the EGF and P4 ($P < 0.05$). On the other hand, oocytes from follicles cultured in the presence of progesterone showed higher mRNA levels for mRNAs of *cyclin B1* and *GDF-9* when compared to the control medium ($P < 0.05$). Levels *of eIF4* mRNA were also higher in the oocytes from follicles cultured in the presence of both EGF and P4 ($P<0.05$). Regarding mRNA *H1Foo* expression, oocytes from follicles cultured with P4 or both EGF and P4 had higher levels of mRNA of *H1Foo* than those cultured in medium containing only EGF ($P < 0.05$). According to this results, it can be concluded that the presence of EGF in the culture medium promotes follicle growth and increase the levels of mRNA for *cMOS*, *eIF4*, *GDF-9* and *PARN*, while progesterone increase the mRNA expression of *cyclin B1* and *GDF-9* in oocytes from cultured bovine secondary follicles.

A096 Folliculogenesis, oogenesis and superovulation

Aspects of superovulatory response and embryo production in holstein heifers

Jairo Pereira Neves[1], Carlos Antônio de Carvalho Fernandes[1,2], Gustavo Henrique de Souza Pereira[1,2], Humberto Luis Del Hoyo Neri[2], Jéssica Ruiz Pereira[2], Ana Cristina Silva de Figueiredo[1,2], Miller Pereira Palhão[1]

[1]Unifenas - Universidade José Do Rosário Velano, Alfenas, MG; [2]Biotran Biotecnologia, Alfenas, MG, Brasil.

The objective of this study was to correlate characteristics of the superovulatory response (SOV) of Holstein heifers with total and viable embryo production submitted to Multiple Ovulation and Embryo Transfer (MOET). Data of SOV and embryo production from 309 donors, from the same farm, over a period of 12 months were used. All donors were superovulated in a same SOV protocol with 180 mg of Folltropin™ (Vetoquinol-Brazil), in decreasing doses. Donors were flushed by the same veterinarian. The number of ovulations (CLs) was determined by ultrasonography (Mindray M5™-China) immediately before flushing. Flushing was performed in all females with two or more ovulations (281 donors - 90.9%). In these animals the mean CLs were 9.6±5.6. A total of 6.7±3.7 embryos and 4.3±3.3 viable embryos per donor were obtained. The rate of embryonic recovery, defined by total recovered embryos divided by total CLs was 62.7%. The percentage of viable embryos in relation to the total was 70.4%. The donors were allocated into 4 groups according to SOV response. G1: 2 to 5 CL (N = 50-17.9%); G2: 6 to 10 CL (N = 97-34.6%); G3: 11 to 15 CL (N = 75-26.8%) and G4: up to 16 CL (N = 56-20.0%). Embryo production, embryo viability and recovery rate in donors of the different groups were analysed by anova and compared using Tukey's test at 5% probability. The number of total embryos was 2.5 ± 1.4^{d}; 5.8 ± 2.0^{c}; 8.3 ± 3.2^{b} and $9.7+4.1^{a}$ ($P<0.05$) and viable embryos was 1.8 ± 1.5^{c}; 4.5 ± 2.2^{b}; 5.7±3.2 and 6.2±4.2 ($P<0.05$) for groups 1, 2, 3 and 4, respectively. The mean recovery rate was 64.9 ± 33.4^{ab}; 73.6 ± 23.7^{a}; 64.5 ± 23.9^{ab} and 53.9 ± 23.3^{b} ($P<0.05$) and the embryo viability 61.1±41.4; 71.9±27.6; 65.3±28.7 and 60.1±28.9 ($P>0.05$) for groups 1, 2, 3 and 4 respectively. The production of embryos is directly related to SOV. The number of viable embryos per donor was positively related to the SOV until the SOV response of 15 CLs per animal. From this, no relation was found. The recovery rate of donors with SOV above 16 CLs (G4) is lower than those with SOV between 6 to 10 CLs (G2). It is concluded that donors that present very large responses to SOV, above 16 CLs, are less efficient in relation to the process, and it may be necessary to reduce the superovulatory stimulus for these animals in a next MOET procedure. Acknowledgment: Biotran, Unifenas, CNPq and CAPES.

A097 Folliculogenesis, oogenesis and superovulation

Varying COC density and culture medium volume during IVM: effects on meiosis progression

Ana Caroline Silva Soares, Jhenefer Bortoleti de Oliveira, Rodrigo Garcia Barros, Jhessica Naomi Sakoda, Isabela Lima Gama, José Buratini Junior

UNESP - Universidade Estadual Paulista campus Botucatu, Botucatu, São Paulo, Brasil.

In vitro maturation (IVM) of cumulus-oocyte complexes (COC) is a crucial and limiting step for successful *in vitro* production of bovine embryos. Oocyte developmental competence is linked to the ability to achieve full cumulus expansion and to complete meiosis. Depending on COC yield following "ovum pick up" (OPU) for a given donor, COC density in the IVM medium may vary largely. However, it is still not clear if individually cultured COC would have their developmental capacity hampered, nor how varying COC density and culture media volume impact on IVM. We hypothesized that individual COC culture and low COC density would reduce the percentage of MII oocytes at the end of IVM. In four replicates, COCs aspirated from slaughterhouse ovaries were grouped to provide different COC density and total medium volume as follows: 1 COC in 20µl (G1; 1:20); 1 COC in 400µL (G2; 1:400), 5 COCs in 400µL (G3; 1:80), 10 COCs in 400µL (G4; 1:40) or 20 COCs in 400µL (G5; 1:20). IVM medium was TCM199 with Earle's salts, bovine serum albumin, amikacin, pyruvate, recombinant human follicle stimulant hormone, amphiregulin, insulin-like growth factor 1, estradiol and progesterone. After 24h of IVM, we assessed cumulus cell expansion and oocyte meiotic status with Hoechst 33342 staining. All groups were similar for cumulus cell expansion. Rates of MII were higher ($P<0.05$) in G3 (65.40%), G4 (61.53%) and G5 (59.69%) compared to G1 (26.34%) and G2 (36.04%). Therefore, individual COC culture provided lower MII rates, suggesting that the interaction between COCs via secreted factors benefit competence to resume meiosis during IVM. Supported by FAPESP 2016/21671-9.

A103 Physiology of reproduction in the male and semen technology

Morphological characterization of brown brocket deer (*Mazama gouazoubira*) sperm ultrastructure by transmission electron microscopy

Duanny Murinelly de Souza Cunha[1], Mírley Barbosa de Souza[2], Vítor Lima Torres[1], Thalles Gothardo Pereira Nunes[1], Leda Maria Costa Pereira[1], Dárcio Ítalo Alves Teixeira[1]

[1]UECE - Ceará State University, Imaging Diagnosis Applied to Reproduction, Fortaleza, CE, Brazil; [2]UNILEAO - University Center Leao Sampaio, Cajuína São Geraldo, Juazeiro do Norte, CE, Brazil.

The present study was conducted to describe the ultrastructure of brown brocket deer (*Mazama gouazoubira*) sperm using transmission electron microscopy (TEM). The study was approved by Ceara State University Ethics Committee (number 7913746-2017) and by the System of Authorization and Information on Biodiversity (number 60925). Before the procedures, animals were sedated with 5 to 10 mg/kg ketamine hydrochloride and 0.5 to 1.5 mg/kg xylazine hydrochloride, both IM (Cursino; Duarte, Royal Society Open Science, 3:1-9, 2016). Two adult animals (2-5 years old) had their semen collected using electroejaculation (n=2 ejaculates). Subjective progressive motility, vigor and viability (using eosin-nigrosin) were immediately analyzed, and an 80 µl aliquot from each animal was separately processed for TEM analysis. The samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.2M; pH 7.4). Three phosphate buffered solution (PBS) washes were performed, followed by post-fixation in osmium tetroxide for 1h and three PBS washes. Then, samples were dehydrated in series of acetone (50%, 70%, 90% and three times in 100%), and embedded in Epon resin. Ultrathin sections (50 nm) were manually stained with uranyl acetate and lead citrate. For both animals, 25 different fields were randomly selected, evaluated by TEM and photographed for later analysis. Progressive motility was 80% and vigor 5 for both collections. Viability was 87% and 90.5% for each animal. In TEM, the head and the flagellum (tail) were tightly surrounded by a plasma membrane (PM). The head contained a nucleus (NU), enveloped by the nuclear membrane, the acrosome (AC) and the sub-acrosomal space (SAS). The NU was electron-dense with smaller electron-lucent areas. The AC was enclosed by inner and outer membranes, and it extended beyond the apex of the head until the equatorial segment where it narrowed caudally. The caudal portion of the NU was covered with a post-acrosomal sheath. The implantation fossa limited the nuclear posterior end and led to the connecting piece which was formed with the capitulum, the proximal centriole and the segmented columns (SC), structures mainly responsible to attach head to tail. The SC ended as the outer dense fibers appeared (ODF) in the midpiece (MP) anterior region, surrounded by the mitochondrial sheath and PM. Enclosed by ODF, there was the axoneme (AX) characterized by nine doublets of microtubules forming a cylindrical bundle arranged around a pair of microtubules. In longitudinal view, 60 mitochondrial spirals were counted in this species. In the principal piece, the AX and ODF were encircled with a fibrous sheath (FS) and PM. Fibers 1,5 and 6 were larger than the others. The FS was attached to the central pair of microtubules in fibers 3 and 8. Characterizing the ultrastructure of *M. gouazoubira* sperm may help not only in sperm morphological studies, but also, in comparative biology studies.

A104 Physiology of reproduction in the male and semen technology

Evaluation of testicular thermoregulation by infrared thermography and its influence on seminal quality in Nellore bulls

Fabio Morato Monteiro[1,2], Luana Gomes Fernandes[2], Marina Oliveira Silva[1], Guilherme Fazan Rossi[2], Naiara Nantes Rodrigues[2], Thais Fernanda Ribeiro[1,2], André Maciel Crespilho[3], Karoline Maria Gil Braz[3], Marcelo Sant'Ana Borges[1], Rogério Ribeiro Vicentini[1], Maria Eugênia Zerlotti Mercadante[1]

[1]IZ - Instituto de Zootecnia, Sertãozinho, SP, Brasil; [2]FCAV - Unesp - Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil; [3]UNISA - Universidade Santo Amaro, Nova Aldeinha Barueri, SP, Brasil.

Testicular temperature should be below body temperature for normal spermatogenesis. An increase in testicular temperature, even if moderate, can cause a drastic decrease in sperm production and seminal quality, leading to a reduction in the bull fertility. Infrared thermography (IRT) can provide important information about the bull's ability to maintain testicular temperature, since the superficial temperature of the scrotum is closely related to the internal temperature of the testicle. Therefore, the objective of the study was to evaluate the relationship between testicular temperature assessed by thermographic images and the seminal quality of Nellore bulls. The experiment was conducted at Instituto de Zootecnia, Sertãozinho-SP. Forty-five semen samples were obtained by 15 Nellore bulls (n=3 ejaculates/bull) with 30 days interval between collections. The surface temperatures of the scrotum were measured by IRT (T300 FLIR Systems®) using two lines, traced across the scrotum image to measure the minimum and maximum proximal (TPMI and TPMX) and minimum and maximum distal (TDMI and TDMX), respectively. Minimum and maximum images of the ocular globe (GBMI and GBMX) and the lacrimal caruncle (CARMI and CARMX), both on the left side of the animals' head were evaluated to verify the thermal state of the bull. Semen samples were collected with electroejaculator. After each collection the sperm kinetics of fresh semen was determined by Computer-Assisted Sperm Analysis (CASA, IVOS-14, Hamilton Thorne Bioscience®, USA) and the following parameters were analyzed: total motility (MT,%), progressive motility (MP,%) and percentage of rapid sperm (RAP,%). For analysis of sperm morphology, aliquos of each semen sample was diluted in 4% saline formalin solution and evaluated by differential interference contrast (DIC) microscopy with 100X. Two hundred cells were counted per sample and the abnormalities classified as major (DEFMA), minor (DEFMI) and total (DEFTO) defects. Seminal parameters (dependent variables) were analyzed with a multiple regression model including all skin surface temperatures as predictor variables. The variation of MT (R^2 = 0.26), RAP (R^2 = 0.28) and DEFMI (R^2 = 0.26) was partial explained by temperature measured at the distal poles of the testis. The temperature of TDMX (32.9±2.2ºC) was negatively related to MT (P=0.023) and to RAP (P<0.001), however CARMI (33.9±2.2ºC) and TPMI (31.4±2,5ºC) were positively related to MT (P<0.001) and to RAP (P=0.064), respectively. TDMI (31.4±2,5ºC, P=0.021) and GBMI (33.4±1.6ºC, P=0.028) were positively related to DEFMI. The results suggest that the temperatures of the distal pole of testis obtained by IRT explain part of the variation of total motility, percentage of rapid sperm, and minor defects of spermatozoa of Nellore bulls. Acknowledgment: FAPESP (process 2014/11.304-3 and 2015/24.174-3) and Botupharma®, Botucatu, São Paulo, Brazil.

A105 Physiology of reproduction in the male and semen technology

Use of low-density lipoproteins and chicken egg yolk as non-penetrating cryoprotectors for cryopreservation of Peruvian Paso Horse semen

Ximena Barriga Marcapura, Simone Herrera Bejar, Victor Pacheco Sanchez, Juan Reátegui Ordoñez

UCSM - Universidad Catolica de Santa Maria, Vicerrectorado de Investigacion, Laboratorio de Biotecnologia Animal, Arequipa, Peru.

To improve cryopreservation efficiency for equine semen, it is important to use new diluents that contain non-penetrating cryoprotective agents. The principal objective of this study was to evaluate semen quality in Peruvian Paso Horse cryopreserved with low density lipoproteins (LDL) and chicken egg yolk. Semen was collected from 10 horses using an artificial vagina, and macroscopically evaluated to determine volume, color, appearance and pH. Semen was diluted with EquiPlus® at 37° C, and then sent to the laboratory to be cryopreserved using the protocol described by Castro et al. (Veterinary Sciences, 2016: 45-64). In the lab, each sample was divided into two aliquots and either 3 gr of LDL or 3 gr of egg yolk were added to each aliquot. The technique developed to obtain LDL was based on the methodology proposed by Gonzales (Gonzales, R., Extraction of low density lipoproteins of egg yolk [Internet, Downloaded May 2019]). After cryopreservation, samples were thawed and microscopically evaluated for motility, viability and morphology, as determined by Eosin Nigrosine for viability, Endosmosis test (HOS) for membrane's functionality, and Bengal Rose for malformations. These tests were based on the standard operating procedures (SOPs, 2018), in the Animal Biotechnology Laboratory, Universidad Católica de Santa Maria. Data were analyzed by central tendency and dispersion statistics. The significance was determined by Student's t-test for independent data at level of $\alpha = 0.05$. The average of macroscopic parameters observed was: volume: 41.5 ml; color: milky white appearance; and pH: 7.46. These parameters were not statistically different ($p > 0.05$) between stallions. Better seminal quality was observed in straws cryopreserved with LDL compared to egg yolk, with statistical differences ($p < 0.05$) for the variables: motility at thawing (48.9% vs. 27.5%), motility at 20 min post thawing (38.2% vs. 19.3%); progressive motility (38.3% vs. 21.5%); viability (45.9% vs. 31.7%); and functional membrane (59.3% vs. 41.6%) for LDL and yolk, respectively. There was no statistical difference for sperm morphology ($p > 0.05$) between groups. In conclusion, the use of LDL improved cryopreservation of Peruvian Paso Horse semen compared to egg yolk. This is likely because of the isolation of the LDL as pure lipoproteins compared to yolk that contains other components.

A106 Physiology of reproduction in the male and semen technology

Stallion sperm capacitation with exogenous modulators: Effect on the conventional *in vitro* fertilization of equine oocytes

Ricardo Felmer[1], Fernanda Fuentes[1], María José Contreras[1], Paulina Cabrera[1], Mauricio Silva[2], María Elena Arias[1]

[1]UFRO - Universidad de La Frontera, Temuco, Chile; [2]UCT - Universidad Católica de Temuco, Temuco, Chile.

A reliable protocol for equine IVF has not yet been established. The aim of the present study was to evaluate sperm capacitation conditions with exogenous cAMP-pathway modulators (dbAMPc and IBMX) and a cholesterol remover (MβCD) on sperm capacitation and assess the functionality of these conditions in a heterologous zona pellucida (ZP)-binding assay and in IVF with *in vitro* matured mare oocytes. Fresh semen was collected from 3 stallions, diluted to $10x10^6$ sperm/mL in non-capacitating and capacitating conditions with inductors and incubated for 0 and 4h at 38°C in air atmosphere using Tyrode´s medium. Membrane fluidity (MC540+), Ca^{2+} levels (FLUO 3-AM), tyrosine phosphorylation (PY mAb) and acrosomal exocytosis (PNA/FITC) were assessed by flow cytometry. Bovine and equine ovaries were collected from local slaughterhouses. In ZP-binding assay, cumulus-oocyte complexes (COCs) were aspirated, selected and denuded from granulosa cells and co-incubated in capacitated sperm for 1 hour and ZP-bound spermatozoa were counted under an epifluorescence microscope. For IVF, collected mare COCs were matured in DMEM-F12 supplemented with FSH and 10%FBS for 32 h at 38.5°C, 5% CO2% and saturation humidity. COCs were co-incubated with capacitated sperm for 18 h and presumptive zygotes were cultured in DMEM-F12 + 10%FBS for 7 days in low O_2 tension. Data are shown as means ± SD of 3 replicates for each stallion. ANOVA was used after arcsine transformation of the proportional data and Tukey's post-test to find difference between groups. Significance difference were considered if $P<0.05$. The results obtained confirmed significantly higher ($P<0.05$) tyrosine phosphorylation, $Ca^{2+}i$, membrane fluidity and acrosomal exocytosis (3300±210AU [arbitrary units], 4493±887AU, 90±5% and 50±6%, respectively) in Tyrode medium containing inductors of capacitation compared to the non-capacitating conditions (1400±40AU, 2615±373AU, 10±4% and 11±2%, respectively). ZP-binding assay revealed higher number of stallion sperm ($P<0.05$) attached to the ZP of bovine oocytes (410) compared to non-capacitating conditions (105). IVF experiments performed only in capacitating conditions showed an average of 31% of cleavage 3 days after insemination and embryos cleaved to the 8 to 16-cell stage. Despite assessing embryonic development until day 7, none of the embryos developed beyond this point. In conclusion, stallion sperm incubated with exogenous inductors of capacitation show cellular and molecular changes consistent with sperm capacitation. Furthermore, ZP and IVF trials confirm the functionality of stallion sperm incubated under these conditions. Acknowledgement: Funding support from CONICYT, Chile grant FONDECYT 1160467, and provision of ovaries from Frigorifico Temuco and Nueva Imperial.

A107 Physiology of reproduction in the male and semen technology

High intensity interval training improves the sperm motility of spontaneously hypertensive rats

Margarete Jardinetti de Oliveira[1], Evellin Heloisa Paulineli Pereira[1], Lauren Chrys Soato Marin Schaffer[1], Kátia Cristina de Melo Tavares Vieira[1], Caliê Castilho[1], Aline de Oliveira Santos[1], Francis Lopes Pacagnelli[1], Ana Paula Alves Favareto[1], Leonardo Oliveira Mendes[1], Giovana Rampazzo Teixeira[2], Ines Cristina Giometti[1]

[1]UNOESTE - Universidade do Oeste Paulista, Presidente Prudente, SP, Brasil; [2]Unesp - Universidade Estadual Paulista, Centro Educacional, Presidente. Prudente, SP, Brasil.

Hypertension is a cause of low fertility in men because it reduces testosterone production and spermatogenesis. Exercises are indicated to decrease blood pressure and improve overall health. The objective of this study is to verify the sperm motility of spontaneously hypertensive rats (SHR) submitted to high intensity interval training (HIIT). For this, Wistar-Kyoto rats, male, adult, with and without spontaneous hypertension were distributed in 3 groups: K-G (control of Wistar-Kyoto rats without hypertension submitted to HIIT, n=5); SHR-G (group of SHR rats, n=9); and SHR-HIIT-G (group of SHR rats submitted to HIIT, n=9). The treadmill HIIT training was realized for 5 days/week for 8 weeks, for 50 minutes approximately, based on the maximum exhaust speed, with active rest intervals according to the protocol of Haram et al. (Cardiovascular Research, 81: 723-732, 2008). The animals initially underwent an adaptation to the HIIT training, which consisted of running on the treadmill for 2 minutes at 0.5 km / h, followed by 5 minutes of rest and soon after 3 minutes at the speed of 0.7km / h, increasing 0.2 km / ha every 3 minutes until it reached 1 mmol / LL above the initial test. After the adaptation period HIIT was performed with 5 minutes of heating at 40% of the lactation threshold. After warming up, the training was started with 3 minutes at 60% of the lactate threshold followed by 4 minutes interval of 85% of the lactate threshold, which was repeated seven times each session. The vas deferens with the sperm were collected. The evaluation of sperm motility was performed immediately at the time of euthanasia. The motility was classified by visual observation under a microscope in: mobile with progressive trajectory; mobile without progression; and motionless. The results were analyzed by analysis of variance (ANOVA), followed by the Tukey test ($P<0.05$). The SHR-G (67.22±1.49) presented lower progressive motility ($P<0.05$) than the K-G (73.10±1.88) and SHR-HIIT-G (73.00±1.11). It is concluded that HIIT improves the sperm motility of SHR rats. Financial support by FAPESP (process number: 2018/22682-0) and PIBIC-EM (CNPq).

A108 Physiology of reproduction in the male and semen technology

Antioxidant effect of caffeine addition to post-thawed equine semen

Natália de Castro Alves[1], Eduardo Damasceno Costa[1], Marina Morra Freitas[2], Raphael Rocha Wenceslau[1], Soraia de Araújo Diniz[1], Virginia Soares Lemos[1], Monique de Albuquerque Lagares[1]

[1]UFMG - Universidade Federal de Minas Gerais, Belo Horizonte, MG; [2]PUC Minas - Pontifícia Universidade Católica de Minas Gerais, Betim, MG, Brasil.

The main objective of this work was to evaluate the antioxidant effect of adding 5mM of caffeine to post-thawed equine semen. One ejaculate of nine stallions was frozen with INRA 82 freezing extender. The straws were thawed performing two treatments: T1) INRA 82, control (without caffeine addition) and T2) T1+5mM caffeine.The antioxidant effect was evaluated after incubation (37 °C, 20min) by nitrite measurement (NO2-), which is an indirect way to investigate the nitric oxide formation (Green et al., Analytical biochemistry, 126: 131-138, 1982) and by hydrogen peroxide concentration (µM / µg protein) by the FOX 2-modified method (Nourooz-Zadeh et al., Analytical Biochemistry, 220: 403-409, 1994). The mean, standard deviation, standard error and variance analysis were performed according to Sampaio (Sampaio, Estatística Aplicada à Experimentação Animal, 17: 189-207, 2002). The NO_2^- and H_2O_2 concentrations presented normal distribution and the mean values were compared by the paired T-test. The significance of $P<0.05$ was considered statistically different. In the present study, lower NO_2^- concentration was observed after the addition of 5mM caffeine to the post-thawed equine semen compared to the control (11.4 ± 2.1^b *vs* 12.8 ± 2.9^a µM/µg of protein, $P<0,05$). However, the H_2O_2 concentration did not differ between the control and the caffeine treated group ($P>0.05$, 36.4±3.6 and 37.0±2.1 µM/µg of protein). Thus, besides increasing stallion sperm motility of thawed semen (Lagares et al., Reproduction, Fertility and Development, 31:142-143, 2019), 5mM caffeine has antioxidant effect, which can be an alternative to increase the fertility rate of post-thawed equine semen.

A109 Physiology of reproduction in the male and semen technology

L-carnitine effects on the intracellular calcium ion concentration and membrane integrity of post-thawed equine sperm

Monique de Albuquerque Lagares[1], Natalia de Castro Alves[1], Marina Morra Freitas[2], Grazielle Caroline da Silva[1], Steyner Franca Cortes[1], Thiago Frederico Diniz[1], Virginia Soares Lemos[1], Raphael Rocha Wenceslau[1], Soraia de Araújo Diniz[1], Angela Maria Quintao Lana, Adalgiza Souza Carneiro de Rezende[1], Rubens Stahlberg[1]

[1]UFMG - Universidade Federal de Minas Gerais, Belo Horizonte, MG; [2]PUC- Minas - Pontifícia Universidade Católica de Minas Gerais, Betim, MG, Brasil.

The aim of this work was to evaluate the addition of L-carnitine (L-C) to the frozen-thawed semen of equine to improve the sperm integrity and metabolism by measuring the intensity of propidium iodide (PI) and the $[Ca_2^+]$i after different periods of time. Semen from five stallions was collected and frozen with INRA 82 extender. After thawing, the samples were distributed according to the treatments: T1) Control, INRA 82 extender (no L-C addition), T2) T1+0.5mM L-C, T3) T1+1mM L-C, and T4) T1+2mM L-C. The samples were analyzed immediately after L-C addition (t0), and post-, 20 (t20) and 40min (t40). An epifluorescent microscope was used to evaluate the $[Ca_2^+]$i with the Alexa Fluo-4AM, and the propidium iodide (PI) dye to evaluate the sperm membrane integrity. After thawing the semen samples were centrifuged, and the pellet was resuspended with a PBS solution with 6 mM Glucose and Fluo-4AM (20 µmol/L), and incubated at room temperature for 30 min. After 20min incubation, the PI was added to the sample and incubated to the last 10 min. It was used an argon laser to excite the Fluo-4AM (488 nm) and emission of 510 nm, with an epifluorescent microscope (400X). The images were captured each 500 msec. and 600 sec. at 400 Hz, 512x512 pixels. The program ImageJ (Wayne Rasband, National Institutes of Health, USA) was used to analyze the sperm cells images. A linear mixed model was adjusted considering Fluo-4 AM and PI fluorescent intensity of sperm as variables according to the treatments and time evaluated. The stallion was considered as random variable. The Tukey test was used to compare the mean values and a significant level of 5% was considered significant. No significant differences until 40 min incubation among the PI intensity of sperm in 1 e 2 mM L-C treatments were observed (54.0 and 50.4 AU), respectively ($P>0.05$). However, at 20 min incubation there was an increase of non-intact sperm membrane in the control (51.5 to 54.7 UA) and with 0.5 mM L-C (52.5 to 56.4 AU, $P<0.05$). In all time evaluated the 2 mM L-C treatment showed lower PI intensity sperm compared to the other treatments ($P<0.05$). Just the 2mM L-C showed a decrease of $[Ca^{++}]$i intensity during 40min incubation (49.4 to 44.6 AU, $P<0.05$). On the other hand, with 1 mM L-C an increase of $[Ca^{++}]$i intensity until 40min was observed (47.4 to 60.6 AU, $P<0.05$). However, it was not associated with an increase of the PI intensity sperm in this treatment. Thus, the addition of 2 mM L-C to the thawed sperm of equine showed higher protection of sperm membrane integrity, while 1 mM was beneficial to sperm metabolism increasing $[Ca^{++}]$i intensity. In conclusion, both 1- and 2-mM L-C improved essentials sperm characteristics, which play an important role to sperm fertilization capability.

A110 Physiology of reproduction in the male and semen technology

Efficiency of sexed semen in the *in vitro* fertilization of oocytes obtained from prepubertal heifers

Larissa Zamparone Bergamo[1], Bruno Valente Sanches[3], Amanda Fonseca Zangirolamo[1], Anne Kemmer Souza[1,2], Fábio Morotti[1], Marcelo Marcondes Seneda[1,2]

[1]ReproA - UEL - Laboratório de Biotecnologia da Reprodução Animal - Universidade Estadual de Londrina Universidade Estadual de Londrina, Londrina, PR; [2]INCT–LEITE - Instituto Nacional de Ciência e Tecnologia para a Cadeia Produtiva do Leite, Universidade Estadual de Londrina, Londrina, PR; [3]IVF, LLC - Vytelle, Hermiston, OR 97838, USA.

The increase investigation for the reduction of the time interval between generations is of great interest in dairy farming. However, lower oocyte competence is well-established for prepubertal heifers. The objective of the present study was to compare the use of sexed semen with conventional semen in prepubertal Holstein calves from 7 to 9 months of age (n = 151), related to the number of embryos produced from oocytes recovered by OPU, with a total of 397 (2.63 ± 0.134 per calf) aspirations. The aspirated oocytes were matured for 24 hours, fertilized with sexed or conventional semen of 60 different bulls with proven fertility and standardized performance in the in vitro production of embryos, and cultured in vitro. Means were compared by analisys of variance and Tukey test with a significance of $P \leq 0.05$. The age of the donors when compared to the average number of embryos did not present difference ($P = 0.803$). There was no difference in the type of semen used and number of embryos produced (sexed semen: n = 156, 1.7 ± 0.25 / conventional semen: n = 241, 2.2 ± 0.19 [$P = 0.091$]). We highlight the optimization of the reproductive techniques in younger animals, especially of the dairy herd, since the use of sexed semen was as efficient as conventional semen.

A124 Embryology, developmental biology and physiology of reproduction

Caloric restriction during gestation in mice decreases ovarian reserve in the offspring

Bianka Machado Zanini, Jorgea Pradiee, Driele Neske, Luís Augusto Cruz, Rafael Mondadori, Kelvin Ruan Andrade, Gabriel Veiga, Kristy Ortale, Olavo Shneider, Augusto Schneider

UFPEL - Universidade Federal de Pelotas, Pelotas, RS, Brasil.

The aim of this study was to evaluate the effect of caloric restriction during gestation on ovarian follicular count in the offspring in mice. For this, we used 14 female mice and 7 male mice of the C57BL/6 lineage maintained with standard diet and water ad libitum under controlled light and temperature conditions. The mice mated at the ratio of one male to two females at the same period in separate cages. Ten days after confirmation of copulation the females were divided into control group (n= 7) and caloric restriction (CR) group (n= 7), which received a diet consisting of 50% of what was consumed by the control group in the day before. Mice were subjected to this restricted diet for 6 days. After delivering and weaning (21 days of age) males and females were separated according to the initial group and received ad libitum diet until 3 months of age. The mice were evaluated every 14 days from weaning to euthanasia, when the ovaries were collected. For histological evaluation the ovarian samples were submitted to serial cut in a microtome, stained with hematoxylin-eosin. Images of ovarian sections captured with a digital camera coupled to a composite light microscope using the 40X objective. Oocytes were classified as primordial, primary, secondary and tertiary. The statistical analyzes carried out used the software GraphPad Prism 6, assuming a level of significance of 5%. The weight gain was increased in the female offspring of the CR group after weaning ($P < 0.0001$). We observed more primordial (1064 ± 128 vs 2740 ± 231; $P<0.0001$) and transition (1042 ± 131 vs 2914 ± 210; $P<0.0001$) follicles in mice subjected to CR during gestation. Primary, secondary, tertiary and total follicles were not different between groups ($P>0.05$). These results suggest that there were less follicles in the reserve of CR mice, but the rate of activation was not different, as we do not see more follicles in the primary stage. This indicates that differences in the ovarian reserve can be occurring during fetal ovarian development. Therefore, CR during gestation negatively affects weight gain and the size of the ovarian reserve of the offspring in mice.

A125 Embryology, developmental biology and physiology of reproduction

In vivo evaluation of the effect of sperm on gene expression in bovine oviductal epithelial cells

José Oliveira Carvalho[1], Luana da Rosa Celin[1], Ligiane Oliveira Leme[1], Nayara Ribeiro Kussano[3], Maurício Machaim Franco[2], Margot Alves Nunes Dode[2]

[1]UFES - Universidade Federal do Espírito Santo, S/n, Alegre. ES; [2]Embrapa - Embrapa Recursos Genéticos e Biotecnologia, Asa Note, Brasília, DF; [3] UnB - Universidade de Brasília, Brasília, DF, Brasil.

The interaction between sperm and bovine oviduct epithelial cell (BOEC) provide a favorable environment to maintain the viability of sperm with a higher potential for fertilization. Studies related to sperm binding to the oviduct, have showed that this binding influences the transcription of genes from these cells, modifying the oviduct environment. The aim of this study was to find out the effect of sperm interaction with oviduct cells 18 h after artificial insemination (AI) on relative expression of genes *FUT 6*, *NQO1*, *CST6*, *B3GNT3*, *CKB*, *RARRES2*, *MIF* and *FOS*. All genes were selected from a RNAseq assessment (data not showed). Nelore heifers were synchronized with an estradiol and P4 based protocol. Heifers identified on estrus were divided into 2 groups: heifers inseminated (n=9) with a total of $8x10^6$ *cryopreserved* semen pool from 6 Nelore bulls (group IA+); heifers inseminated with saline solution (group AI-). Eighteen hours after AI, all heifers were slaughtered, and BOEC from isthmus appraised for any variation in transcripts level of selected genes by real time PCR. For each assay, five different biological replicates were performed. Data were analyzed by analysis of variance (ANOVA) and differences between means were compared by Tukey's test ($P<0.05$). Only the *FOS* gene showed increased on relative abundance of mRNA in the IA+ group ($P=0.03$). All other genes assessed presented similar expression between groups. The gene *FOS* is related to cell proliferation and differentiation, which transcription occurs fast and in a transitory way. Based on these results, it was concluded that interaction of sperm and BOEC from isthmus, modulates the expression of the *FOS* gene. Finnancial support: EMBRAPA, CAPES, FAPDF.

A126 Embryology, developmental biology and physiology of reproduction

Effectiveness of *in vitro* maturation strategies to reduce the lipid accumulation in buffalo embryos

Marivaldo Rodrigues Figueiró[1,2], Joaquim Mansano Garcia[2], Marina Ragagnin de Lima[2], Maite del Collado[3], Clara Slade Oliveira[4], Naiara Zoccal Saraiva[4]

[1]CPATU - Embrapa Eastern Amazon, Belém, Pará, Brazil; [2]FCAV-UNESP - Sao Paulo State University, Jaboticabal, São Paulo, Brazil; [3]FZEA-USP - University of Sao Paulo USP, Pirassununga, São Paulo, Brazil; [4]CNPGL - Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais, Brazil.

In vitro embryo production (IVEP) is a procedure that can promote genetic improvement in a short time frame. However, the success rates obtained with this biotechnology in buffaloes are still inconsistent, which can be associated with the high concentration of lipids in the cytoplasm of oocytes and embryos. Considering the potentially positive impacts of strategies to replace/reduce the supplementation of fetal bovine serum (FBS) during IVEP, the objective of this study was to evaluate the effects of reducing the concentration of FBS and/or use of 5 mM L-carnitine (LC) during *in vitro* maturation on the development and lipid accumulation of buffalo embryos. In the first experiment, we aimed to determine the lowest concentration of FBS in the IVM medium able to maintain the embryo development rate obtained by the control group (10% FBS). Buffalo oocytes were placed in IVM in bovine serum albumin (BSA) medium supplemented with 0%, 2.5%, 5% or 10% FBS for 22 h, and then fertilized in Talp-IVF medium for 24 h, and *in vitro* cultured in modified SOF medium supplemented with 1.5% FBS at 38.5 °C and 5% CO_2 atmosphere in air for 7 days. Blastocyst rates were evaluated and the data analyzed using the analysis of variance (ANOVA) and Tukey test. After defining the lowest effective concentration of FBS as 5% [27/79; 34.18%^{a}, similar to 10% - 52/105; 34.67%^{a} and superior to 0% (11/104 - 10.58%^{b}) and 2,5% (16/83 - 19.28%^{b}) groups], we performed a second experiment in which the 0%, 5% and 10% FBS groups were also evaluated regarding the addition of 5 mM of L-carnitine in the IVM medium. The blastocysts produced in this experiment were submitted to lipid quantification tests, involving staining followed by observation by optical (OilRed O) and confocal (BODIPY 493/503) microscopy. The lipid quantification data were evaluated by the nonparametric Kruskal-Wallis test. All the statistical analyses were performed with the SPSS version 22.0.0.0 software, except for the lipid data, which were evaluated with GraphPad Prism 7 version 7.03. No difference was observed between the 5% (60/184 - 32.61%^{a}) and 10% FBS (82/227 - 36.12%^{a}) groups in blastocyst rate, which were superior to 0% (34/270 - 12.59%^{c}) and groups supplemented with L-carnitine (5% FBS-LC: 32/144 - 22.22%^{b} and 10% FBS-LC: 38/153 - 24.84%^{b}). There was no difference regarding embryo lipid accumulation. The results indicate that it is possible to reduce the FBS concentration to 5% in IVM media for buffalo embryo production and the supplementation of the maturation medium with L-carnitine at a concentration of 5 mM did not cause an increase in the embryo production of this species. Furthermore, alterations in the lipid accumulation during the IVEP were not found, with or without the presence of FBS and addition of L-carnitine during the IVM, indicating the need for further research, mainly involving the *in vitro* culture step of buffalo embryos.

A127 Embryology, developmental biology and physiology of reproduction

Influence of circulating concentrations of estradiol and progesterone on endometrial area and pituitary responsiveness to GnRH

Jéssica Cristina Lemos Motta[1], Guilherme Madureira[1], Lucas Oliveira e Silva[1], Rodrigo Lemos Olivieri Rodrigues Alves[1], Tairini Erica da Cruz[3], Carlos Henrique Paiva Camisa Nova[4], Jessica Nora Drum[1], Mayara Silvestri[1], Carlos Eduardo Cardoso Consentini[1], Alexandre Barbieri Prata[1], Milo Charles Wiltbank[2], Roberto Sartori[1]

[1]ESALQ – USP - Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP; [2]UW - Madison - University of Wisconsin, Madison, WI, EUA; [3]FMVZ – UNESP - Faculdade de Medicina Veterinária e Zootecnia, Botucatu, SP; [4]UFV - Universidade Federal de Viçosa, Viçosa, MG.

The aim was to evaluate the influence of high/low circulating P4 and E2 on endometrial area (EA) and on LH/FSH release after GnRH challenge. In a previous experiment, we developed a proestrus-like hormonal milieu using exogenous hormones (Motta et al. Anim Reprod, 15: 1031, 2018). For the new experiment, 43 nonlactating multiparous Holstein cows were submitted to the following protocol: D-7: 2 mg estradiol benzoate (EB) and 1g P4 implant; D0: implant removal, 0.526 mg PGF and two 2g P4 implants; D1: 0.526 mg PGF; D4 and D5: aspiration (OPU) of all follicles ≥5 mm. On D5 cows were randomized into 4 groups (n=10 or 11 per group): HighE2&LowP4, HighE2&HighP4, LowE2&HighP4, and LowE2&LowP4. LowP4 cows had one implant removed after OPU on D5 and 18 h later the second implant was removed. HighP4 cows had both implants maintained. HighE2 groups received a total dose of 0.8 mg EB divided into 8 treatments given 6 h apart in increasing doses (0.04, 0.04, 0.08, 0.08, 0.12, 0.12, 0.16, and 0.16 mg), starting after OPU on D5. Blood samples for P4 were taken on D5, just before the first implant removal and 18, 30 and 48 h later. The EA was evaluated by ultrasound after OPU on D5 and 12, 24 and 48 h later. The endometrial thickness from uterine horns was measured and then converted to EA (πr^2). On D7, after the last EA evaluation, all cows were treated with 8.4 µg buserelin (GnRH) and blood was collected just before GnRH and at 0.5, 1, 2, 3, 4, 5, and 6 h later for LH and FSH. Data were analyzed by PROC MIXED in SAS and the area under the curve (AUC) by the package MESS in R program ($P \leq 0.05$; tendency = $0.05 < P < 0.1$). Results are presented below in the following order: HighE2&LowP4, HighE2&HighP4, LowE2&HighP4, and LowE2&LowP4. Circulating P4 did not differ within groups with Low or with High P4 concentration. Therefore, data from groups were combined. At time 0, High and Low P4 groups had similar circulating P4 (1.9±0.2 vs 1.8±1.2 ng/mL), but at time 18 (1.2±0.1 vs 2.2±0.2 ng/mL), 30 (0.03±0.01 vs 2.1±0.2 ng/mL) and 48 (0.01±0.0 vs 2.4±0.2 ng/mL), Low P4 cows had lower circulating P4. The EA was similar among groups at time 0 (159.5±12.5 mm^2) and 12 (166.2±10.9 mm^2) but it differed at time 24 (224.4±11.4^a; 152.2±13.5^c; 162.1±11.6^{bc}; 178.6±10.2^b mm^2) and 48 (293.3±22.0^a; 151.5±11.3^c; 163.7±8.5^{bc}; 189.9±16.1^b mm^2). The FSH peak (2.4±0.4^a; 1.6±0.1^b; 0.8±0.1^c; 1.1±0.1^{bc} ng/mL) and AUC (7.3±0.9^a; 5.9±0.4^a; 3.0±0.4^b; 3.9±0.4^b ng^2) differed among groups and the time of peak tended to differ (1.8±0.2^a; 2.2±0.1^{ab}; 2.3±0.1^{ab}; 2.4±0.2^b h). The LH peak (12.2±1.6^a; 8.1±1.4^b; 2.6±0.43^c; 7.3±1.4^b ng/mL), AUC (36.9±5.1^a; 19.9±2.4^b; 8.3±0.9^c; 19.4±2.7^b ng^2) and time of peak (1.8±0.2^c; 2.0±0.0^{bc}; 2.5±0.2^a; 2.3±0.2^{ab} h) differed among groups. In conclusion, EA only changed under high circulating E2 and low P4. Further, the greatest LH and FSH surges after GnRH challenge were obtained when circulating P4 was low and E2 was high. Acknowledgements: FAPESP, CAPES, CNPq, and GlobalGen

A128 Embryology, developmental biology and physiology of reproduction

Pulses of Prostaglandin F2α metabolite during late embryonic development in Bos indicus cattle

Sydney Reese[1], Gessica Franco[1], Ramiro Olivera Filho[1], Lohana Fernandez Montero[1], Gabriela Dalmaso de Melo[3], Ana Beatriz Moraes[4], Don Neuendorff[2], Ron Randel[2], Ky Pohler[1]

[1]TAMU - Texas A&M University, College Station, TX, USA; [2]Agrilife - Texas A&M Agrilife Research, Overton, TX, USA; [3]USP - University of Sao Paulo, Pirassununga, SP, Brazil; [4]UFU - Federal University of Uberlandia, Uberlandia, MG, Brazil.

The period of late embryonic/early fetal mortality (LEM) in cattle occurs simultaneously with initiation of active placentation. The objective of this study was to evaluate differences in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion and pulsatility throughout late embryonic development period in cattle that maintained pregnancy as $PGF_{2\alpha}$ may play a role in the initiation of placental attachment. Pregnancies were established in Brahman females using industry standard estrous synchronization protocols with insemination occurring on day 0 (n = 25). Pregnancy diagnosis was performed on days 28, 40 and 60 via ultrasound. A subset of pregnant heifers (n = 4) and cows (n = 4) were fitted with coccygeal vein cannulas inserted~65 cm into the caudal vena cava for indirect sampling of utero/ovarian drainage. Blood samples were collected every 15 min for 6 h on day 29, day 31, day 34, day 37, and day 39. All animals maintained pregnancy until day 40 when catheters were removed; however, two animals were diagnosed as non-pregnant at the final day 60 pregnancy diagnosis (LEM). Serum concentrations of $PGF_{2\alpha}$ metabolite (PGFM) were measured with a validated ELISA (Interassay CV = 8.76%, Intraassay CV = 6.32%). Data were analyzed using repeated measures in SAS 9.4 and pulses were identified using AutoDecon Pulse2 accounting for unequally spaced sampling days. Average basal PGFM concentrations differed by day ($P<0.01$) with greatest concentration on day 31 (110.36 ± 5.86 pg/mL) and minimal concentrations on day 39 (9.45 ± 7.52 pg/mL) in animals that maintained pregnancy. Heifers had increased ($P = 0.01$) basal PGFM at day 31 (156.86 ± 16.31 vs 78.81 ± 8.89.61 pg/mL) but decreased ($P<0.01$) PGFM at day 34 (26.93 ± 8.49 vs 54.65 ± 6.03 pg/mL) compared to cows. LEM cow had elevated PGFM at day 31, 34, and 37 but not at 39 compared to cows which maintained pregnancy and exhibited twice as many PGFM pulses (8 vs 3) throughout the collection period; however, the LEM heifer exhibited comparable concentrations and pulses to animals that maintained pregnancy on all days. Number of PGFM pulses were increased ($P<0.02$) at day 31 (1.9 ± 0.26 pulses) compared to day 34 (0.17 ± 0.23 pulses), 37 (1.00 ± 0.33 pulses) and 39 (0.33 ± 0.33 pulses) in successful pregnancies. There was no difference in peak amplitude by day ($P = 0.68$) or parity ($P = 0.76$). These data indicate that concentrations and number of pulses of PGFM are increased during the initiation of active placentation during pregnancy; however, more data is needed to determine differences in PGFM in cows undergoing late embryonic/early fetal mortality. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2017-67015-26457 from the USDA National Institute of Food and Agriculture.

A129 Embryology, developmental biology and physiology of reproduction

Profile of type I and II interferon receptor transcripts in peripheral blood mono and polymorphonuclear cells during early gestation in Nelore heifers

Leonardo Marin Ferreira Pinto[2], Gabriela Dalmaso de Melo[1], Igor Garcia Motta[1], Cecília Constantino Rocha[1], Guilherme Pugliesi[1]

[1]FMVZ-USP - Faculty of Veterinary Medicine and Animal Science - University of São Paulo, São Paulo, SP; [2]FZEA-USP - Faculty of Animal Science and Food Engineering - University of São Paulo, Pirassununga, SP, Brazil.

We aimed with this study to analyze the abundance levels of type I and II interferon-tau (IFNT) receptors (IFNAR I and II) in peripheral blood mono (PBMC) and polymorphonuclear (PMN) cells in pregnant Nelore heifers. Twenty-nine heifers (18-20 months) had their estrous cycle synchronized and were subjected to fixed-time artificial insemination (FTAI) on D0. Pregnancy diagnosis was performed by transrectal ultrasonography on D25 and D28 through the detection of the embryonic vesicle and heartbeat. On days 0, 10, 14, 16, 18 and 20, 25 mL of blood were collected in heparinized tubes by puncture of the jugular vein for the isolation of PBMCs and PMNs cells. The isolation was performed by Ficoll® Paque Plus gradient (GE Healthcare, Chicago, USA), in an adapted method (Jiemtaweeboon S et al. 2011. ReprodBiol and Endoc., 9:79-89). Samples from 8 pregnant and 9 non-pregnant heifers were submitted to RNA extraction using the Direct-Zol RNA Miniprep Kit (Zymo Research, Irvine, USA) according to the manufacturer's instructions. The expression of the target genes (*IFNAR I* and *II*) was normalized in relation to the reference genes (*GAPDH* and *PPIA* for PBMCs; and *GAPDH* and *ACTB* for PMNs). For statistical analysis, the transcript abundance levels were evaluated by analysis of variance (ANOVA) with repeated measures of time, considering the random effect of heifer and the fixed effects of group (pregnant or non-pregnant), day and interaction of group by day using the PROC MIXED SAS software (SAS Institute). For PMNs, no significant (P>0.1) differences were detected in the *IFNAR I* expression, while for *IFNAR II*, only a time effect (P= 0.01) was observed, indicating an increase on transcript abundance from D0 to D16, with a progressive decrease on D20 in pregnant heifers. For PBMCs, only a time effect (P= 0.02) was observed for *IFNAR I* expression, characterized by an increase on the transcript abundance between D10 and D16, followed by progressive reduction on D18 and D20. Although an interaction of group by time was not significant (P=0.11), a subsequent analysis indicated that *IFNAR I* abundance on PBMC in the pregnant heifers progressively increased from D0 to D16 and followed a progressive decrease from D16 to D20; whereas, no difference (P>0.05) was detected along the evaluated days in the non-pregnant heifers. Also, the *IFNAR I* abundance on D20 was greater (P=0.04) in the pregnant than non-pregnant heifers. No significant (P>0.1) effects were detected in the *IFNAR II* expression. In conclusion, for PMN, only *IFNAR II* transcript abundance varied during early pregnancy but its expression is independent of the pregnancy status; whereas, for PBMC the pregnancy status may affect the temporal expression of *IFNAR I* at D20, which could be involved in the IFNT signaling mechanisms to guarantee the success of maternal recognition of gestation. Acknowledgments: FAPESP (2015/10606-9; 2017/13472-9; 2018/25393-9).

A130 Embryology, developmental biology and physiology of reproduction

Role of L-carnitine in *in vitro* maturation medium on oocyte nuclear maturation in domestic cats

Maria Clara da Cruz Morais, Nathalia Oliveira Barbosa, Rodrigo Oliveira Cunha, Viviane Lopes Brair, Leticia Pereira Alcaráz de Andrade, Lendel Correia da Costa, Ribrio Ivan Tavares Pereira Batista, Joanna Maria Gonçalves Souza-Fabjan

UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil.

Oxidative stress may have detrimental effects on the oocyte, fertilization process, and subsequent embryo development. L-carnitine is an endogenous substance that, in addition to its significant role in lipid metabolism, has protective effects on the cell through its antioxidant actions. In this sense, data from the literature show that the supplementation of this antioxidant in the range of 0.3 – 0.6 mg/mL during *in vitro* maturation (IVM) exerts a beneficial effect on the progression of meiosis (Zare et al., Int J Reprod Biomed, 15(12):779-786, 2017). The objective of this study was to evaluate the effect of 0.5 mg/mL of L-carnitine during IVM in the resumption of meiosis in domestic cats oocytes. A total of 20 ovaries from queens of reproductive age were collected during elective ovarian hysterectomy at the Veterinary Hospital of Universidade Federal Fluminense, Niterói-RJ, and transported at 4 °C in 0.9% saline. Five replicates were performed. The cumulus-oocyte complexes (COCs) were recovered by slicing and washing each ovary with 1.5 mL PBS, in a 60 mm Petri dish. Subsequently, grade I and II COCs (Wood and Wildt, J Reprod Fertil, 110:355-360, 1997) were washed with TCM199 supplemented with HEPES and $NaHCO_3$ and allocated into one of two groups. Each group was transferred to a well of a 4-well dish with 500 µL of maturation medium (MM) containing TCM199 supplemented with HEPES and $NaHCO_3$, 4 mg/mL BSA, 0.5 µg/mL FSH, 1 µg/mL 17β-estradiol, 0.2 mM pyruvate and 50 µg/mL of antimycotic and antibiotic solution, with or without the addition of 0.5 mg/mL L-carnitine. The COCs were incubated for 24 h at 5% CO_2, 38,5 °C and humidified atmosphere. Afterwards, they were denuded using 100-500 IU/mL hyaluronidase and vortexed for 6 min. The oocytes were washed in PBS and 1% BSA and fixed in 200 µL 4% paraformaldehyde and 1% BSA, where they were kept for at least 24 h. After washing with PBS and 1% BSA, the oocytes were transferred to a slide and stained with 10 µL of 1 µg/µL Hoechst (diluted in TCM199). Then, they were evaluated under fluorescence microscopy, being classified as matured (MII), germinal vesicle breakdown (GVB), germinal vesicle (GV) and degenerated (DEG). For the statistical analysis, the chi-square test ($p<0.05$) was used. Data are presented as mean ± SEM. There was no difference between the parameters of the groups with and without the presence of L-carnitine ($p>0.05$), which were, respectively: MII 51.4 ± 5.6% and 38.2 ± 8.8%, GVB 29.7 ± 7,7% and 26.5 ± 7.4%, GV 2.7 ± 1.2% and 8.8 ± 7.8% and DEG 16.2 ± 4.0% and 26.5 ± 8.2%. In conclusion, although there was no significant difference, the group with the presence of L-carnitine in MM reached a nuclear maturation rate 13.2% higher than the group without the addition of the antioxidant.

A131 Embryology, developmental biology and physiology of reproduction

Expression of interferon stimulated gene 15 in the vaginal mucosa cells as a pregnancy diagnostic methodology during early pregnancy in cattle

Thais Sayuri Imura Oshiro[1], Cecilia Constantino Rocha[2], Gabriela Dalmaso de Melo[2], Leonardo Marin Ferreira Pinto[2], Amanda Guimarães Silva, Guilherme Pugliesi[2]

[1]UNIMAR - Universidade de Marilia, Marília, SP; [2]USP - Universidade de São Paulo, Pirassununga, SP, Brasil.

The aims of the study were 1) to evaluate the using of vaginal cytology as an alternative to determine the expression of interferon stimulated gene 15 *(ISG15)* during early pregnancy, and 2) to compare this method with the isolation of peripheral polimorphonuclear blood cells (PMNs) to determine *ISG15* expression *on day 20 after timed-artificial insemination (TAI) in beef heifers.* Nelore heifers (n=31) weighting 422 ± 47.8 kg were submitted to an estradiol/progesterone based protocol to synchronize ovulation for TAI on day 0. On days 16, 18 and 20, samples of vaginal cells were collected using a cytological brush (Cytobrush; Viamed Ltd, West Yorkshire, UK), which was placed in the fornix surrounding the external cervix os to recover the superficial cells from vagina. On day 20, blood samples (30ml) were also collected from jugular vein for PMNs isolation. The pregnancy diagnosis was performed on day 30 by detection of an embryo with heartbeat, and animals were classified on Pregnant (P; n=16) and Non-Pregnant (NP; n=15) groups. The *ISG15* expression was evaluated by RT-qPCR in the vaginal cell samples on days 16, 18 and 20 in randomly selected animals (n=7/group). On day 20, the relative *ISG15* expression between P and NP groups was compared between the vaginal cells and PMNs (n=13 P and 12 NP/cell type). Two reference genes (*GAPDH* and *ACTB*) selected from five tested genes using Normfinder program were used for normalization of relative expression of *ISG15* in both cell types. For the comparison between the two methods, the relative *ISG15* expression on day 20 in each P heifer was divided by the averaged expression in the NP group. The results were analyzed by ANOVA and PROC MIXED procedure (SAS), considering the main effects of group (G), time (T) and its interaction (TG). No significant effects (P>0.1) of G, T and TG were observed for the *ISG15* expression in vaginal cells. However, when evaluated on day 20 with a large number of animals, a greater abundance of *ISG15* was observed in the P group for the vaginal cells (relative expression to reference genes: 0.22±0.09 vs. 0.05±0.01; P=0.08) and PMNs (2.77±0.42 vs. 1.12±0.36; P<0.008). When compared the relative *ISG15* expression in the P group to NP group in the two cell types, no difference (P>0.1) was found between the PMNs (fold change: 2.47) and vaginal cells (fold change: 4.18). In conclusion, the use of *ISG15* expression in vaginal cells sampled by cytological brush does not show to be an efficient predictor of pregnancy status in cattle, as it presented a high variation among the P samples on day 20.

A132 Embryology, developmental biology and physiology of reproduction

Early or late blastocoel expansion on cytoplasmic lipid content, cell number and apoptosis of *in vitro* produced blastocysts

Diego Müller[1], Mateus José Sudano[2]

[1]UNIPAMPA - Universidade Federal do Pampa, Bagé, RS, Brasil; [2]UFABC - Universidade Federal do ABC, Bangú, Santo André, SP, Brasil.

In vitro embryo production (IVP) is one of the reproductive biotechnologies most applied in the field nowadays, aiming to rapidly increase the number of animals produced and the genetic gain achieved. Although well established, it still has limiting factors for the appropriate embryonic development when compared to in vivo. One of these factors is the increased lipid amount in IVP embryos when compared to in vivo embryos. This altered lipid content of the IVP embryos has been associated with a reduced survival after cryopreservation, hindering a broad commercial application of this biotechnology. The number of blastomers and apoptotic cells are other factors directly related to embryo viability. In this study, the lipid content, number of blastomers and percentage of apoptotic cells were evaluated in bovine expanded blastocysts at day 7 (D7) and 8 (D8) of embryonic culture. Bovine ovaries were recovered from a commercial slaughterhouse and transported to the laboratory, in physiological saline at 35°C. Cumulus oocyte complexes (COCs) were aspirated from follicles of 2 to 8 mm in diameter and only those with uniform cytoplasm and multilayered cumulus cells were selected. The COCs were in vitro matured and fertilized at 38.5°C and 5% CO2 in air. After fertilization, the presumed zygotes were cultivated at 38.5°C and 5%CO2, 5%O2 and 90% N2. The cleavage was evaluated at day 4 and the blastocysts were collected at D7 and D8, and submitted to lipid quantification (by Sudan Black B), nuclear fragmentation and total cell number (by TUNEL). For statistical analysis, the data were submitted to the t-test using PROC GLIMMIX of SAS. The lipid content was higher ($P<0.05$) in blastocyst derived from D8 when compared to D7 blastocysts (5.4± 0.3 vs 7.9 ± 0.2 respectively). Blastocysts derived from D7 presented more ($P<0.05$) blastomeres than the D8 blastocysts (105.9 ± 6.2 blt vs 82.0 ± 6.1 blt, respectively). On the other hand, blastocysts derived from D7 presented less ($P<0.05$) apoptotic cells when compared to the D8 blastocysts (6.1 ± 1.1 blt vs 11.3 ± 1.4 blt, respectively). The late blastocoel expansion of the embryos at D8 was important for increased cytoplasmic lipid accumulation, reduction in the number of blastomeres and increase in the number of apoptotic cells. Therefore, we conclude that the early blastocoel expansion, on D7, favors embryonic quality and can be used as a morphological predictor of competence.

A133 Embryology, developmental biology and physiology of reproduction

Effects of *in vitro* growth and prematuration on mRNA levels for *GDF9*, *CCNB1*, *H1FOO*, *cMOS*, *PARN* and *eIF4E* in oocytes from small bovine antral follicles

Laryssa Gondim Barrozo, Francisco Taiã Gomes Bezerra, Bianca Régia Silva, Pedro Alves Barroso, Anderson Weiny Barbalho Silva, Ana Liza Paz Souza, José Roberto Viana Silva

UFC - Universidade Federal do Ceará, Sobral, CE, Brasil.

During the meiotic maturation of bovine oocytes, the mRNAs are translated between the metaphases and will aid in the later developmental processes. Thus, the objective of this study was to evaluate mRNA levels for *GDF9*, *cyclin B1*, *H1FOO* (oocyte specific binding histone), *cMOS kinase*, *PARN* (poly (A) ribonuclease) and *eIF4E* (cap binding protein) in oocytes derived from small follicles antral (1.0-3.0 mm in diameter) before and after growth, prematuration and *in vitro* maturation (IVM). For this, cow ovaries (n = 40) were obtained from a local slaughterhouse and transported to the laboratory in saline solution. In the laboratory, cumulus-oocyte complexes (COCs) were aspirated from small antral follicles, classified and those that had cumulus compact cells and oocytes with no signs of cytoplasmic degeneration were intended for *in vitro* culture. The COCs were cultured individually for 48 h in TCM-199 supplemented with 4% PVP, 1 µg / ml estradiol, 4 mM hypoxanthine, 0.2 mM pyruvic acid, 2.2 mg / ml sodium bicarbonate, 0 mg / mL LH, 0.5 mg / mL FSH, 5% FBS and 100 IU / mL penicillin and 50 µg / mL streptomycin. The *in vitro* pre-maturation medium was TCM-199 supplemented with 0.2 mM pyruvic acid, 5.0 mg / mL LH, 0.5 mg / mL FSH, 0.4% BSA, 10 µM cilostamide, 100 IU / mL penicillin and 50 µg / mL streptomycin. COCs were cultured for 20 hours. For IVM (22h), the same pre-maturation medium was used, but without cilostamide. Morphology, oocyte diameters, meiotic progression were analyzed and four groups containing 10 oocytes were stored at -80 °C for RNA extraction and subsequent evaluation of mRNA expression before the pre-maturation and maturation period. The percentages of Germinal Vesicle (GV) in the different treatments were evaluated by the Mann Whitney test to analyze the developmental stage of the oocyte. The mRNA levels were quantified at the end of 18 days of culture by the real-time PCR technique. The gene expression data were analyzed by the Kruskal-Wallis test, followed by Dunn's nonparametric multiple comparison test. Differences were considered significant when $P < 0.05$ or $P > 0.05$ when there was no difference. After the growth and pre-maturation of the COCs of the small follicles, only 17.0% of the oocytes had a resumption of meiosis. However, after IVM, meiosis resumption rate was 80%. These pre-mature oocytes showed increased mRNA levels for *GDF9*, *PARN* and *eIF4E* when compared to those before culture. However, there was no significant increase ($p < 0.05$) in mRNA levels for *cMOS*, *CCNB1* and *H1FOO* after pre-maturation. In conclusion, prematuration increased the expression levels of *GDF9*, *PARN* and *eIF4E* genes. Furthermore, the increased expression levels of these genes in oocytes may be a good indicator of oocyte viability during *in vitro* culture. However, further studies are needed to confirm the effect of premature oocyte development.

A134 Embryology, developmental biology and physiology of reproduction

Endometrial expression of oxytocin receptor, and interferon-stimulated gene 15, and circulating PGFM after oxytocin challenge differ between AI and IVP derived pregnancies on days 18 and 32

Jéssica Nora Drum[1,2], Guilherme Madureira[1], Maria da Conceição Gonçalves Macedo[3], Danila Barreiro Campos[3], Jéssica Cristina Lemos Motta[1,2], José Renato Gonçalves[4], Camila Rosa[5], Marcelo Marcondes Seneda[5], João V. R. Vicente[4], Lucas Oliveira e Silva[1], Milo Charles Wiltbank[2], Roberto Sartori Filho[1]

[1]ESALQ - USP - Department of Animal Science, Luiz de Queiroz College of Agriculture, University of São Paulo, Piracicaba, SP, Brazil; [2]UW-Madison - Department of Dairy Science, University of Wisconsin-Madison, Madison, WI 53706, USA; [3]UFPB - Department of Agricultural Sciences, Federal University of Paraíba, Areia, PB, Brazil; [4]FEALQ - "Hildegard Georgina Von Pritzelwiltz" Experimental Station, Londrina, PR, Brazil; [5]UEL - Department of Veterinary Clinics, State University of Londrina, Londrina, PR, Brazil.

The aim was evaluate oxytocin-induced prostaglandin F metabolite (PGFM) in pregnant cows from artificial insemination (AI) or *in vitro* produced (IVP) embryos on d18 and d32, and its association with factors that impact the success of pregnancy, such as circulating progesterone (P4), conceptus length on d18 and embryo size on d32. Moreover, aimed to quantify and localize oxytocin receptors (OXTR) and interferon-stimulated gene 15 (ISG15) expression in uterine endometrium. Non-lactating *Bos indicus* (Nelore) cows (n=142) were submitted to a synchronization protocol, and randomly assigned to one of the following groups: non-inseminated (NI), AI on d0 (48 h after implant removal), or received an IVP embryo on d6.5. Then, NI, AI and IVP cows were slaughtered on d18 or d32, according to AI or IVP groups. One d before slaughter (d17 and d31) cows were challenged with 50 IU oxytocin, i.m., and blood samples were collected before (0 min), 60 and 120 min after oxytocin for circulating PGFM. Samples for P4 were collected on d6.5 and on the d of oxytocin. After slaughter, uterus was collected and dissected for conceptus, embryo and OXTR analysis. Statystical analysis were performed using PROC MIXED of SAS 9.4. There was no difference (P>0.05) between AI vs IVP for conceptus length on d18 (44.6±4.3 vs 53.3±5.9 cm), or P4 on d6.5 and d17. However, embryo size on d32 (1.8±0.2 vs 1.3±0.1 cm) was bigger, and P4 on d31 (8.5±0.9 vs 6.6±0.5 ng/mL; P=0.07) tended to be higher in AI than IVP. For basal circulating PGFM on d31, AI and IVP were similar (47.3±6.4 vs 39.8±4.8 pg/mL), and both were respectively 3 and 2.5-fold higher than NI (15.7±3.6 pg/mL), and 2.6 and 2.2-fold higher than IVP on d17 (17.9±3.9 pg/mL). AI cows on d17 had intermediate basal circulating PGFM (33.3±6.6 pg/mL) which whereas greater than IVP on d17, but similar to NI and both groups on d31. The highest increase in PGFM after challenge was detected at 60 min for all groups. The NI cows had higher PGFM after oxytocin challenge than IVP (7.6- vs 0.2-fold increase). The AI group on this same d had an intermediate response, which did not differ from other groups. Cows from IVP on d17 had lower oxytocin-induced PGFM than AI on the same d, although on d31 both groups had higher PGF release than IVP d17. In addition, OXTR were significantly highly suppressed on pregnant cows on d18, especially in IVP cows, but highly expressed in NI cows and in both groups on d32, being AI higher than IVP. The ISG15 had lower expression in NI and d32 groups, whereas was highly expressed in d18 pregnant cows for both groups. In conclusion, the PGF production pathways are induced after the first month of pregnancy in uterus, suggesting alternative mechanisms for CL maintenance than PGF suppression. Moreover, the signaling differences between IVP and AI pregnancies affected molecular and endocrine environment, influencing PGF release during these two critical time points. Acknowledgements: FAPESP, CNPq, CAPES, FEALQ.

A135 Embryology, developmental biology and physiology of reproduction

In vitro production of bovine thermotolerant embryos

Sheila Costa de Souza Marques[1,3], João Victor Gonçalves da Silva[1,4], Agostinho Jorge dos Reis Camargo[2], Luiz Sérgio de Almeida Camargo[1], Clara Slade Oliveira[1]

[1]EMBRAPA - Empresa Brasileira de Pesquisa Agropecuária, Juiz de Fora, MG, Brasil; [2]Pesagro Rio - Pesagro Rio, Niterói, RJ.Brasil; [3]UBM - Centro Universitário Barra Mansa, Barra Mansa, RJ, Brasil; [4]UGB - Centro Universitário Geraldo di Biase, Barra do Piraí, RJ, Brasil.

Considering the climatic changes and the elevation of temperature, the reproductive indices in dairy herds of countries with tropical climate are critical. High metabolic rate and the high uterine temperature are related to embryonic death. This experiment was designed to develop a heat treatment protocol for bovine embryos in order to induce thermotolerance in Girolando embryos. Subsequently, those embryos will be characterized (HSP protein expression) and tested as an alternative to conventional embryos during the summer season. For this experiment, oocytes were collected from F1 (½ Gir and ½ Holstein) donors by ultrasound guided follicular aspiration (OPU, ovum pick-up) (CEUA EGL 3956180316). Oocytes were in vitro fertilized with Holstein bull semen for production of ¾ Holstein embryos. A mild heat treatment protocol was designed and tested at 96, 120 or 144h. p.i.. The embryos were submitted to the heat treatment at a temperature of 38.5 to 40.5 °C for 6 hours. A homemade incubator was settled with a water filled plastic box placed at a heat stage. Temperature increase or decrease was controlled manually by opening or closing the plastic box. Treated embryos were moved to cryotubes with 200ul medium and 200ul of mineral oil and remained for 40 min in the incubator for gas equilibration with a loose cover. After that, cryotube was tightly closed and sealed with parafilm and transferred to heat treatment chamber. Heat treatment was carried out as six 1h cycles. At each cycle, every 7.5 min a 0.5° C increase was induced up to 40.5°C, and then every 7.5 min a 0.5°C decrease was induced until 38.5°C. Control remained in the incubator at 38.5 °C. Two replicates were performed, and blastocyst rates at d7 were evaluated as well as the number of cells and apoptosis rate of the blastocysts. No differences were observed in the blastocyst rates (C = 28.57, TT96hpi = 35.14, TT120hpi = 23.81, TT144hpi = 19.77, p> 0.05, Fisher's exact test, n = 298 cleaved embryos) or on the apoptosis index (C = 6.47 ± 3.93, TT96hpi = 7.41 ± 4.05, TT120hpi = 7.07 ± 5.21, TT144hpi = 4.54 ± 2.71, p> 0.05, ANOVA and Dunnett, n = 50 blastocysts). The mean number of cells did not differ in any treatment compared to the control group (C = 70.53 ± 20.03, TT96hpi = 67.46 ± 13.65, TT120hpi = 57.80 ± 8.74, TT144hpi = 78, 00 ± 14.87, p> 0.05, ANOVA and Dunnett, n = 50 blastocysts). The results allow us to conclude that heat treatment developed can be used at any of the tested moments without being harmful to embryos. The 144h.p.i. can be preferred due to the proximity to embryo transfer (168 h.p.i.), so that the post-transfer effects are prolonged. Acknowledgements: Fapemig and CNPq.

A136 Embryology, developmental biology and physiology of reproduction

Use of lipolysis supplement during IVM reduces lipid content of bovine oocytes but do not affect blastocyst cryosurvival

Viviane Luzia da Silva Feuchard[1], Sheila Costa De Souza Marques[2,4], Raquel Varella Serapião[1], Naiara Zoccal Saraiva[2], Marina Ragagnin de Lima[3], Joaquim Mansano Garcia[3], Clara Slade Oliveira[2]

[1]PESAGRO-RIO - PESAGRO RIO, Niterói, Rio de Janeiro, Brasil; [2]EMBRAPA GADO DE LEITE - Empresa Brasileira de Pesquisa Agropecuária, Juiz de Fora, MG, Brasil; [3]UNESP - UNESP, Jaboticabal, SP, Brasil; [4]UBM - Centro Universitário Barra Mansa, Barra Mansa, RJ, Brasil.

In vitro produced embryos have high lipid content and this characteristic is related to cryotolerance. Since lipid accumulation first occurs during in vitro maturation (IVM), our hypothesis was that the use of lipolysis regulators during IVM would reduce lipid content of the oocytes and consequently of the embryos and improve cryosurvival. We evaluated the effect of a lipolysis supplement (L-carnitine, linoleic acid and forskolin) during IVM on total lipid content of oocytes and in vitro produced blastocysts and their influence on cleavage, blastocyst and survival rates after vitrification. CCOs were obtained from slaughterhouse ovaries, in 6 replicates, and randomly distributed in two groups: Control (C, TCM 199 + 10% SFB) and Lipolysis supplement (L, TCM 199 + 10% SFB and lipolysis supplement (2,5 mM L-carnitine, 150 µM linoleic acid and 15 µM forskolin) for 24 h IVM. After IVM, part of matured oocytes was denuded with Tryple Express (Gibco, Grand Island, NY) solution and fixed for Oil Red O staining and most followed IVF and IVC for 7 days. All steps were performed at 38.5ºC, 5% CO2 and maximum humidity. Samples of denuded oocytes and day 7 blastocysts from C and L groups were fixed in 4% paraformaldehyde and stained with Oil Red solution for 30 min. Lipid content was estimated by stained cytoplasm area fraction (µm²) and staining levels (pixels) using ImageJ® software. Bl and Bx grade 1 were vitrified in vitrification solution 1 (HSOF + 7.5% ethylene glycol [EG] + 7.5% dimethyl sulfoxide [DMSO]) for 3 minutes and solution 2 (HSOF + 15 % ET + 15% DMSO) for up to 30 seconds. Subsequently, the embryos were transferred to vitrification forks and submerged in liquid nitrogen. The lipolysis supplement reduced lipid content in L oocytes compared to Control group considering staining levels (C- 49.90 ± 1.59, L - 45.00 ± 1.86, P <0.046, T test, n = 192,104-88 per group) and area fraction (C - 127.25 ± 4.06, L – 114.77 ± 4.76, P<0.046, T test, n = 192,104-88 per group). In blastocysts, there was no difference between groups (C - 62.38 ± 2.68, L- 66.78 ± 2.61, P = 0.27, T Test, n = 40, 25 -15 per group). There was no difference in developmental rates or survival rates after vitrification. The mean rate of cleavage (C - 74.88 ± 5.52, L - 78.21 ± 5.76, P = 0.45, T Test, n = 152, 74-78 per group) and blastocyst (C - 42.24 ± 5.75, L - 31.46 ± 3.10, P = 0.09, T Test), as well as the reexpansion rate (C - 64.36 ± 8.58a, L - 54.78 ± 7.73a, P = 0.39, Test T, n = 196, 108-88 per group) and hatching (C - 23.42 ± 5.66a, L - 24.51 ± 6.87a, P = 0.93, Test T, n = 72, 36 per group) of vitrified blastocysts 48 hours after warming did not differ between groups. We conclude lipolysis supplement use during IVM was efficient in reducing the total lipid content of oocytes but levels were similar to control in blastocysts. No effect was detected in developmental rates or survival after vitrification. Acknowledgments: FAPERJ, EMBRAPA, Pesagro-Rio.

A137 Embryology, developmental biology and physiology of reproduction

Monitoring lipids behavior in bovine preimplantation embryos

Camila Bruna de Lima[1,2], Érika Cristina dos Santos[2], Jéssica Ispada[2], Christina Ramires Ferreira[4], Marcella Pecora Milazzotto[2]

[1]USP - Universidade de São Paulo, III Cidade Universitária, São Paulo, SP; [2]UFABC - Universidade Federal do ABC, Santo André, SP; [3]UNESP - Campus Botucatu - Universidade Estadual Paulista "Julio de Mesquita Filho", Rubiao Junior, Botucatu, SP, Brasil; [4]Purdue University - Purdue Univeristy, West Lafayette, IN 47906, EUA.

In addition to their important role as structural units and in cell signaling pathways, lipids are a potential energy source for the embryo. However, an inverse relationship between embryonic quality and lipid content has been established. In general, factors that contribute to compromised viability such as high oxygen tension and non-optimized media culture supplementation also result in an increase in lipid content. It is therefore imperative to overcome the barriers in lipid analysis and understand how the culture system modulates lipid content to enable embryo formation. In this context, this study proposes to comprehensively monitor the impact of the interaction between glucose supplementation and oxygen tension in the lipid profile of individual bovine embryos. Using a factorial experimental design (2x3), embryos were produced *in vitro* by standard protocols and cultured in 20% or 5% O_2, and also in distinct glucose concentrations (0, 2 and 5mM). Blastocysts (n=10/group) were collected on day 7 and submitted to the Bligh&Dyer lipid extraction protocol. Then, using a sensitive profiling method based on mass spectrometry (Multiple Monitoring Reactions), we monitored 178 lipids from triacylglycerol (TAG), free fatty acids (FFA) and cholesteryl ester subclasses. Absolute intensity of each lipid was gathered in a matrix, normalized and submitted to multivariate analysis and univariate statistics. Results show that the augmentation of glucose in the culture media only impacts the lipid profile if the embryos are being cultured in a non-optimized oxygen tension (higher in 2mM and 5mM compared to 0mM in 20% O_2; $p<0.05$). This was confirmed by a non-supervised multivariate analysis (Principal Component Analysis), where the model was able to separate the groups cultured in different oxygen tensions (PC1+ PC2 = 67.5%), but not those produced with distinct glucose supplementation. Therefore, we investigated the general behavior of these lipids in both oxygen tensions and observed that although embryos cultured in 20% O_2 presented generally higher relative amounts of lipids ($p<0.000$), this was not a widespread effect. TAG and Cholesteryl esters were augmented in the embryos cultured in 5% O_2 ($p<0.000$), while only FFA were significantly increased in embryos cultured at 20% O_2 ($p<0.000$). Moreover, important fatty acids such as palmitic and stearic acids were found to have relative amounts around 2 times higher in the groups cultured in 20% O_2. With this results it is possible to point out that (i) glucose supplementation alone is not responsible for an increase in lipid relative amounts; (ii) the higher oxygen tension drives lipid metabolism to the production of free fatty acids that will likely be oxidized to produce energy and (iii) in 5% O_2, lipid metabolism is orientated towards the production of TAGs and Cholesteryl esters, probably representing a protection against mitochondrial damage and a way to ensure correct membrane formation and energy stock.

A138 Embryology, developmental biology and physiology of reproduction

Presence of CL and/or intravaginal progesterone insert affect ovulation and subsequent CL development after gonadorelin treatment

Lucas Oliveira e Silva[1], Rodrigo Lemos Olivieri Rodrigues Alves[1], Natália Picoli Folchini[1], Jéssica Nora Drum[1], Guilherme Madureira[1], Carlos Eduardo Cardoso Consentini[1], Jéssica Cristina Lemos Motta[1], Mateus Anastacio da Silva[1], Amanda Moelemberg Cezar[1], Milo Charles Wiltbank[2], Roberto Sartori Filho[1]

[1]ESALQ-USP - Department of Animal Science, Luiz de Queiroz College of Agriculture of University of São Paulo, Piracicaba, São Paulo, Brazil; [2]UW-Madison - Department of Dairy Science, University of Wisconsin - Madison, Madison, WI, USA.

The aim of this study was to determine the effect of presence of corpus luteum (CL) and/or an intravaginal progesterone (P4) insert on ovulatory response and subsequent CL development, after administration of 100 µg gonadorelin acetate (GnRH). Non-lactating Holstein cows were submitted to a presynchronization protocol: D-17: 2 mg estradiol benzoate and a 2 g P4 insert previously used for 7 d; D-9: 0.530 mg cloprostenol sodium (PGF) and 1 mg estradiol cypionate; D-7: 100 µg GnRH. Only cows that ovulated were used (n = 90, Age = 5.0 ± 2.3 years; BCS = 3.3 ± 0.1; 4 replicates). On D -1.5 cows were randomly assigned to a 2x2 factorial design (CL x P4 insert). Groups designed to have no CL at GnRH challenge received 0.530 mg PGF on D -1.5 and a second dose 24 h later. On D0 all cows were treated with 100 µg GnRH. Simultaneously, cows from P4 insert groups received a 2 g P4 device, that was maintained for 14 d. Dominant follicle (DF) size, ovulatory response, and development of the subsequent CL were assessed by ultrasonography on D0, D2, D7, and D14, respectively. For a subset of cows (n = 35), CL development was evaluated daily from D5 to D14. Statistical analyses were performed by MIXED and GLIMMIX of SAS 9.4 (means ± SEM; $P \leq 0.05$). Only cows with DF ≥ 10 mm on D0 were considered in the analysis. There was no difference on DF diameter on D0 among treatments or between ovulated and non-ovulated cows. There was an effect of presence of CL on the ovulatory response ($P<0.001$), but there was no effect of P4 insert or interaction between these factors: with CL = 58.1% [52.4% (11/21) and 63.6% (14/22) with and without P4 insert, respectively]; without CL = 95.5% [90.9% (20/22) and 100% (22/22) with and without P4 implant, respectively]. The presence of CL at GnRH negatively affected the volume of the new CL on D7. Cows with CL on D0 had smaller subsequent CL than cows without CL (2.9 ± 0.3 vs. 4.2 ± 0.2 cm^3; $P = 0.001$). Moreover, there was an interaction for CL volume on D14. Cows without CL on D0 that did not have a P4 insert had greater CL than cows with a P4 insert at GnRH (6.3 ± 0.4 vs. 3.5 ± 0.6 cm^3; $P = 0.001$). From D10 to D14, all cows from the group without CL and without P4 insert on D0 maintained the CL, whereas the other groups presented a significant decrease in mean CL volume. The results confirm that CL presence at GnRH administration exerts a suppressive effect on ovulatory response of a 7-day old follicle and on subsequent CL development. Although the insertion of an intravaginal P4 device has not affected ovulation, surprisingly, it negatively influenced CL development/maintenance by D14, even in cows that did not have a CL at the time of GnRH challenge. Thus, it is very likely that P4 supplementation at the time of ovulation induction causes a suppressive effect on the subsequent CL lifespan by anticipating luteolysis. Acknowledgments: FAPESP, CNPq, and CAPES.

A139 Embryology, developmental biology and physiology of reproduction

Metabolic gene expression and lipid accumulation in bovine embryos produced *in vitro* from semen of Nellore bulls selected for residual feed intake

Gisele Zoccal Mingoti[1,2], Anelise Ribeiro Peres[2], Giovana Barros Nunes[2,1], Priscila Chediek Dall'Acqua[2,1], Natália Marins Bastos[2], Guilherme Fazan Rossi[2], Fabio Morato Monteiro[3,2], Patrícia Kubo Fontes[4], Marcelo Fábio Gouveia Nogueira[5,4], Flávia Regina Florencio de Athayde[1], Luana Teixeira Rodrigues Rossi[2], Maria Eugênia Zerlotti Mercadante[3]

[1]São Paulo State University (UNESP) - FMVA - School of Veterinary Medicine, Laboratory of Reproductive Physiology, Araçatuba, SP, Brazil; [2]UNESP - FCAV - School of Agricultural and Veterinarian Sciences, Post-Graduation Program in Veterinary Medicine, Jaboticabal, SP, Brazil; [3]Institute of Animal Science (IZ) - São Paulo Agribusiness Technology Agency (APTA), Sertãozinho, SP, Brazil; [4]UNESP - IBB - Institute of Biosciences, Department of Pharmacology, Botucatu, SP, Brazil; [5]UNESP - FCLAs - School of Sciences, Humanities and Languages, Department of Biological Sciences, Assis, SP, Brazil.

Feed costs account for more than half of the total cost of cattle production, therefore improving feed efficiency (FE) is desirable to improve the economy in the livestock sector. FE can be measured by residual feed intake (RFI), which is defined as the difference between the actual and the predicted dry matter intakes based on the body size and performance of each animal. Animals selected for lower RFI (high efficiency) present lower feed consumption with no effect on growth rates, however, small changes in body composition towards greater lean and less fat have already been reported in low RFI animals. Considering the differences in the metabolism of selected animals for FE, this experiment aimed to investigate the expression of genes related to metabolism and intracytoplasmic lipid accumulation in embryos produced *in vitro* from semen of Nellore bulls classified for high (less efficient; n=3) and low (more efficient; n=3) RFI. The semen was cryopreserved when the animals were 24 months old and was used to inseminate *in vitro*-matured oocytes recovered from ovaries obtained in a slaughterhouse. Putative zygotes were culture until day 7 when blastocysts (n=44) were stained with the lipophilic dye Sudan Black B for determination of the intracytoplasmic lipid content and expanded blastocysts (3 pools per treatment, each containing 3 blastocysts of each bull) were collected to assess the abundance of 88 transcripts by RT-qPCR using a microfluidic platform (BioMark HD System™, Fluidigm®). The ΔCt values were calculated relatively to the geometric mean of five most stable reference genes and fold changes were calculated as $2^{-\Delta Ct}$. Data were analyzed by t test ($P<0.05$). Blastocysts rates on day 7 (18.2%; averaged) and intracytoplasmic lipid content (131.3 arbitrary units of pixels; averaged) were unaffected by FE ($P>0.05$). Transcript levels of *CD36* and *ACAT1* genes (lipid metabolism) were up-regulated ($P<0.05$), whereas several other transcripts were down-regulated ($P<0.05$) in low RFI group, including genes related to lipid metabolism (*HMGSC1* and *PPARGC1*), embryo development and quality (*NANOG* and *IFNT2*), epigenetic regulation (*H3F3A*) and stress response (*HSF1*). These genes were uploaded to the R package clusterProfiler for analysis of gene ontology (GO) and the program identified 23 enriched GO biological processes. We also identified 4 KEGG pathways related to *ACAT1* and *HMGCS1* genes. The results suggest that there is a genetic association between paternal dietary efficiency and the expression of genes related to metabolism of descending embryos, and the increase in metabolism is associated with lower RFI (ie, higher efficiency). However, the up-regulation of transcripts related to lipid metabolism did not reflect in an increase on embryonic lipid content. In conclusion, the gene expression and metabolic pathways of *in vitro*-produced embryos are affected by paternal RFI. Financial support: FAPESP (#2015/06733-5 and #2012/50533-2) and CNPq (#307416/2015-1).

A140 Embryology, developmental biology and physiology of reproduction

miRNAs identified in corpus luteum of IVF recipient cows are absent in SCNT recipient animals on day 19 of pregnancy

Alessandra Bridi[1], Ana Clara F.C.M de Ávila[1], Tiago H. C. de Bem[1], Gabriella Mamede Andrade[1], Rafael Vilar Sampaio[1], Guilherme Pugliesi[2], Niamh Forde[3], Flávio Vieira Meirelles[1], Juliano Coelho da Silveira[1], Felipe Perecin[1]

[1]FZEA/USP - Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil; [2]FMVZ/USP - Department of Animal Reproduction, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil; [3]LICAMM/LEEDS - Discovery and Translational Sciences Department, Leeds Institute of Cardiovascular and Metabolic Medicine, Faculty of Medicine and Health Sciences, University of Leeds, Leeds, United Kingdom.

Corpus luteum (CL) is responsible by P4 production and it is necessary for establishing and maintenance of pregnancy in cattle. Normal luteal function is regulated by miRNAs, that are small non-coding RNA molecules involved in post-transcriptional regulation of target genes. However, the role of miRNAs and its potentially regulated pathways are poorly known in the CL of cows carrying conceptus derived from different biotechnologies (IVF or SCNT). Therefore, our hypothesis is that miRNAs are exclusively expressed in the CL of cows carrying IVF or SCNT conceptus on day 19 pregnancy. For this, COCs recovered from ovaries collected at local abattoir were used to produce SCNT blastocyst and, IVF embryos were made from oocytes collected by OPU. Nellore cows had the estrus synchronized and received one embryo (IFV or SCNT), on day 7 after expected estrus. CL were collected on day 19 of pregnancy, in animals carrying IVF (n=3) or SCNT (n=3) conceptus. The CL function was evaluated by P4 concentration in blood serum collected from the jugular vein on days 9, 14 and 19 of pregnancy. Mature miRNAs were reverse transcribed using MiScript HiSpec Buffer. The relative levels of 384 miRNAs were evaluated. The data were normalized by the geometric mean of miR-99b, RNU43 snoRNA and Hm/Ms/Rt U1 snRNA. miRNAs were considered exclusively expressed when the expression was detected in all CL samples of one group and not detected (not expressed) in all samples of the other group. On days 9, 14 and 19, serum P4 concentrations were similar between IVF and SCNT groups (Bridi, et al., Animal Reproduction, 15:480, 2018). A total of 288 mature miRNAs were identified in CL samples from both groups, with one exclusive miRNA in the SCNT-CL and three exclusive miRNAs in the IVF-CL. The identification of bovine genes modulated by each miRNA was performed using TARGETSCAN software. After gene identification, the code Ensembl Transcript ID was used to determine enriched pathways regulated by these miRNAs using DAVID Bioinformatics Resources 6.8, NIAID/NIH. The miRNA bta-miR-129-3p, uniquely detected in the SCNT CL group, modulated signaling pathways that include MAPK (14 genes), oxytocin (9 genes), GnRH (7 genes) and estrogen (6 genes). Moreover, bta-miR-141, bta-miR-302a and bta-miR-875, which were uniquely detected in the IVF CL, regulate PI3K-Akt (40 genes), MAPK (33 genes), Hippo (25 genes) and oxytocin (18 genes) pathways. The results show that, on day 19, the expression of different miRNAs in the CL of recipient cows can be modulate by conceptus of different origins (IVF or SCNT). Furthermore, the exclusive miRNAs of both groups regulate oxytocin signaling pathway in the CL, that have an important biological role in the maintenance of luteal function and, consequently in the establishing of pregnancy. Funding: FAPESP grants 2014/22887-0; 2016/50433-9; 2017/19681-9, 2017/50438-3 and 2018-13155-6. Acknowledgments: WTA.

A141 Embryology, developmental biology and physiology of reproduction

Profile of new early pregnancy markers identified by transcriptomic analysis in peripheral blood immune cells in beef heifers

Cecília Constantino Rocha[1], Sónia Cristina da Silva Andrade[1], Gabriela Dalmaso de Melo[1], Igor Garcia Motta[1], Thais Sayuri Imura Oshiro[2], Angela Maria Gonella-Diaza[3], Luiz Lehmann Coutinho[1], Mario Binelli[3], Guilherme Pugliesi[1]

[1]USP - Universidade de São Paulo, Pirassununga, SP; [2]UNIMAR - Universidade de Marília, Marília, São Paulo, Brazil; [3]UFL - Universidade da Flórida, Gainsville, Flórida, USA.

We aimed with this study in pregnant (P) and non-pregnant (NP) heifers: 1) to discover new pregnancy markers (PM) by RNA sequencing (RNAseq) in peripheral blood mononuclear cells (PBMC) on day 18 post-AI; and 2) to assess the mRNA profile of new PM in PBMC and peripheral blood polymorphonuclear cells (PMN) at early pregnancy. Nelore heifers (N=21) were AI in fixed-time (D0). On D10, 14, 16, 18 and 20, blood was collected for isolation of PBMC and PMN, and P4 concentration assay and color Doppler ultrasonography was performed to evaluate the corpus luteum (CL). Pregnancy diagnosis was done on D28 and heifers were *classified* in P (N=9) and NP (N=12). Heifers (N=6/group) with different (P<0.05) plasma P4 concentration, CL area and blood perfusion on D18 were selected and RNAseq was done on PBMC samples. RNAseq analysis indicated 220 differentially expressed genes (200 up regulated in P). Twenty genes found on RNAseq of PBMC with the highest fold-changes or no overlapping between P and NP, were assessed by qPCR from D10 to 20 (N=6/group). Reference genes were used for expression normalization (*GAPDH* and *PPIA*). Data were analyzed by ANOVA using the PROC MIXED procedure (SAS) and considering the main effects of group (G), time (T) and their interaction (GT). For PBMC, G and T effects (P<0.1) and GT interaction were observed for *IFI6*, *RSAD2*, *IFI44*, *IFITM2*, *TNFSF13B* and *LGALS3BP*, reflecting a greater (P<0.1) expression in the P group on D18 and D20 for *IFI6*, *RSAD2*, *IFI44* and *IFITM2*, and on D16 and D18 for *TNFSF13B*. For *CLEC3B*, *OAS2* and *LOC100139209*, a T effect (P<0.05) and GT interaction (P<0.1) were detected, reflecting a greater (P<0.05) expression in the P group on D20 for *OAS2* and *CLEC3B*. For *DMKN*, a GT interaction (P<0.05) reflects an increase on D16 in the P group. For *A2M*, *BPI*, *ANG*, *PLSCR2*, and *DRAM1*, only a T effect (P<0.05) was observed, reflecting a progressive increase from D10 to D20. For *LIG1*, a greater (P<0.1) expression was observed in the NP group from D10 to D20. For PMN, a T effect and GT interaction (P<0.1) were observed for *IFI44*, *RSAD2*, *OAS2* and *LGALS3BP*, reflecting a greater expression in the P group on D18 and 20 for *RSAD2* and *LGALS3BP*, and on D20 for *IFI44* and *OAS2*. An interaction (P<0.05) was also detected for *IFI6*, *C1R*, *RHOT1* and *LIG1*, indicating an increase in the P group on D16, D18 and D20, respectively, for *RHOT1*, *C1R* and *IFI6*, and a decrease in *LIG1* expression in NP group on D20. However, no effect (P>0.1) was observed for *SIGLEC1*, *SORD*, *C1R* and *RHOT1* in *PBMC* and for *IFITM2* in PMN. In conclusion, 9 genes presented increased expression on PBMC of P in at least one-time point from D16 to D20; but only 4 of these genes retained the expression increased on PMN (*IFI6*, *IFI44*, *RSAD2* and *LGALS3BP*). Thus, results indicate potential genes to be used as novel pregnancy predictors in immune cells in cattle at early gestation. Acknowledgments: FAPESP (2015/10606-9; 2017/13994-5).

A142 Embryology, developmental biology and physiology of reproduction

Influence of apical domain formation on the segregation of cell lineages in early development bovine embryos

Marcelo Demarchi Goissis[1], Samuel Augusto Aguiar dos Anjos[2,1], Mayra Elena Ortiz D'Ávila Assumpção[1], José Antonio Visintin[1]

[1]VRA - FMVZ - USP - Department of Animal Reproduction, College of Veterinary Medicine and Animal Science, University of Sao Paulo, Cidade Universitária, São Paulo, SP; [2]IB - USP - Institute of Biosciences, University of Sao Paulo, São Paulo, SP, Brasil.

The first event of cellular differentiation in mammals consists in the segregation between the inner cell mass (ICM) and the trophectoderm (TE). Biological processes that comprise this event are not yet clear during bovine embryo development and studies in mouse suggest that cellular contractility and formation of an apical domain plays a role in this event. In this study, we tested the hypotheses that blocking cellular contractility would block apical domain and inhibit TE formation or that direct inhibition of apical domain formation would inhibit TE formation in bovine embryos. First, we evaluated the presence of an apical domain during bovine embryo development by immunofluorescence of apical domain proteins PARD6B (Novus Biologicals, Littleton, CO USA) and EZR (Abcam, Cambridge, MA, USA) in IVP embryos. We observed that EZR is present since 8-cell stage while PARD6B becomes apically localized at the blastocyst stage. To test the effect of cellular contractility on TE formation we treated IVP embryos with blebbistatin (Bb), a myosin light chain kinase inhibitor. We assessed embryos at 90 hours post-insemination (90hpi) and those at 8-cell stage or further ahead in development were submitted to the following treatments: control, 25μM Bb (+)- and 25μM Bb (-)- (Cayman Chemical, Ann Arbor, USA). Embryos were kept in treatments until 186hpi when development rates (blastocysts/treated embryos) were assessed and embryos fixed with paraformaldehyde (Merck KGaA, Darmstadt, Germany). Developmental rates were analyzed by ANOVA followed by Tukey's adjustment for comparison of means after 5 replicates. Unexpectedly, no statistical difference ($p<0.05$) was observed considering developmental rates among all three groups: control 47.24±7.30% (45/96), Bb (+)- 60.24±7.30% (58/96) and Bb (-)- 49.50±7.30% (46/96). Immunofluorescence revealed that EZR was practically abolished in Bb (+)- treated embryos while present in the other groups. YAP (Abcam), a HIPPO-pathway related protein, was nearly undetected in Bb (+)- treated embryos while visible in other treatments. Also, CDX2 (Abcam), a commonly used marker for TE cells, was not observed in Bb (+)- embryos. To confirm these results, we used the same experimental design and statistical analysis to test if inhibition of apical domain establishment blocks TE formation. Embryos were submitted to following treatments: Control, vehicle (DMSO, Merck) and 7.5μM U73122 (Cayman Chemical), a phospholipase C inhibitor. No statistical difference was observed considering developmental rates among all three groups: control 41.8±3.27% (51/122), vehicle 35.29±3.27% (44/124) and U73122 35.27±3.27% (45/128). Combined, these results led us to conclude that inhibition of contractility or inhibition of the apical domain do not block formation of the TE in bovine embryos, suggesting that different biological processes are involved in ICM/TE segregation in bovine embryos. Funded by FAPESP grants 2017/09576-3, 2017/25574-0, 2018/08285-8.

A143 Embryology, developmental biology and physiology of reproduction

Effect of cysteamine during *in vitro* maturation of bovine oocytes on embryo development

Thaisy Tino Dellaqua[1], Isabela Lima Gama[1], Ana Caroline Silva Soares[1], Isabella Rio Feltrin[1], Valentina Lodde[2], Alberto Maria Luciano[2], José Buratini Junior[1]

[1]UNESP - Universidade Estadual Paulista - Campus Botucatu, Distrito Rubião Junior, Botucatu, SP; [2]UNIMI - Università degli Studi di Milano, MIlão, Itália.

In in vitro embryo production (IVP), oxidative modifications via increased reactive oxygen species (ROS) represent a major culture induced stress. Anti-oxidant systems such as glutathione (GSH) can attenuate deleterious effects of oxidative stress decreasing ROS thus protecting the zygote and early embryo. Previous studies suggest that addition of cysteamine to in vitro maturation (IVM) medium can increase intracellular GSH synthesis, improving pronucleus formation, cleavage rates and embryo development. The aim of the present work was to investigate the effects of cysteamine during IVM with conventional FSH stimulation or utilizing the IVM phase of the follicular system (FS), recently proposed by Ovarian Molecular Physiology Laboratory research group. The FS base medium consisted of TCM199 (with Earle's salts, bovine serum albumin, amikacin, pyruvate) supplemented with rhFSH, amphiregulin, insulin-like growth factor 1, estradiol and progesterone (Soares et al., Reproduction, Fertility and Development, 29:2217-2224, 2017). Five replicates were performed to compare four experimental groups: FSH (basic medium supplemented with rhFSH 10-1 UI/mL); FSH+C (FSH medium supplemented with cysteamine 1 mM/mL); FS and FS+C (FS medium supplemented with cysteamine 1 mM/mL). Ovaries were obtained from a slaughterhouse and COCs recovered by aspiration were submitted to IVM for 24h, followed by in vitro fertilization for 18h and in vitro culture (IVC) for seven days. Blastocyst rate was calculated in relation to total oocytes subjected to IVM and blastocyst cell numbers were assessed by Hoechst 33342 staining. Rates of expanded and hatched blastocysts were calculated in relation to total blastocysts. Data were arcsine transformed and compared with Tukey (parametric data) or Wilcoxon (non-parametric data) tests. Differences were considered significant when $P \leq 0.05$. Addition of cysteamine did not alter blastocyst rate ($P > 0.05$; FSH 24.67±5.37; FSH+C 33.50±4.95; FS 25.96±4.92; FS+C 22.95±5.56), expanded and hatched blastocysts rates ($P > 0.05$; FSH 94.44±3.51; FSH+C 83.57±5.61; FS 85.83±4.86; FS+C 83.36±7.65), nor the total number of embryonic cells ($P > 0.05$; FSH 118.40±7.41; FSH+C 123.39±7.36; SF 124.97±9.75; SF+C 124.37±9.63). In conclusion, addition of cysteamine to the IVM medium did not improve embryo production. Supported by FAPESP 2017/07588-4.

A144 Embryology, developmental biology and physiology of reproduction

Anisomycin inhibits ERK1/2 MAPK activities in activated bovine oocytes

Cecilia Valencia, Felipe Alonso Perez, Carola Matus, Ricardo Felmer, Maria Elena Arias

UFRO - Laboratorio de Reproducción, Centro de Biotecnología de La Reproducción (CEBIOR-BIOREN), Universidad de La Frontera, Chile.

Pioneering studies by our research group have demonstrated the advantages of using Anisomycin, a protein synthesis inhibitor, in the activation of bovine oocytes in embryos generated by intracytoplasmic sperm injection (ICSI), somatic cell nuclear transfer (SCNT) and parthenogenesis. However, the precise mechanism by which anisomycin releases the oocyte's meiotic arrest and allows the activation of oocytes and further embryonic development is unknown. Therefore, the objective of the present study was to evaluate the effect of the activation of bovine oocytes by anisomycin on the inhibition of the MAPK activities. For this, oocytes were activated using ionomycin (ION), ionomycin plus anisomycin (ION/ANY) and ionomycin plus cycloheximide (ION/CHX; activation control). Also in vitro fertilized oocytes (IVF) at 6 hours and MII-oocytes were included as controls. The evaluations were conducted at 1, 4 and 15 hours post activation (hpa) and 3, 6 and 17 hours post fertilization (hpf, in the case of IVF 2 extra hours to allow the penetration of the spermatozoon), respectively. The status of phosphorylation of ERK1/2 were measured by immunoblotting using GAPDH as loading control. Differences between treatments were analyzed using ANOVA and to identify the differences between groups, Tukey's post-test was performed with a level of significance of $p<0.05$. The preliminary results of two biological repetitions showed no differences in the status of phosphorylation of ERK1/2 at 1 hpa-3 hpf. However, assessment at 4 hpa-6 hpf showed a low level ($p<0.05$) of phosphorylation of ERK1/2 in oocytes activated by ION/ANY (0.03±0.003), compared to MII-oocytes (2.4±0.9) and oocytes activated by ION (2.2±0.5). It was also observed a decreased, although not significant, in the status of phosphorylation of ERK1/2 in oocytes activated by ION/ANY (0.03±0.003), compared to oocytes activated by ION/CHX (0.36±0.2). Oocytes activated by ION/CHX did not show differences in relation to MII-oocytes and oocytes activated by ION, meanwhile, the level of phosphorylation of ERK1/2 in IVF-oocytes (3.3±1.4) was greater ($p<0.05$) than the other treatments. The analysis at 15 hpa-17 hpf showed a low level ($p<0.05$) of phosphorylation of ERK1/2 in oocytes activated by ION/ANY (0.03±0.01) and ION/CHX (0.03±0.02), in relation to MII-oocytes (1.6±0.5), oocytes activated by ION (1.3±0.1) and IVF-oocytes (1.5±0.7). In conclusion, anisomycin showed a similar pattern of phosphorylation than cycloheximide, one of the most common exogenous oocyte activation treatments, since both compounds showed to inhibit the ERK1/2 MAPK activity by dephosphorylation at 4 hpa-6 hpf and 15 hpa-17 hpf. Further studies are necessary to determine the global effect of anisomicyn on the MAPKs. Acknowledgment: The provision of ovaries by our local Slaughterhouse (Frigorífico Temuco) and funding support from FONDECYT 1181453 CONICYT-Chile are gratefully acknowledged.

A145 Embryology, developmental biology and physiology of reproduction

Formulation of a conjugated polymer-drug system for the *in vitro* antibacterial evaluation in the treatment of subclinical endometritis in cows

Simone Herrera Bejar[1], Karin Vera Lopez[1,1], Victor Pacheco Sanchez[1], Arnaud Béduneau[2], Rita Nieto Montesinos[1], Juan Reátegui Ordoñez[1]

[1]UCSM - Universidad Catolica de Santa Maria, Vicerrectorado de Investigacion, Laboratorio de Biotecnologia Animal, Arequipa, Peru; [2]UFC - Laboratorio de Farmacia Galénica y Biofarmacia. Universidad de Franche-Comté, Besançon, Francia.

The objective was to obtain a chitosan-enrofloxacin conjugate system for the treatment of subclinical endometritis (ES) in cows. Chitosan, a biopolymer known for its mucoadhesive, anti-inflammatory and antibacterial properties, which could induce a synergistic effect with enrofloxacin, a veterinary fluoroquinolone known for its good absorption and high bioavailability. The conjugate was achieved through a "crosslinking" reaction, the process of joining two or more molecules through a covalent bond, chitosan and enrofloxacin. The presence of the carboxylic acid in the enrofloxacin and the amino group in the chitosan facilitated the conjugate reaction. The activation of these molecules occurred through the use of the reagents EDC (N- (3-Dimethylaminopropyl) -N'-ethylcarbodiimide)) and NHS (N-hydroxysuccinimide), allowing the formation of an amide bond between the polymer and the drug. A controlled release system is expected after the break of the amide bond thanks to the proteases produced by the bacteria present in the aforementioned disease. To obtain the enrofloxacin-conjugated value, fluorescence quantification was performed by a multipurpose spectral scan plate reader Varioskan Flash (Thermo scientific), using the parameters described by Lihua *et al* 2018. The results shown that a conjugate with reproducible drug values was obtained, 23.1 µg ± 1.95 (n = 3) of enrofloxacin per mL of formulation. Size, polydispersity index (PDI) and zeta potential were measured using the Zetasizer nanoZS® (Malvern Instruments, UK). The size of the conjugated system "Chitosan-enrofloxacin" (459.4 nm), will allow us to use it as a component for a nanoparticle formulation, and thus be able to encapsulate the enrofloxacin bounded effectively. The value obtained from 0.8 of PDI shows us that there are different sizes of particle population. For this reason, it is recommended to work on decreasing this indicator to obtain a more stable conjugate. The zeta potential of the conjugate was positively charged, thus giving good mucoadhesive properties to the formulation by interacting with negatively charged cells present in the endometrial mucosa. Currently, the implementation of the bacterial susceptibility test *in vitro* to the polymer-drug conjugate system is being developed to three bacterial strains (*Trueperella pyogenes, Bacteroides fragilis* and *Fusobacterium necrophorum*) identified in ES, using the method developed by Patel J. *et al* 2015 described as microdilution in MH broth. The *in vitro* antibacterial study will verify if enrofloxacin can be released from chitosan after incubation with bacteria; and if the conjugation method using the carboxyl group of enrofloxacin will not alter its antibacterial activity.

A146 Embryology, developmental biology and physiology of reproduction

Morfofunctional and endocrine aspects related to cloprostenol-induced luteolysis in equine females

Jéssica Ruiz Pereira[1], Rafael Angelo de Sá Teodoro[2], Carlos Antônio de Carvalho Fernandes[2,1], Gustavo Henrique de Sousa Pereira[2,1], Ana Cristina Silva de Figueiredo[2,1]

[1]Biotran - Biotran LTDA, Alfenas, MG, Brasil; [2]Unifenas - Universidade José do Rosário Vellano, Alfenas, MG, Brasil.

The objective of this study was to evaluate variables related to morphological, functional and endocrinological alterations related to cloprostenol sodium induced luteolysis in equine females. Twenty-five females aged between 60 and 182 months, between days 7 and 10 of the estrous cycle were used. After selection, (0h) the animals' blood was collected using vacuum collection tubes without anticoagulant. On the same day, ultrasound evaluation of the ovaries was performed using B-mode and color Doppler technology (Mindray-M5™). They were recorded from each ovary that had the corpus luteum (CL), a sequence of 252 frames in B mode and 150 frames in Doppler mode. Immediately after the evaluations, 0.250mg of Cloprostenol sodium (Clocio™-Bimeda, Monte Mor-Brazil) was applied IM. Blood samples and the same ultrasonographic evaluations were done 12, 24, 36 and 48 hours after luteolytic application. Mode B images were used to measure the perimeter and area of the corpus luteum (CL). The color Doppler images were used to determine the vascularization score on a scale of 1 to 4 (according Siqueira et al., J. Dairy Sci., 96:6461-72, 2013). Serum obtained from the blood samples were used for the measurement of progesterone (P4) via Electrochemiluminescence (ECL) using Cobas E411 equipment and commercial Elecsys ™ kits Progesterone III (Roche - Germany). The B mode CL measurements and P4 concentrations were accessed by Anova and compared between the days using Tukey's test. Vascularization scores between the different days were compared by the Kruskal Wallis test. Significant probabilities less than 5% were considered. The intra-assay coefficient of P4 dosages was 1.67%. The mean P4 concentrations were 6.64 ± 5.18^{a}; 2.59 ± 1.97^{b}; 1.18 ± 0.99^{b}; $0.63+0.49^{c}$ e 0.34 ± 0.30^{c} ng/mL for times 0, 12, 24, 36 and 48 hours ($P<0.05$). The mean CL circumferences were 7.83 ± 1.49^{a}; 7.53 ± 1.32^{a}; 7.29 ± 1.53^{ab}; $7.07+1.51^{b}$ and 5.63 ± 1.63^{c} cm and area 3.99 ± 1.53^{a}; 3.85 ± 1.34^{a}; 3.72 ± 1.51^{ab}; 3.36 ± 1.35^{b} and 2.81 ± 0.66^{c} cm^2 for the times 0, 12, 24, 36 and 48 hours ($P<0.05$). The mean values of CL vascularization score were 3.88^{a}; 3.38^{ab}; 2.38^{b}; 2.19^{b} and 1.25^{c} for 0, 12, 24, 36 and 48 hours, respectively ($P<0.05$). The efficiency of luteolysis was 100%. The reduction of P4 concentration was observed at 12 hours, and reduction of vascularization at 24 hours that is, at the 1st and 2nd evaluation after the application of the product. On the other hand, the morphological regression of CL occurred only in the 3 evaluation, at 36 hours, 24 and 12 hours later than endocrinological functional and regression, respectively. It is concluded that the product used is efficient in causing luteolysis in equine females. Functional regression and reduction of P4 concentrations precede morphological changes in CL. Supported by: Bimeda, Biotran, Unifenas, Capes and CNPq.

A147 Embryology, developmental biology and physiology of reproduction

Lipid content in maturation media alters the lipid profile of oocytes and blastocysts

João Vitor Alcantara da Silva[1,2], Giulia Zanotto Barbosa[3,2], Camila Bruna de Lima[4,2], Kelly Annes[2], Jessica Ispada[4,2], Érika Cristina dos Santos[2], Aldcejam Fonseca Junior[2], R. Graham Cooks[5], Christina Ramires[5], Marcella Pecora Milazzotto[2]

[1]UMC - University of Mogi das Cruzes, Vila Leopoldina, São Paulo, SP; [2]UFABC - Federal University ABC, Santo André, SP; [3]FMU - Faculdade Metropolitanas Unidas, São Paulo, SP; [4]USP - University of São Paulo, Butantã, São Paulo, SP; [5]PUCC - Purdue University, West Lafayette, Indian.

Lipids are an important energy source for oocytes and embryos during in-vitro production, since they are stored as lipid droplets in cytoplasm and can be used according to the energy demand. However, high amounts of lipid droplets may increase apoptosis rates in blastomeres and impact embryonic survival after cryopreservation. Based on that, the aim of this study was to evaluate how the increase or reduction in lipid supplementation during in vitro maturation could affect bovine oocytes and blastocysts development. Bovine CCOs were in vitro matured in maturation media supplemented with 10% FBS (CO group), 10% of delipidated FBS (-lip group) or 10% FBS plus lipid extracted from FBS (+lip group). After maturation, oocytes were collected for analysis or fertilized and cultured until Day 7. Cleavage (Day 3) and blastocyst rates (Day 7) were assessed and the blastocysts were collected for analysis. Lipid profile for both oocytes and blastocysts were determined by the quantification of lipid droplets (Sudan Black B staining) and the characterization of lipid content by MRM-MS (multiple reaction monitoring-mass spectrometry). The results from embryo rates and lipid staining were analyzed by ANOVA followed by Tukey test (5 Prism GraphPad Inc.) and MRM-MS by principal components analysis (PCA). There was no difference in cleavage rates (CO:72±5%; -lip: 74±5%; +lip: 73±4%; P= 0.98), while there was a higher conversion of blastocysts in +lip group (CO: 30±3%; -lip: 30±3%; +lip: 42±3%; P=0.02). In addition, although the quantification of lipid droplets were similar for oocytes (CO: 11.0±1.7AU; -lip: 8.0±0.9AU; +lip: 9.3±1.2AU; P<0.30), blastocyst from CO group presented a higher amount of lipids than the other groups (CO: 7.6±0.8AU; -lip: 4.6±0.8AU; +lip: 4.9±0.5AU; P=0.0107). The PCA analysis of MRM data revealed that, for both oocytes and blastocysts, the lipid profiles were similar between +lip and -lip groups, which differed from CO. Among these lipids, +lip and -lip had an increase in triacylglycerols (TAG) and cholesterol (oocyte - TAG CO: 0.9±0.01AU; -lip: 1.9±0.1AU; +lip: 1.6±0.05AU; P<0.0001; Cholesterol CO: 3.8±0.1AU; -lip: 6.7±0.3AU; +lip: 6.8±0.4AU; P=0.0004/ blastocyst - TAG CO: 8.3±0.3AU; -lip: 22.4±0.8AU; +lip: 17.5±0.4AU; P<0.0001; Cholesterol CO: 4.0±0.1AU; -lip: 8.1±0.3AU; +lip: 7.3±0.3AU; P<0.0001) when compared to CO group. Also, blastocysts from -lip group had lower relative amounts of fatty acids than +lip group (CO: 11.7±1.5AU; -lip: 9.0±0.7AU; +lip: 15.2±0.9AU; P=0.0003). Different lipid supplementation during in vitro maturation impact the lipid composition of oocytes, leading to changes in blastocyst production and metabolism. Surprisingly, maturation in the presence of higher lipid levels seems to contribute positively to the blastocysts development rates. Acknowledgement: FAPESP (2016/23272-4).

A148 Embryology, developmental biology and physiology of reproduction

The increase of lipid levels in oocytes at germinal vesicle stage and its association with mitophagy in obese mice

Maite del Collado[1], Gabriella Mamede Andrade[1], Bruna Martins Garcia[3,2], Thiago Simões Machado[2], Mateus Priolo Grejo[2], Flávio Vieira Meirelles[1], Marcos Roberto Chiaratti[2], Felipe Perecin[1]

[1]FZEA-USP - Department of Veterinary Medicine, Jardim Elite, Pirassununga, SP; [2]UFSCar - Laboratory of Genetics and Biotechnology, Department of Genetic and Evolution, São Carlos, SP; [3]Max Planck Institute for Biology of Ageing - Metabolism of Infection, Köln, Alemania.

The subfertility of obese females and the increase of lipid content in their oocyte has been deeply studied. However, the possible relationship between oocyte lipid accumulation and removal of damaged mitochondria by mitophagy is unknown. This study aimed to evaluate the association between the increase in lipid accumulation with the reduction of mitophagy in obese mice oocytes. For that, six-week-old females were submitted to control or high fatty acid diet for 12 weeks, originating control and obese groups, respectively. Afterwards, mice were superstimulated with intraperitoneal administration of 5 U.I of eCG to collect immature oocytes at the germinal vesicle (GV) stage. Lipid storages were determined in 36 (control) and 35 (obese) oocytes retrieved from 7 females per group using fixed oocytes. The lipid content was estimated based on lipid area/oocyte area using Bodipy 493/503 and confocal microscopy. Moreover, NAD(P)H levels were assessed in 92 oocytes from 4 control mice and 71 oocytes from 3 obese mice by autofluorescence (using DAPI filter in epifluorescence microscopy). To evaluate mitophagy, 30 oocytes from 4 control females and 18 oocytes from 3 obese females were submitted to immunofluorescence to determine COX IV and LC3B. Additionally, 14 out of 30 (control group) and 13 out of 18 (obese group) oocytes were used to perform co-localization analyses of COX IV and LC3B. This was performed using images that were captured every 0.2 μm (z-stack axis). These images were subjected to 3D reconstruction and Mender´s coefficiency using JACoP plugin in FIJI software. The data were submitted to one-way ANOVA, considering a randomized block design using body weight as blocking variable. We detected an increase ($p<0.05$) in lipid content in oocytes from obese group (0.0169±0.0082) when compared to control oocytes (0.0104±0.0080). An increase ($p<0.05$) in NAD(P)H levels was also identified in oocytes from obese group (3.66 ±1.44) compared to control (3.28±1.73). Regarding mitophagy, we observed an increase ($p<0.05$) of LC3B intensity in obese group when compared to control (16.37±9.08 and 7.97±2.38, respectively), but no difference in COX IV levels (28.6±9.90 and 29.98±8.99, for control and obese, respectively). Likewise, it was observed a decrease ($p<0.05$) in COX IV-LC3B co-localization in oocytes from obese (0.307±0.107) in comparison to control group (0.463±0.148), suggesting a reduction of mitophagy associated with obesity. In conclusion, we were able to show the negative effects of obesity in lipid accumulation and in oxidative status in oocytes, and also a potential association of this condition with disrupted mitophagy. Supported by FAPESP (grants 2017/19825-0 and 2018/13155-6).

A149 Embryology, developmental biology and physiology of reproduction

Post implantation measurements of female PIVE embryos can show risk of pregnancy loss in cows

Pedro Henrique Evangelista Guedes[1,3], Hugo Rocha Sabença Dias[2,3], Célio Freitas[3], Agostinho Jorge Dos Reis Camargo[4], Aline Emerim Pinna[1], Luiz Altamiro Garcia Nogueira[1], Clara Slade Oliveira[3]

[1]UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil; [2]UV - Universidade de Vassouras, Vassouras, RJ, Brasil; [3]Embrapa - Empresa Brasileira de Pesquisa Agropecuária, Juiz de Fora, MG, Brasil; [4]Pesagro - Empresa de Pesquisa Agropecuária do Estado do Rio De Janeiro, Niterói, RJ, Brasil.

The aim of this study was to investigate relationships between pregnancy losses and ultrasound size measurements of Girolando female bovine embryos. The study was performed at the Campo Experimental de Santa Mônica – Embrapa Gado de Leite, Valença (RJ), between January and April 2018 (CEUA/EGL – 3956180316). Girolando recipients (n=92) aged 3 to 6 years with body condition score 4 were treated with hormones for estrous synchronization and received fixed-time embryo transfer (FTET) at D7 post-ovulation. Girolando ¾ grade 1 blastocysts (according to International Embryo Technology Society - IETS standards) *in vitro* produced using sexed sorted semen were used. Positive pregnancy diagnosis was performed 24 days after ET (considered as the D31 of gestation) if the visualization of embryonic vesicle in a B-mode ultrasound examination was performed. A Mindray DP2200 with linear transducer at a 7.5 MHz frequency was used. Non-pregnant animals (n = 57) were excluded from subsequent analyzes. We compared the measurements of Embryonic Vesicle Diameter (EVD), Crown Rump Length (CRL) and Biparietal Diameter (BPD) of embryos that completed gestational development (Control group) (n=30) and of embryos whose gestation was lost up to 90 days (Pregnancy Loss group) (n=5). The 35 pregnant animals were followed up by ultrasonography every 6 days up to D90 or until the fetal heart beat ceased. Among the five gestational losses, only one occurred between D43 and D49. The other four occurred between 60 and 90 days of pregnancy. Measurements of EVD and CRL were performed at D37, D43, D49 and D55 of gestation, while those of BPD, at D43, D49 and D55. The results were analyzed by ANOVA repeated measurements. Significance level of 5% was adopted. The results showed higher EVD in the Control group (37.51 ± 3.56 mm) compared to the Pregnancy Loss group (32.92 ± 2.84 mm) at D55 and no difference between the groups was detected at D37 (14.81 ± 2.09 mm vs. 12.30 ± 1.65 mm); D43 (21.22 ± 2.32 mm vs. 21.48 ± 3.46 mm); and D49 (29.36 ± 2.35 mm vs. 27.79 ± 2.92 mm). The CRL measurements showed higher sizes in the Control group at D37 (16.17 ± 1.65 mm vs. 13.14 ± 1.73 mm) and D55 (46.13 ± 2.74 mm vs. 41.88 ± 5.47 mm). No difference was detected at D43 (23.11 ± 1.72 mm vs. 22.20 ± 2.46 mm) and at D49 (31.94 ± 1.78 mm vs. 30.97 ± 0.74 mm). The BPD measurements did not show any differences between the groups at evaluated moments. These findings indicate that decreased embryo/fetal growth during the first two months of pregnancy may suggest pregnancy loss. We suggest the measurement of fetuses and vesicles at D55 and routine assessment of pregnancies with EVD less than 34 mm and fetuses smaller than 43 mm in this period. Acknowledgements to Coordenação de Pessoal de Nível Superior – CAPES (Financial Code 001) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG (CVZ APQ 00972/16).

A150 Embryology, developmental biology and physiology of reproduction

Influences of *in vitro* mimicking of estrus cycle phases on gene expression profiles of bovine oviduct epithelial cells obtained from pre-ovulatory or mid-luteal phase

Patricia Kubo Fontes[1], Bart M Gadella[2,3], Heiko H W Henning[4], Helena T A van Tol[2], Tom A E Stout[4], Mário Binelli[5], Anthony César de Souza Castilho[6]

[1]UNESP - Department of Pharmacology, Institute of Biosciences, São Paulo State University, Botucatu, São Paulo, Brazil; [2]UU - Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [3]UU - Department of Biochemistry and Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [4]UU - Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [5]UF - Department of Animal Sciences, University of Florida, Gainesville, United States of America; [6]UNOESTE - University of Western São Paulo, Presidente Prudente, São Paulo, Brazil.

Estradiol (E2) and progesterone (P4) play key roles on morphological and functional changes of the bovine oviduct epithelial cells (BOEC) *in vivo*. However, few *in vitro* culture systems have been described to reproduce the functional changes on BOEC. Therefore, the aims of the present study were to mimic the estrus cycle phases on a 3D *in vitro* culture system of BOEC derived from pre-ovulatory or mid-luteal phases and verify the expression of genes encoding steroids receptors and proteins related to fertilization. For that, bovine oviducts were collected at a slaughterhouse (n=4 cows for each phase). BOECs from ampulla were collected separately from each animal and cultured in trans-well inserts (3D system) that allows the cell polarization. BOECs from pre-ovulatory or mid-luteal phases were culture for 14 days within four treatments, i) LUT: mimic of the luteal phase [P4 (100 ng/mL) and E2 (75 pg/mL) for 14 days]; ii) LUT_FOL: mimic of luteal phase followed by a follicular phase [luteal phase for 11 days, one transition day (low P4 and low E2), and E2 (300 pg/mL) and P4 (10 ng/mL) for two days]; iii) LUT_FOL_OverE2: mimic of luteal phase followed by a follicular phase with over high E2 levels [luteal phase for 11 days, one transition day, and E2 (600 pg/mL) and P4 (10 ng/mL) for two days], or iv) CONTROL: vehicle of E2/P4 dilution (0.5% ethanol). The relative mRNA abundance of genes related to fertilization (*OVGP1, HSPA5, FUCA1*, and *FUCA2*) and steroid receptors (*ESR1, ESR2*, and *PGR*) were detected by RT-qPCR. The results were normalized with the geometric mean of the two best reference genes (*18S* and *RLP15*). ANOVA followed by the Tukey-Kramer test was used to assess effects of treatments on BOEC culture using $P < 0.05$ as significance level. The effect of treatments was analyzed separately in BOEC from cows at pre-ovulatory or mid-luteal phases. In BOEC from cows at pre-ovulatory, a higher abundance of *OVGP1* was detected in LUT_FOL and LUT_FOL_OverE2 compared with CONTROL and LUT groups, whereas in BOEC from cows at mid-luteal phase, LUT group presented lower *OVGP1* levels compared with CONTROL. In BOEC collected at pre-ovulatory, the *ESR1* abundance was lower in LUT, LUT_FOL, and LUT_FOL_OverE2 compared with CONTROL, whereas in BOEC collected at mid-luteal phase there was no treatment effect on *ESR1* abundance. *FUCA1* and *FUCA2* showed higher abundance in LUT, LUT_FOL, and LUT_FOL_OverE2 compared with CONTROL group in BOECs from both pre-ovulatory and mid-luteal phases. No effect was observed on *ESR2, HSPA5,* and *PGR.* In conclusion, E2 and P4 modulate the gene expression of *OVGP1*, *FUCA1*, *FUCA2*, and *ESR1* during polarized culture system of BOEC. Moreover, this modulation is driven differentially by BOECs from pre-ovulatory and mid-luteal phases, thus it is an important bias to be considered during primary cell culture. To elucidate this, the global gene expression is ongoing in the same samples. Supported by FAPESP (#16/25685-4, 17/13481-8, 18/06674-7).

A151 Embryology, developmental biology and physiology of reproduction

Conceptus-derived products in circulation during early pregnancy in cattle receiving parthenogenetics vs normal embryos

Gessica Franco[1], Gabriela Melo[3,1], Sydney Reese[1], Veronica Negron-Perez[2], Claire Timlin[2], Webb Fields[1], Kyungjun Uh[2], Kiho Lee[2], Vitor Mercadante[2], Ky Pohler[1]

[1]TAMU - Department of Animal Science, Texas A&M University, College Station, Texas; [2]VT - Department of Animal and Poultry Science, Virginia Tech (Blacksburg, Virginia); [3]USP - Departamento de Reproducao Animal, Universidade de Sao Paulo, Pirassununga, Sao Paulo, Brasil.

Establishment of pregnancy in cattle involves regulated interactions between maternal and paternal genetics to obtain proper conceptus development. In rodents, parthenogenetic embryos are known to have well-developed embryo proper but poor placenta proliferation, but limited information is available in cattle. We hypothesized that recipients receiving parthenotes will have decreased interferon-stimulated genes (ISG) expression and decreased circulating pregnancy-associated glycoprotein (PAG) concentration. This experiment aimed to determine differences in pregnancy establishment and conceptus-derived products in parthenogenetic embryos (PA) compared with biparental embryos (CON). Parthenote embryos were produced in vitro using a validated chemical activation method and control biparental embryos produced in vitro using industry standard techniques. Cows (n=30) were synchronized and embryos transferred 7 days after estrus onset (day 0). Experiment was divided in three replicates of embryo transfer, with cows on the PA group receiving 2-3 embryos per round, and CON cows receiving 1 embryo. Blood samples were collected on days 7, 15 and daily from days 21 to 40 for peripheral blood leukocytes and serum. Transrectal ultrasonography was performed daily to monitor conceptus development. Trizol (Invitrogen, Carlsbad, USA) was used to extract RNA from buffy coats, cDNA was synthesized and RT-PCR performed to determine relative expression of ISG15, MX2, OAS1 and PPIA (house-keeping). An in-house ELISA was used to measure serum PAG concentration. Dependent variables (ISG expression levels and PAG) were analyzed using PROC GLM (SAS 9.4) to test difference among groups. As expected, the PA group had decreased pregnancy rate at day 30 (13%, 2/15) compared to CON (33%, 3/9) and pregnancy was maintained up to day 40 of gestation in both groups. Fold change on day 22 over the baseline was decreased in PA for both OAS1 (7.0 vs 2.4, P=0.02) and ISG15 (13.2 vs 4.2, P=0.07) compared to CON embryos, but no difference was observed for MX2. Circulating PAGs increased from days 24 forward in both groups, but the PA group had reduced concentrations (0.81±0.44 vs 5.23±0.44; P=0.01) at day 32 of gestation. These results indicate that parthenogenetic embryos can establish pregnancy in cattle; however, there are significant decreases in conceptus-derived products in circulation. Overall, these findings suggest that this is a suitable model to investigate parental versus maternal contributions to placental development in cattle. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2017-67015-26457 from USDA NIFA.

A152 Embryology, developmental biology and physiology of reproduction

The modulation of the cholesterol biosynthesis pathway impacts metabolism, viability and cryotolerance of *in vitro* produced embryos

Kelly Annes[1,2], Gianluigi Zullo[2], Giuseppe Albero[2], Valentina Longobardi[2], Nunzia Pagano[2], Susan Costantini[3], Alfredo Budillon[3], Mateus José Sudano[1], Bianca Gasparrini[2], Marcella Pecora Milazzotto[1]

[1]UFABC - Universidade Federal do ABC, Santo André, São Paulo, Brazil; [2] UNINA - Università Degli Studi di Napoli "Federico II", Naples, Italy; [3]IRCCS - Istituto Nazionale per lo Studio e la Cura dei Tumori, Mercogliano, Italy.

Phospholipids and cholesterol are the main constituents of membranes and their distribution pattern and abundance may affect the membrane fluidity, permeability and thermal phase behavior, characteristics that are fundamental in cryopreservation. In this study, we modulate the cholesterol biosynthesis pathway (CBP) throughout the *in vitro* embryo development and characterize its impact on embryo viability. Oocytes were in vitro matured, fertilized and, at the timing of *in vitro* culture (SOFaa supplemented with 8% FBS at low oxygen tension) zygotes were divided in 3 groups: C - control, I – inhibition of CBP by simvastatin (20 μM) and S – stimulation of CBP by IGF-1 (100ng/mL). Blastocysts were collected at day 7 and submitted to lipid quantification [Sudan Black B (n=10-20 per group)], nuclear fragmentation and total cell number [TUNEL (n=50 per group)], apoptosis [Caspase-3 (n=50 per group)], cryotolerance [vitrification (n=100 per group)] and lipid profile [NMR (n=3 per group]. Lipid content of the culture media was also evaluated by NMR. Cleavage and blastocyst rates, cytoplasmic lipid droplet content, TUNEL and Caspase were analyzed by ANOVA and Tukey post test. NMR data were submitted to partial least square discriminant analysis (PLS-DA) by using MetaboAnalyst2.0 and survival, development and hatching rates post warming were compared by Chi square Test. Inhibition of CBP had deleterious effects on embryo development and quality since cleavage and blastocyst rates were lower in I group and percentage of nuclear fragmentation and total lipid content were increased when compared to C and S ($P<0.05$). On the other hand, embryos derived from S group presented less apoptotic cells, evidenced by lower nuclear fragmentation and caspase-3 activity when compared to C and I. Modulation of CBP had influence on the overall lipid metabolism, since all lipid classes presented higher relative abundance in blastocysts from C group, except for ômega-3 that were increased in S and I groups and cholesterol that decreased on I group, as expected ($P<0.05$). The lipidome analysis of the spent culture media revealed that the relative abundance of fatty acids and cholesterol were lower and omega-3 and triglycerides were higher on S group ($P<0.05$) when compared to C and I. These changes in cellular viability as well as lipid metabolism led to a diminished survival rate after cryotopreservation for I group ($P<0.05$). Data from pregnancy rates after embryo transfer are still being collected. These data allow us to conclude that the CBP is crucial for the maintenance of different cellular functions. Inhibition of this pathway results in diminished embryo quality and survival after cryopreservation, while the stimuli of CBP improve cellular functions, however without impact cryopreservation (FAPESP: 2018/01965-3, 2016/05986-0, 2017/18384-0).

A153 Embryology, developmental biology and physiology of reproduction

Small extracellular vesicles from follicular fluid modulate EIF4E in cumulus cells during bovine oocyte *in vitro* maturation

Ana Clara Faquineli Cavalcante Mendes de Ávila[1], Alexandre Bastien[2], Claude Robert[2], Felipe Perecin[1], Flávio Vieira Meirelles[1], Juliano Coelho da Silveira[1]

[1]FZEA-USP - Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga, SP; [2]INAF - Université Laval - Centre de recherche en biologie de la reproduction, Institut sur la nutrition et les aliments fonctionnels, Québec, Canada.

Small extracellular vesicles (EVs) are particles secreted by cells that carry bioactive molecules. Small EVs are found in follicular fluid and can be related to oocyte maturation. The objective of this study was to quantify RNA in zona pellucida (ZP, Exp.1) and to evaluate transcripts related to RNA transport pathway in cumulus cells (Exp.2) after small EVs supplementation during bovine oocyte *in vitro* maturation (IVM). For Exp.1 we collected slaughterhouse ovaries and aspirated small follicles (3-6 mm) to obtain follicular fluid and cumulus-oocyte complex (COCs). Follicular fluid was centrifuged twice (120,000 xg, 70 min) to pellet small EVs, which were diluted in maturation medium. COCs were matured in 100 µL drops of maturation medium with or without small EVs. After 0 (immature), 2 and 4 h of IVM, 10 COCs per group were labeled with SYTO RNA Select (ThermoFisher; 0.05nM), a selective dye to probe RNA molecules, during 30 min at 37°C and denuded oocytes were fixed in PFA 4% during 15 min. A total of 21 slices for each oocyte were imaged, with an interval of 1 µm each, using a confocal microscope (Zeiss LSM 700) at 40x/1.2 objective. RNA molecules localized in ZP were counted using ImageJ. For Exp.2 slaughterhouse ovaries were collected in pairs and classified in early or late estrus cycle stage according to corpus luteum morphology. COCs were *in vitro* maturated with small EVs from follicular fluid of different estrus cycle stage (n=20 COCs/group) or without EVs (control). After 24 h of IVM cumulus cells were collected and RNA extraction (miRNeasy Mini Kit; QIAGEN), reverse transcription (High Capacity; ThermoFisher) and quantitative RT-PCR (Power SYBR Green; Applied Biosystems) were performed. We analyzed eight transcripts related to RNA transport pathway, which were normalized by geometric mean of two reference genes (*PPIA* and *YWAHZ).* Six replicates were realized for each experiment. Statistical analysis was performed using SAS by ANOVA following Tukey's test at a significance level of 5%. Results from Exp.1 showed that COCs matured with small EVs increased RNA mean at 2 and 4 h of IVM compared to immature COCs ($p<0.05$). Also, COCs matured without small EVs increased RNA mean at 4 h of IVM compared to immature COCs ($p<0.05$). Results from Exp.2 demonstrated that small EVs from late estrous cycle increased *EIF4E* in cumulus cells, a translation initiator factor, comparing to control group. In conclusion, results showed that RNA molecules increase in the ZP during IVM overtime independent of EVs supplementation. However, small EVs from late estrous cycle increase *EIF4E* levels in cumulus cells, suggesting a role for EVs modulating protein translation. Based on our findings, small EVs did not increase the quantity of RNA in ZP but could modulate RNA transcripts in cumulus cells. Further experiments are necessary to determine the role of this mechanism in oocyte quality (Funding: FAPESP grant 2014/22887-0; 2017/02037-0; 2018/14869-2).

A154 Embryology, developmental biology and physiology of reproduction

Morphometry of reproductive system in Dorper Sheep submitted to 3 feeding plans in pre-puberty

Gabriela Azenha Milani Soriano[1], Felipe Rydygier de Ruediger[1], Marilice Zundt[1], Thiago Martins[3], Ines Cristina Giometti[1], Luís Eduardo Ribeiro Junior[1], Isabela de Almeida Cipriano[1], Claudia Bertan Membrive[2], Caliê Castilho[1]

[1]Unoeste - Universidade do Oeste Paulista, Presidente Prudente, SP; [2]UNESP - Universidade Estadual Paulista, Dracena, SP; [3]UF - University of Florida, Gainesville, FL, USA.

Nutritional status is the main factor that influences the animal's ability to reproduce. The onset of puberty and the maintenance of reproductive function are physiologically linked to nutrition and body condition. Therefore, the aim of the present study was to evaluate the influence of three feeding planes on the morphometry of reproductive system in pre pubertal Dorper sheep. 24 lambs (7/8 Dorper), aged between 6 and 7 months, were randomly assigned to 1 of 3 groups: G1 (70-80% of the requirement of the National Research Council [NRC]), G2 (100-110% [NRC]) and G3 (140% [NRC]). The ewes of G1 (n=8) and G2 (n=8) were maintained on pasture of Panicum maximum cv. Tanzania with access to water and mineral salt ad libitum, and only those in the G2 group received 1.5% of the live weight of commercial 2x daily feed. G3 ewes (n = 8) were confined during the experimental period, receiving a total diet in the following proportions: concentrate of 20:80, 16% CP and 72% NDT, aiming at daily gain of 200g/day according to NRC, being the mineral salt ad libitum. Initially the sheep received 3.5% of the live weight of the total diet (hay + ration), and this percentage increased until reaching an average of 4.5 to 5% of the live weight. Upon reaching body weight of 35 kg, sheep were synchronized by insertion of a vaginal progesterone delivery device (CIDR®, Pfizer, Brazil) for 12 days. On the day of implant withdrawal, 0.075 mg of cloprostenol (Veteglan®, HertapeCalier, Brazil) and 300 IU of equine chorionic gonadotrophin (eCG, Novormon®, MSD Saúde Animal, Brazil) were administered intramuscularly. Eight days after CIDR removal, all animals were slaughtered and the reproductive tract removed for weighing and morphometry. The number of antral follicles was measured. The data were analyzed by ANOVA using the MIXED procedure (SAS, version 9.4). The number of cervix rings were analyzed using non-parametric Kruskal-Wallis test. The number of cervical rings was greater ($p < 0.05$) in G1 (7.66 ± 0.21) compared to G2 (6.57 ± 0.29) and G3 (5.8 ± 0.20). G2 had a greater number of rings than G3. The width of the uterine horn was greater in G1 (2.63 ± 0.15 cm) than in G3 (1.86 ± 0.16 cm) independent of the side of the horn (ipsilateral or contralateral). The length vs width of the ovary of G1 (1.38 ± 0.05 cm) was greater than G3 (1.17 ± 0.04 cm). The corpus luteum weight did not differ between G1 (0.46 ± 0.1 g), G2 (0.42 ± 0.09 g) and G3 (0.6 ± 0.10 g). The number of antral follicles (12.35 ± 1.91 / 15.12 ± 1.78 / 13.22 ± 1.71) did not differ between groups. Based on the results obtained we can conclude that the food restriction does not negatively affect the size and weight of the reproductive organs of Dorper ewe lambs.

A155 Embryology, developmental biology and physiology of reproduction

Effect of protein source during *in vitro* maturation on the development and sex of *in vitro* produced bovine embryos

Nayara R. Kussano[1,3], Ligiane O. Leme[2,3], Margot A.N. Dode[3,1]

[1]UnB - Universidade de Brasília, Brasília, DF, Brazil; [2]UFES - Universidade Federal do Espírito Santo, Alegre, ES, Brazil; [3]Embrapa Recursos Genéticos e Biotecnologia - Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil.

Several studies have shown that the use of different protein sources (PS) such as FBS and BSA during the IVC of bovine embryos affect quality and embryonic development (Nedambale et al., Theriogenology, 62:437-449, 2004). The most affected parameters are, blastocyst rates, total cells number, percentage of apoptotic cells, metabolism, cryotolerance and gene expression. Considering that most of the studies focus on the effect of PS during IVC, the present study aimed to evaluate whether changes in PS during IVM would affect blastocyst rate and sex of bovine embryos. In order to do this, two experiments were carried out. In the first one, COCs obtained from slaughterhouse ovaries were distributed in 2 groups: IVM-FBS (COCs matured in the presence of 10% FBS); and, IVM-BSA group (COCs matured with 0,4% BSA), after this, both groups were fertilized and cultured in the presence of FBS up to day 8 of development. The second experiment was similar to the first one (IVM-FBS and IVM-BSA groups), differing only in fertilization and culture until D8 that were performed in the presence of BSA instead. In both experiments the embryo rate, the development kinetics and the sex of the embryos were evaluated. Sexing was performed in 30 blastocysts / group by PCR. Data of the blastocyst rate and embryo sex were analyzed using chi-square test ($p < 0.05$). The results showed that when IVC was performed in the presence of FBS, the PS during IVM affected ($p < 0.05$) the blastocyst rate in D8 (IVM-FBS = 64.8% n = 105; IVM-BSA = 50.5% n = 105). When embryos were cultured in the presence of BSA, no effect ($p > 0.05$) of PS during IVM was observed at the blastocyst rate at D8 (IVM-FBS = 41.1% n = 350, IVM-BSA = 37.3 % n = 346). The PS used during IVM did not affect the sex of the embryos ($p > 0.05$), regardless of whether the culture medium was supplemented with FBS, IVM FBS (Male 53.33%, Female 46.66% n = 30) and IVM BSA (Male 56.66%, Female 43.33% n = 30) or with BSA, IVM FBS (Male 46.66%, Female 53.33% n = 30) or IVM BSA (Male 56.66%, Female 43.33% n = 30). It can be concluded that the PS during IVM does not influence the sex of the embryos and only affects the embryonic development if the culture is carried out in the presence of FBS.

A204 Cloning, transgenesis and stem cells

Generation of myostatin knock-out cow embryos using crispr/cas9-assisted gene editing and somatic cell nuclear transfer

Juan Baston[1], Tomas Fanti[1], Lucia Moro[2], Victoria Arnold[1], Mariana Suvá[1], Carlos Luzzani[2], Martín Olguín[1], Santiago Miriuka[2], Diego Viale[3], Gabriel Vichera[1]

[1]KHEIRON - Biotech S.A., Buenos Aires, Argentina; [2]LIAN - FLENI, Buenos Aires, Argentina; [3]UNSAM - Universidad Nacional de San Martín, Buenos Aires, Argentina.

Myostatin (MSTN) is a member of the transforming growth factor-β superfamily that inhibits muscle growth. In the European cattle breeds Charolais, Marchigiana and Maine Anjou, natural mutations on the exon 2 of MSTN gene result in greater muscle mass than other cattle breeds. Previous studies on MSTN knock-out (KO) embryos have shown that blocking the MSTN gene expression causes double muscle phenotype, increasing bovine commercial value. In this study we aimed to disrupt the MSTN gene in bovine fetal fibroblasts using CRISPR-Cas9 nuclease and generate cloned embryos with the modified genotype. First, we evaluated four different single guide RNA (sgRNAs), targeting exon 1 (sgRNA-1), exon 2 (sgRNA-2) or exon 3 (sgRNA-3 and sgRNA-4) of the *Bos taurus* MSTN gene. Experimentally, two sets of $5x10^4$ bovine fetal fibroblasts were nucleofected with 500 ng of the plasmid hspCas9-2A-PuroV2.0 which encodes for Cas9 nuclease, one of the above mentioned sgRNAs and Puromycin resistance. The two sets of nucleofected cells were then cultured in the same well of a 12 multi-well plate for 48 h and then treated with 3 µg/ml of Puromycin for another 48 h to select those cells that incorporated the plasmid. Afterwards, we isolated the genomic DNA from the Puromycin-surviving cells and amplified the sequence of the MSTN gene targeted by each sgRNA by PCR to be further sequenced by Sanger method. Sequencing results were evaluated with ICE-Synthego software to determine de gene edition efficiency. The percentage of modified sequences was analyzed with respect to the control sample. Different edition efficiencies were obtained for each sgRNA: sgRNA-1: 6 %, sgRNA-2: 96 %, sgRNA-3: 15 % and sgRNA-4: 5 %. In view of these results, the sgRNA-2-edited cells were used for embryo production by somatic cell nuclear transfer (SCNT). Embryo development for the MSTN-KO group and the wild-type control group was 67% (n=122/182) vs. 70% (n=98/139) cleavage, and 7.7% (n=14/182)^a vs. 15.8% (n=22/139)^b blastocysts ($p<0.05$ Chi-Squared test), respectively. Ten blastocysts were individually genotyped by PCR amplification of MSTN-exon 2 and further Sanger sequencing. The results of the analysed blastocysts were: 100% bi-allelic mutations (4/10 *homozygous* and 6/10 *heterozygous)*. In conclusion, we produced cloned bovine embryos edited on exon 2 of the MSTN gene by CRISPR-Cas9 with a very high efficiency using sgRNA-2. Although the bovine embryos generated with MSTN-edited cells showed a lower blastocyst rate than control fibroblasts of the same cell line, it was probably due to the cellular stress caused by the nucleofection/puromycin treatments and higher cell culture passages. Our future main goal is to achieve the birth of healthy calves with double-muscle phenotype. This represents a step forward towards the production of animals with increased commercial value.

A205 Cloning, transgenesis and stem cells

Successful generation of induced pluripotent stem cells (eiPS) derived from skin fibroblasts of an aged equine

Raquel Vasconcelos Guimarães de Castro[1,3], Naira Caroline Godoy Pieri[2,3], Ramon Botigelli[4,3], Bianca Moutinho Grizendi[3], Renata Gebara Sampaio Dória[3], Paulo Fantinato-Neto[5], Joaquim Mansano Garcia[1], Fabiana Fernandes Bressan[3]

[1]FCAV/UNESP - Faculty of Agricultural and Veterinary Sciences/Department of Preventive Veterinary Medicine and Animal Reproduction, Jaboticabal, SP; [2]FMVZ/USP - School of Veterinary Medicine and Animal Science/Department of Animal Reproduction, Pirassununga, SP; [3]FZEA/USP - Faculty of Animal Science and Food Engineering/Department of Veterinary Medicine, Pirassununga; [4]IBB/UNESP - Institute of Biosciences/Department of Pharmacology, São Palulo State University, Botucatu, SP; [5]CRV Lagoa - Central Bela Vista - CRV Lagoa, Botucatu, SP, Brasil.

Cellular aging is a limitation in cellular reprogramming since it is associated with cell senescence. As the cell ages, an upregulation of pathways such as p53, $p16^{INK4A}$, and $p21^{CIPI}$ occurs leading to cell cycle arrest along with alterations in cell morphology and metabolism. Considering the difficulty on reprogramming of aged cells, the objective of the work was to achieve reprogramming into pluripotency of a more than 20 years old horse. Therefore, a skin fragment was collected from the dorsal lateral metacarpophalangeal region, taken to the lab and fibroblasts were recovered after a 3 hours digestion period with Collagenase IV (#C2674 Sigma Aldrich). The fibroblasts were then seeded in a 6 well plate ($2x10^4$ cells per well) and the lentiviral vector STEMCCA containing the human sequences of OCT4, SOX2, KLF4, and c-MYC was used for transduction. Six days after transduction cells were seeded in mouse embryonic fibroblast (MEF) layer ($4,75x10^4$ cells per well). The reprogramming efficiency was calculated by dividing the number of formed colonies by the number of seeded cells. The eiPS colonies were evaluated regarding their morphology and detection of alkaline phosphatase, immunocytochemistry for Oct4 (#sc8628, Santa Cruz), Sox2 (#ab97958, Abcam), Nanog (#ab21624, Abcam), SSEA-1 (MAB 4301, Millipore), TRA-1-60 (Mab 4360, Millipore) and TRA-1-81 (Mab 4381, Millipore). The transcript levels were determined by RT-qPCR, for pluripotency genes OCT4, REX-1, NANOG, and SOX2. Therefore, the cycle threshold (Ct) values of the target genes were normalized by the average of Ct values of the housekeeping genes (HPRT1 and PPIA) and the fold changes were then calculated using the $2^{(-\Delta CT)}$ equation. After 16 days of the transduction, colonies were visualized, being primarily identified by their typical morphology: tightly packed cells with a high nuclear/cytoplasm ratio. The efficiency of the reprogramming process was 0,059% (28 colonies from $4,75x10^4$ seeded cells). Colonies were positive for alkaline phosphatase at passages 4 and 12. Immunocytochemistry revealed that cells were found to be positive for OCT4, NANOG, SSEA-1, and TRA-1-81. Cells showed endogenous expression of the pluripotency genes OCT4 (0,3670 ± 0,1032, n=3), REX-1 (0,0391 ± 0,0005, n=3), NANOG (0,1421 ± 0,2903, n=3) and SOX2 (0,0034 ± 0,0020, n=3), being the Ct values all minor than 31,8, using specific equine primers. Herein we conclude that although age is considered as a great barrier to the reprogramming of somatic cells, it was possible to achieve successful reprogramming in an animal in advanced age in our conditions. Financial Support: FAPESP (2018/04009-6; 2015/26818-5) and CAPES.

A206 Cloning, transgenesis and stem cells

Transfection of swine oocyte with polyethyleneimine (PEI): a low cost and convenient method to produce genetically modified swine

Andressa Pereira de Souza[2,3], José Rodrigo Pandolfi[1], Emanuelle Coldebella[3], Shaiana Salete Maciag[3], Francisco Noé da Fonseca[1], Carlos André da Veiga Lima Rosa[2], Mariana Groke Marques[1]

[1]Embrapa Suínos e Aves - Embrapa Suínos e Aves, Concórdia, SC, Brasil; [2]UDESC/CAV - Universidade do Estado de Santa Catarina, Lages, SC, Brasil; [3]IFC - Concordia - Instituto Federal Catarinense - Campos Concordia, Concórdia, SC, Brasil.

Oocytes are excellent candidates to produce genetically modified pigs due to their physiology and absence of nuclear envelope, which favors the incorporation of DNA. However, the presence of the zona pellucida and the sensitivity to stressors make transfection a challenge, since the techniques available are labor-intensive and expensive. Therefore, the objective of this work was to develop a protocol for transfection of porcine oocytes using a cationic polymer, polyethyleneimine (PEI). Thus, the branched PEI 25 KDa (100mL, Sigma Aldrich, Saint Louis, USA) was used. Oocyte maturation and in vitro embryo production procedures were performed according to Marques *et al.*, 2011 (Zygote, 19: 331-337). The data (mean minimum squares ± SE) were evaluated using PROC MIXED (SAS®) with 5% significance. In the 1st experiment, the ability of PEI to overcome the zona pellucida and the cytoplasmic membrane of oocytes matured in vitro was evaluated. For that, PEI was labeled with FITC, and oocytes were incubated (30 min) with 4 concentrations of PEI-FITC (10, 20; 40 and 80 μg/mL). The internalization of the PEI-FITC was evaluated by fluorescence microscopy and the pixel quantification performed using the software Image J 1.40g®. It was observed that all concentrations of PEI were able to reach the cytoplasm. The internalization rate was significant ($p<0.001$) and concentration dependent, and the concentration 10 μg/mL resulted in the lowest internalization as the concentration of 80 μg/mL provided the highest one ($19.60\pm0.25x10^3$ and $22.69\pm0.23x10^3$ pixels, respectively). In the 2nd experiment, transfection rates were evaluated using two preparations containing PEI (20 or 80 μg/mL) complexed with the pmhyGENIE-5 vector at 2 N/P ratio, and then incubated with oocytes matured in vitro. Incubations with the respective vector concentration were also performed in the absence of PEI (INC20 and INC80) and a Control group. After 30 min of incubation, the oocytes were fertilized and cultured in vitro until day 7 of development. No effect of the treatments on the cleavage rates ($p=0.8307$) and blastocysts ($p=0.9780$) were observed. The cleavage rates ranges from 41.19±10.55% to 54.83±7.46% and the blastocyst rates from 16.96±7.81% to 23.68±7.81%. Besides, only the PEI20 group presented blastocytes with GFP expression (3.2±1.91%). The data suggest that PEI, unlike other transfectant agents, has the ability to pass the zona pellucida, and the protocol described herein is capable of producing transgenic blastocysts expressing GFP, so that it could be used as a cheap and easy tool for transfection of swine oocytes.

A207 Cloning, transgenesis and stem cells

Induced pluripotent stem cells (iPSCs) derived from urine progenitor cells in the swine: a novel non-invasive method for regenerative medicine

Kaiana Recchia[1], Lucas Simões Machado[1], Ramon Botigelli[2], Naira Caroline Godoy Pieri[3], Flavio Vieira Meirelles[1,4], Simone Maria Martins Kitamura Martins[3], Fabiana Fernandes Bressan[1,4]

[1]USP - FMVZ - Department of Surgery, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, Butantã, São Paulo, SP; [2]UNESP - São Paulo State University, Institute of Biosciences, Department of Pharmacology, Botucatu, SP; [3]USP - FMVZ - Swine Research Center, Faculty of Veterinary Medicine and Animal Sciences, University of São Paulo, Butantã, São Paulo, SP; [4]USP - FZEA - Department of Veterinary Medicine, Faculty of Animal Sciences and Food Engineering, University of São Paulo, Pirassununga, SP, Brasil.

The swine model is of special interest as a biomedical model due to its immunological and physiological similarity with the human model, and non-invasive collection of cells for iPSCs generation would facilitate its use. Herein we aimed to derive urine progenitor cells (UPCs) *in vitro* cultures from urine samples, still unpublished for species other than human, and to reprogram them *in vitro* into pluripotency. For that, urine samples (approximately 250ml) were collected from three females. Isolation and culture were performed following human UPCs protocol (Steichen et al., 2017). Briefly, the urine was centrifuged at 300 x g, the pellet washed in DPBS (Sigma), resuspended and cultured in 45% DMEM high glucose (Life Technologies), 5% FBS, 50% REBM media (renal epithelial basal media, Lonza) supplemented with 1% glutamine, 1% MEM neaa, 1% penicillin/streptomycin (all Life Technologies) and REGM supplements : hEGF, Insulin, Hydrocortisone, GA-1000, FBS, Transferrin, Triiodothyronine, Epinephrine (Lonza) and 10ng/mL bFGF (Peprotech). After approximately one week, epithelial-like cells were observed in colonies. The cells from one female were submitted to transduction of murine OSKM (OCT4, SOX2, KLF4 and C-MYC - STEMCCA lentiviral vector, Millipore). After 4-5 days, cells were plated onto MEFs and cultured in KnockOut DMEM/F12, 20% KnockOut Serum Replacement, MEM neaa, L-Glutamine, 2-Mercaptoethanol and penicillin/streptomycin (all Life Technologies) supplemented with 10ng/ml bFGF (Peprotech). At approximately 12 days after transduction, colonies presenting typical pluripotent morphology were observed and evaluated regarding efficiency of colony formation and alkaline phosphatase detection. Three clonal lineages (C1, C4 and C6) were further maintained *in vitro* and characterized regarding pluripotency markers for more than 20 passages. The overall reprogramming efficiency observed was 8,455% (percentage of colonies observed in relation to the number of transduced cells plated). All three colonies were positive for alkaline phosphatase in passages 22, 21 and 21, respectively. Immunocytochemistry analysis revealed that C6 was positive for the pluripotency markers OCT4 (1: 100, cat # SC8628), SOX2 (1: 500, cat # ab97959; Abcam), SSEA1 (1: 50, cat # SC21702, Santa Cruz), TRA1- 81, and NANOG (1: 100 # catab77095, Abcam), meanwhile the colonies C1 and C4 were positive only for OCT4 and SOX2. In conclusion, it was possible herein to reprogram cells derived from urine samples into iPSCs that were maintained in culture *in vitro* for at least 20 passages. Further analysis is still needed to prove the complete reprogramming of these cells; however, these results already open a new possibility to generate models of *in vitro* diseases from a non-invasive source in an unprecedented way.

Financial Support: FAPESP (2019/02811-2, 2015/26818-5), CNPq 433133/2018-0 and CAPES.

A208 Cloning, transgenesis and stem cells

Isolation, culture and in vitro proliferation of canine mesenchymal stem cells derived from adipose tissue

Patrícia Campos Paolozzi, Jéssica Cristina Stefanutto, Danieli Aparecida Bóbbo Moreski, Márcia Aparecida Andreazzi, Fábio Luiz Bim Cavalieri, Vinicius Eduardo Gargaro Silva, Isabele Picada Emanuelli

UniCesumar - Centro Universitário de Maringá, Maringá, PR, Brasil.

Stem cells (SC) are undifferentiated cells that can be used in various pathologies promoting the healing of chronic patients. The use of adipose surgical waste is a good alternative for the extraction of adipose mesenchymal stem cells (AMSCs) and with few bioethical implications. With differentiation potential similar to that of bone marrow SC, AMSCs are more efficient in terms of ease of collection, abundance of tissue extracted and rate of expansion in vitro. The objective of this study is to describe and validate a simplified method of isolating and primary culture of SC extracted from surgical fat adipose tissue of dogs. Samples of abdominal subcutaneous adipose tissue were collected from surgical dogs rests (n = 4, sample 4.5g each) transported in PBS + amikacin solution (75µg / mL). The AMSCs isolation method consisted of sample washing in the PBS solution, mechanical maceration (clamp and scalpel) and subsequent enzymatic digestion in a conical tube containing 7.5 mL of PBS solution with trypsin (1000U / mL), vortex homogenized (1 min) and incubated at 38.5 ° C for 30 minutes. They were then filtered through filters (75 microns), and centrifuged (10 min at 3000 RPM). The supernatant was discarded and the pellet was resuspended in 1 mL of culture medium customized in the laboratory, called TCM-cell. The TCM-cell medium consists of TCM-199 Earle Salts with bicarbonate (Gibco®) plus 75 µg / ml amikacin, 0.2 mM pyruvate, 5% FBS, 20 µl / ml essential amino acids and 10 µl / ml of non-essential amino acids (both from SIGMA®) and centrifuged again for 10 minutes (6200 RPM). The pellet was resuspended and cells cultured in plate (60mm) containing the TCM-cell medium (38.5 ° C, 5% CO2 atmosphere in air). The TCM-cell medium was renewed every 72 to 96 hours. Cell growth and expansion were monitored under inverted microscopy. The cultures were evaluated according to the minimum criteria for characterization of TCM of the International Society of Cell Therapy (ISCT): to present fibroblastoid format, to adhere to plastic substrate and auto renew. All samples (n = 4) presented adherence to the plastic within the initial 24 hours and presence of colonies with fibroblastoid morphology in 96 hours. The cell confluence in the plates was reached in the 13th day in all samples, in which the first passage was performed and part of the samples were cryopreserved for later characterization study. The primary culture of TCM extracted from surgical abdominal fat adipose tissue in dogs met the minimum criteria for ISCT characterization presenting fibroblastoid format, adhesion to plastic and auto renew, important evaluative aspects in the first stage of TCM characterization. The expansion occurred progressively, being observed already in the third day of culture indicating that the simplified methodology in customized environment was satisfactory for the isolation, cultivation and expansion in vitro.

A215 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Histone post-translational modifications H3K9me3 and H3K27me3 roles in the kinetics of the first cleavages: causes or consequences?

Jessica Ispada[1,2], Otávio Luiz Ramos Santos[1], Aldcejam Martins Fonseca Junior[1], Camila Bruna de Lima[1,2], Kelly Annes[1], Erika Cristina dos Santos[1], James L Chitwood[3], Pablo Juan Ross[3], Marcella Pecora Milazzotto[3]

[1]UFABC - Universidade Federal do ABC, Santo André; [2]USP - Universidade de São Paulo, São Paulo; [3]UC - University of California Davis, Davis.

The timing of the first cleavages is a tool used for selection of human embryos. The same approach has been adapted for cattle and studies identified that the kinetics during the first cleavages generates blastocysts with different gene expression patterns and DNA methylation levels. These differences might be associated with epigenetic changes. Based on that, we investigated the dynamics of histone H3 lysine 9 trimethylation (H3K9me3) and lysine 27 trimethylation (H3K27me3) and transcripts of factors involved in the control of these epigenetic marks in fast and slow developing embryos. For this, bovine embryos were produced in vitro and, at 40 hours post insemination (hpi), were classified as Fast (4 or more cells) or Slow (2-3 cells) and collected at 40hpi (FCL and SCL), 96hpi (FGA and SGA) or 168hpi (FBL and SBL). For H3K9me3 and H3K27me3, embryos (35-62 nuclei at 40hpi, 67-97 at 96hpi and 402-467 at 168hpi from 7-15 embryos from 3 replicates and 21-33 nuclei at 40hpi, 57-83 at 96hpi and 147-286 at 168hpi from 5-9 embryos from 3 replicates, respectively) were immunostained with specific antibodies. The fluorescence intensity of each nucleus was quantified using ImageJ and analyzed by Student's t-test or one-way ANOVA. For transcript quantitation, RNAseq data was accessed from a previous report using the same kinetics classification model (Milazzotto et al, 83(4):324-36 2016). In this study, H3K9me3 increased in fast and slow embryos in the transition from 96 to 168hpi (SGA 8,8±0,3 AU vs FGA 6,7±0,5 AU; FBL 17,8±0,3 AU vs SBL 14,1±0,2 AU). Also, higher levels of H3K9me3 were present at 40hpi in fast embryos (FCL 15,7±0,8 AU vs SCL 9,9±0,9 AU), whereas slow embryos presented its higher intensity during the genome activation stage ($P<0.0001$). Gene expression analysis of proteins involved with H3K9me3 revealed overexpression of KDM3A and KDM7A at 40hpi and SETDB1, SETDB2, SUV39H1, KDM1A, KDM1B, KDM3A and KDM3C at 168hpi in slow embryos. No differences in H3K27me3 fluorescence was observed at 40hpi (FCL 4,8 ±0,8 AU vs SCL 4,0±0,8 AU; P=0,89). At 96hpi, fast embryos presented higher H3K27me3 intensity than slow embryos (FGA 7,8±0,5 AU vs SGA 5,4±0,4 AU) and, in both kinetics groups, the level increased from 96hpi to blastocyst stage, with higher levels detected in SBL than in FBL (FBL 11,2±0,6 AU vs SBL 21,4±1,0 AU; $P <0.0001$). At 40hpi, slow embryos presented higher expression of KDM7A and KDM3A and less of EZH1 transcripts and at blastocyst stage, higher expression of KDM3A and EZH2 and lower expression of KDM6B were observed in slow embryos. It is possible to conclude that the kinetics of the first cleavages is related with histone post-translational modifications in embryos and, apparently, the difference of H3K9me3 levels between fast and slow embryos appears since the first cleavages, probably being inherited from gametes, whereas H3K27me3 became different between groups only at the genome activation stage. Acknowledgements: FAPESP2017/18384-0

A216 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Using pregnancy-associated glycoproteins to provide early pregnancy diagnosis in cattle

Fabio Girardi Frigoni[1], Mariana Dulce Delle Vedove Ortolan Sayeg[1], Luiz Fernando Rodrigues Féres[2], Fernando José Delai Pardo[3], Mariana Pallú Viziack[1], Carlos Alberto Rodrigues[4], Pietro Sampaio Baruselli[1]

[1]FMVZ/USP - Universidade de São Paulo - Faculdade de Medicina Veterinária e Zootecnia, São Paulo, SP; [2]Fazendas do Basa - Fazendas do Basa, Leopoldina, MG; [3]Idexx - Idexx, Itaim Bibi, São Paulo, SP; [4]Agrindus - Fazenda Santa Rita, Descalvado, SP, Brasil.

The objective of this study was to evaluate the viability of PAG (pregnancy-associated glycoprotein) ELISA kit to provide early pregnancy diagnosis. Two experiments were done. Experiment 1: 150 bovine females (78 Gir and 72 crossbreeds) were synchronized with 1.9g progesterone (P4) device (CIDR® Zoetis, Brazil) and 2mg of estradiol Benzoate (BE) (RicBE®, Tecnopec, Brazil) on Day 0. On Day 8, the P4 device was removed, and all animals received 0.5mg Sodic Cloprostenol (Ciosin®, MSD, Brazil), 1mg of estradiol cypionate (CE; ECP®, Zoetis, Brazil) and 300 IU of eCG (Folligon®, MSD). The embryo transfer (ET) was done 9 days after the P4 device removal. Blood samples were collected from all animals on day 19 (n=150) and on day 21 post-ET (n=125), corresponding to 26 and 28 days of gestation. Ultrasound (US) examinations were performed on day 19 post-ET, using a 7.5 MHz linear array transducer. Observation of allantoic fluid or a visible fetus yielded a positive gestation diagnosis. At US, 46.7% (70/150) cows were pregnant and 53.3% (80/150) were not pregnant. Results were classified as positive diagnosis (vp), false positive diagnosis (fp), negative diagnosis (vn), and false negative diagnosis (fn). Sensitivity [100 vp/(vp+fn)], specificity [100 × vn/(vn+fp)], positive predictive value (PPV) [100 × vp/(vp+fp)], negative predictive value (NPV) [100 × vn/(fn+vn)} and accuracy [(vp+vn)/(vp+vn+fp+fn)] were calculated for each PAG test (26 and 28). Statistical were done using PROC FREQ of SAS 9.4 version. Nemar's test compared sensibility and specificity of PAG 26 and PAG 28. US results were used as a gold standard. The PAG26 test yielded 98.7% of sensitivity, 92.0% of specificity, 92.5% of PPV, 98.6% of NVP and 95.3% of accuracy. The PAG28 test reached 100% of sensitivity, 89.6% of specificity, 89.2% of PPV, 100% of NVP and 94.4% of accuracy. No statistical differences were found in sensibility and specificity (P=1.0) between PAG 26 and PAG 28. Experiment 2: 195 Holstein heifers were synchronized with 3mg norgestomet auricular implant (Crestar® MSD, Brazil) and 2mg BE (Sincrodiol®, OuroFino, Brazil) on Day 0. On Day 8, the implant was removed, and 0.5mg i.m Sodic Cloprostenol (Ciosin®, MSD, Brazil), 1mg CE (Sincroecp®, OuroFino, Brazil) and 300IU eCG (Folligon®, MSD) were administrated. FTAI was performed on Day 10 as well 0.1mg Gonadorelin (Fertagyl®, MSD, Brazil) was administrated. Blood samples were collected on day 26 post-FTAI (n=195) and US examinations were done. The PAG ELISA 26 post-FTAI reached 94.2% of sensitivity, 96.0% of specificity, 92.9% of PPV, 96.8% of NVP and 95.4% of accuracy. In conclusion, no differences were found in the efficiency of pregnancy diagnoses comparing PAG 26 and PAG 28 in the ET program. Furthermore, the PAG 26 presents elevated efficiency for early pregnancy diagnosis in the AI program. Therefore, the use of PAG ELISA on 26 days of gestation can be an alternative to early pregnancy diagnose in the AI and ET programs.

A217 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Toxicity of dimethylsulphoxide in cryopreservation of bovine ovarian tissue

Camila Bizarro-Silva[1,2], Marcela Bortoletto Cerezetti[2], Camila Bortoliero Costa[2], Rafael Luiz Stolf[2], Maysa Lopes Orsi[2], Sofia Botsaris Delchiaro[2], Anne Yaguinuma de Lima[2], Higor Souza de Camargo[2], Marcelo Marcondes Seneda[2]

[1]PUCPR - Escola de Ciências da Vida, Pontifícia Universidade Católica do Paraná, Toledo, PR, Brasil; [2]UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

The objective of this study was to use histology for evaluating the toxicity of three different concentrations (1M, 1.5M, and 3M) of dimethylsulfoxide (DMSO) before and during vitrification of bovine ovarian tissue. The ovarian cortex of *Bos taurus indicus* (n=10) was fragmented (6 mm^3, N=7 per animal) and randomly distributed between the control (non-vitrified) and three groups with different concentrations of DMSO: i) 1M; ii) 1.5M or iii) 3M. Before the cryopreservation, a test was performed to assess the toxicity of DMSO to ovarian tissue without vitrification. To achieve this, the fragments were exposed for 20 min at 20°C in 1.8 mL of essential minimal medium (MEM) containing DMSO at the same three concentrations and then fixed in Bouin for 24h for subsequent histologic evaluation. For vitrification, fragments were placed in cryotubes containing 1.8 mL of MEM plus one of three concentrations of DMSO at 4°C for 15 min. The fragments were then exposed to the same concentrations of DMSO supplemented with 0.25 M sucrose and 10% fetal bovine serum for the period of 15 min at 4°C. Subsequently, the ovarian tissues were dried and placed in contact with the metal cube surface partially immersed in liquid nitrogen for vitrification during 30 seconds. Once vitrified, the sample was stored at -196°C for 7 days. After warming, fragments were then fixed in Bouin and processed for classical histology with Schiff Periodic Acid and Hematoxylin staining. The analyzes were performed considering morphological aspects (integrity and follicular degeneration) before and after cryopreservation. The data were submitted to ANOVA and Tukey's test, considering $P \leq 0.05$. The control contained largely intact follicles and a small portion of degenerate follicles, according to physiological patterns. In the histological analysis during the toxicity test all concentrations of DMSO presented a higher level of degenerated follicles, comparing to the control (1M: 28.3% (210/741); 1.5M: 49.8% (252/506); 3M: 65.7% (349/531) and control: 18.7% (80/428); $P<0.05$). The concentration of 3M DMSO during the toxicity test had an injurious effect the integrity on follicles (34.4% (182/531); $P<0.05$) when compared to the other concentrations of DMSO. After vitrification/warming, the fragments in 1M, 1.5M, and 3M DMSO showed similar percentages of intact follicles (1M: 62.0% (345/557); 1.5M: 55.2% (266/482) e 3M: 33.8% (127/375; $P>0.05$). However, 1M of DMSO resulted in percentages of degenerate follicles statistically equivalent to control (38.0% (212/557) and 18.7%, respectively; $P<0.05$; 1.5M: 44.8% (216/482) and 3M: 66.1% (248/375). In addition, 3M of DMSO was more related to follicle degeneration (66.1%; $P<0.05$) in comparison to other groups. Therefore, it is suggested that the concentration of 1M DMSO showed to be less toxic to the preantral follicles of bovines after vitrification and warming of the ovarian tissue.

A218 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Oxidative stress during the cryopreservation of ovarian bovine tissue with dimethylsulfoxide

Camila Bizarro-Silva[1,2], Andressa Guidugli Lindquist[2], Suellen Miguez González[2], Larissa Zamparone Bergamo[2], Denis Vinicius Bonato[2], Ana Carolina Rossaneis[2], Waldiceu Aparecido Verri Junior[2], Marcelo Marcondes Seneda[2]

[1]PUCPR - Escola de Ciências da Vida, Pontifícia Universidade Católica do Paraná, Toledo, PR, Brasil; [2]UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

The objective of this study was to evaluate the oxidative stress caused to bovine ovarian tissue after vitrification with three different concentrations (1M, 1.5M and 3M) of dimethyl sulfoxide (DMSO). The ovaries from *Bos taurus indicus* cows (n = 10) were collected from a local abattoir, fragmented (6 mm^3; n = 4 per animal) and being placed in a control treatment (placed directly on the liquid nitrogen) and three vitrified groups: i) 1M of DMSO; ii) 1.5M of DMSO and iii) 3M of DMSO. For vitrification, the fragments were placed in 2.0 mL cryotubes and submitted to equilibration solution and transferred to the vitrification solution. Thus, the ovarian fragments were exposed to 1.8 mL of Minimum Essential Medium (MEM) containing one of the following DMSO concentrations (1M, 1.5M and 3M) for 15 min at 4°C. After the equilibration, the fragments were transferred to 1.8 mL in MEM with the same concentrations of DMSO, supplemented with 0.25 M sucrose and 10% fetal bovine serum for 15 min at 4°C. Follicles from all fragments (n = 40) were analyzed for oxidative stress to evaluate the presence of reactive oxygen species and antioxidant capacity after vitrification. Samples of cryopreserved ovarian tissue (n=30) were heated and the cryoprotectant removed for the execution of the kinetic-colorimetric assays for determination of superoxide anion production in homogenates of tissue (10 mg /ml in 1.15% KCl) using the modified nitroblue tetrazolium (NBT) assay. The reduction of NBT was measured at 600 nm (Multiskan GO, Thermo Scientific) and tissue weight was used for normalization of the data. The ability of the sample to resist oxidative damage was determined by the neutralization of free radicals (ABTS) by cation sequestration of 2,2 V-azinobis (3-ethylbenzothiazoline-6-sulfonate). For the statistical analysis of the oxidative stress assays, the data were submitted to ANOVA and Tukey's test, considering a value of $P \leq 0.05$. It was observed that the vitrified samples in 1M and 1.5M DMSO presented similar superoxide anion levels when compared to the control (12.05 in 1M; 15.05 in 1.5M, 26.20 in 3M of DMSO and 16.21 OD/mg protein in the non-vitrified control, $P > 0.05$). For the antioxidant capacity of the fragments submitted to vitrification with 1M DMSO presented similar results with the control (151.01 in 1M, 184.35 in 1.5M; 246.98 in 3M of DMSO and 168.77 equivalent of the Trolox/mg protein in the non-vitrified control, $P > 0.05$). Conversely, vitrified ovarian tissue samples analyzed for the identification of reactive oxygen species presented reduction in superoxide anion levels and antioxidant capacity at 1M and 1.5M DMSO concentration when compared to 3M ($P < 0.05$). It is therefore suggested the use of 1M DMSO for vitrification of ovarian tissue because it presents lower levels of reactive oxygen species and a higher antioxidant capacity.

A219 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Metanalyses of RNA-seq data reveal crucial role of lncRNAs during bovine early embryo development

Ricardo Perecin Nociti[1,2,3], Rafael Vilar Sampaio[2], Carlos A O Biagi Júnior[4], Wilson A Silva Jr[4], Vera Fernanda Martins Hossepian de Lima[1], Pablo Juan Ross[3], Flavio Vieira Meirelles[2]

[1]FCAV-UNESP - Sao Paulo State University, Jaboticabal, São Paulo, SP, Brasil; [2]FZEA-USP - University of Sao Paulo, Pirassununga, SP, Brasil; [3]UC-DAVIS - University of California Davis, Davis, CA; [4]FMRP-USP - University of Sao Paulo, Ribeirão Preto, SP, Brasil.

Long non-coding RNAs (lncRNAs) are a relative new class of non-coding RNAs involved with gene regulation in a stage and tissue specific way. However, little is known about their role during the transcriptional events during bovine embryo development. The bovine genome (Bos_taurus.ARS-UCD1.2) has 1480 annotated lncRNAs and 23515 putative lncRNAs (NONCODE). We hypothesized that RNA-seq public data from the NCBI GEO (Gene Expression Omnibus) could be combined to explore the lncRNAs transcriptional changes through embryo development. Furthermore, we used 9 experiments (GSE52415, GSE61717, GSE25082, GSE74675, GSE44023, GSE48147, GSE56513, GSE85563, SRR7757966), a total of 115 samples from gametes (sperm and oocytes) up to day 19 (D19) of development and all development stages were used, and each stage was contrasted with the previous and with the following stage. We used SRA Toolkit to download data and to convert, from SRA format to fastq format; fastQC for quality check; Trim Galore for read trimming; multiQC for report compiling. For read alignment and gene count we used STAR. We used R for all statistical analysis. For differential gene expression we used DESEQ2 using p adjusted2, for enrichment and co-expression analyses we used the clVALID and ClusterProfiler packages respectively. Our results showed that 6457 transcripts were differently expressed among the contrasts evaluated. The blastocyst stage showed the greatest differences among the stages analyzed. We identified 62 transcripts with the highest expression level during maternal to zygote transition stage, that are enriching the DNA replication, modulation, alkylation, methylation, and demethylation pathways. We also found 33 transcripts that had lower expression levels only during the blastocyst stage, enriching the lamelipodium pathway. A total of 189 transcripts had the highest mean levels of expression during the embryo-maternal recognition period enriching the pathways of lipid metabolism, cholesterol efflux, organization to external stimuli, and cell migration. We also identified 11 lncRNAs clusters, in which we detected 1284 lncRNAs broadly expressed. On the remaining cluster we found 121, 7, 15, 40 and 6 lncRNAs that exhibited the highest expression level during GV to 4 Cells stages; at embryonic genome activation period; at sperm and from Blastocyst to D19; from the embryonic genome activation until D19 and only at blastocysts stage, respectively. We conclude that public bovine embryo sequencing data can be used and combined, been a great information source of bovine embryo development. Furthermore, our analysis suggests a crucial role of lncRNAs during maternal to zygote transition and blastocyst formation and development.

Acknowledgments: CNPQ - INCT em Células-Tronco e Terapia Celular no Cancer (465539/2014-9) and FAPESP- CEPID/CTC - Centro de Terapia Celular (2013/08135-2).

A220 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Embryonic Culture Supplementation (ECS) – a new culture system based on oviduct and uterus fluid composition improves bovine embryo production and quality

Erika Cristina dos Santos[1], Aldcejam Martins da Fonseca Junior[1], Camila Bruna de Lima[1], Jessica Ispada[1], Kelly Annes[1], João Vitor Alcantara da Silva[2], Marcella Pecora Milazzotto[1]

[1]UFABC - Universidade Federal do ABC, Santo André, SP, Brasil); [2]UMC - Universidade Mogi das Cruzes, Mogi das Cruzes, SP, Brasil.

The systems of *in vitro* production (IVP) of bovine embryos aim to mimic the female reproductive tract in order to generate blastocysts more similar to those produced *in vivo*. However, the amount and availability of nutrients in a dynamic system, such as the *in vivo,* may be greater than that required for a static *in vitro* system. In fact, in rats, the embryo development in systems with reduced amounts of nutrients increased blastocyst quality and rates. To verify that, we developed a sequential culture media – *Embryonic Culture Supplementation (ECS)* – based on the salt-based composition of SOF (Synthetic Oviduct Fluid) and supplemented with the amount of energy substrates and amino acids present in the composition of bovine oviduct (*ECS1-100*) and uterus (*ECS2-100*) fluids. We also supplemented *ECS* with half of the concentration found in these biological fluids (*ECS1-50* and *ECS2-50*) to verify if the reduction of such substrates could be beneficial to embryo development and quality. All media used in this experiment were supplemented with 8mg/mL of Bovine Serum Albumin (BSA) and antibiotics. Embryos were produced *in vitro* by using conventional protocols and after fertilization they were transferred to one of the following groups: *Control* (C) - SOFaa: supplemented with 2% essential amino acids, 1% nonessential amino acids, 1.5 mM glucose), *ECS100* – zygotes cultured in ECS1-100 or *ECS50* – zygotes cultured in ECS1-50. At Day 4 (D4), cleavage rates were assessed and embryos were transferred to their correspondent group (Control, ECS2-100 and ECS2-50) where they remained until blastocyst (D7). Blastocysts were assessed by means of rates, energy metabolism (mitochondrial activity, NADH, FAD and ATP production) and oxidative stress (generation of ROS). All data were analyzed by ANOVA and Student's T-test (n=128 blastocyst/16 replicates). Embryo production was positively affected by the reduction of energy substrates and amino acids in culture (Cleavage rates – C = 75.9 ± 2.1; ECS50 = 81.6 ± 1.4; ECS100 = 76.8 ± 3.3, p= 0.03/ Blastocyst rates – C = 34.0 ± 1.4; ECS50 = 41.1 ± 1.8; ECS100 = 37.3 ± 1.9, p= 0.03). Blastocysts from ECS 50 presented reduction of ROS comparing to control group (p= 0.03) and increase of mitochondrial activity (p= 0.01) and NADH production (p= 0.01) when comparing with ECS 100. Mitochondrial activity was also higher in control group (p= 0.04) comparing with ECS 100. No differences were found among groups related to FAD and ATP production. Based on these results, we can conclude that ECS is not only able to support embryo development to blastocyst, but the reduction in energy substrates and amino acids concentration (ECS 50) seems to be beneficial for embryo production and blastocyst quality. Acknowledgment: FAPESP 2016/00350-0 and FAPESP 2017/18384-0.

A221 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Applied use of interferon-tau stimulated genes expression to detect pregnancy status in Nelore cattle submitted to timed-AI

Gabriela Dalmaso de Melo, Barbara Piffero Mello, Catia Aparecida Ferreira, Cecilia Constantino Rocha, Amanda Guimarães da Silva, Leonardo Marin Ferreira Pinto, Carlos Alberto Souto Godoy Filho, Leonardo Amaral, Igor Garcia Motta, Gilmar Arantes Ataide Junior, Ed Hoffmann Madureira, Guilherme Pugliesi

USP - University of São Paulo, Pirassununga, SP.

This study aimed to evaluate the use of interferon tau stimulated genes (ISGs) expression in peripheral blood polymorphonuclear cells (PMN) associated with color Doppler ultrasonography (Doppler-US) to detect pregnancy 20 days after timed-AI (TAI) in beef cattle. Nelore cows (n=144) and heifers (n=103) were submitted to TAI (D0). On D20, PMNs were isolated from jugular blood by Ficoll gradient (GE Healthcare), and the RNA was extracted. Expression of target genes (ISG15 and OAS1) was quantified by qPCR and normalized to reference genes (GAPDH and ACTB). Pregnancy diagnoses were performed on D20 by luteolysis detection with Doppler-US, and on D30 and D70 by detection of embryo and fetus with heartbeat, respectively. Animals were classified as: pregnant (P; fetus on D70), non-P (NP; no active CL on D20), early embryo loss (EEL; active CL on D20 but NP on D30) and late embryo loss (LEL; embryo on D30 but NP on D70). ISG expression was analyzed by ANOVA using PROC MIXED of SAS considering the effects of group, category and their interaction. ROC curves were created and the area under the curve (AUC), accuracy (Ac), specificity (Sp) and sensitivity (Se) were calculated for pregnancy predictions on D20 (ISGs and Doppler-US) compared to the standard diagnosis method on D30. Expression of ISG15 and OAS1 were greater ($P<0.01$) in the P (2.0±0.2 and 2.4±0.2, respectively) compared to the NP (0.5±0.1 for both) and EEL (0.9±0.2 and 0.8±0.2, respectively) groups but did not differ from the LEL group (1.6±0.8 and 1.2±0.5, respectively). A greater ($P<0.01$) ISG15 expression was also observed in heifers than cows (fold change: 1.55). ROC analysis indicated that ISG15 and OAS1 were significant ($P<0.01$) predictors of pregnancy in heifers (AUC= 0.81 and 0.86, respectively) and cows (AUC= 0.77 and 0.82, respectively). The optimal cutoff value for ISG15 was 4-fold increased in heifers than cows; whereas for OAS1, the same cutoff value was determined for both categories. Doppler-US presented a higher Ac (89% for heifers and 93% for cows) compared to ISG15 (80% for heifers and 72% for cows) and OAS1 (81% for heifers and 75% for cows). Doppler-US, ISG15 and OAS1 methods resulted, respectively, in Sp of 76%, 82% and 65% in heifers, and 83%, 70% and 75% in cows, and Se of 100%, 78% and 74% in heifers, and 100%, 78% and 75% in cows. When both genes were evaluated only in females with an active CL on D20, the Ac was improved for OAS1 in heifers (91%) and cows (82%) due to reduction of false positive results. In conclusion, heifers have greater ISG15 expression than cows, which resulted in different cutoff values for both categories. ISG15 and OAS1 expression were increased in P than NP and EEL animals, suggesting they are good predictors of pregnancy. However, ISG expression (mainly OAS1) can only be used to reduce false positive results of EEL females, as both genes presented lower Se and Ac compared to Doppler-US method.
Acknowledgements: FAPESP (2015/106069; 2017/134729)

A222 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Can we can predict estrus in Gir (*Bos indicus*) heifers using behavior monitor device system?

Mariana Dulce Delle Vedove Ortolan Sayeg[1], Luiz Fernando Rodrigues Féres[3], Luísa Helena Bartocci Liboni[4], Jessica Cavanelas Fernandes[1], Leonardo Salgado Maia[2], Diego Marcondes Guerra[2], Fabio Girardi Frigoni[1,2], Augusto Rodrigues Felisbino Neto[1], Laís Ângelo de Abreu[1], Marcos Henrique Alcantara Colli[1], Pietro Sampaio Baruselli[1]

[1]USP - Universidade de São Paulo, Butantã, São Paulo, SP; [2]CRV Lagoa - CRV Lagoa, Sertãozinho, SP; [3]Faz Basa - Fazendas do Basa, Leopoldina, MG; [4]IFSP - Instituto Federal de Educação, Ciência e Tecnologia de São Paulo, Jardim Canaa, Sertãozinho, SP, Brasil.

Heat detection is one of the greatest challenges in dairy farms all over Brazil. Technologies like Ovalert device can be used in reproductive management to identify which cows are in heat, increasing service and pregnancy rates and improving the financial performance of the farm. The goal of this study was to evaluate the efficiency of heat detection by the electronic device Ovalert (CRV Lagoa, Brazil) in Gir (*Bos indicus*) heifers based on data of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy. For that, 54 Gir heifers received the Ovalert collar at least 8 days before the beginning of the experiment, then they were synchronized with 1,9g progesterone device (Cidr ® Zoetis, Brazil) and 2mg of Estradiol Benzoate (RicBE®, Tecnopec, Brazil) on day 0 of the protocol. On day 8, the progesterone device was removed, all animals received an Estrotect® device, 0,5mg i.m Sodic Cloprostenol (Ciosin®, MSD, São Paulo, Brazil), 1mg of estradiol cypionate (ECP®, Zoetis, Brazil) and 300 IU of eCG (Folligon®, MSD). Estrotect was used as a control to determinate true estrus and fake estrus on the Ovalert system and they were evaluated twice a day to determinate the presence of the estrus. The heat in Ovalert system was determined by the warning generated by the increase in animal activity. Ultrasound exam was made on day 18 to determine the presence of corpus luteum (ovulation rate). Statistical analyses were performed using WEKA Software. The ovulation rate was 79.6% (43/54). Cows were considered in estrus when Estrotect was positive. Cows with Estrotect positive and Ovalert negative was considered false negative. All cows had positive Estrotect in this experiment (54/54) and 94.4% (51/54) was also positive in Ovalert, so the index of false negative was 5.5% (3/54). In this study, no false positive were found (0% for false positive index). The sensibility, that is the capacity of Ovalert determinate the estrus in the animals that are really in estrus (confirmed by estrotect) was 94.4%. The specificity, that is the capacity of the system to determinate not heat in cows that are not in heat, was unable to determinate because all cows were in heat. Positive predictive value (the probability that the cow with a positive result in Ovalert is actually in heat and the accuracy (probability of the test providing correct results) are also 94.4%. The negative predictive value was 0 because of the absence of false negative animals. In conclusion, indices reveal high sensitivity of the test and high accuracy. In this way, despite the lack of negative individuals, the indexes reveal that the heat can be predicted using a behavior monitor device system in Gir (*Bos taurus indicus*) heifers.

A223 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Development and validation of a low-cost ELISA methodology for bovine insulin quantification

Guilherme de Paula Nogueira, Marco Antonio Maioli, Ana Flavia Tereza Paiva, Waneska Stéfani Spinelli Frizzarini, Devani Mariano Pinheiro

FMVA-UNESP - Faculdade de Medicina Veterinária - UNESP, Araçatuba, SP, Brasil.

The reproductive processes are dependent on numerous variables, including the animal's metabolic status. Monitoring parameters, such as the quantification of metabolic hormones can be decisive in research to improve reproductive efficiency. The aim of this work was to develop and validate a low cost and specific ELISA for insulin quantification in cattle plasma. First, bovine insulin was processed for zinc removal according to Sodoyez et al. (J. Biol. Chem, 250: 4268-77, 1975) and subsequently conjugated to biotin according to Maioli et al. (Vet. Bras., 37: 1545-53, 2017). Anti-insulin antibody production was performed in guinea pigs by intradermally injection of zinc-free bovine insulin emulsified in Freund's adjuvant, complete on the first immunization and incomplete thereafter (every 28 days). The antiserum was obtained by cardiac puncture at 42, 63 and 100 days of treatment. For the assays, the antibody dilution and biotinylated hormone concentration were determined in bidimensional tests, using microwell plates coated with Goat anti-Guinea-Pig IgG (0.25 µg/well), blocked with 1% BSA, as in Kekow et al. (Diabetes, 37: 321-26, 1988), using tetramethylbenzidine as a chromogen. Insulin was quantified in samples from 5 lactating Holstein cows submitted to the glucose tolerance test (Adamiak et al., Biol. Reprod., 73: 918-26, 2005) for biological validation and the results were expressed in µUI/ml. Assay precision was determined by the coefficients of variation (CV) in assay from high (CA) and low (CB) insulin concentrations controls, and the limits of detection (LOD) and quantification (LOQ) were determined according to Shrivastava et al. (Chron, Young Sci., 2: 21-25, 2011). For determination of these parameters, 689 samples were quantified into 20 assays. Basal insulin concentration of the tested animals was close to 20 µIU/mL. Five minutes after intravenous glucose infusion there was a peak in the hormone concentration (136 ± 11.71 µUI/mL), which decreased around 120 min, reaching the basal values. Mean insulin concentration for CA and CB was 121.75 and 26.02 µUI/mL, respectively, with intra-assay CV being 7.1% (CA) and 7.59% (CB) and the inter-assay CVs were 13.92% (CA) and 15.09% (CB). The LOD was 1.57 µUI/mL and the LOQ was 4.76 µUI / mL. In conclusions, the methodology was considered fully developed and can be applied for quantification of bovine insulin with a calculated cost of U$1.00 per sample.

A224 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Relative gene expression of esr1 and esr2 in the uterine horns of bitches along diestrus

Vanessa da Silva Alves Gossler[1], Francislaine Anelize Garcia Santos[2], Aline de Oliveira Santos[1], Ana Paula Mattoso Miskulin Cardoso[3], Lilian Francisco Arantes de Souza[1], Natalia Caroline de Oliveira Marçal[1], Lauren Chrys Soato Marin Schaffer[1], Paula de Carvalho Papa[2], Ines Cristina Giometti[1]

[1]Unoeste - Universidade do Oeste Paulista, Presidente Prudente, SP; [2]USP - Universidade de São Paulo, São Paulo, SP; [3]Unesp - Universidade Estadual Paulista "Júlio de Mesquita Filho", Presidente Prudente, SP, Brasil.

The diestrus stage in the bitch is characterized by the increase of Progesterone (P4) in the first half of diestrus and by fluctuations of Estradiol (E2) in the second half of diestrus. E2 has higher concentrations in the middle of the diesturs around day 40, declining later (Papa and Hoffmann, Reproduction in Domestic Animals, 46: 750-756, 2011). The effects mediated by E2 depend on the binding of E2 to the estrogen receptors. In general, ERα (whose gene is ESR1) promotes cell proliferation and ERβ (ESR2) appears to play an antiproliferation role (Vivar et al., J Biol Chem, 285: 22059-22066, 2010). The objective of this study was to determine the gene expression of ESR1 and ESR2 in the uterus of bitches during diestrus (days 10, 20, 30, 40 and 50 post-ovulation). For this, the uterine horns of 25 clinically healthy bitches of different ages and with no defined racial pattern were used. The uterus was obtained from dogs that underwent ovariohysterectomy (OSH) at 10, 20, 30, 40 and 50 days after ovulation, thus constituting the five groups studied in this experiment (n = 5 / group). The uterine horns were harvested and stored in TRIzol® (Thermo Fisher Scientific, California, USA) in the freezer at -80 ° C and thereafter the relative abundance of mRNA of ESR1 and ESR2 were evaluated by RT-qPCR, using GAPDH as reference gene. The data were evaluated using the Kruskal-Wallis test followed by Student Newman ($p < 0.05$). ESR2 presented higher relative gene expression ($p < 0.05$) at 10, 20 and 30 days after ovulation when compared to 40 and 50 days. There was no significant statistical difference for the relative gene expression of ESR1 in the studied groups. It is concluded that only the gene expression of ESR2 undergoes alteration during diestrus, decreasing after day 30 post-ovulation, and may have been a consequence of the incremental increase of E2. Support: FAPESP (process number: 2014/00739-9) and CAPES - Financing Code 001.

A225 Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Modulation of mitochondrial acetyl-coa production impacts histone acetylation levels of *in vitro* produced bovine blastocysts

Aldcejam Martins da Fonseca Junior[1], Erika Cristina dos Santos[1], Jessica Ispada[1], Camila Bruna de Lima[1], João Vitor Alcantara da Silva[2], Kelly Annes[1], Marcella Pecora Milazzotto[1]

[1]UFABC - Universidade Federal do ABC, Santo André, SP; [2]UMC - Universidade de Mogi das Cruzes, Mogi das Cruzes, SP, Brasil.

Glycolysis is a metabolic pathway that converts glucose into pyruvate that can be driven to the tricarboxylic acid cycle (TCA) by an intermediate step in which it is converted to acetyl-CoA. The TCA cycle is responsible for the production of citrate that can be exported from the mitochondria to the cytosol and also generate acetyl-CoA. Acetyl-CoA is an important regulatory factor for histone acetylation. Lysine 9 of histone H3 acetylation (H3K9ac) is one of the main epigenetic mechanisms responsible for the genome reprogramming after fertilization. We investigated the possible interaction between acetyl-CoA biosynthesis and the epigenetic control H3K9ac. Bovine embryos were *in vitro* cultured in the presence of the following modulators: dichloroacetate (DCA) – inhibitor of pyruvate dehydrogenase (PDH) phosphorylation, carrying more pyruvate to the mitochondria, increasing acetyl-CoA generation or iodoacetate (IA) - inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), diminishing the pyruvate synthesis which leads to a decrease in pyruvate/acetyl-CoA generation. Embryos were produced by conventional protocols and cultured in a SOF based culture media. At D4 of culture, corresponding to the major genome activation turn, embryos were transferred to new drops of media supplemented with the modulators: Control group – no supplementation; DCA1, DCA2, and DCA5 – supplementation with 1mM, 2mM or 5mM of DCA respectively; IA1, IA2, and IA5 – supplementation with 1uM, 2uM or 5uM of IA, respectively. Embryos were cultured until day 7 when collected and fixed for immunostaining of H3K9ac. Images were acquired by software (LAS, v.3, Leica, German) and the quantification of fluorescence intensity was measured by the mean value of pixels of each nucleus (ImageJ v.1.8.0 NIH, EUA). A minimum of 88 nuclei were analyzed per group. All values were submitted to ANOVA with Tukey or Kruskal-Wallis post-test with $P<0.05$ for fluorescent data as well as for embryo production. Blastocyst rates did not differ among groups for DCA (Control: 32.5%±7.9; DCA1: 37.7%±6.8; DCA2: 32.3%±8.0; DCA5: 21.6%±4.0; $P = 0.461$) and IA (Control: 24.65%±4.8; IA1: 40.03%±5.0; IA2: 43.13%±9.3; IA5: 24.80%±8.3; $P = 0.186$. DCA supplementation resulted in increased H3K9ac mark in all doses when compared to control (Control: 22.90±0,4AU; DCA1: 32.6±0.5AU; DCA2: 41.5±0.7AU; DCA5: 35.6±0.9AU; $P <0.0001$). IA1 and IA2 had a diminished H3K9ac mark but, interestingly IA5 did not present differences when compared to control (Control: 43.8±0.6AU; IA1: 34.7±0.5AU; IA2: 30.1±0.6AU; $P <0.0001$/ IA5: 41.2±1.4AU; $P =0.118$). In conclusion, the addition of modulators of acetyl-CoA generation allows blastocyst development in similar rates that the control. In addition, these changes in embryo metabolism directly influence the acetylation profiles for H3K9 in blastocysts, which can further affect cellular differentiation and potentiality, impacting the development. Acknowledgments: FAPESP 2017/18384-0.

A039E TAI/FTET/AI

Comparison of INRA96 and Andromed as an extender for alpaca epididymal spermatozoa

Essraa M. Al-Essawe[1,2], Celina Abraham[1], Panisara Kunkitti[1], Eva Axnér[1], Kerstin de Verdier[3], Renée Båge[1], Jane M. Morrell[1]

[1]SLU, Sweden; [2]Al-Nahrain University, Iraq; [3]National Veterinary Institute, Sweden.

Keywords*:* alpaca epieidymal sperm, Andromed, INRA96.

Breeding animals by artificial insemination rather than by natural mating has many advantages, for example, to prevent the spread of infectious disease and to allow males of superior genetic merit to produce offspring from a large number of females. However, the technique of artificial insemination is not well developed in alpacas for several reasons, one being the difficulty of working with the viscous ejaculate. Thus, it is difficult to develop protocols for semen handling, including choosing a semen extender. A first step in the development of such a protocol could be to use epididymal spermatozoa to test semen extenders. Two commercial semen extenders, Andromed (A; Minitüb; Tiefenbach, Germany) and INRA96 (I; IMV Technologies, L´Aigle, France), were chosen for this study. Neither of these extenders contains material of animal origin. Objective: to compare the two semen extenders for their suitability for alpaca epididymal spermatozoa. Materials and methods: scrotal contents were obtained from castration of males (n=10) for husbandry purposes. After removal from the animal, the organs were placed in a plastic bag containing phosphate buffered saline and were sent overnight to the laboratory at the Swedish University of Agricultural Sciences (SLU) in a Styrofoam box with a cold pack. This type of packaging is used to transport stallion semen and maintains the temperature at approximately 6 ºC overnight. The tunica vaginalis, connective tissues and blood vessels were removed; after isolating the cauda epididymis from the testis, it was placed in warm (37ºC) semen extender. From each animal, one cauda epididymis was placed in A and the other in I; several cuts were made in the epididymis to allow the contents to flow out. After incubation for 10 minutes at 37°C, sperm motility was measured by computer assisted sperm analysis (CASA; SpermVision, Minitüb), membrane integrity (MI) was assessed after staining with SYBR14/propidium iodide (Live-Dead Sperm Viability KIT LIT L-7011; Invitrogen, Eugene, OR, USA), and acrosome status was determined by staining with FITC-conjugated peanut agglutinin (Sigma, St. Louis, USA). The CASA analysis was repeated incubation for 30 minutes. Means were compared by mixed model using the SAS® software (version 9.3); significance was set to $P \leq 0.05$. Results: LSMEAN (± SEM) after 10 minutes for A and I, respectively, were as follows: total motility 19±5% vs. 21±5% (not significant, NS), MI 58±9% vs. 56±9% (NS); intact acrosomes 65±7% vs 54±7% (NS). Total motility in A and I after 30 minutes was 29±4% and 35±4% (NS), respectively. Progressive motility in I after incubation for 30 minutes was 12±4% compared to 25±4% after 10 minutes ($p<0.05$). However, progressive motility in A was not different between the two time points (11±4% vs. 17±4%, respectively). Conclusion: viable epididymal spermatozoa could be obtained from the material even after overnight transport. There were no differences between the two extenders in the sperm parameters evaluated. Therefore, either extender could be used for alpaca spermatozoa. And should be tested in an insemination trial.

Acknowledgements: Thanks to all veterinarians who contributed material. Funded by the Veterinary Faculty, SLU.

040E TAI/FTET/AI

Influence of parturition number of the recipient on an embryo transfer programme in wool type ewes

Sara Miranda-Hernández[1], Raymundo Rangel-Santos[1], Alfredo Lorenzo-Torres[1], Raymundo Rodríguez-De Lara[1], Demetrio A. Ambríz-García[2], María Del C. Navarro-Maldonado[2], Roberto Ramírez-López[3]

[1]Universidad Autónoma Chapingo, Estado de México, México; [2]Universidad Autónoma Metropolitana. Ciudad de México, México; [3]Rancho Poza Rica, Singuilucan, Hidalgo, México.

Keywords*:* ewes, ovulation rate, pregnancy.

Embryo transfer is important for the multiplication and rapid propagation of sheep breeds of high genetic merit. The selection of the most appropriate genotypes of recipient ewes is essential to obtain high pregnancy rates. The objective of this study was to evaluate the fertility of two groups of ewes (nulliparous and multiparous) exposed to an embryo transfer program. The study was conducted from January to February of 2019 at the commercial sheep farm "Poza Rica", which is located in a temperate area named Singuilucan, in central Mexico. A total of 142 healthy and good body condition Hampshire ewes were used, from which 97 were nulliparous and 45 multiparous. The ewes were synchronized with intravaginal sponges containing 20 mg of micronized cronolone (Chrono Gest, Intervet, Netherlands), which were inserted for 12 days. On day 10, the ewes were injected intramuscularly with 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Kathadin rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined by laparoscopy. The recipients received an embryo of transferable quality (compact morula or blastocyst) within 3 h after its collection, coming from Dorper donor ewes using a laparoscope and standardized procedures. The embryos were kept in holding medium (Syngro, Vetoquinol, Canada) were transferred using a capillary glass tube in the ipsilateral horn to the ovary in which ovulation was recorded, and the presence of the best quality corpus luteum was determined based on its size. On day 35, pregnancy diagnosis was carried out using an ultrasound machine and a 3.5 MHz transabdominal probe (Aloka Prosound 2, Japan). The results of the incidence of estrus and pregnancy rate were analyzed as categorical variables with the GENMOD procedure, and ovulation rate with the GLM procedure, both of them available in SAS. All the ewes were detected in estrus in both treatments. The incidence of estrus was different ($p<0.05$) for ewes from nulliparous and multiparous at 24 h (62.9 and 55.5%), 30 h (32.9 and 45.4%), and 36 h (4.1 and 0%) after sponge removal. Ovulation rate was higher ($p<0.05$) in multiparous than nulliparous ewes (2.11 ± 0.12 vs 1.76 ± 0.08). Moreover, the pregnancy rate was lower ($p<0.01$) in nulliparous than multiparous ewes (29.9 vs 68.9%). In conclusion, under the conditions of the study, the results showed the feasibility of using multiparous Hampshire ewes as embryo recipients.

A041E TAI/FTET/AI

Effect of age of the recipient on an Embryo Transfer programme

Raymundo Rangel-Santos[1], Guadalupe Morales-Añorve[1], Alfredo Lorenzo-Torres[1], Demetrio A. Ambriz-Garcia[2], Maria del C. Navarro-Maldonado[2], Roberto Ramirez-López[3]

[1]Universidad Autónoma Chapingo, Estado de México, México; [2]Universidad Autónoma Metropolitana. Ciudad de México, México; [3]Rancho Poza Rica, Singuilucan, Hidalgo, México.

Keywords*:* ewes, synchronization, embryo.

The reproductive technique of embryo transfer has been used to intensively reproduce high genetic merit animals of several species in different countries. The success of the technique relies on the control of every step to achieve high pregnancy rates. The objective of the study was to evaluate the fertility of two groups of ewes (nulliparous and multiparous) subjected to an embryo transfer program. The study was conducted from November to December of 2018 at the commercial sheep farm Rancho Poza Rica, which is situated in Singuilucan, Hidalgo, Mexico including a total of 46 ewes, from which 20 were nulliparous Katahdin ewes (T1) and 26 were multiparous Katahdin ewes (T2). The ewes were healthy, in good body condition (3.0) and were synchronized with intravaginal sponges containing 20 mg micronized cronolone (Chrono Gest, Intervet, Netherlands), inserted for 12 days. On day 10, the ewes were intramuscularly treated with 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Pelibuey rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined as a number of corpora lutea observed in ovaries during laparoscopy. The recipients received one tranferable compact morula or blastocyst within 2 h after embryo recovery from a Charollais donor ewe using laparoscopy. The embryo recovered into holding medium (Syngro, Vetoquinol, Canada) was transferred using a capillary glass tube in the ipsilateral horn to the ovary in which ovulation was recorded or the presence of the best quality corpus luteum was observed, determined on the basis of its size. On day 45, pregnancy diagnosis was conducted using an ultrasound machine and a 3.5 MHz transabdominal probe (Draminski Animal profi 2, Poland). The results of incidence of estrus and pregnancy rate were analyzed as categorical variables with the Proc GENMOD function and ovulation rate with the procedure Proc GLM, both of them available in SAS. It was considered $\alpha 0.05$ to estab lish significant differences between treatments. The general percentage of estrus was similar for ewes from T1 and T2 (76.9% vs 100%). The incidence of estrus was different ($p<0.05$) only for ewes from T1 and T2 at 24 h (50% vs 90%), but similar ($p>0.05$) at 30 h (19.2% vs 10.0%) and 36 h (7.7% vs 0%). Ovulation rate was higher in ewes from T2 compared to ewes from T1 (2.0 ± 0.22 vs 1.27 ± 0.13). Furthermore, 50% of the ewes were pregnant in both treatments. In conclusion, the results showed the feasibility of using Katahdin ewes as embryo recipients regardless of their age and parity.

A042E TAI/FTET/AI

Import of Belgian Blue embryos in tropical Indonesia: birth of first calves

Gretania Residiwati[1], Habib Syaiful Arif Tuska[1], Budiono[2], Bart Leemans[1], Christophe Boccart[3], Ann Van Soom[1], Geert Opsomer[1]

[1]Department of Reproduction, Obstetrics, and Herd Health, Ghent University, 9820 Merelbeke, Belgium; [2]Gajayana University, Malang, East Java 65144, Indonesia; [3]Association Wallonne De L'Élevage, 1300 Wavre, Belgium.

Keywords*:* Belgian blue cattle, Indonesia.

The Belgian Blue breeding program has been started to improve local beef production in Indonesia. Belgian Blue cattle (BB) are characterized by a double-muscled phenotype caused by a deletion within the myostatin gene. Animals present less bone and fat, more muscle, as well as a higher muscle bone ratio than other breeds (Kolkman et al., Reprod Domest Anim 47, 365, 2012). Indonesia is Southeast Asia's biggest economy, and its population growth, rising incomes, and changing public tastes, caused the beef consumption in this country to increase over time (increase of 4.66% per year), while the growth of domestic beef production is only 3.20 % per year. As a result, beef import rose by 21.58% annually (Kusriatmi et al., Journal of the ISSAAS 20, 115-130, 2014). One of the efforts that the Indonesian government did was importing BB frozen semen and embryos from Belgium into Indonesia (Agung and Syahruddin, 16th AAAP Animal Science Congress 2, 10, 2014). Consequently, the first BB calves were born in South Asia following successful embryo transfer (ET). The result of BB born by ET is 94, with the total of pregnant cow is 138 from 588 of total pregnant checked by rectal palpation; while the result of BB born by artificial insemination (AI) is 168, with the total of pregnant cow is 278 from 545 of pregnant checked by the same method (Indonesian Animal Husbandry, 2019). From those result, we could find that the successful percentage of AI is higher than the ET application with 51% and 23% respectively. Furthermore, all of BB calves by AI was born by normal parturition, while the BB calves by ET was born by C-section. In order to follow up this program and to predict the future of BB in this tropical environment, we compared the birth weight of BB pure breed in Indonesia (by ET) with the crossbreeds (by AI with several Indonesian local cattle) with a total sample size of 105 calves. Furthermore, we also compare the birth weight of BB calves born by ET in Indonesia versus calves born in Belgium, with a total sample size of 115 new born calves. The results indicated that the mean of BB pure breed birth weight in Indonesia 51,23 kg is higher rather than the crossbreed of BB with Friesian Holstein, Simmental, Limousine, Peranakan Ongole, Angus and Madura; with their mean of birth weight 44,80 kg; 43,5 kg; 36,14 kg; 29,59 kg; 46,6 kg; and 25,5 kg respectively. In addition, the result of birth weight of BB in Indonesia versus in Belgium showed that there are a significant different in their birth weight ($\alpha = 0{,}042 < 0{,}05$), which the mean of BB birth weight in Belgium (52,39 kg) is higher rather than the mean of BB birth weight in Indonesia (52,00 kg). This significant difference might be related to the tropical condition in Indonesia. Based on (Brody S, Journal of Dairy Science 39, 6, 715-725, 1956), the environmental comfort zone for European cattle ranges between -1 and 15℃, while the temperature in Indonesia is on average 18°C-30°C (Indonesian Directorate of Animal Husbandry, 2019). In addition, the BB is assumed to be more susceptible to heat stress than most other breeds, owing to the reduced oxygen transport efficiency, caused by the relatively small volume of their heart and lungs in comparison with their body volume (Grobet et al., Mammalian Genome 9, 210, 1988).

A068E OPU/IVF/ET

Effects of an oil covered culture system on bovine *in vitro* produced embryos

Mariya Aravina, Sophie Diers, Christoph Knorr, Jens Tetens, Carina Blaschka

Division of Biotechnology and Livestock Reproduction, Department of Animal Sciences, Georg-August-University Goettingen, Germany.

Keywords*:* embryo, oil, cattle.

Embryos are usually produced in culture systems with an oil overlay, which conveys protection against the evaporation of water and microbial contamination. The oil can also release toxic substances and absorb essential components, such as hormones, which adversely affect the quality of the oocytes and the development of embryos in vitro. In addition, an oil overlay can be prohibitive when applying particular analysis such as concentration of lipophilic substances in medium, as steroids. The aim was to validate an oil-free bovine IVP system. This study compares bovine IVP with and without an oil overlay. Groups of 20 cumulus-oocyte-complexes (COC) collected from abattoir-derived ovaries were matured in tissue culture medium with BSA and eCG/hCG for 24 h with 5% CO2, fertilized in Fert-TALP for 19 h with 5% CO2 and cultured in SOFaa with 5% CO2 and 5% O2, all steps took place at 39°C. The quantity of medium in both groups (with and without an oil overlay) and throughout all stages of IVP was maintained at a volume of 100µl. The oil group was covered with 75µl paraffin oil (IVF Bioscience, Falmouth, UK). The maturation stage of oocytes was assessed using fluorescence staining (Hoechst 33342) after 24h of maturation. The developmental stage (number of blastocysts) were evaluated on day 8. The morphological quality of expanded day 8 blastocysts was determined by live-dead-staining (total cell number as well as ratio of live and dead cells). At least ten replicates were done. The statistical analysis was performed with 'R'. Evaluation of maturation and development rates were analysed using a binomial test. Data obtained from the live-dead-staining were analysed using a t-test. Oocytes matured in the absence of an oil overlay had significantly ($p < 0.05$) higher maturation rates (71.5 ± 6.8%) when compared against matured in medium with an oil overlay (60.2 ± 9.3%). The developmental rate was significantly higher after culture without oil overlay (without oil: 38.4 ± 14.8%, with oil: 33.5 ± 12.6%; $p < 0.05$). The total cell number and the live-dead-ratio was not significantly ($p > 0.05$) different (total cell number: without oil: 130.0 ± 30.2, with oil: 119.0 ± 30.0; live-dead-ratio: without oil: 20.5 ± 11.5, with oil: 19.0 ± 8.0). The osmolarity did not differed between both groups during the IVP. Currently, the medium is analysed with regard to steroid concentrations via radioimmunoassay. So far, based on the higher maturation and development rates, bovine oil-free IVP-systems can be suggested as an alternative to oil covered medium, especially for maturation.

We gratefully acknowledge our colleagues H. Hellmold and D. Teuteberg.

A069 E OPU/IVF/ET

Individual serum-free and oil-based oocyte-to-embryo *in vitro* culture system is yielding high blastocyst rates and can be used as a basic system for individual follow-up

Nima Azari-Dolatabad[1], Annelies Raes[1], Anise Asaadi[1,2], Petra Van Damme[1], Ann Van Soom[1]

[1]Ghent University, Belgium; [2]Shiraz University.

Keywords*:* serum- free and individual culture system, embryo development, bovine.

Bovine *in vitro* embryo production (IVP) is routinely performed by culturing oocytes in group at ratio 1:2 (25/50µL droplets). We have recently shown that individual culture of bovine embryos in SOF-medium supplemented with 0.4 % BSA and insulin, transferrin and selenium (SOF-ITS-BSA) is yielding day 8-blastocyst rates over 40 %. However, in order to get these high blastocyst rates, *in vitro* maturation and fertilization still have to be performed in group culture. Several groups have attempted to develop an *in vitro* maturation-fertilization-culture system allowing individual follow-up from oocyte until embryo. Different approaches such as attaching the oocytes to the bottom of the Petri dish with Cell-Tak®, using a mesh grid or culturing oocytes and embryos in the well-of-the-well system have been attempted. These systems work well but are technically often challenging. Here we describe a simple individual oocyte-to-embryo culture system which is yielding routinely over 30 % blastocyst rates. *In vitro* maturation, fertilization and culture were either performed in group or in individual culture. For group culture, sixty cumulus-oocyte complexes were aspirated from ovaries derived from cows slaughtered in a local abattoir and matured in 500 µL TCM199 supplemented with 20 ng/mL EGF for 22h. Next, mature oocytes were incubated in 500 µL IVF-TALP with 1×10^6 spermatozoa/mL for 20h and then denuded and cultured in groups of 25 presumed zygotes in 50 µL droplets of SOF-ITS-BSA under paraffin oil (7.5 mL) overlay. For individual culture, 3 dishes (60×15 mm) with 20 µL droplets under paraffin oil overlay were used, each droplet containing one cumulus-oocyte-complex for maturation and subsequent fertilization (in the same media as described for group culture), and after denudation, presumed zygotes were cultured individually in 20 µL droplets SOF-ITS –BSA under paraffin oil overlay until day 8. Each dish contained 17 droplets. Blastocysts were then subjected to differential staining. Blastocyst rates (5 replicates) were significantly lower for individual compared to group culture (32 % (79/244) versus 47 % (146/314)) (Independent sample t-test, SPSS 20; $P<0.05$), but higher than 30 % so still acceptable. Blastocyst quality was also significantly lower, with a lower total cell number (90 ± 1.31 vs. 118 ± 1.16) and higher apoptotic cell ratio (8.4 ± 0.25% vs. 5.2 ± 0.19%) for individual versus group culture respectively. This indicates that despite the high overall blastocyst rates, there is still room for improvement in the individual culture system. In conclusion, the serum-free and oil-containing individual culture system we describe here is yielding acceptable blastocyst rates and can as such be used as to investigate (1) how differences in initial oocyte quality can affect embryo outcome; and (2) how addition of specific biochemical factors to the single oocyte maturation medium can be used in order to improve oocyte maturation. We are now testing the addition of different components derived from bovine follicular fluid to maturation medium in order to evaluate their possible effect on individual oocyte maturation and further embryo development.

A070E OPU/IVF/ET

Nobiletin supplementation prior to EGA improves development and quality of bovine blastocysts *in vitro*

Yulia N. Cajas[1], Karina E. Cañón-Beltrán[1], Claudia L.V. Leal[1,2], Encina González[3], Dimitrios Rizos[1]

[1]Department of Animal Reproduction, National Institute for Agriculture and Food Research and Technology (INIA), Spain; [2]Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, Brazil; [3]Department of Anatomy and Embryology, Veterinary Faculty, Complutense University of Madrid (UCM), Madrid, Spain.

Keywords*:* Nobiletin, embryo, quality.

One of the most important events in early embryo development is the maternal-to-embryonic transition when maternal transcripts and proteins are gradually degraded and the embryonic genome is activated (EGA). In bovine embryos, major EGA occurs at the eight- to 16-cell stage. At the same time, an increase in ROS levels during embryo culture in vitro induces oxidative stress leading to failed embryonic development and low quality of the blastocysts produced. Nobiletin is a polymethoxyflavone with antioxidant properties in different cell types. Therefore, we aimed to evaluate the effect of nobiletin supplementation to the culture medium of bovine embryos before major EGA on their development and quality. In vitro produced zygotes were cultured in four-well plates with 500 µl SOF+5% FCS (control), control with 5, 10 or 25 µM nobiletin (MedChemExpress, MCE, Sweden) (Nob5, Nob10 and Nob25 respectively) or control with 0.03% dimethyl sulfoxide (CDMSO vehicle for nobiletin dilution) from 18 to 54 hours post-insemination (hpi) at 38.5ºC, 5% CO_2, 5% O_2 and 90% N_2. For all groups, the speed of development was considered and embryos that reached ≥8 -cells at 54 hpi were selected and cultured in control medium until Day 8. Cleavage rate (54 hpi) and blastocyst yield (D7-8) were evaluated, while quality of embryos were determined by assessing their total cell number, lipid content and mitochondrial activity (fluorescence intensity recorded in arbitrary units (a.u)). For this purpose, a representative number of D7 blastocysts (n=30/group/treatment) were fixed and stained with Hoëscht, Bodipy, and MitoTracker DeepRed, respectively. The images were obtained by confocal microscopy and analysed using Image J. Data obtained from 6 replicates were analysed using one-way ANOVA. No differences were found in cleavage rate while blastocyst yield at D8 was higher ($P<0.001$) for Nob5 (42.9±1.4%) and Nob10 (45.3±2.1%) compared to control (32.9±1.1%), CDMSO (32.6±1.4%) and Nob25 (34.2 ± 1.0%). For embryo quality evaluation, both controls and Nob groups with higher development were used. The number of intact cells per embryo was increased ($P<0.001$) in Nob5 (137.3±0.6) and Nob10 (126.7±0.8) compared to control (105.7±0.8) and CDMSO (106.4±0.8). The lipid content was significantly reduced ($P<0.001$) in Nob5 and Nob10 compared with both controls. For mitochondrial activity, fluorescence was significantly higher ($P<0.001$) in blastocysts from Nob5 and Nob10 compared with both controls. In conclusion, supplementation of nobiletin 5 or 10 µM/mL improves embryo development and the quality of blastocysts in terms of mitochondrial activity and cell numbers, while it reduces their lipid content.
Funding: MINECO-Spain AGL2015-70140-R; Yulia N Cajas, SENESCYT-Ecuador; Claudia LV Leal, FAPESP-Brasil 2017/20339-3

A071E OPU/IVF/ET

Effect of sperm selection using microfluidic sperm sorting chip on bovine embryonic development *in vitro*

Oguz Calisici[1], Adriana R. Camacho[1], Seckin Salar[2], Utkan Demirci[3], Árpád Csaba Bajcsy[1]

[1]University of Veterinary Medicine Hannover, Foundation, Germany; [2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; [3]Demirci Bio-Acoustic-MEMS in Medicine Labs (BAMM), Canary Center at Stanford for Cancer Early Detection, Department of Radiology, Stanford University School of Medicine University of Stanford, U.S.A.

Keywords*:* IVF, microfluidic sperm sorting chip, cattle.

The preparation of bovine sperm for in vitro fertilization (IVF) requires procedures such as a density-gradient centrifugation, which enables to select sperm cells with a higher rate of progressive motility and those that are morphologically intact. Microfluidic sorting devices have been demonstrated to effectively select motile human sperm without centrifugation. The aim of this study was to examine embryonic development after using a microfluidic sperm sorting chip for the selection of bovine sperm cells by the IVF procedure. Bovine ovaries were collected at slaughterhouse and placed and transported at 30°C in phosphate buffer solution until laboratory processing. In total 234 Cumulus-oocyte complexes (COCs) were obtained using the slicing method. The sperm samples were collected from a sire using an artificial vagina at artificial insemination centre. Sperm motility was assessed subjectively, and motile sperm (with more than 70% progressive motility) was prepared and used for IVF. After maturation, COCs (15-20 COCs in each drop) were fertilized with sperm cells that were selected after thawing at 30°C using either a standard density gradients (DG) protocol (SpermFilter®, GYNEMED GmbH & Co. KG, Lensahn, Germany) or a microfluidic sperm sorting (MSS) chip technique (Fertile Plus®, KOEK EU GmbH, Hannover, Germany). Motile sperm cells were added to the IVF drops to reach a final concentration of 1 × 106 cells/mL and were incubated with the COCs (19h, 5% CO2, 39°C). At the end of this co-incubation, presumptive zygotes were denuded using vortex, were washed and then placed into a synthetic oviduct fluid (SOF) under silicone oil. Cleavage and embryonic development rates (blastocyst formation) were recorded. For both treatment groups four replicates were performed. Statistical analysis was performed using Chi-square test with significance at $p < 0.05$. The MSS chip technique did not affect cleavage rates (MSS chip: 75.0% vs. DG: 71.2%, $p > 0.05$) and blastocyst rates at Day 7 (MSS chip: 18.1% vs. DG: 15.3%, $p > 0.05$). At Day 8, blastocysts rates were higher for oocytes, that were fertilized using MSS chip sorted sperms as compared to those selected by the DG technique (MSS chip: 33.6% vs. DG: 22.0%, $p = 0.048$). In conclusion, MSS chip sorted sperm may increase embryonic development rates and outcomes in routine IVF procedure. A larger number of sperm samples of different bulls will be studied in future studies to demonstrate the sperm quality and IVF outcomes after usage of MSS device.
Authors acknowledge KOEK EU GmbH for providing the MSS chips for this study.

A072E OPU/IVF/ET

Melatonin increases the number of trophectoderm cells and total embryonic cells in *in vitro*-derived bovine blastocysts

Juan C. Gutiérrez-Añez[1,2], Patrick Aldag[1], Andrea Lucas-Hahn[1], Heiner Niemann[3]

[1]Department of Biotechnology, Friedrich Loeffler Institut, Mariensee, Lower Saxony, Germany; [2]Medical Surgical Department, College of Veterinary Medicine, University of Zulia, Maracaibo, Venezuela; [3]Hannover Medical School, Hannover, Hannover, Lower Saxony, Germany.

Keywords*:* Melatonin, inner cell mass, trophectoderm cells.

It has been shown that *in vitro*-derived embryos have fewer trophectodermal (TE) cells than *in vivo*-derived embryos. The TE cells are important for attachment of the embryo to the uterine endometrium, the formation of the fetal placenta and ultimately pregnancy establishment. An aberrant allocation of inner cell mass (ICM) and TE cells could be related to insufficient placentation and thus embryonic/fetal losses. Excess of oxidative stress under *in vitro* conditions can alter many important reactions affecting the embryonic development. Recently attention has been directed towards melatonin as a non-expensive broad-spectrum antioxidant. In the present study, we investigated the effects of melatonin on allocation of ICM and TE cells in *in vitro*-derived bovine embryos. A total of 97 blastocysts (Day 8) produced *in vitro* in the presence or absence of two concentrations of melatonin (MT) [MT 0.01nanomolar (nM): n=25 and MT 1.0 nM, n=21], were differentially stained to determine the number of cells (ICM and TE cells). As melatonin has to be dissolved in ethanol a "sham" group containing ethanol (ETOH; n=27) and a standard control group (Control: n=24) were also included in the experimental setting. A modified differential staining technique was applied (Thouas et al., Reprod Biomed Online 3(1): 25-29, 2001). Cells were counted via fluorescence microscopy (470-490 nm excitation filter) (Olympus BX60F, Tokyo, Japan) at 400-fold magnifications. The chromatin in nuclei of TE cells and ICM cells was stained and visualized by red/pink or blue color, respectively. Data were statistically analyzed using the SAS/STAT® software (version 9.3) with the general linear model (PROC GLM). Significant differences were defined at $p<0.05$. The general mean for TE, ICM, and total embryonic cells were 88.9±2.6, 41.9±1.3 and 130.8±3.2; respectively. The number of TE cells was significantly higher ($p<0.05$) in MT 0.01 nM and MT 1.0 nM groups compared to the control and ETH groups (101.3±11.8 and 101.6±8.6 vs. 86.5±12.2 and 83.6±12.2, respectively). No differences ($p>0.05$) were observed in the number of TE cells in sham controls and controls, as well as between both melatonin concentrations. There were no differences ($p>0.05$) regarding the number of ICM cells between the different experimental groups (Control: 43.8±6.9; ETOH: 39.0±6.9; MT 0.01 nM: 42.0±4.9 and MT 1nM: 46.1±6.7). Supplementation of the media with melatonin at 1.0 nM and 0.01 nM increased ($p=0.05$) total cell number compared with control and ethanol groups (147.3±14.6 and 143.7±10.7 vs. 130.3±15.1 and 122.5±15.1, respectively). No differences ($p>0.05$) were found between the control and the ETOH group, neither between both concentrations of melatonin. In conclusion, these data indicate that the presence of melatonin in in vitro embryo production media increases the allocation of embryonic to the trophectoderm, as well the total number of embryonic cells. The physiological importance of this finding warrants further study and could have an important implication to reduce early embryo/fetal losses observed after in vitro embryo production.

A073E OPU/IVF/ET

Perfluorooctane sulfonic acid (PFOS) affects early embryonic development in a bovine *in vitro* model

Ida Hallberg[1,7], Jonna Swahn[1], Sara Persson[1,2], Pauliina Damdimopoulou[3], Matts Olovsson[4], Joëlle Rüegg[5], Marc-André Sirard[6], Ylva Sjunnesson[1,7]

[1]Department of Clinical Science, Swedish University of Agricultural Science, Uppsala, Sweden; [2]Swedish Museum of Natural History, Department of Environmental research and monitoring, Stockholm, Sweden; [3]Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden; [4]Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden; [5]Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden; [6]Department of Animal Sciences, Laval University, Quebec, Canada; [7]Centre for Reproductive Biology in Uppsala, University of Agricultural Sciences and Uppsala University, Uppsala, Sweden.

Keywords*:* Oocyte maturation, cattle, in vitro embryo production, PFOS.

Perfluorooctane sulfonic acid (PFOS) is a chemical that has been widely used in products like food packaging, textile, impregnations and firefighting foams. It is now banned in many countries, including the EU, but still present in nature, animals and humans due to its persistent and bioaccumulating properties. The average reported human serum PFOS levels vary from 4-70nM (median 30 nM) in the literature (and higher in especially exposed groups). PFOS is endocrine disruptive and has toxic effects on reproduction in research animals although human data remains contradictory. The aim of this study was to examine the effect of environmentally relevant concentrations of PFOS on bovine early embryo development *in vitro* as a possible model for human early embryo development. This model was chosen as the process of maturation and fertilization in bovine oocytes shows more similarities to the human process compared to the murine. Abattoir derived oocytes were matured, fertilized and cultured in a bovine *in vitro* system. Oocytes were randomly divided into two treatment groups exposed to either 20 nM PFOS (P20) or 200 nM PFOS (P200) during *in vitro* maturation and to one control group (C) without PFOS. Cleavage rate as well as stage and grade of day 7 and 8 blastocysts were assessed. Further, neutral lipids were analyzed using HCS LipidTOXTM Green Neutral Lipid Stain (ThermoFisher Scientific, Waltham, USA) and nuclei were stained with Hoechst 33342 in paraformaldehyde-fixed day 8 blastocysts. Evaluations of the number of nuclei, total lipid volume, lipid volume of each blastocyst and lipid droplet size were performed using confocal microscopy. Mixed effect logistic regression was used to calculate the effect of treatment on the number of cleaved embryos and developed blastocysts. The effect of treatment on lipid droplets was performed using a linear mixed effect model with log-transformed values to assume normal distribution. From 13 batches with a total of 847 oocytes, 162 blastocysts were developed. Cleavage rate and cleavage rate above the 2-cell stage were significantly lower in the P200 group compared to the control group, although no significant difference could be seen on blastocyst development or grade on day 7 and 8 neither in P20 or P200. The blastocyst stage of development was significantly lower in the P200 group compared to the C group. In addition, in the P200 group PFOS had an effect on lipid droplet size in the early blastocyst stage where the lipid droplets were larger. The results from this experiment indicate that human relevant concentrations of PFOS impair bovine early embryo development. PFOS exposure delays development and affects the size of lipid droplets. These findings are in line with epidemiological studies linking PFOS exposure to lipid metabolism in adults. More studies are needed to elucidate the mechanisms and effects of PFOS on the early embryo development.

Funded by Formas 942-2015-476 and Stiftelsen Nils Lagerlöfs fond (KSLA, GFS2017-0032).

A074E OPU/IVF/ET

Quality of mouse IVF blastocysts after addition of quercetin to the culture media at the morula stage

Nuria Hernández Rollán[1], Marta López Morató[1], José Mijares Gordún[1], Soledad Sánchez Mateos[1], Francisco Miguel Sánchez Margallo[1], Ignacio Santiago Álvarez Miguel[2]

[1]Jesús Usón Minimally Invasive Surgery Centre, Spain; [2]Cell Biology Department. Faculty of Medicine. University of Extremadura.

Keywords*:* IVF, culture-medium, blastocysts.

Quercetin is a plant flavonol found in many fruits and wine, which has an important role as antioxidant in many pathological pathways associated with oxidative stress. The effects of the quercetin have been studied on the *in vitro* maturation, the competency of the oocyte and during the whole embryo development in different animal species. Nonetheless its effects have not been studied on mouse embryos obtained by IVF only during the stage from morula to blastocyst. The aim of this study was to examine the embryo development, cell death and cell number on mouse blastocysts obtained by IVF and cultured under conditions of normoxy, hypoxy and with different concentrations of quercetin. B6D2 strain female mice were hormonally stimulated to activate the recruiting of the follicles and to trigger the ovulation. Mature cumulus-oocyte complexes were obtained, used to perform IVF, cultured in KSOM and divided into 6 groups when they reached the morula stage (day 3): IVF_{KSOM}: embryos were cultured in KSOM until the blastocyst stage (control group); $IVF_{50\mu M}$, $IVF_{10\mu M}$,$IVF_{5\mu M}$ and$IVF_{1\mu M}$: morulae were cultured for 4 hours in KSOM media supplemented with 50 µg/ml, 10 µg/ml, 5 µg/ml or 1 µg/ml of quercetin, respectively, in an atmosphere of 5% CO_2, and transferred back to normal KSOM and cultured until the stage of blastocyst; $IVF_{3\%}$: morulae were cultured for 4 hours in KSOM in an atmosphere of 3% of O_2 (to resemble the uterus condition after the morula stage) and 5% of CO_2 until the stage of blastocyst. The blastocysts were used to study the embryo development (n=15 IVFs/group), the total number of cells (trophoblast cells and ICM by DAPI staining, n=30 blastocysts/group) and the cell death (studied by TUNEL assay, n=25 blastocysts/group). Our results showed that the mean of embryos that developed to blastocyst was 59.90% $\pm$ 25.83 for IVF_{KSOM}, 61.04% $\pm$ 25.9 for $IVF_{50\mu M,}$ 72.14% $\pm$ 22.69 for$IVF_{10\mu M,}$ 62.27% $\pm$ 29.59 for $IVF_{5\mu M}$,68.57% $\pm$ 20.55 for $IVF_{1\mu M}$ and 63.76% $\pm$ 26.86 for $IVF_{3\%}$ ($p>0.05$). The mean of the number of cells per blastocyst was 84.1 $\pm$ 7.82 for IVF_{KSOM}, 82.23 $\pm$ 11.33 for $IVF_{50\mu M}$, 89 $\pm$ 15.6 for $IVF_{10\mu M}$, 86.7 $\pm$ 9.5 for $IVF_{5\mu M}$, 87.56 $\pm$ 10.99 for $IVF_{1\mu M}$ and 88.2 $\pm$ 12.89 for $IVF_{3\%}$ ($p>0.05$). Results for the cell death showed that the mean of dead cells per blastocyst was 0.10 $\pm$ 0.27 for IVF_{KSOM}, 0.12 $\pm$ 0.33 for $IVF_{50\mu M}$, 0.04 $\pm$ 0.2 for $IVF_{10\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{5\mu M}$, 0.08 $\pm$ 0.27 for $IVF_{1\mu M}$ and 0.30 $\pm$ 0.45 for $IVF_{3\%}$ ($p>0.05$). One-way ANOVA test was used for the statistical analysis and a $p<0.05$ was considered statistically significant. Based on the p values, there were no statistically significant differences between the groups in any assay. It is worth mentioning though that in embryos cultured with 10 µM of quercetin the number of cells per blastocyst was higher, the cell death was lower and a higher number of embryos reached the blastocyst stage. These results show that, even though the differences found were not statistically significant, the enrichment of the embryo culture media with 10 µM of quercetin at the stage of morula slightly improves the mouse blastocyst quality, hence showing potential to increase the implantation rates. Further studies are required though to verify this hypothesis.

A075E OPU/IVF/ET

Hormone levels differ between cow recipients carrying *in vivo* or *in vitro*-derived conceptus during early pregnancy

Jordana S. Lopes[1,2], Estefanía Alcázar-Triviño[3], Cristina Soriano-Úbeda[1], Evelyne París-Oller[1,2], Pilar Coy[1,2]

[1]Department of Physiology, Faculty of Veterinary Medicine, University of Murcia - Campus Mare Nostrum, Murcia, Spain; [2]Institute for Biomedical Research of Murcia, IMIB-Arrixaca, Murcia, Spain; [3]El Barranquillo S.L., Torre Pacheco, Murcia, Spain.

Keywords*:* Embryo transfer, reproductive fluids, anti-mullerian hormone.

Bovine embryo production is still far from optimal. In order to better mimic the natural environment, natural reproductive fluids (RF), collected from reproductive tracts, have been proposed as additives for embryo culture and results from RF-derived embryos after transfer (ET) have not shown adverse effect on pregnancy rate at day 30 when compared to a control (BSA) (Lopes, Animal Reproduction, v15, nr3, p550, 2018). However, pregnancy maintenance is dependent on several factors and hormones such as progesterone (P4), estrogen (E2), cortisol or, more recently studied, anti-Mullerian hormone (AMH), play an important role. P4 and E2 are protagonists during pregnancy and P4 has been related to early growth of conceptus in uterus (Shorten, J Dairy Sci, 101:736-751, 2018). Cortisol, on the other hand has been tagged as a meaningful participant in intrauterine regulatory system of early pregnancy in cattle (Majewska, J Rep Imuno, 93:82-93, 2012). AMH levels are known to influence the pregnancy maintenance, being low levels associated with pregnancy loss (Ribeiro, J Dairy Sci, 97:6888–6900, 2014). Therefore, we hypothesised that P4, E2, cortisol and AMH levels might have an influence on recipient's pregnancy outcomes. To test our hypothesis bovine IVP-blastocyts cultured with or without RF (RF and BSA groups) were transferred to synchronized recipients. An in vivo control was added by artificial insemination (AI) of recipients with frozen-thawed semen from the same bull used to produce IVP embryos. At day 30, pregnancy diagnosis was performed, embryos measured and blood samples from pregnant recipients were taken (Pregnancy/ET or AI: 12/54 from RF, 10/45 from BSA and 8/35 from AI group). Blood collected on the day of ET and 7 days post-AI was also analysed. Statistical analysis consisted of Pearson's correlation between variables (group, hormones, embryo size, day) followed by one-way (groups) or t-test (day) when significant correlation ($p<0,05$) was found. At day 7, P4 was significantly lower in AI vs. both RF/BSA. Cortisol had a tendency to be lower for RF group ($p=0,052$) but was not confirmed by post-hoc. AMH and E2 were not different between groups. At day 30, AMH and E2 were lower and higher, respectively, between AI & BSA, but RF had intermediate concentrations. P4 and cortisol were not different between groups. No correlation was found between embryo' dimensions vs. hormone levels in any day nor group. P4 was, independently of the group, significantly lower at day 7 vs. day 30, whereas cortisol, E2 and AMH remained similar. In conclusion, in our study we observed that AMH and E2 levels at day 30 were significantly different between recipients holding AI vs. BSA embryos while recipients from RF group showed an intermediate value. P4 values, 7 days post-AI vs. day of ET were significantly lower for AI vs. ET recipients, but those differences disappeared by day 30. How the embryo influences hormonal levels remains to be further investigated and more analyses to the pregnant recipients as well as non-pregnant animals should be further addressed.

Supported by European Union, Horizon 2020 Marie Sklodowska-Curie Action REPBIOTECH675526; AGL2015-66341-R MINECO-FEDER; 20040/GERM/16 Fundación Seneca.

A076E OPU/IVF/ET

Use of in-estrus heifer serum on *in vitro* culture of sheep embryos

Alfredo Lorenzo[1], Raymundo Rangel[1], Rodolfo Ramírez[1], Demetrio Alonso Ambríz[2], José Ernesto Hernández[2]

[1]Universidad Autónoma Chapingo, Estado de México, México; [2]Universidad Autónoma Metropolitana, Ciudad de México, México.

Keywords*:* embryo culture, supplement, blastocyst.

Fetal bovine serum (FBS) is a universal supplement for embryo culture; however, alternatives have been sought to replace it. The study aimed to evaluate the estrus heifer serum (EHS) as a supplement of the culture medium, and to evaluate the differences between sera from donor Holstein heifers used individually or mixed in the development of ovine embryos to blastocyst stage cultured *in vitro*. A total of 1105 oocytes from ovaries of ewes obtained from a commercial slaughterhouse were used. The oocytes were *in vitro* matured (IVM) in TCM 199 (In vitro S.A., Mexico), supplemented with: 10% of FBS (Biowest, Mayimex, Mexico), 5 μg mL^{-1} FSH (Folltropin-V, Bioniche, Canada), 5 IU mL^{-1} hCG (Chorulon, Intervet, Netherlands) and 1 μg mL^{-1} 17-β estradiol (E8875, Sigma, Mexico). After 24 h of IVM at 5% CO_2 in air, at 38.5 °C and saturated humidity, the oocytes were fertilized (IVF) in commercial medium (In vitro S.A., Mexico) using fresh spermatozoa ($1x10^6$ mL^{-1}) from a Rideau Arcott ram of known fertility. The zygotes were cultured using Cleavage medium (Cook IVF, Brisbane, Australia) for 72 h until 16-cell stage. The rate of IVM, IVF and embryos in the 16-cell stage were registered (85.5, 67.0 and 65.4%, respectively). The embryos in the 16-cell stage (723) were randomly assigned to one of five treatments with Blastocyst culture medium (Cook IVF, Brisbane, Australia) plus 10% serum of different types: T1: FBS (control, n= 146); T2: EHS1 (n= 144); T3: EHS2 (n= 143); T4: EHS3 (n= 143) and T5: EHS mixture (T2, T3 and T4) (n= 147). The embryos were cultured for 96 h until blastocyst stage. The development and quality of the blastocysts were evaluated according to their morphology, while the diameter blastocyst was measured with a digital camera (AmScope, MU1803, China) using an inverted microscope (Nikon, Eclipse TS100, Japan). These variables were analyzed using the SAS program. The development data consider a comparison of binomial proportions with the construction of confidence intervals using GENMOD. Analysis of variance was used to analyze the diameter results with a classification criteria and fixed effects using GLM, while blastocyst quality was modeled according to a multinomial distribution and analyzed with GENMOD procedure. The percentage of blastocysts was similar between treatments (41.8, 40.3, 39.9, 50.4 and 43.5% for T1, T2, T3, T4 and T5, respectively, $p>0.05$). For blastocyst diameter, T4 and T5 were larger than T1 (238 *vs* 223 μm, $p= 0.007$, 234 *vs* 223 μm, $p=0.04$, respectively). Likewise, T4 was larger than T2 (238 *vs* 226 μm, $p=0.03$). No differences were observed among treatments for blastocyst quality ($p>0.05$). In conclusion, the estrus heifer serum used has similar effects as fetal bovine serum when the culture medium is supplemented at 72 h. Moreover, the individual use of serums may be better as a supplement than using a mixture under the conditions of this study.

A077E OPU/IVF/ET

The efficiency of collecting *in vivo*-developed porcine zygotes is not affected by 3-to- 5-days weaning-to-estrus interval

Cristina A. Martinez[1,2], Josep M. Cambra[1], Inmaculada Parrilla[1], Jordi Roca[1], Emilio A. Martinez[1], Heriberto Rodriguez-Martinez[2], Cristina Cuello[1], Maria A. Gil[1]

[1]Dept. of Medicine and Animal Surgery, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain; [2]Dept. of Clinical & Experimental Medicine (IKE), Linköping University, Linköping, Sweden.

Keywords*:* zygote, pig, embryo collection.

The recently developed genome-editing (ZG-E) technology for pig zygotes, opening a new revolution in agriculture and biomedicine, depends of the efficient collection of large numbers of zygotes of the highest quality. Because IVP of zygotes in pigs still is inefficient, *in vivo*-collection remains as major source of zygotes. Little information is available on the efficiency of the collection procedures for in vivo-derived zygotes. Since the interval between pronuclear formation and the first division is very short in pigs, the collection of zygotes must be performed within a very narrow window. While the weaning is an efficient method to synchronize estrus and ovulation in sows, the weaning to estrus interval (WEI) can, due to its inverse relation with length of estrus and time of ovulation, interfere with ovulation and make it asynchronous. In addition, individual sows show variability in ovulation time, even after hCG treatment, which reduces the probability of obtaining zygotes during collection. This study compared the effects of three WEIs: 3d (N=57), 4d (N=131) or 5d (N=29) on the efficiency of zygote collection *in vivo*. The donors were super-ovulated with eCG (1,000 IU; i.m.) 24 h after weaning. Estrus was checked twice per day when allowing snout-to-snout contact of sows and a mature boar while applying manual backpressure. Sows in estrus at 48–72 h post-eCG were treated with hCG (750 IU; i.m.) at the onset of estrus. The donors were inseminated at 6 and 24 h after the onset of estrus and subjected to a laparotomy on Day 2 (Day 0: onset of estrus). After counting the number of corpora lutea, each oviduct was flushed with 20 mL of Tyrode's lactate-HEPES-polyvinyl alcohol medium. Collected structures were evaluated for morphology under a stereomicroscope and only those with a single cell and two visible polar bodies were considered as zygotes. Results were expressed as percentages or means±SD. Differences among groups were analyzed using Fisher's exact test or ANOVA as appropriate and were considered significant when $P<0.05$. A total of 217 out of 223 donors (97.3%) had embryos at collection. The mean ovulation rate (27.3±7.6 corpora lutea) and the mean number of structures (25.2±9.4) collected in these sows did not differ between groups. Of all recovered structures (N=5,468), 67.4%, 31.1% and 1.5% were zygotes, two-to-four cell embryos and oocytes-degenerated embryos, respectively. The different WEIs did neither affect the percentages of collected zygotes (range: 64.1% to 70.0%) nor the percentages of sows with zygotes at the collection (range: 70.2% to 73.3%). In conclusion, these results indicate that neither fertilization rates nor the number of zygotes collected at Day 2 of the cycle from superovulated sows were affected by a WEI of 3 to 5 days.

Supported by MINECO-FEDER (AGL2015-69735-R), Seneca Foundation (19892/GERM/15) and FORMAS (2017-00946).

A078E OPU/IVF/ET

Comparative study of growth parameters in piglets derived from embryos produced *in vitro* with or without reproductive fluids, and piglets derived from artificial insemination

E. París-Oller[1,3], R. Belda-Pérez[1], C. Soriano-Úbeda[1,3], L. Sarrías-Gil[1], A. Canha-Gouveia[1,3], R. Latorre[2], L.A. Vieira[1,3], O. Simonik[1], S. Ruíz[1,3], J. Gadea[1,3], C. Matás[1,3], P. Coy[1,3]

[1]Department of PhysiologyVeterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [2]Department of Anatomy and Compared Pathology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [3]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords*:* Growth, Reproductive Fluids, pig.

Different studies have suggested that the use of assisted reproductive technologies (ART) is associated to a higher incidence of low birth weight (Castillo et al., Hum Reprod doi:10.1093/humrep/dez025, 2019) as well as to alterations in the growth curve (Donjacour et al., Biol Reprod 90:80, 1–10, 2014). Recently, it has been described that porcine blastocysts produced *in vitro* with reproductive fluids (RF) in the culture medium show DNA methylation and gene expression patterns more similar to those produced in vivo than their counterparts produced without RF (Cánovas et al, eLife 1: e23670, 2017). However, it is unknown whether the presence of RF during fertilization and embryo development *in vitro* affects offspring growth-related parameters such as weight and length. The objective of this work was to compare growth parameters of piglets born after the transfer of embryos produced *in vitro* with RF (F-IVP, N=19) or without RF (C-IVP, N=29) added to the culture media with the same parameters in animals derived from artificial insemination (AI, N= 57). After birth, piglets were weighed at different days (0, 3, 9, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180) with a mobile scale and average weight daily gain (AWG) was calculated from total weight values (W) at every two consecutive days. All animals were measured with a zoo-metric tape from the frontal region of the cranium to the beginning of the rump (Cranium-rump length, CRL). All weight data and female (N=42) CRL data were analyzed by robust mixed ANOVA test while mixed ANOVA test was used for male CRL data (N=32) because Mauchly's test for sphericity came out significant but Greenhouse-Geisser (GGe) and Huyhn-Feldt (HFe)corrections were not valid. A total of 4 litters per group were studied, with the litter sizes differing between them (4.75±1.71, 7.25±2.06, 14.25±6.55, for F-IVP, C-IVP, and AI, respectively). Significant differences were reported most days studied for W, AWG and CRL measurements ($P \leq 0.05$) when they were compared by genders in AI piglets versus F-IVP and C-IVP groups, being these last two heaviest and longest than the first, although F-IVP showed intermediate values that could be related to a phenotype more similar to that obtained through AI. Similarly, significant differences were also observed between F-IVP and C-IVP for W, AWG, and CRL from day 9 to day 75. However, due to the low number of piglets under study and to the high differences in litter sizes between groups, further analyses are necessary to elucidate the influence of co-variables such as the mentioned litter size, with a possible strong influence in growth rates. Though preliminary, these are the first data in a large animal model, up to our knowledge, comparing growth parameters between ART-derived and AI derived offspring. In addition, they shed light on possible future phenotypic differences between ART-derived animals produces with or without RF.

Acknowledgements: Sección de Apoyo Estadístico (SAE), Universidad de Murcia (www.um.es/web/acti). Supported by MINECO-FEDER (AGL 2015-66341-R) and Fundación Séneca (20040/GERM/16).

A079E OPU/IVF/ET

Differences in glucose tolerance between piglets born after *in vitro* fertilization/ embryo transfer and relatives born after artificial insemination

E. París-Oller[1,3], S. Navarro-Serna[1,3], C. Soriano-Úbeda[1,3], J.S. Lopes[1,3], E. Rodríguez-Tobón[1], F.A. García-Vázquez[1,3], O. López-Albors[2], R. Romar[1,3], P. Coy[1,3], S. Cánovas[1,3]

[1]Department of Physiology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [2]Department of Anatomy and Compared Pathology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", Murcia, Spain; [3]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords*:* glucose, reproductive fluids, ART.

Increasing evidence indicates a higher incidence of glucose metabolism abnormalities in children derived from Assisted Reproductive Technologies (ART) (Chen et al., Diabetes, 63:3189–3198,2014). Monophasic and biphasic patterns of blood glucose curves have been identified, biphasic curves being associated with better glucose tolerance and beta-cells function, increasing insulin sensitivity in humans (Bervoets et al., Horm Metab, 47:445–451, 2014). On the other hand, the addition of reproductive fluids (RF) to in vitro culture media used during ART has been proposed as a possible way to avoid ART-derived abnormalities in pigs (Cánovas et al., eLife 1: e23670, 2017). The aim of this study was to evaluate the response to oral glucose tolerance test in young growing pigs born from embryos produced in vitro with (F-IVP) or without RF (C-IVP), compared to animals born by artificial insemination (AI). Four litters of relatives animals per group were used. At 45 days of life, the two males and two females of highest and lowest weight per litter were selected for the study (N=14, N=15, and N=16 for F-IVP, C-IVP, and AI, respectively). After 18h overnight fast, water was withdrawn and 1h later, pigs drank 1.75 g/kg BW of glucose solution. Blood samples were collected from the auricular lateral vein before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after glucose intake. Blood glucose concentration (GC) was immediately determined by test strips with a glucometer (GlucoMenLX Plus+). One way ANOVA and Tukey post-hoc tests were applied ($P<0.05$). Pearson correlation coefficient was used to detect litter influence, resulting in a positive correlation between GC value and weight. A monophasic GC curve was observed in the three groups. GC steadily increased reaching a maximum at 45 min after glucose intake, thereafter, it decreased until basal values (range 74,13-78,67mg/dl). Significant differences between AI and F-IVP groups were observed at 15, 20 and 30 min, with F-IVP showing higher values. When the analyses were repeated splitting the animals by sex, males showed a monophasic curve with similar basal levels, and the glucose peak at 45 min in all groups (range 98,43-115,0mg/dl). Significant differences between AI and F-IVP groups were present at 20 min and between F-IVP vs.AI and C-IVP groups at 30 min. In contrast, females showed significant differences between AI and F-IVP groups before glucose intake and at 20 min, while F-IVP was different vs.AI and C-IVP at 15 min. In addition, females on average from F-IVP group presented a biphasic curve, with two peaks at 15 (range 83,50- 128,67mg/dl) and 45 min (range 100,88-128,20mg/dl) compared with other groups. In conclusion, glucose tolerance in growing piglets is affected by sex and by the origin of the embryo, although all the basal and peak values are always into the physiological range.

Acknowledgments: Sección de Apoyo Estadístico (SAE), Universidad de Murcia (www.um.es/web/acti). Supported by MINECO-FEDER (AGL 2015-66341-R) and Fundación Séneca (20040/GERM/16).

A080E OPU/IVF/ET

Effect of type of recipient on an embryo transfer programme in sheep

Raymundo Rangel-Santos[1], Raymundo Rodríguez-De Lara[1], Demetrio A. Ambríz-García[2], María Del C. Navarro-Maldonado[2], Roberto Ramírez-López[3]

[1]Universidad Autónoma Chapingo. Chapingo Estado de México, México; [2]Universidad Autónoma Metropolitana. Ciudad de México, México; [3]Rancho Poza Rica, Singuilucan, Hidalgo, México.

Keywords*:* ewes, embryo transfer, pregnancy.

The implementation of embryo transfer is important to help in the multiplication of high genetic merit animals, through the selection of the best males and females. The success of the technique depends on the possibility of achieving high levels of efficiency in each step to get high rates of pregnancy. The objective of this study was to evaluate the fertility of two groups of ewes (hair and wool types) exposed to an embryo transfer programme. The study was conducted from September to December of 2017 at the commercial sheep farm "Poza Rica", which is located in a temperate area named Singuilucan, in central Mexico. A total of 60 multiparous healthy and in good body condition (3.0) ewes were used, from which 30 were Suffolk x Hampshire (T1; wool type) and the other 30 were Katahdin x Pelibuey (T2; hair type). The ewes were synchronized with intravaginal sponges containing 20 mg of micronized cronolone (Chrono Gest, Intervet, Netherlands), which were inserted for 12 days. On day 10, the ewes received intramuscularly 400 IU eCG (Novormon, Sanfer, Mexico). The estruses were detected every 6 h with two fertile Katahdin rams equipped with an apron, starting 18 h after sponge removal. The time of estrus was recorded. On day 6 after estrus detection, just before embryo transfer, ovulation rate was determined as the number of corpora lutea observed in ovaries during laparoscopy. The recipients received two embryos of transferable quality (compact morula or blastocyst) within 3 h after its collection, coming from Charollais donor ewes using a laparoscope and standardized procedures. The embryos recovered into holding medium (Syngro, Vetoquinol, Canada) were transferred using a Tom catheter in the ipsilateral horn to the ovary in which ovulation was recorded, and the presence of the best quality corpus luteum was determined based on its size. On day 60, pregnancy diagnosis was performed using an ultrasound and a 3.5 MHz transabdominal probe (Aloka Prosound 2, Japan). The results of the incidence of estrus and pregnancy rate were analyzed as categorical variables with the CATMOD procedure, and ovulation rate with the ANOVA procedure, both of them available in SAS. The total percentage of estrus was similar ($p>0.05$) between ewes of T1 and T2 (95 and 100%). The incidence of estrus was also similar ($p>0.05$) for ewes from T1 and T2 at 24 h (75 and 85%) and 30 h (25 and 15%). Ovulation rate for ewes of T2 was higher ($p<0.05$) than for ewes of T1 (2.26 ± 0.21 vs 1.80 ± 0.15). Also, pregnancy rate was lower ($p<0.10$) for ewes of T1 than for ewes of T2 (60 vs 80%). In conclusion, under the conditions of the study, the results showed the superiority of using hair type multiparous ewes as embryo recipients.

A081E OPU/IVF/ET

Prediction of pregnancy after transfer of bovine *in vitro* produced embryos based on recipients' blood plasma metabolomics

Pascal Salvetti[1], Julie Gatien[1], Susana Carrocera[2], David Martín-González[2], Marta Muñoz[2], Enrique Gómez[2]

[1]ALLICE, Nouzilly, France; [2]SERIDA, Gijón, Spain.

Keywords*:* cattle, embryo recipients, metabolomics.

In association with genomic selection schemes, embryo transfer (ET) of *in vitro* produced embryos (IVP) is steadily increasing worldwide in cattle, although calving rates remain lower than from *in vivo* embryos. Thus, identification of competent embryos and recipients able to reach pregnancy at term is a major objective in reproductive biotechnology. However, practitioners lack indicators to select suitable recipients, often leading to the exclusion of fertile animals. In that context, this study aimed to identify metabolite biomarkers in blood plasma of recipients belonging to several breeds (dairy, beef and crossbreed), that could predict pregnancy after ET of fresh or vitrified IVP embryos. Blood plasma of 130 recipients (67 Holstein for the training dataset; 63 for the validation dataset including 17 Holstein, 21 Asturiana de la Montaña and 25 crossbred) was collected at Day 0 (estrus) and Day 7 (4 to 6 hours prior to ET) and stored at -150º C until nuclear magnetic resonance (NMR) analysis. On Day 7, fresh (N=67; 34 for training and 33 for validation) or vitrified/warmed (N=63; 33 for training and 30 for validation) IVP embryos were transferred and pregnancy status was evaluated by trans-rectal ultrasound scanning at Day 40, 62 and at birth. NMR analysis led to absolute quantification of 36 metabolites. Average pregnancy rates were respectively 53.8 (50.7 for fresh and 57.1 for vitrified), 49.2 (44.8 for fresh and 54.0 for vitrified) and 43.8% (40.3 for fresh and 47.6 for vitrified) at Day 40, 62 and birth with no statistical differences between fresh and vitrified embryos. Data were examined for normality with Univariate procedure (SAS/STAT software). Thereafter, metabolites differentially expressed between pregnant and open recipients were identified by General Linear Model for each metabolite and each pregnancy checkpoint. Differences were considered significant at $p<0.05$ and FDR<0.05. Interestingly, putative biomarkers were only identified on Day 7 or by subtracting Day 0 and Day 7 (only for vitrified embryos) but not at Day 0. Biomarkers for fresh embryos were consistently identified on Day 40, Day 62 and birth, while vitrification led to a marked drop in biomarker abundance at birth. Overall classification accuracy was calculated to identify three types of biomarkers: 1) independent of breed and embryo type (2-Oxoglutaric acid; Ornithine); 2) specific for fresh embryos (L-Alanine, Ketoleucine, L-Threonine, 3-methyl-2-oxovalerate, Propionic acid); and 3) specific for vitrified embryos (L-Glycine, L-Glutamine, L-Methionine, L-Lysine). Metabolic enrichment analysis distinguished between recipients for fresh (enriched energy oxidative metabolism from fat in pregnant recipients) and vitrified embryos (low enrichment in lipid metabolism in pregnant recipients). Recipient selection by their pregnancy probability in a defined cycle seems to be possible using the biomarkers here identified for the first time. These findings may allow reliable recipient selection according to the cryopreservation status of the embryo, thus optimizing the efficiency of breeding programs.

Acknowledgements: MINECO (AGL2016-78597-R and AGL2016-81890-REDT). Grupos de Investigación en el Principado de Asturias 2018-2020 (IDI/2018/000178). FEDER. COST Action 16119 (Cellfit). ASEAVA. ASCOL.

A082E OPU/IVF/ET

Short term temperature elevation during IVM affects embryo yield and alters gene expression pattern in oocytes, cumulus cells and blastocysts in cattle.

Constantina Stamperna[1], Eleni Dovolou[1], Themistoklis Giannoulis[1,2], Zissis Mamuris[2], Katerina Dadouli[1,3], Georgios S. Amiridis[1]

[1]Univeristy of Thessaly, Veterinary Faculty, Karditsa, Greece; [2]University of Thessaly, Faculty of Biochemistry and Biotechnology, Larissa, Greece; [3]University of Thessaly, Faculty of Medicine, Larissa, Greece.

Keywords*:* heat stress, embryo, cattle.

Heat stress causes subfertility in cattle by inducing endocrine disruptions and deteriorating of oocyte and embryo quality. In this study we evaluated the effects of short lasting, moderate temperature elevation during IVM, on embryo yield, and on the expression of various genes. Abattoir derived oocytes were matured for 24 hours in TCM199 plus FCS and EGF at 39^{o}C (controls n=549) or at 41^{o}C from hour 2 to hour 8 of IVM (treated, n =867). Matured oocytes were fertilized by frozen/thawed swim-up separated sperm. Presumptive zygotes were denuded and cultured at 39^{o}C in SOF supplemented with FCS for 9 days in microdroplets in groups of 25. In 8 replicates, cleavage and blastocyst formation rates were evaluated at 48 hours PI and on days 7,8,9 respectively. Cumulus cells, oocytes and blastocysts from 5 replicates were snap frozen for gene expression. qRT-PCR was used for analysis of expression pattern of genes related to metabolism, thermal and oxidative stress response, apoptosis, and placentation in oocytes (7 genes), cumulus cells (12 genes) and blastocysts (11genes). Three reference genes (YWHAZ, EEF1A1, UBA52) were used to normalize gene expression values per sample using their geometric mean and their suitability for normalization was checked with the geNorm program. Cleavage, embryo formation rates and gene expression between treated and control groups were tested by 2tailed students t-test. Correlation analysis was performed by bi-clustering the samples according to their origin and their condition, which is an appropriate method for functionally heterogeneous data. Correlation and regression analysis were performed using gene expression data between groups, by the functions cor and rcorr implemented in R.

In treated group, cleavage and embryo formation rates were lower compared to controls (cleavage 86.7% vs 74.2%; blastocysts: day 7, 29.9% vs 19.7%, day 8, 34.2% vs 22.9% and day 9 35.9% vs 24.5%), in all cases $p<0.001$. Relative mRNA abundance of *HSPA1A, HSPB11, HSP90AA1, GPX1, GLUT1, PTGS2, GREM1, CPT1, G6PD, LDHA, CCNB1, MnSOD* in cumulus cells, *HSPA1A, HSPB11, HSP90AA1, G6PD, GPX1, CCNB1, MnSOD* in oocytes and *HSPA1A, HSP90AA1, DNMT3A, PTGS2, ACR1B1, PLAC8, GPX1, MnSOD, GLUT1, IGF2R, BAX* in day 7 blastocyst was measured by RT-PCR. No statistically significant difference was detected in any gene between treated and control groups. Heat treatment affected ($p<0.05$) the correlation of expression between *HSPB11 and G6PD, GPX1* and CCNB1 in oocytes. In cumulus cells HSP90AA1 was negatively correlated with *HSPA1A, LDHA* and *CCNB1,* while *CCNB1* was positively corelated with *HSPA1A, LDHA, GPX1* and *G6PD*. In blastocysts, heat treatment caused a negative correlation between *HSP90AA1, AKR1B1* and *PLAC8*. These results imply that exposure of oocytes to elevated temperature even for only 6 hours dramatically reduces the developmental competence of the oocytes, suppresses blastocyst yield and disrupts the coordinated pattern of a series of gene expression.

Acknowledgments: This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH – CREATE – INNOVATE (project code:T1EDK-1078).

A083E OPU/IVF/ET

Effect of antioxidant α-tocopherol on bovine oocyte's maturation

Loide Valadão, Maria Moreira da Silva, Fernando Moreira da Silva

University of the Azores, Faculty of Agrarian Sciences and Environment - IITAA - Portugal.

Keywords*:* cattle, oocyte maturation, alpha-tocopherol.

Vitamin E is an important natural antioxidant, and its most common and biologically active form is the α-tocopherol, being well known as a scavenger of free radicals in a hydrophobic milieu. As the chain-breaking of this antioxidant has not been reported to be present in mammalian spermatozoa, the present work was designed to evaluate the effect of the α-tocopherol on the maturation rate of bovine oocytes. For this purpose, 194 bovine ovaries divided by 12 replications, were collected at the slaughterhouse in the Terceira Island and transported to the laboratory. Follicles 2 to 8 mm in diameter were punctured, 779 cumulus-oocyte complexes (COCs) considered of quality 1 and 2 according to their morphological aspect, were assigned to maturation and randomly divided into 4 groups. Each oocyte group was maturated in a standard TCM 199 medium supplemented with fetal bovine serum, FSH/LH, Estradiol, Glutamine and Sodium pyruvate, added with different concentrations (0, 0.5, 1 and 2 mM) of α-tocopherol for 24 h at 38.5 °C, with 5% CO2 in the air and saturated humidity. Then, COC's, cumulus cells were removed, the oocytes were stained with aceto-orcein, observed under the phase contrast microscope, and the different nuclear phases were evaluated from profase I to Metaphase II. *In vitro* maturation results are expressed as a percentage of oocytes reached the Metaphase II stage. Statistical differences among treatments were evaluated by the ANOVA test. α-Tocopherol at a concentration of 0.5mM increased significantly ($P<0.05$) the maturation rate, relative to the control group, 68.0% vs 60.8%, respectively. At a concentration of 1 and 2 mM, no significant differences were observed when compared to the results obtained in the control group, with maturation rates of 64.3 % and 60.6%, respectively. This study clearly suggests addition of **α**-tocopherol at a concentration of 0.5 mM increases the maturation rate of bovine oocytes, despite it did not reveal the mechanism by which the antioxidant acts to improve maturation results. Further studies on possible effects of different concentrations of this antioxidant on the developmental competence of *in vitro* produced embryos (IVM/IVF), and the viability of these embryos after transfer to recipient heifers on Day 7 post-estrus will be evaluated in our future research. Studies on vitamin E supplementation of bovine females will be also implemented.

This project was financed in 85% by FEDER and in 15% with regional funds through the Programa Operacional Açores 2020 (Operational Program Azores 2020), in scope of the project «BEMAP-ET - ACORES-01-0145-FEDER-000026».

A084E OPU/IVF/ET

Influence of selected factors on the effectiveness of the embryo transfer in cows

Maria Wieczorkiewicz[1], Magdalena Herudzińska[1], Jarosław Czeladko[1], Jakub Kulus[1], Ewa Wędrowska[3], Bartłomiej Maria Jaśkowski[2], Magdalena Kulus[1], Marek Gehrke[1], Jędrzej Maria Jaśkowski[1]

[1]Center for Veterinary Sciences, Nicolaus Copernicus University in Torun, Torun, Poland, Poland; [2]Department of Reproduction and Clinic of Farm Animals of the Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland; [3]Department of Applied Informatics and Mathematics in Economics, Faculty of Economic Sciences and Management, Nicolaus Copernicus University in Torun, Torun, Poland.

Keywords*:* embryo transfer, recipients, risk factors.

The objective of the study was to identify risk factors associated with CR(conception rate) after ET(embryo transfer) in cows. It involved 952 cases of ET. Embryos were obtained from previously selected, hormonally synchronized (200 μg of cloprostenol) and superovulated (30 mg of pFSH in 8 decreasing intramuscular doses within 4 days and then 2 intramuscular injections of 500 μg cloporstenol) donors using a bloodless method (embryo flushing). Embryos' selection was carried out according to the evaluation of their morphology. Only embryos in stages of development 3-6 and quality 1-3 were used in the study (Bo 2013). Excellent quality embryos in the morula stage (n= 667) were intended for the direct transfer. Other embryos (n= 285) were destined for freezing and prepared for direct transfer after thawing. The group of embryo recipients consisted of 952 heifers of the Polish Holstein-Friesian breed with mean age 15.5 months and mean body condition score (BCS) 3.02. The heat was synchronized using 2 intramuscular injections of 500 μg cloprostenol given at 14-day intervals. Based on the ultrasound examination females having corpora lutea with diameter above 15 mm qualified for the ET. Embryos were placed in the recipients' uteruses between 6 and 8 days after synchronized or natural heat (757 vs. 195 heifers). The transfer procedure was performed with the use of Wörrlein gun (Goldenpick type) placed in the plastic sanitary case (Minitüb). Embryos were always inserted in the uterine horn ipsilateral to the ovary with the CL. After 2 months all recipients were clinically examined (transrectal palpation) for the pregnancy. It investigated the effect of season on ET, embryo quality or type (fresh vs. frozen), recipient's age and BCS, day of embryo introduction, depth of embryo insertion in the uterine horn, duration of the gun passage through the cervix, horn of the uterus (left vs. right), size and type of the corpus luteum (solid vs. cavernous) and type of treatment used before ET (hCG, flunixin meglumine (FM) and a combination of FM and hCG).To identify the determining factors of CR, the multivarious logit model was estimated using STATA software. The pregnancy was confirmed in 419 after fresh and 159 after frozen embryo transfer cases out of 952 of recipients (CR respectively: 62.8 and 55.8; total CR= 60.7%). The season as well as the embryo development stage were distinguished as statistically significant ($p < 0.05$). The best results (CR= 70%) were obtained in spring, the weakest in summer (CR= 50.2%). Most embryos were transferred at the stages "5" and "6" (n= 451, CR= 62.9). The more developed the transferred embryo was, the higher the CR in recipients. While embryos at the stage "4" led to pregnancy of 53% of the recipients (n=114), embryos at the stage of development "7" and "8" resulted in 66.7% and 83.3% of pregnancies respectively. However, embryos in these stages amounted only to 1.89% (n= 13). The significant ($p < 0.01$) impact of the 'condition' variable was also observed. The highest CR (67.6%) was noticed in recipients with 3.5 points (n= 157) and the lowest - 45.2% - in recipients with BCS higher than 4 (n= 42). The influence of other analyzed variables was not statistically significant.

A098E Folliculogenesis, oogenesis, and superovulation

Bisphenol S affects *in vitro* early developmental oocyte competence in ewe

Alice Desmarchais, Ophélie Téteau, Pascal Papillier, Sébastien Elis

UMR 85 PRC, CNRS, IFCE, INRA, Université de Tours, 37380, Nouzilly, France.

Keywords*:* Bisphenol S, oocyte, ewe.

Bisphenol A (BPA), an estrogenomimetic endocrine disruptor, causes deleterious effects on oocyte meiosis and maturation (Machtinger, R. Reprod Biomed Online. 29(4): p. 404. 2014). BPA was banned from food industry and replaced by structural analogs including Bisphenol S (BPS). Some studies on fish and rodent species, reported BPS effects on reproduction similar to BPA effects (Uzumcu, M. Reprod Toxicol. 23(3): p. 337. 2007; Giulivo, M. Environ Res. 151: p. 251. 2016; Ullah, H. Chemosphere. 152: p. 383. 2016). This study aims, therefore, to assess the acute effects of low doses of BPS during *in vitro* maturation on oocyte developmental competence in ewe, as bisphenol resistance was previously reported in rodent species. Cumulus-oocyte complexes (COC) were collected from ovine follicles > 2 mm (n=3789 ovaries). First, COC underwent *in vitro* maturation (IVM) for 24h (Paramio, M.T. Theriogenology. 86(1): p. 152. 2016), in untreated condition (control) or in presence of BPS (Sigma, Saint Quentin Fallavier) at low doses (1 µM, 10 µM) and at environmental doses (1 nM, 10 nM, 100 nM) (Liao, C. Environ Sci Technol. 46(12): p. 6860. 2012). Oocyte viability was assessed with Live dead® staining (Thermofischer, Illkirch, France) and fluorescence microscope observation (Zeiss, Munich, Germany) (n= 1159 oocytes). Nuclear oocyte maturation rate was evaluated by metaphase II oocyte count after chromatin Hoechst staining (Sigma, Saint Quentin Fallavier, France) (n= 978 oocytes). Then, matured COC were *in vitro* fertilized (IVF) and developed (IVD) during 7 days in SOF medium (Zhu, J. Int J Vet Sci Med. 6(Suppl): p. S15. 2018). Cleavage and blastocyst rates were determined on day 2 and on day 7 post-IVF respectively, by microscope observation (n= 2280 oocytes). Data were analyzed using logistic regression and generalized linear model (R package Rcmdr, R version 3.5.3).

Our results showed that, BPS 1 µM and 10 µM do not affect oocyte viability rate (98% [n = 245] and 97.2 % [n = 282] respectively) compared to control (99 %, n = 289). Metaphase II oocyte rate is decreased by 13 % with BPS 10 µM (76,6%, p = 0.0008) compared to control (88%). Among fertilized COC (about 300 per condition), the very low dose BPS 1 nM significantly increased cleavage rate by 28.4 % (70.1%) compared to control (54.6%, p= 0.0003). On the contrary, BPS 1 µM decreased by 12.7 % the cleavage rate (47.6%) compared to control (p = 0.004). Among cleaved embryos, BPS 10 nM and BPS 1 µM decreased blastocyst rate respectively by 34.8% (14.2%, p = 0.046) and by 42.6 % (12.5%, p= 0.017), compared to control (21,8%). Particularly, BPS 1µM significantly reduced blastocyst hatching rate by 65 % (3.3%, p = 0.032) compared to control (9.4%). BPS during *in vitro* maturation negatively affects ovine cleavage and blastocyst rates. Our data suggest BPS negatively influences early developmental oocyte competence. Further studies are needed to investigate the potential BPS effect on estrogen receptors transcripts and on signaling pathways.

Supported by INRA, Region Val-de-Loire (project BEMOL) and the French National Research Agency (project ANR-18-CE34-0011-01 MAMBO). We thank A. Arnould and T. Delpuech for ovine ovary collection.

A099E Folliculogenesis, oogenesis, and superovulation

Global transcriptome alterations in porcine oocytes with different developmental competence

Ahmed Gad[1,2], Lucie Nemcova[1], Matej Murin[1], Jiri Kanka[1], Jozef Laurincik[1,3], Radek Prochazka[1]

[1]Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Liběchov, Czech Republic; [2]Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt; [3]Constantine the Philosopher University in Nitra, Nitra.

Keywords*:* oocyte, transcriptome, follicle size.

Although our knowledge regarding oocyte quality and development has improved significantly, molecular mechanisms regulating and determining oocyte developmental competence are still unclear. Therefore, the objective of this study was to identify and analyze the transcriptome profiles of porcine oocytes derived from different sized follicles and exhibited different developmental competence using RNA high throughput sequencing technology. Cumulus-oocyte complexes (COCs) of the same grades were aspirated from medium (MO; 3-6 mm) or small (SO; 1.5-1.9 mm) ovarian follicles and tested for developmental competence and chromatin configurations. After aspiration and removal of cumulus cells, oocytes were stained with Hoechst 33342 and chromatin configurations were assessed under a fluorescence microscope. COCs from the two groups were matured and cultured in vitro after parthenogenetic activation according to our previous protocol (Prochazka *et al.* 2011*, Reproduction* 141:425-435). After 144 h, the ability of embryos to reach the blastocyst stage was analyzed. For RNA sequencing, RNA libraries were constructed from both oocyte groups (three replicates each, n= 360) and then sequenced on an Illumina HiSeq4000. Raw expression data were normalized using the trimmed mean of M-values (TMM) normalization method. The differential expression analysis was done using the statistical Bioconductor software package EdgeR. Oocytes of MO group showed significantly higher blastocyst rate compared to the SO group (33.41±7.82 vs 15.51±3.44, respectively). MO group exhibited a significantly higher proportion of surrounded nucleolus chromatin configuration compared to the SO group which exhibited a higher percentage of the non-surrounded nucleolus configuration. Transcriptome analysis showed a total of 14,557 genes were commonly detected in both oocyte groups. A group of 930 genes was representing the top highly expressed genes (>5000 reads in each replicate) including genes related to cell cycle and oocyte meiosis and quality (*CCNB1, CCNB2, ESPL1, CPEB1, CUL1, CDC25B, CDC27, BMP15*, and *GDF9*). Differential expression (DE) analysis revealed 60 up- and 262 down-regulated genes (FDR< 0.05, FC≥ 1.5) in MO compared to SO group. *ACOD1, TNFSF11, and OAZ3* were among the top up-regulated genes, while *KCNJ14, IQCA1, CLDN15,* and *IGFBP2* were among the top down-regulated genes. Ontological classification of DE genes indicated that regulation of actin cytoskeleton, oxidative phosphorylation, and ECM-receptor interaction were among the significantly enriched pathways. In addition, biological processes related to cell growth and signaling, transcription, cytoskeleton, and extracellular matrix organization were among the highly enriched in DE genes. In conclusion, this study provides new insights into the transcriptome alterations of oocytes in relation to developmental competence.

This work was supported by projects CZ.02.1.01/0.0/0.0/15_003/0000460 from OP RDE, VEGA 1/0022/15, VEGA 1/0327/16 and APVV-14-0001.

A100E Folliculogenesis, oogenesis, and superovulation

PGE2 concentration of the follicular fluid as a measure of heterogeneity of the response to hormonal stimulation of the bovine ovarian follicle

Christophe Richard[1], Gilles Charpigny[1], Olivier Dubois[1], Valérie Gelin[1,2], Fabienne Nuttinck[1]

[1]UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy en Josas, France; [2]INRA, UCEA Bressonvilliers, 91630 Leudeville, France.

Keywords*:* ovarian-stimulation, PGE2, bovine.

The LH surge promotes prostaglandin E2 (PGE2) production within the preovulatory follicle. Oocyte microenvironment levels of PGE2 affect the developmental competence after fertilization. This study aimed to characterize the follicular fluid PGE2 enrichment during superovulation treatment. Six heifers (Holstein, 20.3 +/-0.85 months old) received FSH (Stimufol®, Reprobiol, Belgium): half dose, ie, 250 µg of porcine follitropin (pFSH), combined with 50 µg of porcine lutropin (pLH). At the rate of 8 injections, in degressive dose, spread over 4 days. PGF2α (Estrumate®, MSD Santé Animale, France) was injected at the same time as the 5th injection of Stimufol®.LH peak was assumed to occur between 35 and 40 hours after the PGF2α injection. Individual sampling of fluid from antral follicles was performed by ovum pick up 12 hours before PGF2α injection and 60 hours after PGF2α injection. This protocol was designed to allow the collection of fluids from ovarian follicle containing either a pre-matured or a matured oocyte. Each heifer was his own control as we took the "pre-matured" follicular fluid on a first ovary and the "matured" follicular fluid on the 2nd ovary, 3 days later. The punctures were repeated twice and were cross-checked for the next repetition to evaluate the impact of the ipsi or contralateral side of the corpus luteum (CL) on the follicular fluid composition. The volume of fluid was measured for each punctured follicle. The PGE2 concentration of the follicular fluid was measured by Elisa (Cayman Chemical) to determine the progress of terminal follicular differentiation. An average of 13 +/- 5.06 and 28 +/-13.9 follicles were punctured per session/heifer for respectively pre-matured (n=78) and matured (n=169) follicles. The mean collected volume differed between the two groups (pre-matured: 0.229+/-0.213 ml/follicle; matured: 0.575+/-0.379 ml/follicle; two samples t-test, pval<0.0001). No effect of the side of CL on fluid volume was detected (2-way Anova, p=0.397). The PGE2 concentration was determined in 25 pre-matured follicles and 127 matured follicles. The mean PGE2 concentration significantly differed between the two groups of follicular fluids (pre-matured: 7.2 +/-7.5 ng/ml; matured: 60.2 +/-58.6 ng/ml) No effect of the side of CL was detected (p=0.278). Surprisingly, there was no linear relationship between fluid volume and PGE2 concentration (adjusted R-squared: -0.0002, p-value=0.327). PGE2 concentrations were very spread out within the matured group. This important dispersion (Interquartile range=58.6 ng/ml) indicated that despite follicle growth in response to hormonal stimulation (FSH/LH) the ability of follicular granulosa and cumulus cells to synthesize PGE2 was imperfectly achieved. Only 48% of the follicular fluids in the mature group had higher PGE2 levels than those in the premature follicle group. In conclusion, despite the ability of the stimulation treatment to promote growth of many follicles, there was a great heterogeneity in terms of PGE2 synthesis. This alteration could represent defective signaling mechanisms that could impact the developmental competence of the oocyte.
Acknowledgements: APISGENE.

A101E Folliculogenesis, oogenesis, and superovulation

Effects of bisphenol S on ovine primary granulosa cells *in vitro*

Ophélie Téteau, Manon Jaubert, Alice Desmarchais, Pascal Papillier, Sébastien Elis

INRA Centre Val de Loire, France.

Keywords*:* Bisphenol S, Granulosa Cells, Steroidogenesis.

Bisphenol A (BPA), a plasticizer used in food and drink packaging, medical devices and paper products (Giulivo M. et al., Environmental Research, Vol. 151: 251-264, 2016), has been prohibited in food industry due to its deleterious endocrine effects on both male and female reproduction (Bloom M.S. et al., Fertility and Sterility, Vol. 106: 857-863, 2016). Thus, BPA has been recently replaced by a structural analogue: bisphenol S (BPS). While its presence is exponentially increasing, BPS use is not regulated and its effects are still poorly understood, particularly on female reproduction. Several studies, especially in fish and rodents, already showed that BPS exhibits impacts similar as BPA in terms of both effects and intensity on the reproductive functions of these species, but this comparison BPA vs BPS was not yet study on granulosa cells (GCs) (Chen D. et al., Environmental Science & Technology, Vol. 50: 5438-5453, 2016 ; Rochester J.R. et al., Environmental Health Perspectives, Vol. 123: 643-650, 2015). GCs are essential for female reproductive function. They proliferate and secrete the hormones: progesterone and estradiol to allow the growth and maturation of the follicle and oocyte. The aim of this study is thus to investigate the *in vitro* effects of both BPS and BPA on ovine primary GCs. The ewe model was chosen as it is a relevant animal model for women reproduction. After follicle aspiration of approximately 1000 ovaries from local slaughterhouses, GCs were collected, purified and treated in complemented serum-free Mc Coy Medium, in absence (control) or presence of increasing concentration of BPS or BPA (1 nM, 10 nM, 100 nM, 1µM, 10 µM, 50 µM, 100 µM and 200 µM) for 48 hours. Progesterone and estradiol levels (12 and 5 independent cultures respectively) were measured by ELISA in the supernatant and normalized to the protein concentration of each well. Cell proliferation (13 independent cultures) was measured by ELISA assay after BrDU (BromoDesoxyUridine) incorporation. Data were analyzed using non-parametric permutational ANOVA and Tuckey post-hoc test. Our results showed that BPS did not affect cell proliferation, in contrast to BPA which significantly reduced cell proliferation at 50 µM ($P = 0{,}0007$) compared to the control. On the other hand, BPS significantly decreased progesterone secretion from 10 µM onwards (- 22 %; $P = 0{,}0038$) , whereas BPA lowered the level of progesterone only at 100 and 200 µM ($P < 0{,}0001$) compared to the control. BPS and BPA significantly increased estradiol secretion similarly from 10 µM onwards (+ 198 % $P = 0{,}0075$ vs. + 259 % $P < 0{,}0001$, respectively) compared to the control. These first results showed that BPS exhibits similar effects as BPA on steroidogenesus in ovine primary GCs, but not on cell proliferation. BPS even affected progesterone secretion at lower dose compared to BPA. Thus, BPS is probably not a safe alternative to BPA. Mechanisms disrupted by these molecules are currently studied in ovine primary GCs.

Supported by INRA, Region Val-de-Loire (project BEMOL) and the French National Research Agency (project ANR-18-CE34-0011-01 MAMBO).

A102E Folliculogenesis, oogenesis, and superovulation

Effect of LIF, IL-6 and IL-11 on microRNA expression of bovine cumulus cells and oocytes matured *in vitro*

Meritxell Vendrell-Flotats[1,2], Leanne Stalker[3], Manel Lopez-Bejar[2], Marc Yeste[4], Teresa Mogas[1], Jonathan LaMarre[3]

[1]Dept. Medicina i Cirurgia Animals, Universitat Autonoma de Barcelona, Spain; [2]Dept. Anatomia i Sanitat Animals, Universitat Autonoma de Barcelona, Spain; [3]Dept. Of Biomedical Sciences, University of Guelph, Canada; [4]Dept. de Biologia, Universitat de Girona, Spain.

Keywords*:* oocyte, microRNA, cytokines.

Members of the interleukin (IL-6) family of cytokines including leukemia inhibitory factor (LIF), IL-6 and interleukin-11 (IL-11) are important for reproductive function, and the expression of specific cytokines is actually required for ovulation. They participate in follicular growth and development, oocyte maturation and implantation. IL-6 cytokines stimulate the intracellular Janus kinase/signal transducer, thereby activating JAK/STAT, MAP-kinase and PI(3)-kinase pathways. This alters downstream expression of genes and microRNAs (miRNAs) in oocytes and follicular cells, creating a microenvironment that improves oocyte quality and competency. However, the putative involvement of miRNAs in the JAK/STAT signal transduction pathway activated by members of the IL-6 family has not been fully elucidated. We, therefore, characterizedthe effects of LIF, IL-6 and IL-11 on miRNA expression in bovine cumulus-oocyte complexes matured*in vitro*. We assessed the expression of *miR-21*, *miR-155*, *miR-34c*and *miR-146a*, miRNAs previously implicated in oocyte maturation and cumulus expansion. Oocytes were distributed in 5 groups: GV (germinal vesicle), Control (matured in TCM199 + 10% FBS + FSH + LH + E2), LIF (TCM199 + 25 ng/mL LIF), IL-6 (TCM199 + 10 ng/mL IL-6), IL-11 (TCM199 + 5 ng/mL IL-11) and non-supplemented (TCM199). After 24h of IVM, cumulus cells were stripped from oocytes and both cumulus cells and oocytes were collected for miRNA extraction and qPCR analysis. The effects of treatment were analyzed by one-way ANOVA followed by a Sidak test ($p<0.05$). *MicroRNA-21*expression was significantly higher in cumulus cells from the control (FBS) and LIF groups and was higher in LIF-treated oocytes compared to TCM199 alone. IL-11 treatment increased *miR-146a*expression in oocytes while no significant differences were observed in the levels of *miR-146a*in cumulus cells. In cumulus cells,*miR-155*was significantly higher in controls, compared to oocytes, where no differences were observed between groups. The presence of cytokines during maturation had no effect on *miR-34c*expression in cumulus cells or oocytes in any group. miR-21 seems to be one of the most relevant miRNAs in oocyte function. It is the most abundant miRNA in cumulus cells in bovine. It is considered as an indicator of oocyte quality because it increases along oocyte maturation, when the oocyte becomes competent for fertilization. And also, for its anti-apoptotic role, as some of its target genes are related to apoptosis. miR-21 inhibition leads to an increase of active caspase 3 in granulosa cells, what results in an increased apoptosis. In conclusion, LIF addition to the maturation media may improve oocyte quality through increased expression of*miR-21*.It is relevant that LIF without serum and hormones could create a response in miR-21 similar to that in the controls. Further studies to evaluate the potential effects and mechanisms of action of LIF on bovine oocytes are warranted.
Supported by MCIU, Spain (AGL2016-79802-P) and by the OECD.

A111E Physiology of reproduction in the male and semen technology

Transcript abundance and antioxidant biomarker of buck semen cryopreserved with melatonin supplementation

Gamal Ashour[1], Sherif Mohamed Dessouki[1], Nasser Ghanem[1], Motaz El-Gayar[2], Fakhri El-Hadi El- Azzazi[2], Elias Michael Kodi[1,3]

[1]Cairo University; [2]Suez Canal University; [3]Bahr El-Ghazal University.

Keywords*:* Keywords: bucks, melatonin, motility, total antioxidant capacity, gene expression.

This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants with two doses of melatonin as antioxidant. Pooled samples from four sexually mature Egyptian Baladi Bucks were used in this experiment. Semen was diluted (1:8) of Tris-fructose-citric extender containing egg yolk. Either Glycerol or Dimethyl sulfoxide (DMSO) was used as cryoprotectant at a final concentration of 5 %. Extended semen was supplemented with different levels (10^{-6}mM and 10^{-9}mM) of melatonin (M5250, Sigma-Aldrich, St Louis, MO, USA) in addition to control group and cooled at 5 °C for 4h before deep-freezing at -196°C. Extended semen of all groups was adjusted to the same concentration and finally packed in 0.25 ml French straws (IMV).Computer assisted semen analysis (CASA) was used to evaluate semen after cryopreservation. Data was analyzed using the SAS GLM procedure (SAS, 2004) and applying the following model. Duncan's multiple range test was used to detect differences among means, the significance level was set at $P<0.05$. Quantitative real-time PCR data was analyzed using delta delta Ct method and values were reported as relative expression of target genes to the calibrator after normalization to reference gene (GAPDH). the progressive motility was higher ($p < 0.05$) in control sample extended with glycerol (71.6±2.3%) than that supplemented with DMSO (32.9±2.5%). The progressive motility was higher ($P<0.05$) in samples supplemented by low dose of melatonin (10^{-9} mM) compared with high dose (10^{-6}mM) in glycerol (74.4±2.4% and 64.4±2.5%, respectively) and in DMSO based extender (35.5 ±2.4% and 32.9 ±2.5%, respectively). The CASA parameters (VAP, VCL and VSL um/s) were significantly different in low melatonin dose from high melatonin dose in glycerol based extender being (57.4±1.1, 103.5±2.9 and 42.5±0.8) against (51.3±1.2, 91.8±3.0 and 37.7±0.8) respectively. The activity of total antioxidant capacity (TAC) was significantly greater in DMSO group supplemented with the low melatonin dose (0.49 mM/L ±0.09) than high melatonin dose (0.16 mM/L ±0.09) group. While, there was no significant differences in TAC between glycerol extender groups. Transcript abundance of genes enhancing mitochondrial activity CPT2, ATP5F1A and SOD2 was significantly ($p < 0.05$) increased in glycerol based extender groups and this was more apparent in low melatonin dose compared with all other glycerol based extender groups. On contrast, gene regulating oxidative stress (NFE2L2) was up-regulated ($p < 0.05$) in groups cryopreserved with DMSO extender compared with those cryopreserved in glycerol based extender. It could be concluded that using glycerol based extender supplemented with low concentration of melatonin would be recommend for enhancing the fertilizing ability of buck semen.

A112E Physiology of reproduction in the male and semen technology

Effects of increasing concentrations of LPS on *in vitro* ovine oocyte developmental competence

Sara Ataei Nazari[1], Sepideh Heydari[2], Atieh Hajarizadeh[2], Maryam Rahimi[3], Abdollah Mohammadi Sangcheshmeh[1], Ail Fouladi Nashta[4]

[1]Tehran university, Tehran,Iran; [2]Islamic Azaqd University, Tehran, Iran; [3]Tehran university, Karaj,Iran; [4]The Royal Veterinary College, London, United Kingdom.

Keywords*:* inflammation, lipopolysaccharide, oocyte developmental competence.

Negative energy balance (NEB) during early lactation in dairy cows leads to an altered metabolic state that has major effects on animal reproduction. Feeding high concentrate diet, a common strategy for mitigation of NEB, enhance the risk of ruminal acidosis. Both ruminal acidosis and infectious diseases can enhance the concentration of lipopolysaccharide (LPS), an important bacterial component in circulation leading to disturbed reproductive performance. Nevertheless, some degree of LPS induced inflammation be beneficial through triggering antioxidant process to protect cell from oxidative stress. Although considerable number of researches investigated the effects of LPS on reproductive performance of dairy cows, the response of sheep to the increasing concentrations of environmental LPS is not defined yet. Ewes ovary were collected from slaughterhouse, sliced and the oocytes with more than three layers of cumulus cell and integrated cytoplasm were matured for 24 h under increasing concentrations of LPS (0, 0.01, 0.1, 1 and 10 µg/mL). In order to measure the intracellular glutathione (GSH) content, a number of matured oocytes were denuded and stained with cell tracker blue and then observed using an epifluorescence microscope and were analyzed by ImageJ software. A number of matured oocytes also were fertilized using frozen ram semen. Then, the rate of oocytes reached to the blastocyst stage were recorded at day 8 post-insemination. Data were analyzed with GLM procedure of R software. Our data showed that there was no difference ($P \geq 0.05$) between the groups in GSH content, although it was higher in medium with 10 µg/mL of LPS. Addition of LPS reduced the number of fertilized oocytes reached to blastocyst stage in a dose dependent manner (36.69, 34.21, 35.41, 16.66 and 14.28 % of oocytes reached to blastocyst stage, respectively for 0, 0.01, 0.1, 1 and 10 µg/ml of LPS; $P<0.05$). It has been shown that LPS induces the production of pre-inflammatory cytokines such as (Interleukin 6) IL-6 and (Interleukin-8) IL-8 from variety of cells. In mammals, transcription factors such as (nuclear factor-κB) NF-κB and IFN are activated after recognition of LPS by Toll like receptor (TLR-4). Moreover, bovine granulosa cells express TLR4 receptor complex and response to LPS through phosphorylation of TLR signaling components p38 and extracellular signal-regulated kinase and increase the IL-6 and IL8 transcripts. LPS was reported to affect intracellular redox status and increase apoptosis through enhancing pro-apoptotic factors. A group of antioxidant enzymes and non-enzymatic processes protects gametes and embryos against ROS damage during oocyte maturation and early stage of development. In this study, although the difference between groups in regards to GSH content was not significant but maybe higher concentration of glutathione in response to high level of LPS was a mechanism for confronting the inflammatory response created in those groups. In conclusion, our results demonstrate that LPS in 1 and 10 µg/mL concentrations may have detrimental effects on oocyte developmental competence in ovine.

A113E Physiology of reproduction in the male and semen technology

The CatSper inhibitor effect on porcine sperm in the presence of higher chemotactic activity of the follicular fluid

Alessia Diana[1], Carmen Matàs[1,2], Jon Romero-Aguirregomezcorta[3], Luis A. Vieira[1,2]

[1]University of Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.; [3]Department of Physiology Faculty of Medicine and Nursing University of the Basque Country (UPV/EHU), Spain.

Keywords*:* Boar spermatozoa, NNC effect, follicular fluid.

Several components in follicular fluid (FF), in particular progesterone, have chemotactic capacity and depend on the entry of Ca2+ through membrane or CatSper channels in some species (Lishko P.V. et al., Nature, 471(7338):387–91 (2011)). On the other hand, P4 does not seem to be a clear CatSper agonist in porcine (Vicente-Carrillo A. et al., ReproBiol, 17 (1): 69-78 (2017)). The NNC 55-0396 inhibitory effect on CatSper in sperm has been demonstrated. The aim of this study was to investigate NNC effect in the presence or not of FF. The chemotaxis system used consists of two wells (A and B) connected by capillaries. Four wells (A) were filled with fresh sperm were first washed in a discontinuous gradient of Percoll®, followed by TALP medium (20x106/mL diluted in 500 µL) from proved fertility boars (N=4) previously incubated or not with NNC alone, NNC and 0.25% of FF, while the opposite wells (B) were filled with TALP (control group) and TALP supplemented with 0.25% of FF. NNC (2 µM) without cytotoxic effects and 0.25% of FF were used. Two experiments were performed: Experiment I: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: FF- (TALP+NNC (A) – FF (B)). Experiment II: the A and opposite B were filled with 1: Control group (TALP (A) – TALP (B)), 2: FF+ (TALP (A) – FF (B)), 3: NNC (TALP+NNC (A) – TALP (B)), 4: NNC+FF (TALP+NNC+FF (A) – FF (B)). In experiment I, treatment 3 and 4 were preincubated (10 min) with NNC before chemotaxis, likewise, for treatment 3 in experiment II. However, the treatment 4 in experiment II was preincubated (10 min) at the same time with NNC and 0.25% of FF before chemotaxis. After 20 min of chemotaxis, the sperm concentrations (%) from wells B were evaluated using the free statistical software, Sas University Edition (SAS, 2016). In experiment I, the highest percentage of attracted sperm was seen in FF+ (9.1%b) versus control group TALP (7.5%a), NNC group (5.6%a), and FF-group (6.8%a) ($p<0.05$). Similarly to experiment II: FF+ (5.7%b) versus control group (4.6%a), NNC group (3%c), and NNC+FF group (3.9%a) ($p<0.05$). These results may indicate the chemotactic effect of FF on boar spermatozoa. Moreover, NNC inhibited sperm chemotaxis even in the presence of higher chemotactic activity of FF, which suggests that at least the chemoattractant components in FF might act via CatSper. Further studies should be carried out to test this hypothesis.

Supported by Fundación Séneca, Saavedra Fajardo (20020/SF/16). MINECO-FEDER (AGL 2015-66341-R).

A114E Physiology of reproduction in the male and semen technology

Factors affecting the sperm concentration assessment in commercial seminal doses in pigs

Carmen Escudero, Sergio Navarro-Serna, Joaquín Gadea

University of Murcia Dept. Physiology, Murcia, Spain. International Excellence Campus for Higher Education and Research "Campus Mare Nostrum" and Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords*:* sperm assessment, quality control, sperm concentration.

Artificial insemination is widely implemented worldwide with more than 90% of the sows inseminated with fresh semen. However, up to now, a quality control system for swine artificial insemination centers has not been internationally established. Evaluation of the sperm concentration in commercial seminal doses is a key point in the control of the dose's quality, since a clear relationship is established between total sperm number in the insemination dose and fertility outcome. The use of cell counting chambers is a cheap and simple methodology. Nevertheless, it tends to be less precise than other more sophisticated and expensive methodologies (Hansen, Theriogenology, 66, 2188. 2006). The aim of this study was the evaluation of different factors (dilution rate, pipetting repeatability, microscopy magnification, time of sample resting before evaluation and chamber area evaluated) that could modify the results in sperm concentration of commercial seminal doses, for further proposal of a scientific base a standardized protocol. Sperm concentration in 27 seminal doses was evaluated by one observer by dilution (1/10 or 1/20 rate) in saline solution (0.3% formaldehyde), pipetting by triplicate, disposed in a Neubauer chamber and observed after 1 or 5 minutes by contrast phase microscopy (x100, x200 or x400 magnification) and counting the number of spermatozoa present in 0.12 or 0.2 mm^2. Data were expressed as the mean ± SEM and analysed by ANOVA, considering the specific factors (dilution, pipetting, time, objective, area) as the main variable and sample as covariate. Bland-Altman analysis was applied to assess the degree of agreement, showing the bias (mean±SD). The pattern of relationship between difference and average was evaluated by lineal regression as quality of the agreement ($p<0.05$). Sperm concentration was not affected by dilution rate 1/10 or 1/20 (bias 1±5.42, p=0.74). No differences were found for concentration of samples by 3 pipetting procedures (p=0.81), between 1 or 5 minutes in the chamber before examination (p=0.73) and between counting areas of 0.12 vs. 0.20 mm^2 (p=0.69). However, the concentration measured using x10 objective was higher (41.56±2.54x10^6/ml) than using x20 (37.81±1.95, p=0.03) and x40 objectives (38.03±1.89, p=0.02). These differences were confirmed with significant regression for the difference x40-x10 (bias -3.53±9.14, p=0.01) and 20x-10x (bias -3.75±9.76, p=0.02). The overestimation with x10 objective could be related to difficulties to observe with precision the limits of the counting area or measuring as spermatozoa other different particles. These problems are minimized when higher magnification is used, although time consuming is higher with higher magnification. So, according to the obtained results, we propose a standard procedure with the selection of x20 contrast phase objective, dilution 1/20, 1 min sample resting and 0.2 mm^2 to optimize precision and time consuming.
Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480).

A115E Physiology of reproduction in the male and semen technology

L-carnitine supplementation to UHT skimmed milk-based extender improves motility and membranes integrity of chilled ram sperm up to 96 h

Diego A. Galarza[1,2], Antonio López-Sebastián[1], Julián Santiago-Moreno[1]

[1]Department of Animal Reproduction, INIA, 28040 Madrid, Spain; [2]Faculty of Agriculture Sciences, University of Cuenca, EC010205, Cuenca, Ecuador.

Keywords*:* L-carnitine, sperm, ram.

The addition of new additives as L-carnitine (LC) to extenders could mitigate reactive oxygen species (ROS) production and improved motility and viability in chilled ram sperm as has been demonstrated in other species (e.g. human, bull, and mice). The aim of this work was to evaluate the antioxidant effect of LC on motility variables and integrity of plasma, acrosomal, and mitochondrial membranes of chilled (5 ºC) ram sperm up to 96 h. Twelve pools from 36 semen ejaculates were collected by artificial vagina from 12 Merino rams (3-9 years) in four sessions during non-reproductive season (June to August). Each pool was divided into 6 aliquots and then diluted at $200x10^6$ sperm/ml in UHT-based extender (skimmed milk-6% egg yolk) supplemented either 1mM (LC1), 2.5mM (LC2.5), 5mM (LC5), 7.5mM (LC7.5), and 10mM (LC10) of LC. A control group without LC was included in each pool. Sperm motility variables were assessed by CASA system (SCA®) and total sperm with intact plasma membrane / intact acrosome / intact mitochondrial membrane (IPIAIM,%) was assessed by triple fluorescence association test (PI/PNA-FITC/Mitotracker green) at 0, 48, and 96 h. The effects of LC concentration and cold-storage time were analyzed by one-way ANOVA and Bonferroni´s test ($p<0.05$). Overall, the results showed that kinetic variables and integrity of sperm membranes decreased ($p<0.05$) as cold-storage time increased in all groups. The results revealed a higher ($p<0.01$) sperm motility (SM,%) in all LC groups than control group at 48 h. However, at 96 h, both LC5 and LC10 groups showed a SM higher ($p<0.001$) than both LC7.5 and control group (87.9±2.2 and 88.0±1.8 vs 82.9±2.1 and 82.5±3.1, respectively). Progressive sperm motility (PSM,%) was higher with LC5 group than control group at 48 h (42.2±2.9 vs 36.7±1.8) and 96 h (35.7±3.4 vs 29.0±1.7). Surprisingly, straight line velocity (VSL, µm/s) was improved with all LC groups compared with control group at 0h ($p<0.01$), 48h ($p<0.001$) and 96 h ($p<0.001$). Moreover, at 96h VSL (µm/s) value was higher with LC7.5 group than all LC groups ($p<0.5$) and control ($p<0.001$) (LC7.5: 87.2±4.9 vs LC1: 75.1±4.5, LC2.5: 78.6.2±5.9, LC5: 79.4±5.0, LC10: 79.3±5.2, and control: 65.4±3.4). Likewise, IPIAIM percentage was higher ($p<0.001$) in all LC groups than control group during at 48 h and 96 h (LC1: 62.3±2.0, LC2.5: 66.3±1.7, LC5: 63.3±2.8, LC7.5: 66.5±2.4, and LC10: 66.3±1.9 vs control group: 49.2±2.9). These results revealed a kinetic-enhancer effect of LC supplementation to UHT skimmed milk-based extender, which might improve fertility following cervical insemination of sheep.

Supported by the European Union's project Horizon 2020, (677353 IMAGE) and pre-doctoral scholarship ARSEQ-BEC-008856-2016 SENESCYT, Ecuador

A116E Physiology of reproduction in the male and semen technology

Identification and localization of NADPH oxidase 5 in ram spermatozoa

Silvia Gimeno-Martos, Blanca Pina-Beltrán, Adriana Casao, Jose A. Cebrián-Pérez, Teresa Muiño-Blanco, Rosaura Pérez-Pé

Dpto. Bioquímica y Biología Molecular y Celular (BIOFITER). Instituto de Investigación en Ciencias Ambientales de Aragón (IUCA). Facultad de Veterinaria. Universidad de Zaragoza.

Keywords*:* ram sperm, NADPH oxidase, melatonin.

The aim of this study was to identify the presence of NADPH oxidase 5 (NOX5) in ram spermatozoa and to investigate if melatonin could modulate this enzyme during *in vitro* capacitation. Semen from nine *Rasa Aragonesa* rams was collected and pooled. Seminal plasma free spermatozoa were selected by a swim-up procedure (control sample). Spermatozoa were then incubated in TALP medium without (TALP sample) or with cAMP-elevating agents (cocktail sample, Ck) for 3 h at 39 °C and 5% CO_2. 1 µM melatonin was added to TALP and cocktail samples (TALP-Mel and Ck-Mel) (n=6). Capacitation status was evaluated by chlortetracycline (CTC) staining. Identification and distribution of NOX5 in ram spermatozoa was investigated by western-blot and indirect immunofluorescence (IIF) with the anti- rabbit NOX5 C-terminal antibody (ab191010, Abcam, Cambridge, UK). At least 200 spermatozoa were scored per sample in CTC and IIF assays. Differences between experimental groups in CTC staining and NOX5 immunolabeling were compared by means of chi-square test using GraphPad InStat software (Version 3.01). As expected, the inclusion of cAMP-elevating agents in the cocktail sample increased the capacitated-sperm pattern by CTC compared with TALP sample after *in vitro* capacitation ($p< 0.001$), whereas the presence of melatonin at 1 µM in both samples increased the non-capacitated-pattern relative to samples without hormone ($p<0.001$). Regarding the presence of NOX5 in ram spermatozoa, Western blot analyses revealed a band of 86 kDa compatible with that reported to NOX5 in human (Musset et al., The journal of biological chemistry, 287: 9376-9383,2012) and equine (Sabeur and Ball, Reproduction 134:263-270, 2007) spermatozoa. IIF revealed six differences immunotypes depending on the presence of NOX5 in the ram sperm: I: apical region II: acrosome , III: post-acrosome, IV: apical and post-acrosomal, V: acrosome and post-acrosome (all subtypes with midpiece labelling) and VI: labelling in the midpiece of the spermatozoa. In swim-up selected (control) ram spermatozoa, the predominant NOX5 immunotypes were I and II. After incubation in capacitating conditions, these inmunotypes decreased in TALP samples and increased those III and V ($p< 0.001$) when compared to control. In cocktail samples, there was also an increase in the rate of spermatozoa with labelling only in the midpiece of the flagellum (type VI, $p<0.001$). However, the presence of melatonin in TALP medium (TALP-Mel) increased II subtype and in cocktail sample (Ck-Mel) increased V immnutype ($p< 0.001$),spermatozoa presented a NOX5 distribution very similar to that observed control and TALP samples respectively. In conclusion, these preliminary results reveal for the first time that NOX5 is present in ram spermatozoa, and that melatonin can prevent the NOX5 distribution changes associated with sperm capacitation.

Acknowledgements: AGL2017-83799-R, DGA-A07_17R , BES-2015-07203, red PIVEV AGL2016-81890-REDT.

A117E Physiology of reproduction in the male and semen technology

Effect of repeated ejaculates on seminal plasma composition and semen liquid storage in INRA180 ram

Naima Hamidallah[1], Anass Benmoulaa[1,2], Abderaouf El Hilali[1], Abdelmoughit Badi[1,2], Kaoutar EL Khalil[1,2], Bouchra El Amiri[2]

[1]Faculté des Sciences et Techniques, Université Hassan Premier,Settat, Morocco; [2]INRA-Centre Régional de la Recherche Agronomique de Settat, Marocco.

Keywords*:* repeated ejaculate, semen storage, seminal plasma INRA 180 ram.

In sheep, superior rams are used extensively for mating or as sperm donors for genetic improvement. Nevertheless, sperm production capacity and its storage are a major factor confining extensive use of rams over numerous ewes for a long period of time. The objective of this experiment is to evaluate the effect of repeated ejaculates on liquid storage sperm motility, and seminal plasma biochemical composition in INRA180 ram. Five INRA180 rams were collected weekly for 4 weeks at a rate of three ejaculates each 20 minutes. Concentration of total protein (g / l) (Prot) (Lowry et al.,1951. *J. Biol. Chem. 193, 265-275*), total lipid (g / l) (Lip) (Woodman and Price, 1972. *Clin. Chim. Acta., 38, 39-43*) and fructose concentration (g / l) (Fruc) (Mann, 1948, *J. Agric. Sci., 38, 323-331*) were evaluated. Immediately after collection and initial evaluation, the semen was extended in skim milk (SM) at 15°C to reach 0.8×10^9 spermatozoa/ml. Thereafter, the samples were evaluated at different storage times (0, 8, and 24 h). A CASA system was used to determine total (TM%) and progressive (PM%) motilities. All analyses were carried out using a statistical software program JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA). Variance analysis (one-way ANOVA) was performed. The statistical model included fixed effects of repeated ejaculates (first, second and third). When statistically significant differences were detected, the Tukey's post hoc, was used to compare the means and standard errors for Prot, Lip and Fruc in seminal plasma, TM and PM in each storage duration (0, 8 and 24h) considering the significance level of $P < 0.05$. The results showed that, in seminal plasma, the total protein concentration was significantly higher in the first (25.30±0.22 g/l) and second ejaculates (25.17±0.29 g/l) compared to the third (24.36±0.23 g/l). The highest total lipid concentration was recorded in the first ejaculates (3.75±0.07 g/l) ($P <0.05$), followed by the second ejaculates (3.63±0.08 g/l) ($P <0.05$), while the third ones (3.46±0.09 g/l) recorded the lowest total lipid concentrations ($P <0.05$). The fructose concentration was higher in the second ejaculates (5.49±0.16 g/l) ($P <0.05$), followed by the first ejaculates (5.39±0.14 g/l) ($P <0.05$), while the third ejaculates recorded the lowest fructose concentrations (4.96±0.17 g/l) ($P <0.05$). Regarding semen liquid storage, the results indicated that the second ejaculate has significantly better sperm motility compared to the first and the third ones and this still true until 8 h of liquid storage. While at 24 h, the first ejaculate gives the best results ($P <0.05$). To conclude, our results recommend the use of the second ejaculate for artificial insemination before 8 h of storage and the first ejaculate until 24 h of storage.

A118E Physiology of reproduction in the male and semen technology

Effects of semen collection methods and equilibration times on post-thaw sperm kinematic parameters of Saanen bucks

Khoboso C. Lehloenya[1], Kambulu Lukusa[2], Fhulufhelo V. Ramukhithi[3], Matshidiso B. Matabane[3], Abubeker Hassen[2]

[1]1Department of Agriculture, University of Zululand, KwaZulu-Natal, South Africa; [2]Department of Animal & Wildlife Sciences, University of Pretoria, South Africa; [3]Germplasm Conservation and Reproductive Biotechnologies, Agricultural Research Council Animal Production Institute, Pretoria, South Africa.

Keywords*:* electro-ejaculation, equilibration time, sperm kinematics.

The success of AI depends on semen quality and female fertility. Buck semen is commonly collected by artificial vagina (AV). However, when bucks cannot be trained for semen collection or semen is collected to evaluate fertility of bucks before mating season, the electro-ejaculation (EE) method is usually utilized. However, differences on sperm characteristics between ejaculates collected by AV and EE have been reported. The equilibration process and type of extender are known to affect the quality of post-thaw sperm quality and this study hypothesize that semen collection methods also have confounding effects. The present study was conducted to investigate the effects of semen collection methods and equilibration times on sperm kinematic parameters of Saanen bucks. Eight bucks were divided into two equal groups (4 bucks) based on semen collection methods; AV or EE. A total of 12 ejaculates (collections) per buck were collected at weekly intervals. Freshly collected ejaculates were pooled per group and extended with clarified egg-yolk tris extender (CEY). Pooled semen samples were cooled to 4^{o}C within 2 h and equilibrated at 4^{o}C for 2, 4 and 6 h in separate aliquots. Thereafter, the samples were then frozen using standard procedure after completion of each equilibration time. Four straws of frozen semen per group per collection were thawed at 33°C for 30 sec and evaluated for post-thaw sperm motility and kinematic parameters using CASA system 24 h after freezing. Semen collected with AV had significantly ($p<0.001$) higher sperm curvilinear velocity (VCL: 122.21±1.23 μm/s), straight line velocity (VSL: 89.24±0.11 μm/s), linearity (LIN: 64.23±0.91 %), beat/cross-frequency (BCF: 7.21±0.02 Hz), total motility (98.12±0.34 %), rapid sperm (66.26±0.11 %) and progressive motility (77.51±1.12 %) compared to semen collected with EE method. Post-thaw sperm curvilinear velocity (VCL: 65.52±0.02 μm/s) were higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods compared to other equilibration times. Straight line velocity (VSL: 49.15±0.92 μm/s) was higher ($p<0.001$) for sperm equilibrated for 2 h in semen collected with AV methods. Average path velocity (VAP: 64.65±0.43 μm/s) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV. Linearity coefficient (LIN: 74.34±1.01 %) and straightness coefficient (STR: 77.89±0.45 %) in semen collected with AV were higher ($p<0.001$) for sperm equilibrated for 2 h compared to semen collected with EE and other equilibration times (4 and 6 h). Amplitude of lateral head displacement (ALH: 3.46±0.98; 3.39±0.02 μm) was higher ($p<0.001$) for sperm equilibrated for 4 h in semen collected with AV and 2 h equilibration in semen collected with EE. The AV method and equilibration times for 2 or 4 hours preserved sperm motility and kinematic parameters post-thaw.

A119E Physiology of reproduction in the male and semen technology

Description of porcine spermatozoa-interacting proteins after contact with male and female reproductive fluids

Chiara Luongo[1], Paula Cots Rodriguez[2], Leopoldo Gonzalez Brusi[2], Manuel Aviles Sanchez[2,3], Maria Jose Izquierdo Rico[2,3], Francisco Alberto Garcia-Vazquez[1,3]

[1]Department of Physiology, Veterinary School, University of Murcia, Murcia 30100, Spain. International Excellence Campus for Higher Education and Research (Campus Mare Nostrum); [2]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia, Murcia 30100, Spain; [3]Institute for Biomedical Research of Murcia, IMIB-Arrixaca, Murcia, Spain.

Keywords*:* porcine, biological fluids, sperm proteome.

After ejaculation, sperm are deposited within the female genital tract by natural or artificial insemination, starting their journey towards the fertilization site. Along the way, sperm take contact with reproductive fluids, and only few of them reach the oocyte. The first fluid in which sperm are immersed during ejaculation is seminal plasma (SP), involved in aiding sperm transport and survival through the female genital tract by SP-proteins, improving their fertilizing ability (Bromfield, Animal, 104-109, 2018). Once deposited within the uterus, sperm contact with uterine fluid (UF), a dynamic female milieu that changes its proteome during the oestrus cycle (Soleilhavoup, Mol Cell Proteomics, 93-108, 2016). In sow, sperm-UF interaction is still unknown, but UF exerts a cytotoxic effect on sperm cells unprovided of SP (Kawano, Proc Natl Acad Sci, 4145-50, 2014). After crossing the uterus, selected sperm reach the oviduct, interacting with the oviductal fluid (OF), inducing sperm functional changes (Perez-Cerezales, Biol Reprod, 262-276, 2018). Since sperm interaction with fluids may change sperm proteome, the aim of this study was to identify proteins that adhere to ejaculated sperm (S) after contact with different reproductive fluids [SP, UF and OF (collected in slaughtered sows)] for a better understanding of sperm behavior during their journey previous to fertilization. The experimental groups used were: 1) S group (control): sperm without reproductive fluids; 2) SP group: S incubated with 20% SP; 3) UF group: S incubated with 20% UF (late follicular phase); 4) OF group: S with 20% OF (late follicular phase); 5) UF-SP group: S with 20% UF and 20% SP; 6) OF-SP group: S with 20% OF and 20% SP. All the groups were incubated for 180 min at 38°C, then centrifuged at 600 *g* for 5 min and the pellet was used for protein extraction, carefully performed to detect surface proteins. Sperm proteome was assessed by HPLC-MS/MS analysis. The total number of proteins identified was 88. Among these, 56 proteins were detected in S group and in UF, SP and UF-SP groups. 72 proteins were detected in S group and in OF, SP and OF-SP groups. This study has also allowed to identify a higher number of proteins in common between OF and OF-SP groups (29), than in UF group and UF-SP group (3 proteins in common). Furthermore, sperm incubated with UF-SP showed a lower number of proteins (17) than when incubated with SP (32) or UF (42). Instead, sperm showed 44 proteins when incubated with OF-SP, 32 proteins with SP and 42 proteins with OF. One of detected proteins, sperm acrosome membrane-associated protein 1, was expressed in all the groups except in OF-SP group. Moreover, sperm equatorial segment protein 1 was detected in all the groups except in UF-SP group. The combined use of SP with UF or OF suggests an interaction between these fluids that modify the sperm proteins probably caused by a steric hindrance. In conclusion, this study highlights how sperm proteome changes after interaction with different reproductive fluids, with a potential physiological impact during the *in vivo* fertilization process. Supported by MINECO and FEDER (AGL2015-66341-R and AGL2015-70159-P) and Fundación Séneca (19357/PI/14).

A120E Physiology of reproduction in the male and semen technology

Nuclear morphometrics and chromosome positioning in boar sperm

Anjali A. Mandawala[1], Benjamin M. Skinner[2], Grant A. Walling[3], Simon C. Harvey[1], Katie E. Fowler[1]

[1]Canterbury Christ Church University, United Kingdom; [2]Department of Pathology, University of Cambridge, Cambridge, UK; [3]JSR Genetics Ltd, Driffield, East Yorkshire, UK.

Keywords*:* sperm, nucleus, morphometrics.

The predicted increase in the global population and changes in dietary preferences have led to a rise in the demand for meat products. Artificial insemination is routinely used in commercial pig breeding, for which the use of high-quality semen samples during insemination is crucial. With an aim to reduce inter-operator variability and the laborious nature of manual semen analysis, we have developed a fast, unbiased software-based approach which allows comprehensive analysis of pig sperm nuclear morphometrics. Fresh ejaculated semen samples were identified as either fertile or sub-fertile using a combination of computer assisted sperm analysis (CASA) and manual assessment prior to use of samples in this study. Using CASA, 'normal morphology' was assigned to samples if less than 30% of the sample contained morphological defects such as bent tails, coiled tails, distal midpiece reflex (DMR), proximal droplets and distal droplets. Subjective manual assessment was used to score motility from 1 to 5, 1 being dead and 5 being excellent. Samples were categorised as fertile if more than 70% of the sample had 'normal morphology' and if at least 85% of the sample had a motility score of 4 or above. Those falling below these criteria were categorised as sub-fertile. Analysis of nuclear morphology from 50 fertile and 50 sub-fertile samples yielded measures from 11,534 and 11,326 nuclei respectively. Cluster analysis using measures of Area, Circularity, Variability, Bounding height and Bounding width by Ward linkage using squared Euclidean distance and standardised variables supported the existence of three clusters with different membership for fertile and sub-fertile sperm. Specifically, sperm heads from fertile animals were overrepresented in one cluster and underrepresented in another. The cluster in which sperm heads from fertile samples were overrepresented was characterised by a high mean nuclear area, which was a consequence of greater head width, and by low variability between sperm heads. We extended this analysis to determine if chromosome positioning in pig sperm also varies between fertile and sub-fertile samples. In a preliminary study, two fertile and two sub-fertile semen samples from Pietrain boars were analysed using fluorescence *in situ* hybridisation with locus-specific subtelomeric probes, and the position of pig chromosomes 10 and X were determined. This suggested that chromosomal position also differs between nuclei from fertile and sub-fertile samples. Based on this preliminary finding, we are currently extending this study to perform a complete analysis of nuclear organisation using a larger sample size of 20 samples and imaging more cells per sample. In conclusion, we show that there are morphological and chromosome positioning differences between sperm nuclei from fertile and sub-fertile samples. This approach therefore has the potential both to be used as a tool for sperm morphology assessment and as a way to investigate the causes of fertility differences.

A121E Physiology of reproduction in the male and semen technology

Season affects refrigerated-stored semen doses from a commercial stud AI centre: A flow cytometry study of sperm physiology and chromatin status

Felipe Martínez Pastor[1,2], Andrea Núñez-González[1], Estela Fernández-Alegre[1], Carlos Vega-Gutiérrez[1], Beatriz de Arriba[1], Beatriz Martín-Fernández[1,2]

[1]INDEGSAL, Universidad de León, Spain; [2]Molecular Biology, Cell Biology, Universidad de León, Spain.

Keywords*:* spermatozoa, pig, seasonality.

The pig industry rely on the production of semen doses in stud centers, which are distributed to the production farms. Advances in boar selection, extender formulation and storage allow for consistency on sperm quality and artificial insemination (AI) results. However, seasonality still affects semen quality (Porcine Health Manag 3:15, 2017). Our objective was to characterize the influence of the season in the doses produced in a modern center (NE Spain). We tested two hypotheses: Sperm quality was affected by the season, and this effect followed a yearly sinusoid pattern. Semen doses (40×10^6 ml^{-1}) were produced from 236 Pietrain boars in routine semen production (extender from Magapor, Zaragoza, Spain). The doses (436 from early 2017 to early 2019) were sent at 17 °C to the laboratory, being analysed by 48 h of storage. An aliquot was added to the staining solution at 10^6 ml^{-1} (BTS with Hoechst 33342, PNA-FITC, merocyanine 540, propidium iodide and Mitotracker deep red; ThermoFisher, Waltham, MA), for viability, acrosomal status, capacitation, and mitochondrial activity assessment (Theriogenology 80, 400–410, 2013). Another aliquot was submitted to ORT (osmotic resistance test, 15 min in 150 mOsm/kg BTS before staining). After 15 min at 37 °C, samples were run in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). Sperm chromatin was assessed by SCSA (Sperm Chromatin Structure Assay; Methods Cell Sci 22:169–189, 2000), obtaining %DFI (DNA fragmentación) and %HDS (chromatin immaturity). Data were analyzed with the R statistical package, testing season effects with linear mixed-effect models (calendar season as fixed and male as grouping factors) and cosinor regression. We also tested the relationship between physiological and chromatin variables by Pearson correlations. Doses collected were 111 in spring, 96 in summer, 117 in fall and 112 in winter. Sperm quality was overall good (interquartile ranges, viability: 79.6, 89.6; intact acrosomes: 88.4, 93.2; viable-capacitated: 2.9, 5.5; viable-active mitochodria: 71.1, 97.2; ORT: 61.6, 87.1; %DFI: 0.4, 0.7; %HDS: 0.5, 1.6). We detected a season effect in viability, acrosomal integrity, mitochondrial activity, and %HDS ($P<0.001$) , and in viable capacitated ($P=0.003$). Cosinor detected a yearly sinusoid pattern ($P<0.025$) (peak and low-point indicated) for: viability and mitochondrial activity (mid-spring/mid-fall); acrosomal integrity (early-fall/early-spring); capacitated (late fall/late spring); and %HDS (early summer/early winter). We also found significant correlations of %HDS with acrosomal integrity (-0.66, $P<0.001$) and mitochondrial activity (0.40, $P=0.048$). Overall, the effect size of the calendar season was small. However, its influence on the %HDS, being a chromatin structure parameter, merits study. Stud centers should take these results into account, since the season effect may increase in suboptimal situations, affecting the adherence to quality standards. Supported by RTI2018-095183-B-I00 (Ministry of Science, Innovation and Universities, Spain) and AGL2016-81890-REDT (MINECO, Spain). We thank EVB (Spain) and Lucía Tejerina for their collaboration in this study.

A122E Physiology of reproduction in the male and semen technology

SLO1 channels are essential for acrosome reaction during *in vitro* capacitation of boar spermatozoa

Yentel Mateo, Ariadna Delgado-Bermúdez, Sandra Recuero, Marc Llavanera, Beatriz Fernandez-Fuertes, Isabel Barranco, Sergi Bonet, Marc Yeste, Elisabeth Pinart

University of Girona, Spain.

Keywords*:* sperm; capacitation; acrosome reaction; pig; SLO1 channels.

The aim of the present study was to determine whether SLO1 channels, also known as big potassium (BK) or maxi K^+ channels, are involved in sperm capacitation and acrosome reaction in boar spermatozoa. With this purpose, we incubated semen samples from five boars in *in vitro* capacitation medium plus paxilline 100 nM (PAX), a specific blocker of SLO1 channels. Sperm samples were incubated in capacitation medium at 5% CO_2 and 38.5ºC for 240 min, with or without PAX. At 240 min, progesterone was added to control and PAX samples to induce the acrosome reaction. Samples were incubated for further 60 min (300 min). After 0, 60, 120, 180, 240, 250, 270 and 300 min of incubation, total and progressive motility were measured by Computer Assisted Sperm Analysis (CASA), and acrosome integrity, permeability of plasma membrane, and intracellular calcium levels measured by Fluo3 and Rho5 were determined by flow cytometry. After confirming that data distributed normality and variances were homogenous, a mixed model followed by post-hoc Sidak test was run. Total and progressive motility, as well as calcium levels measured by Fluo3, which preferentially stains calcium residing in the mid-piece, did not differ significantly between control and PAX samples at any incubation time ($P>0.05$). Despite permeability of plasma membrane and acrosome integrity being lower in PAX than in control samples after the addition of progesterone, these differences were not significant ($P>0.05$). In contrast, intracellular calcium levels measured by Rhod5, which has more affinity for calcium residing in the sperm head, and acrosome reacted spermatozoa were significantly ($P<0.05$) lower in PAX than in control samples after 250 and 300 min of incubation. We can thus conclude that, while SLO1 channels do not seem to play a key role for motility regulation of boar spermatozoa during capacitation and progesterone-induced acrosome exocytosis, they are essential for triggering the acrosome reaction. This involvement appears to be related with the modulation of calcium stores present in the sperm head.

A123E Physiology of reproduction in the male and semen technology

In vitro assessment of sperm characteristics using semen from Norwegian Red bulls with high and low fertility

Birgitte Narud[1,2], Abdolrahman Khezri[2], Else-Berit Stenseth[2], Teklu T. Zeremichael[2], Frøydis D. Myromslien[2], Bjørg Heringstad[3], Elisabeth Kommisrud[2]

[1]Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; [2]Department of Natural Sciences and Technology, Inland Norway University of Applied Sciences, Hamar, Norway; [3]Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Norway.

Keywords*:* bull, non-return rate, sperm characteristics.

The aim of this study was to investigate possible associations between *in vitro* parameters and fertility by assessment of several *in vitro* sperm characteristics and IVF using cryopreserved semen from Norwegian Red bulls of contrasting fertility. The bulls were characterized as low- or high-performing bulls based on non-return rate after 56 days (NR56) for an average of 1132 first AIs per bull, ranging from 47% to 79%. NR56 was calculated as LSmean for 507 bulls used in AI from 2013-2018, based on a General Linear Model (PROC GLM in SAS®) including the following parameters: bull, AI month and year, parity and double AI within 1-4 days. Totally 37 bulls with contrasting NR56 were selected for analyses, 19 bulls with NR56 LSmean ranging from 0.76 to 0.78 and 18 bulls ranging from 0.46 to 0.65. Cryopreserved semen doses were analysed for total sperm motility, progressive motility and hyperactivity by computer-assisted sperm analysis. Additionally, the ATP content was assessed using the CellTiter-Glo® Luminescence assay. Sperm chromatin, acrosome and plasma membrane integrity were analysed by flow cytometry using the Sperm Chromatin Structure Assay, Alexa 488 conjugated peanut agglutinin and propidium iodide, respectively. Furthermore, semen from selected bulls from the contrasting fertility groups were used for IVF. *In vitro* production of embryos were performed with media from IVF Bioscience using four well plates containing 500 µL of the respective media. Bovine ovaries were collected at a local slaughterhouse, transported to the laboratory and cumulus–oocyte complexes (COCs) were aspirated from follicles sized 3 to 15 mm in diameter. Groups of high quality COCs were matured for 22 h (6% CO_2, 38.8°C). Spermatozoa prepared at a concentration of 1×10^6 /ml were added to each group of oocytes followed by 18 h incubation (6% CO_2, 38.8°C). Cumulus cells were removed from the presumptive zygotes by vortexing prior to cultivation in a humidified atmosphere (7% O_2, 6% CO_2 and 87% N_2). At day 3 post-fertilization, the cleavage rate was evaluated. Further, the blastocyst rate at day 7 and day 8 was recorded. Cryopreserved semen from each bull was used in three replicate experiments including 180 oocytes per bull. Statistical analyses were performed by linear mixed models in Rstudio (v 1.1.463) using the *in vitro* sperm parameters and LSmean for NR56 as dependent and independent variables, respectively. In addition, bull, age and season at the time of semen collection were included in the model. Total motility, progressive motility and hyperactivity was positively associated with NR56 ($p<0.05$). Furthermore, sperm chromatin integrity, calculated as DNA fragmentation index and high DNA-stainable sperm, showed a negative association with NR56 ($p<0.05$). The ATP content and acrosome integrity were not associated with NR56. Preliminary results from IVF indicate that bulls of similar fertility (NR56) obtain different blastocyst yields *in vitro*. In conclusion, the results of the study showed that NR56 was associated with several *in vitro* sperm parameters. Prediction of fertility might be possible combining *in vitro* sperm analyses, where the use of IVF could provide valuable additional information.

A156E Embryology, developmental biology and physiology of reproduction

Embryo-induced alterations in the endometrial transcriptome of prepubertal bovine heifers

Sandra Bagés Arnal[1], José M Sánchez[1], Beatriz Fernandez Fuertes[2], Michael McDonald[1], Colin J Byrne[3], Alan K Kelly[1], David A Kenny[3], Susanta K Behura[4], Tom E Spencer[4], Trudee Fair[1], Pat Lonergan[1]

[1]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland; [2]Biotechnology of Animal and Human Reproduction (TechnoSperm), Department of Biology, Institute of Food and Agricultural Technology, University of Girona, Spain; [3]Teagasc Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland; [4]Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA.

Keywords: endometrium, prepubertal, calves.

Advancing the age at which puberty is reached in replacement heifers is central to the financial and environmental sustainability of cattle production systems. Puberty onset is regulated by a complex network of biochemical processes and involves interaction between key metabolic, neuroendocrine and reproductive tissues. Most components that regulate the hypothalamic-pituitary-ovarian axis are in place before the occurrence of puberty. However, it is unclear if the prepubertal uterus is capable of responding to the presence of an embryo or conceptus. Thus, the objectives of this study were to determine the response of the endometrium of 5-month-old prepubertal heifers to i) Day (D) 7 blastocysts (Experiment 1), and ii) a D14 conceptus or 100 ng/ml of interferon tau (IFNT) (Experiment 2), and to compare this response to that of a postpubertal endometrium. Angus X Holstein-Friesian heifer calves (prepubertal group; n= 9) were euthanized at 21 weeks of age. Reproductive tracts were recovered to obtain endometrial explants. For Experiment 1, crossbred postpubertal beef heifers (n= 5) were synchronized and slaughtered on D7 of the cycle (D0 = expected ovulation) to obtain endometrial explants. Twenty D7 in vitro produced (IVP) blastocysts were placed on top of an explant from prepubertal (PreP-D7; n= 5) or postpubertal heifers (PostP-D7; n= 5), and co-cultured for 6 h. For Experiment 2, crossbred postpubertal beef heifers were synchronized and either used to generate D14 conceptuses following the transfer of IVP blastocysts on D7 (n= 9; 15 embryos/recipient) or were used to obtain D14 endometrial explants (n= 5). Conceptuses were recovered on D14 by post-mortem uterine flushing and placed individually on top of explants from prepubertal (PreP-D14; n= 4) or postpubertal heifers (PostP-D14; n= 5) and co-cultured for 6 h. In both experiments, endometrial explants were cultured with medium alone as a negative control (PreP-CTRL and PostP-CTRL; n= 4-5 /group). All explants were snap frozen for subsequent RNA-seq. Despite a large number of differentially expressed genes (DEG) between PreP-CTRL and PostP-CTRL on D7 (n= 6063), the response to D7 blastocysts was similar: 27 DEG between PreP-D7 and PreP-CTRL and 5 DEG between PostP-D7 and PostP-CTRL (all 5 also upregulated in the prepubertal endometrium). All D7 embryo-induced DEG were interferon-stimulated genes (ISG). Similarly, while a comparison between PreP-CTRL and PostP-CTRL on D14 revealed 3544 DEG, endometrial response to a D14 conceptus was similar: 42 DEG in PreP-D14 and 61 DEG in PostP-D14 (37 genes in common). All genes upregulated in PreP-D14 and 57 of the 61 DEG in PostP-D14 were ISG. Exposure to exogenous IFNT increased the expression of a similar number of genes (165 in PrepP-IFNT and 168 in PostP-IFNT, relative to the controls, 113 of which were shared). Of the 27 DEG induced in PreP endometrium by a D7 blastocyst, 26 were common with those induced by a D14 conceptus. In conclusion, prepubertal endometrium is capable of responding to D7 blastocysts, a D14 conceptus, and IFNT in a manner similar to that of postpubertal endometrium.

Supported by EU Horizon 2020 Marie Sklodowska-Curie REPBIOTECH 675526 and Science Foundation Ireland 13/IA/1983.

A157E Embryology, developmental biology and physiology of reproduction

Anti-cancer potential of pomegranate peel on human ovarian carcinoma cells OVCAR-3

Simona Baldovská[1], Jan Nevoral[2], Katarína Michalcová[1], Michal Ďuračka[1], Adriana Kolesárová[1]

[1]Department of Animal Physiology, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic; [2]Biomedical Center, Charles University, Pilsen, Czech Republic.

Keywords: pomegranate, cancer, ovary.

Pomegranate (*Punica granatum*) is a unique and potent source of biologically active substances including flavonoids, anthocyanins, and especially ellagitannins and punicalagins with many beneficial properties. Furthermore, several scientific studies have focused on the bioactivity of pomegranate peel extracts, which possess remarkable antioxidant, antibacterial, anti-inflammatory and anti-cancer activities. In accordance with anti-cancer potential of pomegranate fruits, the aim of our study was to examine the *in vitro* effect of pomegranate peel extract at the different concentrations (0; 25; 50; 100; 200 μg/ml), in short-term application (for 24 h) on a human ovarian carcinoma cell model system (OVCAR-3). Analysis were focused on cell viability, production of reactive oxygen species (ROS), and expression of NAD-dependent deacetylase SIRT1 and histone γ-H2AX as a marker DNA double strand breaks. For this experiment, the ethanol extract from lyophilized pomegranate peel was prepared. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control) for the experiments. The metabolic activity was evaluated by AlamarBlue™ cell viability assay; the ROS production was quantified by chemiluminescence and the protein expression was detected by Western Blot analysis. Band intensity was quantified using Image Lab™ software (Bio-Rad, CA, US). Statistical significances were established by using One-way ANOVA along with Dunnett's test. All experiments were done in triplicate. The pomegranate peel extract significantly ($P \leq 0.001$) inhibited the viability of OVCAR-3 cells at all used concentrations in comparison to control. Moreover, ROS generation was significantly ($P \leq 0.01$) increased at all used concentrations of pomegranate peel extract in a dose-dependent manner. Interestingly, evaluation of the level of SIRT1 showed significant ($P \leq 0.05$) decrease in ovarian cancer cells OVCAR-3 in comparison to healthy cells human ovarian granulosa cells (HGL5). On the other hand, SIRT1 expression was significantly increase after pomegranate peel extract treatment at the concentrations 100 and 200 μg/ml in OVCAR-3 cells ($P \leq 0.05$). Additionally, all used concentrations of pomegranate peel extract led to significant ($P \leq 0.01$) γ-H2AX over-expression in ovarian cancer cells OVCAR-3 as a response to DNA damage. In conclusion, our data suggested that oxidative stress due to pomegranate-induced ROS production resulted in a decrease in the number of viable OVCAR-3 cells. The results show dose-dependent effect of pomegranate peel extract on human ovarian carcinoma cells OVCAR-3 and the potential role of pomegranate in the prevention or treatment of cancer by regulation of various signal pathways. Further studies are essential to understanding the therapeutic potential of pomegranate peel extract, however, it might serve to be a potential chemoprotective agent.

Acknowledgment: This work was supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic projects APVV-18-0312, APVV-15-0543, VEGA 1/0039/16, Center of Animal Reproduction CeRA, Tatra bank Foundation and EU project no. 26220220180: Building Research Centre AgroBioTech.

A158E Embryology, developmental biology and physiology of reproduction

A proteomic approach to decipher embryo-maternal interactions in the oviduct

Charles Banliat[1,2], Guillaume Tsikis[2], Ana-Paula Teixeira-Gomes[3,4], Valérie Labas[2,4], Emmanuelle Com[5], Charles Pineau[5], Pascal Mermillod[2], Benoît Guyonnet[1], Marie Saint-Dizier[2,6]

[1]Evolution XY, Noyal-Sur-Vilaine, France; [2]INRA, UMR PRC, Nouzilly, France; [3]INRA, UMR ISP, Nouzilly, France; [4]INRA, University of Tours, CHU of Tours, platform CIRE, Nouzilly, France; [5]Protim, Inserm, UMR S1085, Irset, Rennes, France; [6]University of Tours, Tours, France.

Keywords*:* oviductin, mass spectrometry, morula.

In vivo, the bovine embryo develops in contact with the oviductal fluid (OF) up to the 8-cell or morula stage. Oviduct proteins are known to be highly regulated across the estrous cycle. However, up to now, using immunohistochemistry, only few proteins, such as oviductin and osteopontin, have been identified as interacting with the developing embryo. The aim of this study was to use two complementary proteomic approaches: (i) bottom-up using nanoliquid chromatography coupled to tandem MS (nanoLC-MS/MS), and (ii) profiling by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry (MS), to characterize new OF proteins interacting with the early bovine embryo. Pairs of bovine oviducts were collected at a local slaughterhouse and transported to the lab on ice. Only oviducts at the post-ovulatory stage (small hemorrhagic *corpus luteum* (CL)) and ipsilateral to the CL were used for OF collection by squeezing (n=22 cows). After 2 centrifugations (2000 g, 10 min then 12000 g, 10 min, 4°C), the OFs were pooled, aliquoted in small volumes and stored at −80°C. *In vitro* matured oocytes from slaughterhouse ovaries were fertilized with frozen semen. Zygotes were then cultured in SOF medium without proteins for 5 days. Pools of 25 embryos at the morula stage were incubated in 25 µL of OF (treated) or SOF (control group) for 6 h at 37°C then rinsed 3 times and stored at −80°C before MS analyses. For each proteomic approach, morulas from four replicates were analyzed. Bottom-up analyses were performed on pools of 25 embryos after protein extraction and trypsin digestion (n=4 pools/condition) and nanoLC-MS/MS (Tims-TOF, Bruker). Profiling analyses by MALDI-TOF (UltrafleXtreme, Bruker) in the 2-30 kDa mass range were performed on intact individual embryos (n=40 embryos/group). In parallel, the OF was analyzed both by nanoLC-MS/MS and MALDI-TOF MS. Proteins were considered as embryo-interacting proteins if they were detected in the OF and detected in treated but not in control embryos, or detected with significantly higher abundance in treated *vs*. control embryos (fold-change of mean normalized spectral counts > 2; p-value of t-tests < 0.05). By the bottom-up approach, a total of 561 proteins were identified, among which 21 OF interacting with embryos, including oviductin (OVGP1), galectin-3, transgelin-2, and several annexins (ANXA1, 2, 4). Among interacting proteins, seven had a signal peptide or were reported as secreted via non-classical secretory pathways. By the profiling approach on single embryos, a total of 221 masses were detected, among which five OF interacting with embryos. These masses were annotated as gluthatione S-transferase and several ribosomal proteins. In conclusion, high throughout proteomic methods were successfully used to identify embryo-interacting proteins originating from OF. Further analyses are requested to specify in which embryo compartments (zona pellucida, perivitelline space, blastomeres) these proteins are localized and which roles these interactions could play.

A159E Embryology, developmental biology and physiology of reproduction

Sexual dimorphism during early embryo development in the bovine: differential gene expression in relation with oxidative stress and culture conditions

Matthew Dallemagne, Laura Chabotier, Laura Saerens, Catalina De Schrevel, Isabelle Donnay

Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, Belgium.

Keywords: bovine blastocyst, RT-qPCR, sex ratio.

Female and male mammalian embryos differ from the onset of the embryonic genome. This is mainly due to differential gene expression related to sexual chromosomes. Indeed, large parts of X-linked genes are overexpressed in female embryos up to the inactivation of one X chromosome. Therefore, metabolism and adaptation to environmental conditions differ between sexes. Using the IVP bovine embryo as a model, we showed that culture conditions and induced oxidative stress differentially impact male and female embryos at the early blastocyst stage: male embryos survived better an oxidative stress induced by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) in the presence of serum (FCS medium; sex ratio: 61 vs 46% in control embryos; $p<0.05$), while female embryos were more resistant than males in serum-free conditions (BSA-ITS medium; sex ratio: 44 vs 59% in control embryos; $p<0.05$). The quality of the surviving blastocysts, i.e the apoptotic rates, also differed between sexes (Dallemagne et al., Theriogenology, 2018, 117, 49). In order to decipher the origin of those differences, the relative level of expression of several genes was evaluated by RT-qPCR on single blastocysts obtained in the same culture conditions. Briefly, bovine embryos were cultured in FCS or BSA-ITS medium. Oxidative stress was induced from D5 post-insemination (pi) using 0.1 or 1mM AAPH, respectively. At D7 pi, blastocysts were recovered and individually snap frozen in liquid nitrogen until RNA extraction. RNA extraction and reverse transcription were performed using the RNeasy Plus Micro kit from Qiagen and the iScript cDNA Synthesis kit from Bio-Rad, respectively. PCR was first performed on *DDX3Y* gene to sex the embryos. Then the samples were submitted to qPCR for 2 reference genes and 5 genes of interest (StepOnePlus, Applied Biosystem; 109 embryos; 3 replicates; 3 to 15 embryos per condition). The two reference genes, *YWHAZ* and *H2AFZ*, were selected for their stable expression whatever the condition (female or male, with or without oxidative stress, in FCS or BSA-ITS medium). Four X-linked genes (*AIFM1, XIAP, G6PD, HPRT*) and one autosomal gene (*BAX*) were selected based on the literature for their roles in the control of apoptosis or oxidative and their potential implication in the observed differences between the tested conditions. Statistical analysis was performed with the Standard Least Squares method (fixed effects: stress, sex, stress*sex; random effect: replication). All X-linked genes showed a higher expression in female embryos, whatever the culture medium ($p<0.01$; between 1.4 and 2.5 fold). AAPH treatment significantly decreased the expression of *XIAP* only in FCS containing medium (on average 0.78 fold), while it increased the expression of *BAX* (1.3 fold) and *HPRT* (1.3 fold) only in BSA-ITS medium ($p<0.01$). In this last medium, the impact of stress on *AIFM1* expression tended to depend on the sex of the embryo ($p=0.068$; females: on average 1.2 fold increase vs male 0.9 fold). In conclusion, the study confirms the higher expression of the tested X-linked genes in female embryos. The few differences observed between culture and stress conditions did not allow linking the expression of the studied genes to the sexual dimorphism observed for the developmental and apoptotic rates in the tested conditions.

A160E Embryology, developmental biology and physiology of reproduction

Trolox during *in vitro* maturation of bovine oocytes can protect embryos from palmitic acid induced lipotoxicity during development: effects on mRNA transcript abundance

Jessie De Bie, Waleed F.A. Marei, Peter E.J. Bols, Jo L.M.R. Leroy

Department of Veterinary Sciences, Gamete Research Centre, University of Antwerp, Wilrijk, Belgium.

Keywords*:* fatt acids, trolox, in vitro production of bovine embryos.

Maternal metabolic disorders are associated with elevated concentrations of palmitic acid (PA), which is known to jeopardize bovine oocyte and embryo development and quality. Molecular analyses of PA exposed bovine oocytes and embryos point towards oxidative stress (OS) pathways. Previous research has shown that the detrimental effects of PA-exposure during oocyte IVM cannot be alleviated by antioxidant (AO) supplementation, e.g. Trolox (TR, water soluble VitE), during IVM or IVC. Exposing oocytes with TR during IVM protected subsequent embryo development under PA conditions (De Bie *et al.* 2018, AETE). In the present study, we examined the effects of TR on the quality of the produced blastocysts at the transcriptome level. Bovine COCs were matured, fertilized and cultured in 2 different experiments (min 3 repeats each). In EXP1, COCs (n=1565) were exposed to pathophysiological follicular PA concentrations (150 µM, Sigma-Aldrich, BE), subsequent embryos were cultured under solvent control (ethanol) conditions (PA-SC). TR was added during IVM or IVC (100 µM, Thermo Fisher, BE; PATR-SC, PA-TR). In EXP2, COCs (n=1477) were matured under solvent control conditions, subsequent embryos were exposed to pathophysiological oviductal PA concentrations (230 µM; SC-PA). TR was added during IVM or IVC (100 µM; TR-PA, SC-PATR). In each experiment, a solvent control was included (SC-SC). Pools of min 10 day 8 blastocysts per treatment were examined for relative transcript abundance of genes (normalized to *H2AFZ* and *YWHAZ*) involved in OS (*CAT, GPX, SOD1, SOD2, PRDX1, PRDX3, NRF2*), mitochondrial function (*TFAM, HSPD1*), lipid metabolism (*PPARg*) and apoptosis (*BAX*) and analyzed by one-way ANOVA.A significant increase in *NRF2* and *TFAM* was found in blastocysts from PA exposed COCs (PA-SC) and embryos (SC-PA) compared with controls (SC-SC). Increased *NRF2* in blastocysts from PA exposed COCs (PA-SC) returned to control levels when TR was added during IVM or IVC (PATR-SC, PA-TR). In contrast, when embryos were exposed to elevated PA (SC-PA), adding TR during IVM or IVC (TR-PA, SC-PATR) was not able to alleviate elevated *NRF2* expression to control levels, suggesting activation of OS defense mechanisms. The addition of TR in each EXP significantly reduced *TFAM* gene expression to levels similar to controls (SC-SC), suggesting normalization of mitochondrial biogenesis. In EXP1, a significant increase in *CAT* was found in PA exposed oocytes (PA-SC) compared with their control counterparts. Adding TR during IVM or IVC (PATR-SC, PA-TR) significantly reduced blastocyst *CAT* expression to levels lower than controls. No significant PA-induced changes were found in the expression of other genes. In conclusion, the enhancement of the developmental capacity of PA-exposed bovine oocytes and embryos by TR is most promising when oocytes are protected by TR prior to the PA insult. Moreover, subsequent blastocysts appear to have control levels of expression of genes related to OS and mitochondrial function and increased expression of genes involved in OS relief.

A161E Embryology, developmental biology and physiology of reproduction

Effect of Temopl on *in vitro* oocytes maturation in Egyptian Buffalo

Marwa Said Faheem[1,2], Osama Galal Sakr[1], Nasser Ghanem[1]

[1]Faculty of Agriculture, Cairo University., Egypt; [2]Cairo University Research Park (CURP), Faculty of Agriculture, Cairo University, Egypt.

Keywords*:* Tempol, in vitro maturation, Buffalo oocytes.

Oxidative stress is a major biological threat that negatively affect oocytes quality and subsequent maturity competence. Cellular antioxidant system, like superoxide dismutase (SOD), plays a substantial role for maintaining redox balance against the excessive accumulation of reactive oxygen species (ROS). Consequently, the antioxidants are frequently used in the in vitro culture system to promote the oocyte's maturation. Tempol is is a single chemical compound that facilitates hydrogen peroxide metabolism, scavening ROS, and functionally similar to SOD. Therefore, the current study was conducted to study the effect of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) as an exogenous antioxidant on in vitro oocyte maturation rate of Egyptian buffalo. Cumulus-oocyte complexes (COCs) were recovered from animals slaughtered at local abattoir and grade A and B were used in this study. The maturation rate was assessed by nuclear status (oocytes reached metaphase II stage) and cumulus expansion of the oocytes as well as on the molecular level. Three different concentrations (0.5, 1 and 2 µM) of Tempol (Sigma-Aldrich, Cat no 176141,) were added to the buffalo oocyte maturation medium (Tissue Culture Medium, TCM-199 HEPES medium supplemented with 2% fetal bovine serum, 5µg/ml of FSH, 1µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22µg/ml Na-pyruvate, 50µg/ml gentamycin). By the end of maturation period (22-24 h), COCs expansion rate and nuclear maturation rates were evaluated. After oocytes denudation, the oocytes from all experimental groups were stored at – 80°C for further genetic analysis. Two candidate genes regulating metabolic activity (CPT2) and antioxidant status (NFE2L2) were profiled using Real-Time PCR find out molecular action of Tempol on COCs during maturation. The GAPDH was used as a reference gene for relative expression quantification. The results revealed that 0.5 µM of Tempol (88.0±4.0 %) enhances buffalo COCs expansion rate comparing to control (75.7±3.0 %) and the other two concentrations of Tempol (1 and 2 µM were 79.9±4.1 and 68.1±4.2 %, respectively). While, the differences were significant ($P \leq 0.05$) between the higher and lower concentration of Tempol. Moreover, the metaphase II (indicator of maturation rate) oocytes were higher in 0.5 µM concentration (84.4 %) in comparison to 1 and 2 µM concentrations (68.9 and 76.7 %, respectively) and control group (75.8 %). For the molecular analyses, the transcriptional abundance of CPT2 showed a significant ($P \leq 0.05$) decline trend with the increase of Tempol concentration in the maturation medium being in addition significantly lower than control group. While, for NEF2L2, the expression level of NEF2L2 gene was comparable between the control group and the groups of oocytes matured with 0.5 and 1 µM of Tempol, However, 2 µM of Tempol concentration showed lower level of expression of NEF2L2 in comparison with all experimental groups. Moreover, the expression pattern of CPT2 and NEF2L2 genes strengthened the results of oocyte maturation rate in vitro. In conclusion, the 0.5 µM concentration of Tempol revealed potential significance for oocyte maturation competence as well as on the molecular level.

A162E Embryology, developmental biology and physiology of reproduction

Embryo vitrification device has consequences at birth and adulthood

Ximo Garcia-Dominguez, David S Peñaranda, Jose S. Vicente, Francisco Marco-Jimenez

Universitat Politècnica de València, Valencia, Spain.

Keywords*:* embryo, vitrification, phenotype.

The development of assisted reproductive technologies (ART) over the past decades has provided tremendous advantages in livestock. Cryopreservation of reproductive cells is the second most used ART, which allows for long-term storage gametes/embryos by cooling them to subzero temperatures. This technique has become essential to enable the banking and the transport of embryos from high genetic value animals around the world. In this field a progressive replacement of slow freezing by vitrification methodologies has occurred. However, rather than a standardized method, an explosion of vitrification methods has been appeared over the last decade, using extremely variable vitrification media and more than 25 different vitrification devices. Moreover, it is increasingly common to find evidences that ART conditions can affect embryo development and, ultimately, the adult phenotype via epigenetic mechanisms that vary depending on the nature of the procedure (embryonic developmental plasticity). Therefore, using the rabbit as a model, the aim of this study was evaluate the effect over the growth performance of two vitrification devices, both based on the use of the minimum volume strategy but composed of different material (metal or plastic), which brings different cooling/warming rates. To assess this issue, 72-hours embryos (late morula/early blastocyst) were vitrified using metallic loops (n=102; ML) and Cryotop® (n=100; CP). Embryos were vitrified in a two-step addition procedure; equilibrium (10% EG + 10% DMSO + 10% Dextran) for 2 minutes and vitrification (20% EG + 20% DMSO + 10% Dextran) for 1 minutes. After thawing, embryos were transferred into the oviducts of 16 foster mothers. Birth rate (animals born / transferred embryos) was recorded and the pups were identified and weekly weighted until adulthood. Gompertz growth curve equation [$y= a \cdot EXP(-b \cdot EXP(-k \cdot t)]$] was used to determine the growth rate (k). Statistical analysis was performed through a general lineal model (considering $p<0.05$). The results showed that ML birth rate (44.1±4.67%) was lower compared to CP group (65.0±4.72%; $p<0.05$). However, CP animals were smaller (-9.6±2.69g; $p<0.05$) at birth, even after using the litter size as covariable. Finally, although growth rate was similar between both groups (average k value: 0.16±0.01), data suggest that differences at birth cannot be restored later in life. Therefore, CP animals showed lower body weight (-318.9±103.86g; $p<0.05$) at adulthood. In conclusion, the vitrification device does not seem to be trivial. Our results increase the studies that reports a significant effect of the vitrification device in the achievement of a successful vitrification. In addition, our findings provide evidences of an embryonic plasticity in response to the vitrification device, which modify the birth weight and the late growth performance. Therefore, we show for the first time to our knowledge that phenotype can change in response to a vitrification device.
Research supported by Ministry of Economy and Competitiveness, Spain (AGL2017-85162-C2-1-R).

A163E Embryology, developmental biology and physiology of reproduction

Culture under the physiological temperature registered along the reproductive tract of female pigs improves the blastocysts yield *in vitro*

Soledad García-Martínez[1], Octavio López-Albors[2], Rafael Latorre[2], Raquel Romar[1], Pilar Coy[1]

[1]Department of Physiology, University of Murcia, Spain; [2]Department of Anatomy & Comparative Physiology University of Murcia, Spain.

Keywords*:* temperature, assisted reproductive technology, pig.

Despite temperature being one of the main external environmental factors that affects gene expression, thereby influencing the way an organism develops (Lobo, I. Nature Education vol. 1(1), p39, 2008), its oscillation pattern is barely taken into account in *in vitro* embryo production (IVP). Few studies obtaining direct measurements of temperature within the reproductive tract of the female pig have been reported and the procedures used in those previous studies have been surpassed today by the use of cutting-edge devices. While no reference temperature values have been published to date in the pig uterus, a temperature gradient within oviduct and ovary was described (Hunter et al., Reprod Biomed, vol. 24 (4), p. 377, 2012). We hypothesized that transferring physiological temperature conditions given in nature to IVP could help to reduce the prevalence of polyspermy after insemination in swine and improve the efficiency of that biotechnology in this species. Hence, we aimed to measure temperature within the isthmus, ampulla and uterus of sows (n=15) and use these values in IVP protocols. To this end, oviductal and uterine temperature was monitored adopting a laparo-endoscopic single-site surgery assisted approach along with a flexible and thin miniaturized probe previously used by López-Gatius and Hunter (López-Gatius and Hunter, Reprod Dom Anim, vol. 52(3), p. 366, 2017). The same temperature was retrieved in ampulla and isthmus (37.0 ± 0.5ºC) whereas a significantly higher value was found in uterus (38.7 ± 0.1ºC).This finding suggest that a lower temperature is required during IVF, while a higher temperature is needed during the embryo development. To test this assumption, *in vitro* matured oocytes were inseminated at two different temperature conditions: the routinely used value in pig IVP (38.5ºC) and a lower value (37ºC), recorded in the oviduct. At 18-20 hours post-insemination (hpi), putative zygotes were transferred to embryo culture medium and maintained at 38.5ºC in both groups. A sample of presumptive zygotes (n=218) was fixed and stained to assess the fertilization rates. At 180 hpi, development to blastocyst stage was evaluated. Data were analysed by one-way ANOVA. A P-value <0.05 was considered to denote statistical significance. Monospermy rate was significantly higher at 37ºC compared to 38.5ºC (65.0 ± 6.1% *vs.* 46.0 ± 6.1%), not being enough to improve the IVF yield. However, an increase in blastocyst yield when embryos were fertilized at 37ºC (39.0 ± 3.6%) was observed compared to those fertilized at 38.5ºC (24.0 ± 2.8%). Our study supports the recent data published by Hino and Yanagimachi in mice (Hino and Yanagimachi, Biol Reprod, in press, 2019) claiming that peristaltic movement within the oviduct and the continue ad-ovarian transport of oviductal fluid make a temperature gradient within the oviduct unlikely existent. In contrast, we found a temperature gradient between oviduct and uterus.

Supported by MINECO-FEDER (AGL 2015-66341-R) and Fundación Séneca, 20040/GERM/16.

A164E Embryology, developmental biology and physiology of reproduction

Immunohistochemical identification of CIRBP in bovine ovary and testicle

Jaume Gardela[1], Josune García-Sanmartín[2], Mateo Ruiz-Conca[1], Manuel Álvarez-Rodríguez[1,3], Alfredo Martínez[2], Teresa Mogas[4], Manel López-Béjar[1]

[1]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Bellaterra, Barcelona,Spain; [2]Oncology Area, Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain; [3]Department of Clinical and Experimental Medicine (IKE), Linköping University, Linköping, Sweden; [4]Department of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.

Keywords*:* CIRBP, stress-induced protein, cattle.

The cold-inducible RNA-binding protein (CIRBP) is a highly conserved stress-induced protein that helps cells to resist adverse environmental conditions via stabilizing specific mRNAs and facilitating their translation. CIRBP participates in anti-apoptotic and anti-senescent cytoprotective processes. In relation with gametes, CIRBP improves the developmental competence in vitrified-warmed yak oocytes and exerts a protective effect against spermatogenic injury caused by heat stress and cryptorchidism in mice. The purpose of this study was to identify the expression of CIRBP on different cell populations in adult bovine ovary and testicle. Tissues were obtained from healthy slaughtered animals (non-pregnant heifers and sexually matured males). Paraffin blocks containing tissue sections of ovary and testicle were processed for immunohistochemistry. Tissue sections were dewaxed, blocked for intrinsic peroxidase (15 min, 3% H_20_2 in methanol), and subjected to antigen retrieval (10 mM sodium citrate, pH 6.0, 30 min, 95°C). Nonspecific binding was blocked with 10% normal donkey serum (30 min). Tissues were incubated with two dilutions (1/250, 1/500) of two primary antibodies against CIRBP overnight at 4°C: monoclonal rabbit anti-CIRBP [EPR18783] (ab191885, Abcam) or polyclonal goat anti-CIRBP (ab106230, Abcam). Antibodies were detected with a commercial peroxidase kit and diaminobenzidine. Sections were lightly counterstained with hematoxylin. Some sections were processed for immunofluorescence and analyzed through confocal microscopy, using two secondary antibodies at 1/600: Alexa Fluor® 546 donkey anti-rabbit IgG or Alexa Fluor® 633 donkey anti-goat IgG (Invitrogen). Fluorescent sections were counterstained with DAPI. Sections stained on the absence of primary antibody (negative controls) demonstrated a lack of unspecific binding for the detection system. Both immunohistochemistry and immunofluorescent sections were analyzed with ImageJ Software. Two-Way ANOVA analysis was performed with GraphPad Prism Version 8.0.2. *Post hoc* comparisons were performed using the Tukey test. In ovary, CIRBP was present in follicular cells of primordial follicles and in the granulosa and theca cells of the subsequent follicular stages. Oocytes presented less intensity compared to follicular cells. Granulosa cells presented more intensity than theca cells. In testicle, CIRBP was present in Leydig, Sertoli, and spermatogenic cells but not in mature spermatozoa. Dilutions at 1/250 presented better-contrasted images in both primary antibodies. In conclusion, CIRBP was present in bovine male and female gonads as seen in other species. Slight differences can be found using monoclonal or polyclonal antibodies against CIRBP in both tissues. Despite CIRBP being known to play an important role in spermatogenesis, little is known about its role in folliculogenesis and developmental competence of oocytes. Further studies are needed on the function of CIRBP on bovine follicles, oocytes, and developing embryos.

Project AGL2016-79802-P and AGL2016-81890-REDT supported this study. JG is founded by 2018 FI_B 00236, MRC by FPU2015/06029, and MAR by IJCI-2015-24380.

A165E Embryology, developmental biology and physiology of reproduction

Metabolomic profiling of oviductal extracellular vesicles across the estrous cycle in cattle

Julie Gatien[1], Carmen Almiñana[2,3], Sarah Janati Idrissi[1], Ophélie Bernardi[2], Daniel Le Bourhis[1], Pascal Salvetti[1], Pascal Mermillod[2], Marie Saint-Dizier[2,4]

[1]Allice, Nouzilly, France; [2]UMR85 PRC, INRA, Nouzilly, France; [3]University of Zurich, VetSuisse Faculty, Zurich, Switzerland; [4]University of Tours, Tours, France.

Keywords*:* Oviduct, extracellular vesicles, metabolomics.

Extracellular vesicles (EVs) in oviductal secretions have been suggested to play major roles in the cross-talk between gametes/embryo and the oviduct. The aims of the present study were to determine the metabolomic profile of bovine oviductal EVs and to examine whether the metabolic content of oviductal EVs varies according to the stage of the estrous cycle and the side relative to ovulation.
Bovine oviducts were collected at a local slaughterhouse, transported on ice, and classified into 4 stages of the estrous cycle according to the ovarian and corpus luteum morphologies (n=34-54 cows/stage): post-ovulatory (Post-ov; Days 1-4 of estrous cycle, coinciding with the time of embryo presence in the oviduct), mid luteal (Mid-lut; Days 5-11), late luteal (Late-lut; Days 11-17) and pre-ovulatory (Pre-ov; Days 18-20, coincinding with the time of estrus). Additionally, follicular fluid was collected from the Pre-ov follicles to exclude animals with cystic follicles (intra-follicular concentrations of progesterone > 160 ng/ml and estradiol < 40 ng/ml). Oviductal fluids (OF) were collected from contra- and ipsi-lateral oviducts by squeezing. Then, OF was separated from cells and cell debris by centrifugation (10 min at 2,000 g then 15 min at 12,000 g). Oviductal EVs were isolated from pools of OF (8-19 cows per pool; 3-4 pools per stage and side; 397 ± 15 µl of OF per pool) by ultracentrifugation (90 min at 100,000 g twice) and resuspended in PBS. Finally, EV samples (with not trace of oviductal fluid) were assayed for protein concentration (12.9 ± 0.5 mg/ml per pool) and stored at – 80°C for metabolic analysis. Samples of EVs were analyzed by proton nuclear magnetic resonance spectroscopy (NMR) as previously described (Lamy et al. Reprod. Fertil. Dev. 2018). The concentration of each metabolite was normalized to 1 mg of protein. Normalized values were compared between stages of the cycle and sides relative to ovulation using two-way analysis of variance (ANOVA) followed by Tukey's tests with $P < 0.05$ considered significant. NMR identified 22 metabolites in oviductal EVs, from which 15 could be quantified. Among them, 5 were amino acids (alanine, glycine, isoleucine, methionine and valine) and 9 energy substrates including lactate, myoinositol, glucose-1-phosphate and maltose as the most abundant metabolites. With the exception of maltose, all metabolites identified in oviductal EVs were previously identified in the OF (Lamy et al. 2018). Except for maltose, the side relative to ovulation had no effect on metabolite concentrations. Interestingly, levels of methionine were significantly higher at Pre-ov compared to Late-lut ($P<0.05$). Furthermore, glucose-1-phosphate and maltose concentrations were greatly affected by the stage of the estrous cycle ($P<0.0001$), showing 10- to 40-fold higher levels at Mid-lut and Late-lut than at Pre-ov and Post-ov. The metabolites identified in the present study could be taken up by gametes/embryos via EVs and play key roles in gamete maturation, fertilization and/or embryo development.
This study was funded by APIS-GENE under the name Embryomimetisme.

A166E Embryology, developmental biology and physiology of reproduction

Parental contribution of splicing factors ZRSR1 and ZRSR2 in early embryo development

Isabel Gómez Redondo, Priscila Ramos-Ibeas, Eva Pericuesta, Benjamín Planells, Raúl Fernández-González, Ricardo Laguna-Barraza, Alfonso Gutiérrez-Adán

Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Spain.

Keywords*:* minor splicing, embryogenesis, zygotic gene activation.

ZRSR1/2 have been implicated in 3' splice site recognition of U12 introns, a minor intron class (<0.4% of all introns) which is conserved across eukaryotic taxa with important roles in developmental processes. *Zrsr1* is a imprinting gene which is paternally expressed in mice, and *Zrsr2* is in the X-chromosome in all mammalian species analyzed. To determine the implications of minor splicing in early embryo development, CRISPR technology was used to produce *Zrsr1/2* mutant mice (*Zrsr1*^{mu} *and* *Zrsr2*^{mu}) that were viable with normal lifespan. We crossed homozygous *Zrsr2*^{mu} female with *Zrsr1*^{mu} male, being the double mutation (Zrsr1/2^{mu}) lethal, giving rise to embryos that stopped developing mainly between the 2- and 4-cells stages, just after zygotic gene activation (ZGA). This indicates that embryos need at least one normal *Zrsr1* allele from the father or one *Zrsr2* allele from the mother to survive. Rescue experiments in which *Zrsr1* mRNA was injected into 1-cell Zrsr1/2^{mu} embryos allowed the development of mutant embryos to blastocyst stage, revealing that minor splicing is essential for ZGA. To investigate the molecular basis of impairing the minor spliceosome machinery during embryo development, 3 pools of 100 *Zrsr1/2*^{mu} 2-cell embryos and 2 pools of 100 wild-type 2-cell embryos were used to purify their RNA and perform RNA-seq analysis. Differential gene expression (DGE) was evaluated with two independent software (DESeq2 and edgeR) (adjusted p-value <0.01) to improve the reliability of our findings. Differential alternative splicing (AS) events was determined using vast-tools software, considering events as modified when the difference in their average inclusion levels was above 10%. DGE analysis showed 3423 upregulated and 1446 downregulated genes in Zrsr1/2^{mu} embryos, which could indicate that the degradation of the maternal mRNAs is impaired. Genes with lower expression in Zrsr1/2^{mu} embryos were enriched in translation, rRNA processing and splicing, and cell cycle GO terms, indicating an essential role of minor splicing during ZGA. Differential AS analysis revealed 2645 upregulated and 1717 downregulated events in *Zrsr1/2*^{mu} embryos, being all categories of alternative splicing affected. Intron retention events were then checked to determine if they were U2- or U12-type intron events, as well as those U2 intron events that occur in genes that contain U12 introns. Seven percent of the intron retention events identified correspond to U12-introns, which represents a significant enrichment when compared with the overall proportion of U12 introns in the mouse genome (7% vs 0.04% expected), showing a crucial role of the U12-type introns during early embryo development. This study identifies paternal ZRSR1 and maternal ZRSR2 as essential factors for efficient U12 intron splicing, highlighting their crucial role on early preimplantation embryo development. Research supported by the Spanish Ministry of Economy and Competitiveness through the project AGL2015-66145-R and BES-2016-077794 grant.

A167E Embryology, developmental biology and physiology of reproduction

Ovulation mediated changes in the transcriptomics of the rabbit isthmus

Leopoldo González-Brusi[1], María José López-Andreo[2], Paula Cots-Rodríguez[1], Rebeca López-Úbeda[1], Carla Moros-Nicolás[1], José Ballesta[1], María José Izquierdo-Rico[1], Manuel Álvarez-Rodríguez[3], Manel López-Béjar[3], Manuel Avilés[1]

[1]Department of Cell Biology and Histology, Universidad de Murcia and IMIB-Arrixaca; [2]Molecular Biology Section (ACTI), University of Murcia; [3]Department of Animal Health and Anatomy, Veterinary Faculty, Universitat Autónoma de Barcelona.

Keywords*:* isthmus, transcriptomics, rabbit.

The isthmus is the section of the oviduct where sperm is stored forming the oviductal reservoir, before capacitation takes place and spermatozoa are freed to fecundate the oocytes within the ampulla. This study shows transcriptomics changes in the isthmus shedding light on the molecular processes taking place near the ovulation in rabbit species (*Oryctolagus cuniculus*). Six sexually mature New Zealand rabbit does received 0.02 mg Gonadorelin (im; Fertagyl, Merck & Co., Inc., Kenilworth, USA) at insemination time (t=0 h) to induce ovulation. Tissues were collected after euthanasia at 10 h (pre-ovulatory; n=3) and 20 h (post-ovulatory; n=3). RNA was extracted and samples with a RNA integrity number > 7 were analyzed by microarray platform GeneAtlas System (chip Rabbit Gene 1.1 ST Array Strip, Affymetrix). Data analysis was done with the Partek Genomics Suite software, raw intensities were background corrected and RMA normalized. Differentially expressed genes (DEGs) were defined as those with a fold change > |1.5| and FDR < 0.05 obtained through a variance analysis, and false discovery rate adjustment with RankProd. Biological meaning was assessed with Gene Ontology (GO) enrichment, and pathway analysis with KEGG database. There were 86 genes upregulated at the pre-ovulatory stage. Pathway analysis was enriched in terms such as "protein processing in endoplasmic reticulum", "ECM-receptor interaction" and "MAPK signalling pathway". GO analysis revealed enrichment in extracellular proteins, with 18 gen products labelled as “extracellular exosome” and 28 being secreted proteins. Among the extracellular proteins there were some previously reported as oviductal fluid (OF) proteins in rabbit and other species (cow, pig and mouse). There were upregulated chaperones (HSPA5, DNAJB11, HYOU1 and HSPB7), integrins (ITGA5, IGTA2 and IGTB3), neuropeptide Y (NPY), and protease inhibitor SERPINE1. On the other hand, there was an upregulation of 33 genes at the post-ovulatory stage. GO anotation revealed the presence of eight secreted proteins, among the two proteins associated with fertility, PLAT and SPP1. PLAT was previously reported to be necessary for the success of *in vitro* fertilization in mice, while osteopontin (SPP1) has shown a positive effect on spermatozoa, *in vitro* fertilization and embryos in cattle. In the present study, an overexpression of exosomal proteins was reported for the pre-ovulatory stage. Enrichment in proteins previously detected in murine oviductosomes (OVs), including those integrins mediating spermatozoa-OVs fusion, suggest that this molecular mechanism might be conserved in rabbits, and thus participate in modification of the spermatozoa located in the isthmus. Further experiments are required to characterize these oviductosomes their evolution over the doe reproductive cycle, and investigate their role in the modulation of the spermatozoa fertilization ability. Moreover, there are secreted proteins in the rabbit oviductal fluid previously detected by proteomics that show a differential expression pattern before and after ovulation whose role in reproduction remains unknown, requiring additional functional studies.
Supported by Fundación Séneca 19357/PI/14 and AGL2016-81890-REDT.

A168E Embryology, developmental biology and physiology of reproduction

Selected physicochemical parameters of serum and fluid from the cavity of corpora lutea and their connection to the mechanisms of its formation in cattle

Bartłomiej Maria Jaśkowski[1], Magdalena Herudzińska[2], Maria Wieczorkiewicz[2], Jakub Kulus[2], Magdalena Kulus[2], Jędrzej Maria Jaśkowski[2], Maciej Rogoziewicz[2], Marek Gehrke[2]

[1]Department of Reproduction and Clinic of Farm Animals of the Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland; [2]Center for Veterinary Sciences, Nicolaus Copernicus University in Torun, Torun, Poland.

Keywords*:* cavitary fluid, serum, corpus luteum with a cavity, progesterone, cattle.

It is well known that cows have two morphologically different forms of the corpus luteum - solid and cavernous. In the studies presented in previous years, among others in the AETE meeting (Jaskowski 2016), we have shown that the presence of the cavity is accompanied by an increased level of progesterone in the blood and better results of conception. Perhaps the presence of the cavity is an additional factor that increases the probability of pregnancy in its early stage. The assessment of the nature and some of the fluid components could help to explain the positive effect of cavities on the CL function. The aim of the study was to determine some physicochemical parameters of the fluid from the CL cavities and serum in connection with the presence of the cavernous form of the corpus luteum. The study material from 30 Polish Holstein Friesian cows aged from 43 to 148 months, obtained in one of the cattle slaughterhouses in Poland, was used. After ultrasound examination performed before slaughter and finding the cavity inside CL, blood was collected (tail vein, 7.5 ml tubes, EDTA), and after slaughter liquid from cavities of corpora lutea (eppendorf tubes). The samples were transported to the laboratory under chilled conditions, and the analyzes were held at -80 ° C. The specific gravity, total protein content (Danlab refractometer), glucose concentration (Reflovet Plus, Roche) and progesterone (RIA) were determined in the fluid and serum samples. Data were analysed by logistic regression using the STATISTICA 9.0 software PL. The correlation between the size of cavities and the specific gravity, protein and glucose concentration was clearly established. While the increase in cavity diameter corresponded to the increase in glucose concentration (0.5116, p=0.015), specific gravity and protein concentration were negatively correlated with the size of the cavity (-0,5192, p=0.013 and -04813, p=0.023, respectively). There were no significant correlations between the fluid from the cavities and the amount of luteal tissue, as well as serum and fluid from the cavities. There was no correlation between the concentration of progesterone in the cavity and serum fluid, however the mean P4 values obtained from cavitary fluid were significantly higher than from serum (4612±3847.6 to 28.6±15.2 ng/ml, p<0.001). It is known that progesterone has an autocrine luteotropic effect on the luteal cells positively affecting its own concentration measured in blood. The P4 values in the cavities which is many times higher than in serum may explain the higher concentrations of P4 in the blood of cows with cavitary corpora lutea in relation to those with solid counterparts. Physical parameters of the fluid from the cavities suggest its exudative character. Higher specific gravity and protein concentration in smaller sized cavities compared to the large ones may indicate the angiogenesis related background of cavity formation.

A169E Embryology, developmental biology and physiology of reproduction

Mono (2-ethylhexyl) phthalate induces transcriptomic and proteomic alterations in bovine oocytes

Dorit Kalo[1,2], Anais Vitorino Carvalho[3], Catherine Archilla[3], Veronique Duranthon[3], Marco Moroldo[4], Yishai Levin[5], Meital Kupervaser[5], Yoav Smith[6], Zvi Roth[1,2]

[1]Department of Animal Science, Robert H.Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel; [2]Center of Excellence in Agriculture and Environmental Health, The Hebrew University of Jerusalem, Israel; [3]UMR BDR, INRA, ENVA, Université Paris Saclay, Jouy-en-Josas 78350, France; [4]Animal Biological Resources for Integrative and Digital Genomics Facilities, Jouy-en-Josas 78350, France; [5]The de Botton Institute for Protein Profiling, The Nancy and Stephen Grand Israel National Center for Personalized Medicine, Weizmann Institute of Science, Rehovot 76100, Israel; [6]The Bioinformatics Unit, Faculty of Medicine, the Hebrew University, Jerusalem 91120, Israel.

Keywords*:* phthalates, oocyte developmental competence, transcriptome.

Phthalates are plasticizers, used in a variety of industrial plastics. Di(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticizer, and its main metabolite, mono(2-ethylhexyl) phthalate (MEHP), are known reproductive toxicants. A residual concentration of MEHP (~20 nM) has been found in follicular fluid aspirated from IVF-treated women and DEHP-treated cows. Previously we have reported that exposure of oocyte during maturation to MEHP impairs nuclear maturation, reduces clevage and blastocyst formation rates. However, the effect of 20 nM MEHP on the transcriptomic profile of oocytes and their derived blastocyts is not entirely clear. Bovine oocytes were in-vitro matured with or without 20 nM MEHP for 22 h. At the end of maturation, they were collected for transcriptomic (by microarray; n = 20 per sample; 4 replicates) and proteomic (n = 200 per group) analyses to examine a possible direct effect of MEHP on the oocyte transcriptomic and proteomic profiles. The remaining oocytes were in-vitro fertilized and embryonic development was recorded 42–44 h and 7 days postfertilization. Blastocysts were also collected for microarray analysis (n = 10 per sample; 4 replicates). Transcriptomic data were analyzed using Partek Genomics Suite software. Control probes were removed; signals were log2 transformed followed by interslide quantile normalization. Genes were considered differentially expressed if the *P*-value by one-way ANOVA was lower than 0.05 and absolute fold change was 1.5 between the control and MEHP-treated group. Proteomic raw data were imported into Expressionist® followed by Mascot software. Data were searched against the bovine sequences from UniProtKB. Proteins were considered differentially expressed at a fold change of ±1.5 with at least 2 unique peptides. Oocyte transcriptome analysis revealed MEHP-induced alterations in the expression of 456 genes. The differentially expressed genes were associated with actin cytoskeleton (n = 47 genes; e.g., *ACTG1*), metabolic pathway (n = 43) including oxidative phosphorylation (n = 12; e.g., *ND5*), oocyte maturation (n = 9; e.g., *PIK3CA*), and embryonic development (n = 14; e.g., *SOX10, NOTCH*); 191 proteins were affected by MEHP in mature oocytes, associated with methylation and acetylation (n = 51), metabolic pathway (n = 33) including mitochondrial oxidative phosphorylation (n = 7; e.g., ATP5E), and cytoskeleton structure (n = 32; e.g., ACTN1, EGFR). In control vs. MEHP-derived blastocysts, 290 genes were differentially expressed, associated with transcription process, cytoskeleton regulation and metabolic pathway; 9 of these genes were impaired in both oocytes exposed to MEHP (i.e., direct effect) and blastocysts developed from those oocytes (i.e., carryover effect). The study explores, for the first time, the risk associated with exposing oocytes to relevant MEHP concentrations (i.e., those found in the follicular fluid) to the maternal transcripts. Although it was the oocytes that were exposed to MEHP, alterations carried over to the blastocyst stage, following embryonic genome activation, implying that these embryos are of low quality.

A170E Embryology, developmental biology and physiology of reproduction

Atrazine-induced DNA fragmentation in bovine spermatozoa is associated with alterations in the transcriptome profile of in-vitro-derived embryos

Alisa Komsky-Elbaz[1,2,3], Zvi Roth[1,2,3]

[1]Department of Animal Sciences; [2]Animal Sperm Research Center, Robert H. Smith Faculty of Agriculture, Food and Environment; [3]Center of Excellence in Agriculture and Environmental Health, The Hebrew University of Jerusalem, Israel.

Keywords*:* ATZ, spermatozoa, microarray.

Atrazine (ATZ) is an extensively used herbicide, considered a ubiquitous environmental contaminant. ATZ and its major metabolite, diaminochlorotriazine (DACT), cause several cellular and functional alterations in spermatozoa, involving viability, acrosome integrity, and mitochondrial membrane potential. Our aim was to examine the effect of ATZ/DACT exposure on DNA integrity of bovine spermatozoa, fertilization competence, embryonic development and transcriptome profile of in-vitro-produced embryos derived from fertilization with pre-exposed sperm. Three experiments were performed with fresh semen. Statistical analysis was performed using JMP-13 software. Comparison of treatments was performed by one-way ANOVA followed by *t*-test. In the first experiment, spermatozoa (25 x 10^6 cells/mL) were capacitated (4 h) in presence of 0.01% DMSO (solvent; control), 0.1 µM ATZ or 1 µM DACT and were examined for DNA fragmentation (acridine orange dye). Exposure to ATZ and DACT increased the proportion of cells with fragmented DNA compared to control (26 and 25.8 *vs* 84.5% respectively; $P < 0.0001$). Spermatozoa were separated using annexin V micro-bead kit. DNA-fragmentation index was higher among annexin-positive (AV+) vs. negative (AV-) spermatozoa (99.5 ± 0.05 *vs* 20.8 ± 4.29 % respectively; $P < 0.0001$). In the second experiment, fertilization competence of AV+ and AV- spermatozoa was examined with in-vitro-matured (22 h) oocytes (1,051 oocytes, 4 replicates). In the control group, AV+ spermatozoa gave lower cleavage rate (31.3 ± 2.92 %) relative to AV- spermatozoa (78.6 ± 2.18 %; $P < 0.0001$). No blastocysts were recorded in the AV+ group. ATZ/DACT-treated spermatozoa defined as AV- had a significantly lower cleavage rates relative to the control (59.8 ± 4.04 and 65.3 ± 3.33 *vs* 78.6 ± 2.18 %, respectively; $P < 0.005$). The proportion of the developed blastocysts did not differ between groups. In the third experiment, in-vitro fertilization was performed with non-separated spermatozoa. Blastocysts (n=4 per sample; 4 replicates) were collected on day 7 post fertilization and subjected to microarray analysis to identify differentially expressed genes (DAVID). Transcriptome analysis revealed that 139 and 230 genes were differentially expressed (up- or down-regulated) in blastocysts derived from spermatozoa treated with ATZ and DACT, respectively. In particular, alterations were found in genes involved in pregnancy (*IFNT2*, *IFNT3*, *IGFBF5*) and in-utero embryonic development (*YBX3*, *ANKRD11*, *PDGFRA*, *VIM*), pluripotency (*MYF5*), apoptosis (*THEM4*, *BCAD29*, *EIF2AK2*), and methylation and acetylation (*H2B*, *RAB27B*, *H4*, *HIST1H1C*, *LOC616868*). In conclusion, DNA damage induced by ATZ and DACT might explain, in part, the reduced fertilization competence of treated spermatozoa, reflected by lower cleavage rates. Given that blastocyst-formation rate did not differ between groups, other mechanisms cannot be ruled out. Here we report on alterations in the genomic profile of embryos developed from ATZ/DACT-treated spermatozoa, suggesting alterations in some cellular processes, including genetic and epigenetic modifications.

A171E Embryology, developmental biology and physiology of reproduction

Endoplasmic reticulum stress: is it induced in heat-shocked bovine oocytes?

Lais B. Latorraca[1], Kethelyn L. Gomes[2], Weber B. Feitosa[2], Fabiola F. Paula Lopes[1,2]

[1]Institute of Bioscience, Sao Paulo State University, Botucatu, São Paulo, Brazil; [2]Federal University of São Paulo (UNIFESP), Diadema, São Paulo, Brazil.

Keywords*:* heat stress, salubrinal, in vitro maturation.

The endoplasmic reticulum (ER) is a multifunctional organelle that plays a role in protein synthesis and modification, calcium homeostasis, and lipid synthesis. It also acts as a sensor of environmental stress. Accumulation of unfolded protein in the ER lumen leads to ER stress and activation of the Unfolded Protein Response. The bovine oocyte is very susceptible to environmental stress such as elevated temperature. However, the role of ER stress on heat-shocked oocytes has not been investigated. Therefore, the objective of this study was to determine the role of ER stress on bovine COCs exposed to heat shock (HS) during IVM. The first experiment was conducted to determine whether HS induces ER stress on bovine oocytes. COCs obtained from slaughterhouse ovaries were distributed on the following groups: Control (IVM at 38.5ºC for 22 h) and Heat Shock (IVM at 41ºC for 16 h followed by 6 h at 38.5ºC). After IVM, oocytes were denuded and stored at -80ºC for Western Blotting analysis. This experiment was replicated 5 times using 60 oocytes/treatment/replicate. Exposure of bovine oocytes to HS increased ($P<0.05$) the abundance of spliced X-box-binding protein-1 (sXBP1: ER stress marker) compared to control group, indicating the occurrence of ER stress. The second series of experiments (experiment 2, 3 and 4) were conducted to determine the role of ER stress on fertilization, kinetics and developmental competence of heat-shocked oocytes. COCs were matured in IVM medium containing 0 or 400 nM Salubrinal (ER stress inhibitor) under control and HS temperatures. After IVM, COCs were submitted to IVF and IVC. The fertilization rate was determined at 18 h after insemination (hai) and preimplantation developmental kinetics was determined by evaluating the cell number of each embryo at 26, 29, 32, 35, 38, 41 and 48 hai. These experiments were replicated 5 times using 30 COCs/treatment/replicate. There was not effect of temperature on fertilization rate. However, ER stress inhibition at 38.5ºC increased ($P<0.05$) fertilization rate compared to HS in the absence of salubrinal. Moreover, exposure of oocytes to HS in IVM medium caused a delay on embryonic developmental kinetics reducing ($P<0.05$) the percentage of 2-cell embryos at 29, 35 and 38 hai, as well as the percentage of 4-8 cell embryos at 41 hai and >8-cell embryos at 48 hai. Consequently, HS impaired cleavage rate ($P<0.05$) at 32-48 hai. On days 3 and 8 after insemination, cleavage and blastocyst rates were also reduced ($P<0.05$) in heat-shocked oocytes. Addition of Salubrinal to heat-shocked oocytes had a negative effect on embryonic development reducing ($P<0.05$) the percentage of 3-cell embryos at 38 hai and cleavage rate from 32 to 41 hai compared to 38.5ºC. However, cleavage and blastocyst rates at days 3 and 8 were not different between those groups. In conclusion, the present study demonstrated for the first time that HS induces ER stress on bovine oocytes during IVM. Moreover, HS retarded kinetics of embryonic development, reducing cleavage and blastocyst rates. ER stress inhibition on heat-shocked oocytes with Salubrinal was not as benefic as expected, but further studies are necessary to determine the importance of ER stress on bovine oocytes exposed to HS during IVM.

A172E Embryology, developmental biology and physiology of reproduction

Bta-miR-10b secreted by bovine embryos negatively impacts preimplantation embryo development

Xiaoyuan Lin[1], Krishna Chaitanya Pavani[2], Katrien Smits[2], Dieter Deforce[3], Petra de Sutter[4], Ann Van Soom[2], Luc Peelman[1]

[1]Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke, Belgium; [2]Reproduction, Obstetrics and Herd Health, Ghent University, Merelbeke, Belgium; [3]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium; [4]Department of Uro-gynaecology, Faculty of medicine and Health Sciences, Ghent University, Ghent, Belgium.

Keywords*:* Bovine embryos, Secreted miR-10b, HOXA1.

In a previous study, we found miR-10b to be more abundant in the conditioned culture medium of degenerate embryos compared to that of blastocysts. In this study, miR-10b mimics, double-stranded and chemically synthesized RNAs ordered from the company, were supplemented to the culture medium (SOF+BSA+ITS) at 21-hours post insemination with a final concentration of 1 μM. The expression of miR-10b in embryos was evaluated by RT-qPCR and was found to be approximately 70 times higher expressed in embryos treated with mimics compared to the control embryos, indicating that miR-10b mimics can be taken up by embryos. Additionally, this uptake results in an increase in embryonic cell apoptosis (2.15 times) using TUNEL staining and aberrant expression of DNA methyltransferases (*DNMTs*) using RT-qPCR. Using several computational methods Homeobox A1 (*HOXA1*) was identified as one of the potential miR-10b target genes and dual-luciferase assay, which measures firefly and Renilla luciferase, confirmed *HOXA1* as a direct target of miR-10b in bovine embryos. Microinjection of si-*HOXA1* into embryos also resulted in an increase in embryonic cell apoptosis (4.44 times) and downregulation of *DNMTs*. Overall, this work demonstrates that miR-10b negatively influences embryonic development and might do this by targeting *HOXA1* and/or influencing DNA methylation.

A173E Embryology, developmental biology and physiology of reproduction

Effects of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEndMSCs) on porcine embryo development *in vitro*

Beatriz Macías-García[1,2], Federica Marinaro[3], Verónica Álvarez[3], Lauro González-Fernández[1]

[1]Research Group of Intracellular Signaling and Technology of Reproduction (SINTREP), Research Institute of Biotechnology in Livestock and Cynegetic (INBIO G+C), University of Extremadura, Cáceres, Spain; [2]Department of Animal Medicine, Faculty of Veterinary Medicine, University of Extremadura, Cáceres, Spain; [3]Stem Cell Therapy Unit, CCMIJU, Cáceres, Spain.

Keywords: porcine, IVF, embryo culture.

In vitro fertilization in pigs is an assisted reproductive biotechnology that is still developing to optimize its efficiency. Porcine IVF is a very labour-intensive technique that yields inconsistent results between IVF sessions due to low initial oocyte quality, polyspermy and suboptimal composition of embryo culture media among others. Many different approaches are being tested to increase the quality and quantity of embryos produced by IVF in this species. Specific commercial embryo culture media are not available in pigs, being the North Carolina State University 23 (NCSU-23) the most commonly used medium. Combinations of NCSU-23 and different macromolecules, growth factors, hormones or oviductal fluid have been shown to improve blastocysts yield *in vitro*. In this regard, supplementation of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEnd-MSCs) to the zygote culture medium has demonstrated to increase embryo yield and quality in mice (Marinaro F. et al., Biology of Reproduction, 2019, 100(5), 1180–1192). Therefore, in the present work we aimed to improve blastocyst formation in swine using evEnd-MSCs in the embryo culture medium. To test this, EndMSCs were isolated from menstrual blood from four healthy women and characterized according to multipotentiality and surface marker expression. Extracellular vesicles from 4 donors were pooled, purified and characterized by nanoparticle tracking and CD9/CD63 expression by flow cytometry. Porcine oocytes were retrieved at a slaughterhouse, matured *in vitro* and fertilized with 1 x 10^5 spermatozoa/ml for 4 hours in a humidified atmosphere at 38.5°C in a 5%CO_2/95% air incubator. After IVF, the presumptive zygotes were randomly allocated to one of the following groups and cultured for 7 further days: A) Bovine specific medium: BO-IVC medium (IVF Bioscience, Barcelona, Spain); B) BO-IVC + evEnd-MSCs: BO-IVC added with 40 µg/ml of evEnd-MSCs; C) NCSU-23 and D) NCSU-23 + evEnd-MSCs: NCSU-23 added with 40 µg/ml of evEnd-MSCs. The evEnd-MSCs batch used was the same for all the experiments. Four IVF trials were performed and a minimum of 82 oocytes per group were evaluated. Blastocyst rates relative to initial oocyte number were compared among groups by a Chi-Square test, $P < 0.05$ was considered significant; results are presented as embryos obtained/initial oocyte number. Blastocyst rates were: (8/87) for BO-IVC, (5/82) for BO-IVC + evEnd-MSCs, (13/90) for NCSU-23 and (20/87) for NCSU-23 + evEnd-MSCs; no statistically significant differences were observed among groups ($P > 0.05$). In our setting, the use of evEnd-MSCs seemed to yield better results when combined with media specifically developed for porcine, not being able to help to overcome suboptimal culture conditions. More experiments need to be performed to fully corroborate our observations.

Funded by: AGL2015-73249-JIN and RYC-2017-21545 (AEI/FEDER/UE); ISCIII (PI18/0911, CP17/00021 and MS17/00021) co-funded by ERDF/ESF, "Investing in your future"; GR18094 and IB16168 from Junta de Extremadura (FEDER/UE).

A174E Embryology, developmental biology and physiology of reproduction

Protective effects of Mitoquinone during *in vitro* maturation of bovine oocytes under lipotoxic conditions

Waleed F.A. Marei, Lotte Van den Bosch, Peter E.J. Bols, Jo L.M.R. Leroy

Gamete Research Centre, University of Antwerp, Belgium.

Keywords*:* Mitochondria, antioxidants, embryo development.

Oxidative stress and mitochondrial dysfunction in oocytes play a central role in the pathogenesis of several conditions associated with infertility. Upregulated lipolysis during negative energy balance can directly increase oxidative stress and alter mitochondrial functions in oocytes. Furthermore, *in vitro* maturation (IVM) following ovum pick up has been shown to increase gene expression of markers of cellular stress in oocytes. This leads to reduced developmental competence and reduced production efficiency. Mitochondrial targeted treatments containing co-enzyme Q10 are used to increase the anti-oxidative capacity within the mitochondrial matrix and enhance mitochondrial activity, however their efficiency in assisted reproduction to enhance oocyte developmental competence has not been investigated. In the present study, we tested the effect of different concentrations of Mitoquinone (MitoQ; 0, 0.1, 0.5, 1.0 μM) during bovine oocyte IVM, then we tested the effect of MitoQ (0.1 μM) in the presence or absence of palmitic acid (PA)-induced lipotoxicity (150μM) as a model (Marei *et al.* 2019, Sci. Rep. 9:3673). The effect of the carrier molecule of MitoQ, triphenyl-phosphonium (TPP) was also tested. A total of 2823 bovine oocytes from slaughterhouse ovaries were used. All data were derived from at least three replicates and were compared by linear logistic regression (categorical data) or ANOVA (numerical data) with Bonferroni post-hoc corrections. MitoQ supplementation at 1 μM significantly ($P<0.05$) reduced cleavage (50.8±6.81 vs. 78.7±5.17), and blastocyst rates (6.7±0.98 vs. 27.4±6.07) compared with solvent control (ethanol 0.01%). TPP (1 μM) also induced similar toxic effect ($P<0.05$). This was associated with, and probably caused by, a reduced mitochondrial inner membrane potential (J-aggregates: monomer intensity ratio of JC-1 staining) ($P<0.05$). Lower concentrations of MitoQ and TPP had no effects on developmental competence. PA increased the levels of oxidative stress in oocytes (43±2.39 vs. 28.4±2.36, CellRox Deep Red pixel intensity) and reduced cleavage (56.6% vs. 69%) and blastocyst (13.9% vs. 24%) rates compared with the controls ($P<0.05$). These negative effects were ameliorated in the presence of 0.1 μM MitoQ (CellRox, 30.5±2.30; cleavage, 69.4%; and blastocysts, 24.2%, $P<0.05$). In contrast, 0.1 μM TPP alone did not enhance cleavage (55.8%) and blastocysts rates (20.2%) compared to the PA group ($P>0.1$). In conclusion, low concentrations of MitoQ can protect against induced oxidative stress during oocyte IVM, and enhance developmental competence under lipotoxic conditions. These effects are specific to the CoQ10 content of MitoQ since the carrier molecule TPP had no protective effects. In contrast, higher doses of MitoQ and TPP are toxic for oocytes.

A175E Embryology, developmental biology and physiology of reproduction

Prolonged transportation of ovaries negatively affects oocyte quality and *in vitro* embryo production in sheep

Alicia Martín-Maestro, Carolina Maside, Irene Sánchez-Ajofrín, María Iniesta-Cuerda, Patricia Peris-Frau, Daniela A. Medina-Chávez, Eric Marín, Beatriz Cardoso, José Julián Garde, Ana Josefa Soler

SaBio Group (CSIC-UCLM-JCCM), ETISAM, Albacete, Spain.

Keywords*:* sheep, oocyte quality, ovary transport.

The first step during *in vitro* embryo production (IVP) is the collection of developmentally competent oocytes. For wild species, such as Iberian red deer, the recovery of oocytes represents a problem because the slaughtering usually takes place far away from the laboratories and transport times are usually much longer. This may negatively influence developmental competence of immature oocytes and, therefore, their quality. The aim of this work was to examine the effects of different ovary storage times and media composition, using sheep as a model, with a view to achieving better results for wild species in which long transport times are inevitable. Adult sheep ovaries were recovered and randomly assigned to the Control (saline solution) or TCM (medium TCM199) groups and maintained for 13 h at 30 ºC. At 3, 7 and 13 h since ovary collection, sixty cumulus–oocyte complexes (COCs) were denuded and late stages of apoptosis were detected by TUNEL staining. Remaining 889 COCs (467 for Control and 422 for TCM) were matured, fertilized and cultured *in vitro* in order to examine oocyte maturation and sperm penetration rates by staining with Hoechst 3342 fluorescent dye, cleavage and blastocyst rates. Generalized linear model was used to study the influence of medium composition and storage time on oocyte quality and embryo production. When the analysis revealed a significant effect ($P<0.05$), a post hoc test with Bonferroni correction was carried out. Immature oocytes retrieved from ovaries stored during 13 h showed higher apoptosis ($P<0.05$), regardless of the medium composition. After fertilization, the proportion of inseminated oocytes with two pronuclei (2PN) was significantly higher ($P<0.05$) in the Control group compared to TCM (29.43% ± 4.54 vs 13.33% ± 4.54) and after 3 h of storage ($P<0.05$) compared to 7 and 13 h (47.58% ± 5.57 vs 14.46% ± 5.57 and 2.08% ± 5.57, respectively). Although maturation and embryo production did not show differences ($P>0.05$) in terms of medium composition and between 7 and 13 h of ovary storage, the percentage of Metaphase II (MII)-oocytes, cleavage and blastocyst rates were significantly higher ($P<0.05$) when ovaries were stored during a short period of time (3 h) compared to long periods of 7 and 13 h (maturation: 70.83% ± 7.96 vs 32.64% ± 7.96 and 6.25% ± 7.96; cleavage: 73.17% ± 8.43 vs 18.20% ± 8.43 and 1.02% ± 8.43; blastocyst rate: 32.78% ± 2.21 vs 5.82% ± 2.21 and 1.02% ± 2.21, respectively). In summary, although ovary storage medium composition had an influence on oocyte quality, the most prominent effect was found with transport times. Prolonged transportation of ovaries increased oocyte apoptosis and decreased maturation, sperm penetration, cleavage and blastocyst rates. Therefore, for wild species such as Iberian red deer, the optimization of these conditions is necessary to maintain oocyte quality and ensure a successful outcome during IVP.

This work was supported by Spanish Ministry of Economy and Competitiveness (AGL2017-89017-R).

A176E Embryology, developmental biology and physiology of reproduction

Evaluation of the cumulus cells viability and its oxidative state by flow cytometry from ovaries subjected to prolonged storage in sheep

Daniela A. Medina-Chávez, Alicia Martín-Maestro, Irene Sánchez-Ajofrín, María Iniesta-Cuerda, Patricia Peris-Frau, J.Julián Garde, Ana J. Soler, Carolina Maside

SaBio Group (CSIC-UCLM-JCCM), ETSIAM.

Keywords*:* cumulus cells, sheep, long storage.

Cumulus cells (CCs) have an important role during oocyte maturation and competence acquisition. Assessment of viability and oxidative status of CCs may be potential non-invasive predictors of oocyte quality in *in vitro* maturation (IVM) routines. In addition, the time intervals required by transport of ovaries from the slaughterhouse to the laboratory may adversely affect the oocyte quality. In this context, the current study aimed to evaluated the impact of ovaries storage duration on viability and oxidative status of CCs during *IVM* in sheep oocytes. Adult sheep ovaries were collected in saline solution with antibiotics at 30ºC and storage for 3, 7 or 13 h. Cumulus-oocytes complexes (COCs, n= 2436, four replicates) were collected after these times and placed in TCM199 supplemented with 10 ng/mL FSH/LH, 100 µM cysteamine and 10% fetal calf serum for 24 h. After IVM, COCs were denuded and CCs were collected. Denuded oocytes were stained with Hoechst3342 to assess *in vitro* maturation rate and CCs were evaluated with flow cytometry using specific fluorophores for viability, intracellular generation of reactive oxygen species (ROS), glutation content (GSH) and mitochondria activity. A general linear model was used to study the influence of storage time on CCs quality and IVM rates. When the analysis revealed a significant effect ($P<0.05$), values were compared by Bonferroni test. The percentage of live CCs was greater ($P<0.05$) when ovaries were stored for 3 h (63.0 ± 8.0) compared to 7 h (19.4 ± 8.0) and 13 h (22.4 ± 8.0) of storage while, at 13 h (56.1 ± 6.5) the dead CCs percentages was significantly higher than for 3h (20.9 ± 6.5) and 7 h (35.6 ± 6.5). After 7h of ovary storage, a significantly higher ($P<0.05$) percentage of apoptosis was observed compared to 3h of storage (45.0 ± 6.6 and 16.2 ± 6.6, respectively). The storage of ovaries for long time period (13 h, 779.3 ± 78.8) produced less ROS levels in CCs than short time periods, 3 h (1 067.8 ± 78.8) and 7 h (1 052.0 ± 78.8). However, no difference in GSH content (670.2 ± 54.3, 687.7 ± 54.3, 543.5 ± 54.3) and mitochondria activity (10.7 ± 2.8, 6. 5± 2.8, 4.7 ± 2.8) was shown for CCs from ovaries stored for 3, 7 and 13 h, respectively. In addition, *in vitro* maturation rate was found significantly higher after 3 h (69.2 ± 6.5) of storage followed by 7 h (34.0 ± 6) and 13 h (4.68 ± 6.5), respectively) ($P<0.001$). In conclusion, ovary storage time negatively influenced CCs viability that may be responsible for alters in oocyte quality and the *in vitro* maturation parameters in sheep.

Supported by Junta de Comunidades de Castilla-La Mancha-FEDER and by Spanish Ministry of Economy and Competitiveness (SBPLY/17/180501/000500, AGL2017-89017-R)

A177E Embryology, developmental biology and physiology of reproduction

Effect of vitrification of prepubertal goat oocytes matured with melatonin on embryo development after Parthenogenic Activation

Irene Menéndez-Blanco[1], Sandra Soto-Heras[1], Maria Gracia Catalá[1], Anna Rita Piras[2], Dolors Izquierdo[1], Maria Teresa Paramio[1]

[1]Autonomous University of Barcelona, Spain; [2]University of Sassari, Italy.

Keywords*:* Melatonin, Vitrification, Goat oocytes.

It is known that vitrification negatively affects oocyte quality. Melatonin added to the *in vitro* maturation (IVM) medium helps to improve embryo development of prepubertal goat oocytes (Soto-Heras, Reprod Fertil Dev;30(2):253-261.2018). The aim of this study was to assess the effects of vitrification of IVM-oocytes matured with melatonin on; a) intra-oocyte Reactive Oxygen Species (ROS) levels, b) oocyte apoptosis and c) blastocyst development after Parthenogenic activation (PA). Prepubertal goat oocytes were subjected to IVM in our conventional conditions. Three experimental groups were designed: 1) Control group (CG): oocytes after IVM. 2) Vitrified Group (VG): oocytes vitrified after conventional IVM and 3) Melatonin Vitrified group (MVG): oocytes matured with 10^{-7} M melatonin and vitrified after IVM. After 22 h of IVM, oocytes from VG and MVG groups were vitrified in an open-system using CVM™ Cryologic devices (IVF Bioscience; UK) and vitrification and warming protocol and solutions as described by Kuwayama (Kuwayama, Reprod Biomed Online;11:300-8. 2005). At the end of IVM, intra-oocyte ROS level was measured by staining denuded oocytes during 30 min with 10 μM 2',7' dichlorodihydrofluorescein diacetate (Molecular Probes Inc., OR, USA) (36-37 oocytes per group in 3 replicates). Oocyte apoptosis was analysed using eBioscience™ Annexin V Apoptosis Detection kit (Invitrogen. USA). 98-118 oocytes per group in 5 replicates were classified as viable (unstained), early apoptotic (stained with annexin), dead non-apoptotic (stained with propidium iodide) and necrotic (stained with both: annexin and propidium iodide). PA of mature oocytes was performed by 4 min incubation with 5 μM Ionomicyn followed by 4 h incubation with 2 mM 6-(Dimethylamino) purine (Sigma-Aldrich®Chemical,St. Louis.USA) (83-151 oocytes per group in 4 replicates). Presumptive zygotes were *in vitro* cultured in BO-IVC medium (IVF Bioscience; UK) for 8 days. Data were analyzed by two-way ANOVA followed by Tukey's multiple-comparison test (SAS® software version 9.4). ROS levels were lower in CG than VG oocytes (21327 ± 3309. vs 36959 ± 4336; $P<0.05$). No differences were found between VG and MVG groups. The percentage of viable oocytes after IVM was significantly higher ($P<0.05$) in the CG (67.0%±3.5%) than in both VG (50.8%±2.9) and MVG (39.0%±5.6) vitrified groups. After PA, CG showed higher cleavage rate than VG (80.1 ± 5.3 vs 53.4 ± 9.9, $P<0.05$) and a tendency to higher cleavage rate than MVG (55.6 ± 1.2 %, $P=0.055$), but no differences were found between vitrified groups. However, we did not find any blastocyst development in any of the vitrified groups regardless of the presence of melatonin, whereas in the CG we obtained 19.72%±3.38 blastocysts per oocytes. In conclusion, the vitrification process increased ROS levels and apoptosis in prepubertal goat oocytes and hinder the blastocyst development. Melatonin supplementation during IVM did not prevent these negative effects.

Funding: Spanish MINECO Grant AGL2017-85837-R and Spanish MECD predoctoral grant FPU15/00773.

A178E Embryology, developmental biology and physiology of reproduction

In silico prospection reveals evolutionary divergence in the cattle DNA methylation pathway

Marcelo T. Moura, Fabíola F. Paula-Lopes

Universidade Federal de São Paulo (Unifesp), São Pualo, SP, Brazil.

Keywords*:* bioinformatics, bovine, epigenetics, thermoregulation.

DNA methylation is an epigenetic mechanism that controls gene activity by bookmarking CpG-enriched DNA sequences which recruits methyl-binding trans-acting factors mostly for gene silencing. Multiple reports have demonstrated compelling data that environmental factors – such as heat stress - affect the DNA methylation patterns of germ cells and early embryos. Nevertheless, little is known about the intrinsic epigenetic divergences between taurine - *Bos taurus taurus (B. taurus*) and zebu - *Bos taurus indicus (B. indicus*) cattle, in spite of the greater adaptability of *B. indicus* to harsh environments. Therefore, a preliminary study using *in silico* tools was conducted to prospect evolutionary differences in the components of the DNA methylation pathway (DNA methyl-transferases, transcription factors, and co-factors) by sequence homology approaches between *B. taurus* and *B. indicus*. Initially, DNMTs were scrutinized for fishing possible divergences in DNA methylation activity. The genome anchorage carried out using the "genome data viewer" was similar between subspecies, where DNMT1 is localized on chromosome 7, DNMT2 and DNMT3B on chromosome 13, DNMT3A on chromosome 11, and DNMT3L on chromosome 1. Both *B. taurus* and *B. indicus* hold an identical number of DNMT isoforms (DNMT2: 2, DNMT3A: 2 and DNMT3B: 6), except DNMT1 (*B. taurus*: 10 vs. *B. indicus*: 1) and DNMT3L (B. taurus: 2 vs. *B. indicus*: 1). DNMTs display similar size and sequence between subspecies (DNMT2: 391 Aa, DNMT3A: 909 Aa, DNMT3L: 417 Aa). However, one DNMT3L isoform in *B. indicus* displays four additional amino acids (Aa) in the N-terminus. Six DNMT3B isoforms were found with variable size (*B. taurus*: 773 - 844 Aa vs. *B. indicus*: 733 - 842), with a divergent 10-Aa stretch in the N-terminus and another of 100 Aa in the C-terminus. The larger enzyme is DNMT1 (*B. taurus*: 1,611 Aa vs. *B. indicus*: 1,644). Remarkably, the *B. indicus* DNMT1 N-terminus displays an alanine-rich sequence, which may confer greater structural or thermal stability. No difference was found at the protein domain level of DNMTs between *B. taurus* and *B. indicus* by the CD-search tool. Additional DNA methylation pathway components involved in DNA methylation maintenance were thus addressed. The UHRF1, ZFP57, DPPA3/STELLA, and ZNF445 genes did not show any sequence difference between *B. taurus* and *B. indicus* for the aforementioned protein traits. There is evidence that the expression of DNMT1 is enriched in *B. taurus* spleen and testis within nine tissues investigated (expression atlas - www.ebi.ac.uk). Moreover, DNMT1 mRNA levels have been shown to fluctuate in *B. indicus in vitro* produced embryos and in oocytes and embryos of unknown genotype under heat shock. Even though Dnmt1 protein is found throughout oocyte maturation, it is only present in the cytoplasm of early embryos up to the 8-to-16 cell stage when genome activation is established, probably contributing to imprinting maintenance. In conclusion, the components of the DNA methylation pathway are highly conserved between *B. taurus* and *B. indicus*, although DNMT1 displays evolutionary-driven variation that deserves further experimental investigation.

A179E Embryology, developmental biology and physiology of reproduction

The potential role of isoquercitrin by interfering with cellular reactive oxygen species and growth factors in human ovarian cancer cells

Katarína Michalcová[1], Simona Baldovská[1], Eva Tvrdá[1], Jaromír Vašíček[2], Ľuboslav Sanisló[3], Vladimír Křen[4], Adriana Kolesárová[1]

[1]Department of Animal Physiology, Slovak University of Agriculture in Nitra, Slovak Republic; [2]Institute for Farm Animal Genetics and Reproduction, National Agricultural and Food Centre, Lužianky, Slovak Republic; [3]St. Elizabeth Cancer Institute Hospital, Bratislava, Slovak Republic; [4]Institute of Microbiology, the Czech Academy of Sciences, Prague, Czech Republic.

Keywords*:* isoquercitrin, OVCAR-3, ROS production.

Nowadays, many studies have reported that beneficial properties of flavonoid compounds from medicinal plants can be attributed to isoquercitrin. Isoquercitrin is powerful phytocompound that have been shown to exhibit disease prevention and health promotion properties. The aim of our study was to investigate the impact of isoquercitrin (purity 96.5 %, prepared by selective enzymatic derhamnosylation of rutin) treatment at the concentrations 5, 10, 25, 50, and 100 μg/mL on a human ovarian carcinoma cell culture (OVCAR-3) *in vitro.* Cell viability, cell death, apoptosis, the release of human epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), insulin-like growth factor I (IGF-I), and the production of reactive oxygen species (ROS) by cells after short-term application of 24 h were analyzed. The metabolic activity was determined by AlamarBlue^{TM} assay, the apoptotic assay using flow cytometry, presence of growth factors was detected by ELISA method, the ROS production was quantified by chemiluminescence. One-way ANOVA along with Dunnett's test was used to establish statistical significance at $P<0.05$. All experiments were done in triplicate. Isoquercitrin caused any significant changes neither in metabolic activity of OVCAR-3 cells, nor in the proportion of live, dead and apoptotic cells, nor in the release of EGF, TGF-β1, and IGF-I ($P>0.05$). However, tendency of a slight increase of TGF-β1 level after isoquercitrin application at the highest concentration 100 μg/mL was detected. Interestingly, our results showed, that lower concentrations (5, 10 and 25 μg/mL) significantly ($P<0.01$) inhibited the production ROS. On the other hand, ROS production observed in OVCAR-3 cells after isoquercitrin treatment was significantly ($P<0.001$) increased at the high concentrations (50 and 100 μg/mL). In conclusion, the results of our *in vitro* study show antioxidant and pro-oxidant activities of isoquercitrin in dose-dependent manner. ROS production by OVCAR-3 cells was increased at hight concentrations of isoquercitrin and decreased at lower concentrations. Isoquercitrin at any used concentrations did not interfere with growth factors in human ovarian carcinoma cells, however, further studies are required to better understandig of biological actions of isoquercitrin.

Acknowledgment: This work was supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic projects APVV-18-0312, APVV-16-0170, APVV-15-0543, VEGA 1/0039/16, Center of Animal Reproduction CeRA, Tatra bank Foundation and EU project no. 26220220180: Building Research Centre „AgroBioTech".

A180E Embryology, developmental biology and physiology of reproduction

Inhibition of miR-152 during *in vitro* maturation enhances the developmental potential of porcine oocyte

Matej Murin[1], Ahmed Gad[1,2], Lucie Nemcova[1], Radek Prochazka[1], Jozef Laurincik[1,3]

[1]The Czech Academy of Sciences, Institute of Animal Physiology and Genetics, Liběchov, Czech Republic; [2]Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt; [3]Department of Zoology and Anthropology, Faculty of Natural Sciences, Constantine the Philosopher University in Nitra, Nitra, Slovak Republic.

Keywords*:* oocyte, transfection, miRNA.

Oocyte and embryo development are regulated by complex molecular mechanisms. Several molecules are involved in these regulation mechanisms including microRNAs (miRNAs). MiR-152 is well known as a tumor suppressor in human cancer by inhibiting cell proliferation and suppressing the PI3K/Akt and MAPK signaling. We have shown previously that miR-152 is upregulated in fully-grown compared to growing oocytes. Several genes required for progression through different stages of meiosis are putatively targeted by this miRNA. However, its specific role in oocyte and embryo development is still unknown. In this study, we evaluated the developmental potential of porcine oocytes after manipulation of miR-152 abundance during in vitro maturation using mimic, inhibitor or random sequence miRNA as negative control. Cumulus-oocytes complexes isolated from 3 – 6 mm follicles of premature gilts have been cultivated in maturation medium TCM 199 (Sigma-Aldrich, Munich, Germany) without hormonal stimulation but with dibutyryladenosine cyclic monophosphate (dbcAMP) and miR-152 mimic (40nM), inhibitor (600nM) or negative control (40nM) in the presence of Lipofectamine 3000 (Thermo Fisher Scientific, Massachusetts, USA) for 4 hours. Then transfection was continued in TCM 199 with hormonal stimulation for 44 hours and metaphase II (MII) rate was calculated. Some of the matured oocytes were parthenogenetically activated and cultivated in porcine zygote medium 3 (PZM 3) for 6 days until blastocyst stage. The abundance of miR-152 was analyzed in MII oocytes using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) on droplet digital PCR (ddPCR) system (Bio-Rad Inc.). Our preliminary results showed that mimic and inhibitor treatments change the abundance of miR-152 in MII oocytes by 2.2 and -2.5 folds, respectively, compared to the negative control group. There were no differences in MII rate among negative control, mimic and inhibitor group (92±1%, 92±2%, and 91±5%, respectively) or in cleavage rate of parthenotes of the same treatment groups (71±12%, 77±3%, and 82±7%, respectively). On the other hand, the blastocyst rate of parthenotes was significantly higher in the inhibitor group (48±3%) compared to negative control (28±5%) or mimic group (22±9%). In conclusion, inhibition of miR-152 during oocyte maturation could enhance the developmental rate of porcine parthenotes. More studies are required to understand the exact function of miR-152 during oocyte and embryo development.

A181E Embryology, developmental biology and physiology of reproduction

Establishment of a single embryo sequential culture system for cell free DNA (cfDNA) based genetic screening in cattle

Preetha Nathan[1], Giuseppe Silvestri[2], María Serrano-Albal[2], Melinda Jasper[3], Gary Harton[3], Simon Harvey[1], Katie Fowler[1], Darren Griffin[2]

[1]Canterbury Christ church University, United Kingdom; [2]School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NH, UK; [3]PerkinElmer Health Sciences, 40-46 West Thebarton Road, Thebarton, 5031, Australia.

Keywords*:* Culture media, sexing PCR, blastocyst.

Recent studies have demonstrated the natural release of genomic DNA by embryo during culture. This cell free DNA (cfDNA) could be employed in whole genome amplification (WGA) strategies to obtain representative quantities of embryonic DNA without the need for invasive techniques, minimising damage to the blastocyst intended for embryo transfer, and provides an easily scalable platform for cattle breeders interested in screening large numbers of *in vitro* produced (IVP) embryos for sex or genetic value, a growing trend in the current market. As a result, the development of efficient single embryo culture systems to obtain cfDNA from spent culture media has immense potential for the genetic screening of IVP blastocysts. The present study aims to optimise a culture system, free from foreign DNA contaminants, for the *in vitro* culture of cattle zygotes, to demonstrate presence of amplifiable quantities of cfDNA in this system, and to obtain sexing diagnoses by PCR. Oocytes from abattoir material across two replicates (n=146) were matured for 22 h and fertilised with frozen/thawed bull sperm. Presumptive zygotes derived from the same dam were co-cultured for 3 days in 100 µl of Synthetic Oviduct Fluid (SOF) medium supplemented with 6 mg/ml BSA. The zygotes were then cultured in individual SOF droplets of 25 µl for a further 4 days when blastulation rates were recorded. Following this, individual media samples of different volumes (2 or 5 µl) from each drop and the corresponding blastocysts (n=5) were collected separately on day 7 post-IVF and subjected to WGA by DOPlify® kit (Perkin-Elmer, Waltham, MA), as per manufacturer's instructions. Quantification of the WGA products was obtained by Qubit dsDNA HS fluorometry assay (Thermo Fisher Scientific, Waltham, MA) as per manufacturer's instructions using a 1:200 dilution of the original WGA sample. Following amplification, sexing was performed by PCR using chromosome Y (SRY primers) and bovine autosomal primers (BSPF primers). In this pilot study, culture in single droplets produced a blastocyst rate of 36% per cleaved embryo (n=21 blastocysts and n=58 cleaved embryos). The average cfDNA yield per 25 µl following WGA was 0.92 ± 0.15 µg, while whole blastocysts produced a similar amount of 1.08 ± 0.33 µg (t-test, P= 0.66). There was no statistically significant difference in the total yield of DNA amplified when either 2 or 5µl of spent culture medium were used (paired t-test, P=0.31). The proportion of WGA samples producing a sexing PCR result was 50% and the concordance for sex determination between the blastocyst and its medium was 25% (n=12). While presence of cattle specific DNA was confirmed by PCR, the results presented suggest DNA cross-contamination may affect WGA and PCR efficiency. To address this, future tests will replace BSA with recombinant human serum albumin (HSA) and will include media changes during culture. To improve PCR specificity we will employ shorter amplicons since cfDNA is known to be of very low molecular weight. Following optimisation, we will use these cfDNA samples for single nucleotide polymorphism typing to develop a non-invasive aneuploidy screening strategy for cattle IVP.

A182E Embryology, developmental biology and physiology of reproduction

Timing of pronucleus formation and first DNA replication in porcine IVP zygotes using frozen-thawed spermatozoa

Sergio Navarro-Serna, Alba Pérez-Gómez, Raquel Romar, Joaquin Gadea

University of Murcia Dept. Physiology, Murcia, Spain. International Excellence Campus for Higher Education and Research "Campus Mare Nostrum" and Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain.

Keywords*:* pig, embryo, DNA replication.

Improvements in the efficiency and repeatability of porcine IVP is necessary for the application and optimization of new technologies as gene edition of embryos by CRISPR/Cas9. Our lab has optimized IVF procedures like sperm selection by swim-up, the addition of natural fluids (Cánovas, eLife. 6:e23670, 2017) and variations of atmosphere conditions in IVF (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). For future experimental designs and optimize the IVF system could be useful to know the kinetic of penetration, pronuclear formation and DNA replication. To determinate starting of DNA replication 441 porcine oocytes were *in vitro* matured in NCSU-37 medium (Cánovas, eLife. 6:e23670, 2017) and were inseminated in TALP with frozen-thawed boar spermatozoa selected with NaturARTs-Pig sperm swim-up medium (EmbryoCloud, Murcia, Spain) and cultured at 38ºC, 5% CO_2 and 7% O_2 (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). Groups of 25 oocytes were fixed every hour from 4 hours to 12 hours and 24 hours after IVF and were staining with Click-iT™ EdU Alexa Fluor™ Imaging Kit (Invitrogen, Spain) to determinate penetration rate (PEN), spermatozoa/oocyte (S/O), male pronucleus formation (MPF) and rate of zygotes with DNA replication. PEN already started at 4h after IVF (15.79%) and was increasing to 55.00-66.67% at 5-8h until reaching 80.43% at 9h and without significant differences with the following hours. Pronuclei formation started at 6h after IVF (MPF = 12.00%), increasing to 52.78% at 7h and near 100% since 8h (93.33%). DNA replication was not detected until 10h after IVF (20.00%) with a slight increase at 11h (37.78%) and 12h (43.48%) to reach 100% at 24h. The kinetic of penetration and MPF depend on various factors like male, sperm preservation (fresh or frozen-thawed), sperm origin (epididymal or ejaculated), sperm selection and capacitation method. Our swim-up method has similar time of penetration rate and pronucleus formation respect other fresh sperm preparation and capacitation method described in the literature but Percoll gradient advance both parameters (Matás, Reproduction. 125(1), 133-41, 2003). Regarding the beginning of DNA replication, our study had a delay of 2h respect the commence of DNA replication in porcine embryos produced by IVF previously reported (Jeong, Dev Dyn 236(6), 1509-16, 2007). Know the time when first DNA replication takes place in the zygote is important to plan strategies of technology applications like genetic engineering, for which gene edition previous DNA replication is an important objective to reduce mosaicism in the future organism. Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

A183E Embryology, developmental biology and physiology of reproduction

In vitro production of the first reindeer (Rangifer tarandus tarandus) blastocysts

Jaana Peippo[1], Heli Lindeberg[2], Mervi Mutikainen[1], Tuula-Marjatta Hamama[1], Jouko Kumpula[3], Oystein Holand[4]

[1]Natural Resources Institute Finland (LUKE), Jokioinen, Finland; [2]Natural Resources Institute Finland (LUKE), Maaninka, Finland; [3]Natural Resources Institute Finland (LUKE), Inari, Finland; [4]Norwegian University of Life Sciences (NMBU), Ås, Norway.

Keywords*:* ruminant, ex situ conservation.

Reindeer herding is economically, societally, culturally and ecologically important livelihood in northern Eurasia. In the future, climate and socio-economical changes in the arctic region may challenge the vitality of the reindeer populations. Optimized reproductive technologies could be used both for the conservation of the reindeer genetic resources and to facilitate successful breeding of reindeer in the future. The aim of this study was to test the suitability of optimized bovine *in vitro* embryo production protocol for reindeer embryo production *in vitro.* Reindeer ovaries were collected during the period of cyclic ovarian activity which extends from September to February in reindeer females. At the slaughterhouse, reindeer ovaries were sliced after collection in EMCARE^{TM} Biofree Flushing solution (ICPbio Reproduction, Spring valley, WI, USA). Recovered and washed oocytes were placed in tubes containing Hepes-buffered TCM199 supplemented with glutaMAX-I (100 × stock solution, Gibco™; Life Technologies Limited, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss, Netherlands), 1 µg/ml β-estradiol (E-2257) and 10% heat inactivated FBS (Gibco™, New Zealand) for maturation for 24 h at 38.5°C in air while transported to laboratory. Matured oocytes were fertilized for 20 h with Sperm-TL washed (2×4 ml) frozen-thawed semen (Lindeberg H, Nikitkina E, Nagy Sz, Krutikova A, Kumpula J, Holand Ø 2019, Abstract book of the 10th Circumpolar Agriculture Conference, 13th – 15th March 2019, Rovaniemi, Finland. p. 87) in IVF-TL supplemented with heparin (10 µg/ml) and PHE having 1×10^6 spermatozoa/ml as a final concentration. Denuded zygotes were cultured in G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with FAFBSA (4 mg/ml) and L-carnitine (1.5 mM) at 38.5°C in maximal humidity in 5% O_2, 5% CO_2 and 90% N_2. Cleavage rates were recorded at 42 hpi. Blastocysts were recorded on days 7 and 8 (IVF=day 0). Unless otherwise stated all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The average cleavage rate was 28.4% for the 162 best quality oocytes selected for maturation during the two slaughterhouse visits, one in December and the other in January. Altogether four and two blastocysts (3.7%) were produced by day 7 and 8, respectively. In conclusion, bovine *in vitro* embryo production protocol may also be used to produce reindeer embryos *in vitro*. However, the challenge is the oocyte quality as most of the slaughtered females are either prepubertal or old.

Acknowledgements: Nordforsk and ReiGN (Reindeer husbandry in a Globalizing North – resilience, adaptations and pathways for actions) project are acknowledged for the funding. The reindeer slaughterhouse in Ranua is acknowledged for the ovary collections.

A184E Embryology, developmental biology and physiology of reproduction

Gene expression profiles of bovine genital ridges during sex determination and early differentiation of the gonads

Benjamín Planells[1,2], Isabel Gómez-Redondo[1], José María Sánchez[2], Michael McDonald[2], Ángela Canovas[3], Patrick Lonergan[2], Alfonso Gutiérrez-Adán[1]

[1]School of Agriculture and Food Science, University College Dublin, Dublin, Ireland; [2]Departamento de Reproducción Animal, INIA, Madrid, Spain; [3]Centre for Genetic Improvement of Livestock, Department of Animal Biosciences, University of Guelph, Guelph, Ontario.

Keywords*:* sex determination, Rna-seq, cattle.

Sex determination in mammals depends on a complex interplay of signals that promote the bipotential fetal gonad to develop as either a testis or an ovary. Most knowledge of this process has arisen from experiments in the mouse model. (Sekido and Lovell-Badge 2008; Kim et al. 2007). In mice, the differentiation of supporting cell progenitors into male-specific Sertoli cells or female-specific granulosa cells is controlled by SOX9 presence or absence. However, there is scarce information concerning the process of sex determination in livestock species, especially in cattle. In order to clarify the process of sex determination in cattle, we used an RNA sequencing (RNA-seq) strategy to analyze the transcriptome landscape of male and female bovine fetal gonads collected *in vivo* at important developmental stages before, during, and after *SRY* activation. The estrous cycles of cross-breed heifers were synchronized followed by AI, and heifers were slaughtered at 35 (n=12; bipotential gonad formation), 39 (n=12; *SRY* peak of expression) and 43 (n=9; early gonad differentiation) days later. At each time-point, genital ridges were dissected from mesonephros and RNA was extracted using a Direct-zol^{TM} RNA MiniPrep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. After PCR sexing of the fetuses, RNA-seq libraries were prepared from 3 male and female samples and were sequenced using a HiSeq2500 v4 chemistry system at the Centre of Genomic Regulation (Barcelona, Spain). Differential gene expression analyses were performed independently using DESeq2 v.1.20 (adjusted p-value < 0.05). Firstly, we identified the differentially expressed genes (DEGs) between male and female gonads (sex analysis at D35, D39 and D43). Secondly, we identified DEGs during the period of transition between sex determination and differentiation within each sex (time-couse analysis between D35 and D39, and between D39 and 43). We also used a hierarchical clustering approach to cluster genes with similar expression profiles during the period from D35, D39 and D43. We performed western blot and immunofluorescence analysis of SOX9 and SOX10 to check if expression was also evident at the protein level. Gene analysis identified 143, 96 and 658 DEGs between males and females at D35, D39 and D43, respectively. Regarding the time-course analysis, 767 DEGs were identified in the comparison between D35 vs D39 male gonads, and 545 DEGs in the female gonads. In the comparison between D39 vs D43, 3157 DEGs were identified in males, and 2008 DEGS were identified in females. We found expression of several Y chromosome genes before SRY (that are absent in mice and human), *SOX9* and *SOX10* expression in both somatic and germinal cell lineages in the XY genital ridge during sex determination, the nuclear and higher expression of SOX10 instead of SOX9 in Sertoli cells during male determination and early differentiation, a lack of nuclear internalization of SOX9 in Sertoli cells during early sex differentiation and no early expression of the WNT/β-catenin pathway repressing *SOX9* in gonads. In conclusion, our data indicate that sex determination and early gonad differentiation in cattle exhibit some unique characteristics.

A185E Embryology, developmental biology and physiology of reproduction

Metabolic changes in uterine fluid collected from cyclic heifers at different stages of embryo receptivity

Alejandra C Quiroga[1], Ismael Lamas-Toranzo[1], José María Sánchez[2], Michael McDonald[2], Pat Lonergan[2], Pablo Bermejo-Alvarez[1]

[1]Animal Reproduction Department, INIA, Madrid, Spain; [2]School of Agriculture and Food science, University College Dublin, Belfield, Dublin 4, Ireland.

Keywords*:* Metabolism, elongation, uterine fluid.

Preimplantation embryo development relies on the metabolites, hormones and growth factors present in oviductal and uterine fluids. *In vitro* culture conditions partially recapitulate the natural milieu where embryos develop, but a culture system capable of supporting bovine embryo development beyond the blastocyst has not been yet established, suggesting that medium composition requires further optimization. The objective of this study was to characterize the changes in biochemical compounds in bovine uterine fluid (UF) at different stages of embryo receptivity. To achieve this, 22 crossbred heifers were synchronized and UF was collected post-mortem by uterine flushing using 20 ml of DPBS at specific days after ovulation: 1) Day 0, when UF does not support embryo development, 2) Day 7, when UF supports blastocyst development, 3) Day 10, just prior to when UF triggers conceptus elongation, and 4) Day 14, when UF supports the exponential growth phase during conceptus elongation. UF (n=6, 5, 6 and 5 samples for Days 0, 7, 10 and 14, respectively) were centrifuged at 1500 g for 15 min at 4ºC to remove cell debris and supernatants were kept at -80 ºC until analyses. Metabolomic analyses were performed by Metabolon Inc., which provides an unbiased metabolite analyses based on Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). Welch´s two-sample t-test was used to identify biochemicals that differed significantly between groups ($p<0.05$). The analysis identified 359 compounds of known identity. The most dramatic changes in the abundance of different compounds occurred between D7 and D14, suggesting that elongation requires significant modifications in UF composition. Biochemicals related to glucose metabolism showed significant changes over time. For instance, glucose, fructose, mannitol/sorbitol and pyruvate increased over time (1.5-, 2.3-, 190- and 5-fold increases between D7 and D14). Significant changes were also noted for glutamate metabolism, with a steady increase of beta-citrylglutamate (24-fold increase between D7 and D14). Compounds involved in Krebs cycle also exhibited significant variation between days, with increases in citrate, aconitate and 2-methylcitrate/homocitrate (8-, 12- and 11-fold increases between D7 and D14). These results highlight that embryo metabolic requirements vary greatly between blastocyst hatching and conceptus elongation and provide relevant insights to develop an *in vitro* system to achieve the cell proliferation, differentiation and migration events involved in this process.
Acknowledgements: This study is supported by projects AGL2017-84908-R from the Spanish Ministry of Economy and Competitiveness, 757886-ELONGAN from the European Research Council, and 13/IA/1983 from Science Foundation Ireland. ILT is supported by a FPI contract.

A186E Embryology, developmental biology and physiology of reproduction

Post-hatching *in vitro* bovine embryo development inside agarose tunnels or without physical constriction

Priscila Ramos-Ibeas[1], Ismael Lamas-Toranzo[1], Álvaro Martínez-Moro[1,2], Celia de Frutos[1], Alejandra C. Quiroga[1], Esther Zurita[1], Pablo Bermejo-Alvarez[1]

[1]Animal Reproduction Department, INIA, Madrid, Spain; [2]Procreatec, Calle Manuel de Falla 6-8, 28036 Madrid, Spain.

Keywords*:* Elongation, conceptus, hypoblast.

The greatest gestational losses in cattle occur during the second week of pregnancy, when critical developmental events take place: hypoblast migrates to cover the entire inner surface of the embryo, and epiblast forms a flat embryonic disk. Previous studies have established an *in vitro* post-hatching development system based on agarose gel tunnels and glucose-enriched medium. This system achieves some expansion of the trophoblast and hypoblast proliferation. However, embryonic disc formation is not achieved and it remains unclear whether the hypoblast covers entirely the inner surface of the embryo. An open question about this system is whether embryo culture inside tunnels is actually required for development or it just shapes the embryo to a tubular shape by mechanical constriction. The objective of this study has been to compare post-hatching development inside agarose tunnels or free-floating in an agarose-coated dish. *In vitro*-produced E11 blastocysts were measured and cultured in Synthetic Oviduct Fluid (SOF) supplemented with 27.7 mM glucose and 10 % FCS inside ~1mm agarose tunnels or over an agarose surface until E15. At the end of the culture period, embryo area was calculated using Fiji software and the development of specific lineages was assessed by immunostaining for SOX2 and NANOG (epiblast), SOX17 (hypoblast), and CDX2 (trophectoderm). No differences were found on embryo survival until E15 and the main factor determining survival was the initial embryo size at E11. In particular, when <0.5 mm E11 were cultured, only 1/16 (6 %) embryos cultured free-floating or 1/18 (6 %) cultured in tunnel survived, while when E11 embryo diameter was ≥ 0.5 mm, 12/17 (71 %) and 16/21 (76 %) survived when cultured free-floating or inside tunnel, respectively. Surviving embryos showed a cylindrical shape when they developed inside the tunnels and spherical when they develop without physical constriction, but area and volume were significantly smaller in embryos cultured inside a tunnel (2.19±0.21 vs. 4.76±1.14 mm^2 and 1.71±0.40 vs. 9.32±2.92 mm^3, for embryos cultured in tunnel or free-floating, respectively, t-test $p \leq 0.05$). A layer of hypoblast cells (SOX17+) was detected inside the trophectoderm, but, irrespective of the culture system, that layer did not cover the entire inner surface of the embryo. Similarly, although a compact cell structure was detected in some developmentally advanced embryos, no SOX2 or NANOG-positive epiblast cells were detected in any of the culture systems. In summary, post-hatching blastocyst culture inside agarose tunnels shapes embryo morphology by physical constriction, but it may restrict embryo growth and does not seem to provide any significant advantage in terms of development of hypoblast and epiblast lineages. The partial hypoblast migration and the absence of embryonic disc highlight that post-hatching culture conditions still requires significant optimization.
Acknowledgements: This study is supported by projects AGL2017-84908-R from the Spanish Ministry of Economy and Competitiveness and 757886-ELONGAN from the European Research Council. PRI is supported by a talent attraction fellowship from Madrid region and ILT is supported by a FPI contract.

A187E Embryology, developmental biology and physiology of reproduction

Changes in the transcriptome of ovine MII oocytes caused by lipopolysaccharide

Mahsa Rasekhi[1], Morteza Daliri[1], Abdollah Mohammadi Sang Cheshmeh[2], Pablo J. Ross[3], Mohammad Reza Bakhtiari Zadeh[2], Vahid Shariati[1]

[1]National Institute of Genetic Engineering and Biotechnology, Iran, Islamic Republic of; [2]Department of Animal and Poultry Sciences, Aburaihan Campus, University of Tehran Pakdasht, Tehran, Iran; [3]Department of Animal Science, UC Davis, California, USA.

Keywords*:* ovine oocyte, lipopolysaccharide, differential gene expression.

Increasing low fiber high fermentable carbohydrate diets increase the ruminal lipopolysaccharide (LPS) derived from gram negative bacteria cell walls. LPS was detected in plasma and follicular fluid of ruminants with endometritis and mastitis and disturbed the reproduction performance. While several studies have examined the effect of LPS on oocyte maturation and developmental competence, limited knowledge is available on potential effect of LPS on transcript aboundance of ovine oocytes. Thus, transcriptome profiles of MII oocytes matured in presence or absence of LPS were compared using 3'tag digital gene expression method. Cumulus oocyte complexes collected from salaughterhouse-derived ovaries were matured either in media supplemented with 0.1 μg/mL, 1 μg/mL, 10 μg/mL of LPS (Sigma Aldrich Inc) or in media without LPS (control). After in vitro maturation, the cleavage and blastocyst formation following parthenogenetic activation were determined for each group. Subsequently, three biological replicates of 36 oocytes cultured in 1 μg/mL LPS and controls were subjected to 3'tag digital gene expression profiling. Differential expression analysis was performed using the R Statistical Programming Language and limma package (using voom method, an animal as a blocking factor and treatment in the model). Functional enrichment analysis of the differentially expressed genes was further performed using Enricher database. Our results showed that maturation rate (determined based on first polar body extrusion), was not significantly different between the groups. The lowest LPS dose that significantly affected developmental competence to blastocyst stage was 1 μg/mL of LPS (unpublished data). After culturing ovine oocytes in vitro for 22 hours in the presence of 0 μg/mL (control) and 1 μg/mL, a total of 7887 gene transcripts were detected and only eight genes were differentially expressed. Of these, seven genes were down-regulated (two-fold or greater) in LPS-treated group (adjusted $p< 0.05$). Down regulated genes were the following: (Tripartite motif containing 25 (TRIM25), Tripartite motif containing 26 (TRIM26), Zona Pellucida glycoprotein 3(ZP3), Family with sequence similarity 50-member A (FAM50), Glyoxalate and hydroxy pyruvate reductase (GRHPR), cornichon family AMPA receptor auxiliary protein 4 (CNIH4) and NADH ubiquinase oxireductase subunit A8 (NDUFA). Functional analysis showed that these genes were significantly enriched in immune response, oxidation-reduction process as well as oocyte development. It is worth to note that *TRIM25* and*TRIM26* were reported as important genes in innate immune response. In addition, *ZP3* is well known as a positive regulator of inflammation, interferon gamma and interleukin 4 production, oocyte and blastocyst development. Moreover, NDUFA8 is involved in electron transport process which could be related to decreased mitochondrial membrane potential in matured oocytes in the presence of LPS. Accordingly, LPS is associated with impaired developmental competence.In conclusion,our results expand our knowledge of the genes transcribed of non-LPS and LPS treated in MII oocytes, which can shed light on molecular mechanisms of LPS-induced infertility in ruminants.

A188E Embryology, developmental biology and physiology of reproduction

Comparing the ability of epididymal or ejaculated sperm to elicit endometrial transcriptomic changes in cattle

Sandra Recuero[1], José María Sánchez[2], Yentel Mateo[1], Sandra Bagés-Arnal[2], Michael McDonald[2], Ariadna Delgado-Bermúdez[1], Marc L. Lavanera[1], Isabel Barranco[1], Susanta K. Behura[3], Tom E. Spencer[3], Marc Yeste[1], Pat Lonergan[2], Beatriz Fernandez-Fuertes[1]

[1]Department of Biology, University of Girona, Girona, Spain; [2]School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland; [3]Division of Animal Sciences, University of Missouri, Columbia, Missouri, USA.

Keywords*:* seminal plasma, sperm, endometrium.

In mice and pigs there is robust evidence of seminal plasma (SP) modulating the maternal environment, which positively impacts embryo survival and development. However, similar evidence in cattle is sparse. Both mice and boars deposit the ejaculate inside the uterus, while bovine semen deposition takes place in the vagina, and it is questionable whether any SP reaches the uterus. However, at ejaculation, sperm encounter SP, leading to proteins binding tightly to their plasma membrane, which probably allows them to act as a vehicle for SP proteins. Based on the beneficial effect of SP observed in other species, we hypothesised that ejaculated sperm, through SP proteins, elicit a different response in the endometrium than epididymal sperm (which have never been exposed to SP). To test this, a model of endometrial explants, which has been previously used to study embryo-maternal interaction, was used. Six crossbreed heifers were oestrous synchronised and slaughtered 12 h after the onset of oestrus. Three explants from the uterine horn ipsilateral to the preovulatory follicle were obtained from each animal. Epididymal sperm were collected and pooled from the cauda epididymis of three beef bulls slaughtered in a commercial abattoir. In addition, ejaculates were collected by artificial vagina from three Holstein bulls. After pooling, they were washed through a density gradient to isolate ejaculated sperm. Endometrial explants were incubated for 6 h with: 1) medium alone (control); 2) epididymal sperm (10^6 sperm/ml) or 3) ejaculated sperm (10^6 ejaculated sperm/ml). After incubation, they were snap frozen for subsequent RNA sequencing. Strikingly, explants exposed to ejaculated sperm had no differentially expressed genes (DEG) in comparison with control explants. In contrast, explants incubated with epididymal sperm exhibited 48 DEG (32 down and 16 up) in comparison with control explants. For the annotated genes (n=35), the most represented Gene Ontology (GO) terms were “binding” (n=12; 9 down and 3 up) and “catalytic activity” (n=13; 10 down and 3 up) for the molecular function, whereas “cellular process” (n=13; 10 down and 3 up) was the highest represented term in the biological process category. When explants exposed to ejaculated sperm were compared with those exposed to epididymal sperm, 80 DEG were identified (72 up and 8 down). For the 64 annotated genes, “binding” (n=21; 19 up and 2 down), “biological process” (n=29; 26 up and 3 down) were again the most represented GO terms, together with “catalytic activity” (n=17; 16 up and 1 down), and “biological regulation” (n=13; 13 up and 0 down) in the biological process category. In conclusion, these data do not support a role for bovine SP in the regulation of endometrial function. In contrast, the results suggest that SP may mask sperm surface proteins, inhibiting their interaction with the endometrium. Because the media used in this experiment does not support sperm capacitation, it remains to be determined whether this process, which is triggered in the female reproductive tract, can reshape the sperm surface in a way that it enables sperm-endometrium interaction.

Supported by EU Horizon 2020 Marie Sklodowska-Curie (No 792212).

A189E Embryology, developmental biology and physiology of reproduction

Improvement of pig embryonic development after the addition of haptoglobin (Hp) to the culture medium

Ernesto Rodríguez-Tobón[1], Rebeca López-Úbeda[2], Francisco A. García-Vázquez[1], Manuel Avilés[2], María J. Izquierdo-Rico[2]

[1]Department of Physiology, Veterinary School, Campus Mare Nostrum, University of Murcia, and IMIB-Arrixaca, Spain; [2]Department of Cell Biology and Histology, Faculty of Medicine, University of Murcia and IMIB-Arrixaca, Spain.

Keywords*:* embryo, haptoglobin, porcine.

Haptoglobin (Hp) is an acute phase protein recently detected in female reproductive structures (ovary, endometrium and decidua) and fluids (vaginal and amniotic). We previously described the presence of mRNA and detection by immunohistochemistry of HP in the porcine oviduct along of the oestrous cycle, especially in postovulatory and luteal phases. The upregulation of HP gene in these phases could indicate a function in embryo development. The aim of this study was to evaluate the effect of Hp protein on pig embryonic development. Oocytes were matured in NCSU-37 medium at 38.5ºC, 5% CO_2 and 95% humidity. Later on, *in vitro* matured oocytes were mechanically stripped, transferred to TALP medium and co-incubated at 38.5°C, 5% CO_2 with $1x10^4$ spz/ml porcine sperm selected by a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (45/90%). At 18h post-insemination putative zygotes were transferred to NCSU23a medium supplemented and incubated for 22-24h. Subsequently, the percentage of cleavage was evaluated and only those zygotes that presented 2 to 4 cells were transferred to NCSU23b medium (in which the sodium pyruvate and lactate of the NCSU23a medium were replaced by D-glucose 5.55mM) for another 120h under the same conditions previously mentioned, to complete a development of 7 days post insemination. In the case of the Hp group, TALP and NCSU23a culture media were supplemented with purified pig Hp protein (HGLB12-N-25. Alfa Diagnostic Internacional, San Antonio, EEUU) at a final concentration of 10 µg/ml. Blastocysts obtained were photographed and their diameter was evaluated by ImageJ® program. Finally, blastocysts were stained with Hoechst 33342 to evaluate the total number of cells per blastocyst. The data were analyzed by Chi-square test ($p<0.05$). Our results showed that cell division was similar in both experimental groups (control: 35.60% vs. Hp: 32.97%). However, the blastocyst development was higher in the Hp group in comparison with the control (control: 37.83 % vs. Hp: 64.50 %). In the case of the embryo quality, both the diameter (control: 357.86 ± 8.98 µm vs. Hp: 373.13 ± 5.40 µm) and the number of cells per blastocyst (control: 52.46 ± 2.73 vs. Hp: 56.11 ± 2.50) were identical in both groups (control: N=14 and Hp: N=20 blastocysts evaluated respectively). In conclusion, adding Hp protein into the culture medium increases the number of embryos that reach the blastocyst stage.
Supported by MINECO-FEDER (AGL2015-70159-P) and Fundación Séneca (19357/PI/14).

A190E Embryology, developmental biology and physiology of reproduction

Immunofluorescence analysis of NR3C1 receptor following cortisol exposure during bovine *in vitro* oocyte maturation

Mateo Ruiz-Conca[1], Jaume Gardela[1], Manuel Alvarez-Rodríguez[1,2], Teresa Mogas[3], Manel López-Béjar[1]

[1]Dept. of Animal Health and Anatomy, Universitat Autonoma de Barcelona, Spain; [2]Dept. of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden; [3]Dept. of Animal Medicine and Surgery, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.

Keywords*:* oocyte, cortisol, NR3C1.

Glucocorticoid hormones (GCs) play a key role in a various set of important cellular and physiological functions such as stress signaling, lipid and carbohydrate metabolism, apoptosis and mitochondrial activity. However, the mechanisms by which stress and glucocorticoids damage or protect the oocyte are largely unknown. Current knowledge has reported differences in the effect of cortisol exposure depending on the species. As completely opposite examples, pig oocyte in vitro maturation (IVM) was inhibited by cortisol (Yang, Biol Reprod 60:929–936, 1999), whereas equine was not impaired (Scarlet, Dom Anim Endocrin 59:11-22, 2017) and a previous study in bovine reported beneficial effect of cortisol in the IVM medium on blastocyst rate (daCosta, Theriogenology 85(2):323-329, 2016). Here we studied the nuclear maturation rates and the levels of the glucocorticoid receptor (NR3C1) after exposure of bovine oocytes to cortisol during IVM. Briefly, cumulus-oocyte complexes (COCs) were cultured for 24h with cortisol (Control (n=374) (C): 0µg/mL, Control vehicle (n=371) (CV): ethanol, CORT1(n=372): 0.1µg/mL, CORT2 (n=370): 0.25µg/mL) at 38.5ºC in an atmosphere of 5% CO2 in humidified air. After 24 h of IVM, oocytes were denuded and fixed in 4% paraformaldehyde (30 min at 38.5ºC) and permeated (0.3% Triton-X100, 30 min, room temperature). For nuclear maturation assessment, oocytes were mounted in DAPI medium (Vector labs, Burlingame, USA) for chromosome staining and coverslipped. Metaphase II achievement status was checked. For immunofluorescence determination of NR3C1 glucocorticoid receptor presence, permeated oocytes were incubated overnight at 4ºC with anti-rabbit primary antibody (1:500) (GR/NR3C1 NBP2-42221, Novus Bio, Centennial, USA), washed 5x in PBS and then incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (1:1000) for 1h at room temperature for subsequent washing and immunofluorescence semiquantitative assessment. Results showed that nuclear maturation of cortisol treated groups was improved (C: 61.5±1.5; CV: 59.8±3.7; CORT1: 75.3±2.0; CORT2: 76.8±0.5; $p < 0.01$) compared to control ones. NR3C1 expression was 40.1% and 40.9% times more expressed in CORT1 and CORT2, respectively, compared to control groups; while whole intensity of the oocyte was 5.7% and 5.4% increased. Cortisol seems to play a role in the oocyte developmental competence and may be acting directly on oocyte maturation. We hypothesize that this may be due to the preparation of the oocyte for the following stressing phenomenons, acting as a sublethal stress for the acquisition of stress tolerance (Pribenszky, Biol Reprod 563;83:690-7, 2010). Further studies are needed in order to elucidate the specific mechanism by which the glucocorticoid receptor affect the development of oocyte competence on different species.

This study was supported by the Gov. of Spain, Ministry for Science (AGL2016-79802-P). M.R-C. holds a FPU predoctoral fellowship (FPU2015/06029) from MECD; M.A-R. holds a Juan de la Cierva Incorp. (IJCI-2015-24380). J.G. holds an AGAUR predoctoral fellowship (2018 FI_B 00236).

A191E Embryology, developmental biology and physiology of reproduction

Production of cloned and fertilized embryos of a cloned bull of buffalo (Bubalus bubalis)

Monika Saini, Rasika Rajendran, Naresh Selokar, Seema Dua, Nidhi Rawat, Rakesh Sharma, Dharmendra Kumar, Prem Singh Yadav

ICAR-Central Institute for Research on Buffaloes, India.

Keywords*:* buffalo, re-cloning, IVF, and embryos.

Buffalo cloning has gained popularity in India as a valuable tool to make genetically identical copies of superior bulls to mitigate demand of quality semen for breeding schemes. However, it is unknown how the somatic cells of cloned buffalo will behave in re-cloning experiments and fertility of cloned bulls. Also, no data is available about the in vitro fertilization success rate using cloned bull semen. As a step towards answering these apprehensions, we performed re-cloning of the cloned bull that was produced in 2015 by us, and the semen of this cloned bull was used to produce in vitro fertilized embryos. Briefly, the somatic cells of cloned bull and its donor were used for nuclear transfer experiments. Three independent experiment data were used and data were analyzed by Student's t-test using SPSS software (SPSS.com). Following cloning, the blastocyst rate (39.6±1.1 vs 41.2 ±1.2), total cell number (322.0 ±18.2 vs 333.1±28.8) and apoptotic index (3.9±0.5 vs 3.1±0.3) of blastocysts were similar between the donor and cloned bull, respectively. Similarly, there was comparable blastocyst rate (17.5±1.4 vs 16.5±4.8), total cell number (234.8±30.9 vs 202±22.5), and apoptotic index (2.4±0.3 vs 2.5±0.4) of blastocysts produced from IVF procedure using donor and cloned bull semen, respectively. In addition, at the time abstract submission, we established one pregnancy from the transfer of cloned blastocyst of cloned bull and 8 pregnancies following artificial insemination using cloned bull semen. In conclusion, the somatic cells of a cloned bull can be used for re-cloning experiments; whereas, sperms can be used to produce in-vitro fertilized embryos and to impregnate females using artificial insemination.

A192E Embryology, developmental biology and physiology of reproduction

In vivo characterization of pH, CO2 and O2 analytes in the bovine uterus: preliminary study

Jorge X Samaniego[1], Salvador Ruiz[2], Mª Carmen Rizo[1], Ángela Marín[2], Rafael Latorre[1], Joaquín Ortuño[3], Octavio López Albors[1]

[1]Dept. Anatomy and Comparative Pathology. Veterinary Faculty. Campus of Excellence Mare Nostrum. 30100. University of Murcia, Spain; [2]Dept. Physiology. Veterinary Faculty. Campus of Excellence Mare Nostrum. 30100. University of Murcia, Spain; [3]Dept. Analytical Chemistry, Campus of Excellence Mare Nostrum. 30100. University of Murcia.

Keywords*:* uterus, pH, oxygen.

A precise knowledge of the physiological level of certain environmental parameters such as pH, CO_2 and O_2 are of high relevance for in vitro production of embryos (IVP). The uterus is the site for the transport of sperm, early embryonic development and gestation. However, its physiological environment is poorly defined yet, and in cattle only pH values have been reported (Hugentobler et al., Theriogenology 61, 1419, 2004). By new cutting-edge devices and a non-invasive approach, we aimed to define a new method to record *in vivo* data of pH, CO_2 and O_2 in cows during the ovulation day (Ovul) and luteal (5 days after ovulation, Lut) phases. For this, 3 multiparous Holstein females of 6-8 years under the same conditions of feeding and handling were used. The synchronization of ovulation in all the animals was induced through administration of GnRH at day 0 (0.2 mg i.m., Dalmarelin®, Fatro Ibérica, Barcelona, Spain), together with the application of a progesterone-releasing intravaginal device (1.38 g, CIDR®, Zoetis, Madrid, Spain). On day 7 the intravaginal device was removed and PGF2α (25 mg i.m., Dinolytic®, Zoetis, Madrid, Spain) was administered twice (days 7 and 8, 24 hours interval), plus a final injection of GnRH i.m. (day 9) to induce ovulation. Monitorization of the estrous cycle was verified on a daily basis to detect ovulation time and the existence of corpora lutea through a portable ultrasound scanner (ImaGo®, ECM, Angoulême, France) equipped with a linear transducer from 5 to 7.5 MHz. To facilitate the measurement procedure, all the animals were immobilized in a cattle cage and calmed with 0.20 ml/100 kg of xylazine i.m. 2% (Nerfasin®, Fatro Ibérica, Barcelona, Spain), followed by lidocaine 2% (Anesvet®, Laboratorios Ovejero S.A., León, Spain) epidural (5 ml/animal). Miniaturized (0.3 mm diameter) luminescent probes of pH, CO_2 and O_2 (PreSens®, Regensburg, Germany) were inserted in the caudal-middle part of the ipsilateral uterus horn to ovulation through an insemination steel catheter of 70 cm and 6 mm outer diameter. At Ovul y Lut stages instant values of these parameters were taken simultaneously every 5 seconds for a total of 15 minutes in one cycle per animal. Data were then processed for basic statistics (mean±SD). No inference tests were used due to the low number of animals. The continuous records of pH, CO_2 and O_2 showed variable oscillatory patterns over time. Average pH was 7.13±0.11 (6.99-7.23 range) and 6.98±0.04 (6.95-7.0 range) at Ovul and Lut phases, respectively; CO_2 was 4.21±0.74 % (3.19-4.81 % range) and 5.75±1.55 % (3.67-6.27 % range); and O_2 was 4.35±0.56 % (2.89-5.21 %) and 10.98±0.78 % (8.64-12.30 %). These preliminary results showed that the methodology used can provide an effective characterization of the uterine environmentt of cattle with minimal iatrogenesis. Increasing the number of measurements is necessary to better define the oscillatory patterns of each parameter and ascertain potential differences between the stages of the estrous cycle. Finally, this information might be helpful to optimize IVP protocols in cattle. Acknowledgements.
Supported by MINECO-FEDER (AGL 2015-66341-R)

A193E Embryology, developmental biology and physiology of reproduction

Embryo quality in relation to endometrial health in cows with problematic reproductive anamnesis intended for MOET

Ilga Sematovica, Olga Ponomarjova, Vita Antane, Guna Ringa-Karahona, Aida Vanaga, Toms Martinsons, Mara Mangale

Latvia University of Life Sciences and Technologies, Latvia.

Keywords*:* embryo quality, subclinical endometritis, somatic cell count.

The quality of embryos is strongly related to the donor cow's overall and reproductive health, age and management. Poor health of the endometrium is a detrimental factor for embryo development, quality and harvest. Periparturient reproductive problems may predispose dairy cows to subclinical endometritis (SE). The diagnosis of SE is proven if more than 5% of polymorphonuclear leucocytes (PMNL) are present in the cytological sample of endometrium >8 weeks post parturition (pp) and >14% of PMNL 4 weeks pp. An inflamed uterus is a poor environment for the developing embryo. The aim of this study was to investigate embryo harvest in Latvian native breed donor-cows with problematic reproductive anamnesis in relation to endometrial health and somatic cell count (SCC) in the milk. Ten Latvian native breed donor-cows which had a problematic reproductive anamnesis (repeated artificial insemination, difficult parturition, stillbirth or elevated somatic cell count in the milk (SCC) and had no any signs of illness were included in this study. Milk recording data from Agricultural Data Centre of Latvia were used to establish productivity and milk quality. Cytological samples were obtained using a uterobrush (Mekalasi, SAXO, Finland) and blood samples were taken to establish count of white blood cells (WBC) after the embryo flushing procedure (7^{th} day after AI). Diff-Quick stain (Sysmex, Japan) was used to visualize cells (PMNL, epithelial cells, lymphocytes, eosinophils (Eo), monocytes). Cytological samples at 400x magnification using immersion oil were investigated. One hundred somatic cells were counted and the percentage of cells was determined in each sample. Results were analysed in relation to obtained transferable and damaged embryos in healthy cows and cows with SE. Average ± standard deviation (SD) was calculated, two-independent samples t-test (Mann-Whitney U test), two-tailed bivariate correlation was performed using *SPSS 17*. Cows were 6.3±2.71 (average±SD) years old (min. 3, max. 12 years), in 3.5±1.90 lactation (min.1 and max. 6 lactation). Productivity was 18.7±6.16 kg/day, milk fat 4.7±0.84%, milk protein 3.6±0.63%, SCC 821.4± 1505.15 thousand/ml (min. 50.0 and max. 5010.0 thousand/ml). Total embryo harvest from all cows was 8.0±6.67 embryos per cow (min. 0, max.18 embryos per cow); 4.7±5.3 embryos per cow were transferable (min. 0, max. 13 embryos) and 2.1±4.10 embryos per cow (min. 0, max. 13 embryos) were degenerated. Subclinical endometritis was diagnosed in 70% of cows on the embryo flushing day. Healthy cows, in comparison to SE cows, had no significant differences regarding WBC in blood (8.0±5.05 vs 7.1±1.40 $x10^3/mm^3$), age (4.8±2.26 vs 6.9±2.80 years), productivity (19.9±6.8 vs 18.2±6.40 kg/day), milk fat (4.9±1.35 vs 4.6±0.65%) and milk protein (3.6±0.25 vs 3.6±0.75%) ($P>0.05$). Cows with SE, in comparison to healthy cows, had statistically significant lower total embryo count (6.1±6.57 vs 12.3±5.50 embryos), transferable embryo count (2.6±4.76 vs 9.7±2.52 embryos) and higher degenerated embryo count (3.6±4.50 vs 1.3±2.30 embryos) ($P<0.05$). SCC in milk strongly correlated with PMNL in the endometrium ($r=0.99$; $P<0.05$) despite an optimal count of WBC in all cows' blood (7.4±2.80 $x10^3/mm^3$). SCC in cows without SE was 253.3±135.24 thousand/ml, but in cows with SE it was 1064.9±1778.09 thousand/ml ($P>0.05$). The count of degenerated embryos correlated with Eo in endometrium ($r=0.97$; $P<0.05$). In conclusion, a cytological investigation of the endometrium should be performed before cows with problematic reproductive anamnesis are considered for use as embryo donors. Studies must be continued to establish at what level increased SCC in milk affects the quality of the embryos in donor cows, because it may be an early marker for successful MOET.

Acknowledgements. Financial support was provided by ERAF project Nr.1.1.1.1/16/A/025

A194E Embryology, developmental biology and physiology of reproduction

SNP based Preimplantation Genetic Testing for Aneuploidy (PGT-A) to improve pregnancy outcomes in cattle IVP: a blind retrospective study

María Serrano-Albal[1], Giuseppe Silvestri[1], Lucy M Vining[2], Remi Labrecque[3], Gabriele Marras[3], Steven Larmer[3], David H Black[4], Patrick Blondin[3], Alan H Handyside[1], Kevin D Sinclair[5], Darren K Griffin[1]

[1]School of Biosciences, University of Kent, Canterbury, Kent, UK; [2]School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, Kent, UK; [3]Boviteq, Saint-Hyacinthe, Quebec, CANADA; [4]Activf-ET, Dalston, Carlisle, UK; [5]School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire, UK.

Keywords*:* bovine, pregnancy rates, blastocyst.

Currently, the ability to produce Genomic Estimated Breeding Values (GEBVs) from Single Nucleotide Polymorphism (SNP) data acquired from live animals plays a key role in guiding the selection process operated by the cattle breeding industry. Increasingly, this technology is being applied to *in vitro* produced (IVP) embryos as a way to increase genetic gain rates and avoid the birth of unwanted animals. However, a significant proportion of the recipients of a SNP typed embryo will not become pregnant, resulting in a waste of time and resources for breeders. Aneuploidy is the most common cause of early developmental arrest and implantation failure in IVP embryos. Thus, supplementing GEBVs with the use of preimplantation genetic testing for aneuploidy (PGT-A) may ensure the selection of embryos with desirable traits which stand a high chance of returning a pregnancy. Here we employed a new PGT-A algorithm (Handyside *et al*. 2010, J Med Genet, 47:651-8) to obtain ploidy diagnoses from the same SNP data used to establish GEBVs. Heterozygous loci in one parent that are homozygous in the other were used as markers to trace chromosome inheritance across generations. The analysis of haploblock patterns in the chosen embryo was made possible by comparing embryonic and parental SNP information with data acquired from a full sibling (either another embryo or a live-borne). To test the hypothesis that the selection of euploid embryos by our PGT-A algorithm would benefit pregnancy rates, we performed a blind retrospective study analysing the SNP and pregnancy data provided by two commercial cattle breeders: Boviteq (Saint-Hyacinthe, Canada) and Activf-ET (Carlisle, UK). The analysis of 66 embryos revealed that 18.2% of them were aneuploid. When individually transferred, 75.0% of the aneuploid embryos did not result in pregnancy, compared to a rate of just 46.3% for euploid embryos (chi square, P=0.05). One of the pregnancies from aneuploid embryos (n=3) resulted in a miscarriage, effectively increasing aneuploid embryo failure rate to 83.3%. If only euploid embryos had been transferred in this cohort, the average pregnancy rate would have increased from 48.5% to 53.7%. When an embryo transfer resulted in a pregnancy, our PGT-A algorithm identified an euploid embryo in 90.6% of cases; conversely, when there was no pregnancy, aneuploidy was identified in 26.5% of cases. In conclusion, when embryos are euploid, our PGT-A algorithm cannot reliably predict pregnancy outcomes as ploidy is just one aspect of a complex system. Nevertheless, our preliminary study seems to suggest that euploid embryos are more developmentally competent and their elective transfer might offer better value for money to breeders. However, our statistical analysis did not provide a robust answer in this pilot study, and the test of a larger sample is likely necessary to achieve clear statistical significance. In our future work, we will analyse a much larger sample database for a more in-depth analysis. We will then investigate whether certain chromosomal abnormalities are more often associated with reductions in pregnancy rates, and which, if any, can be tolerated by the embryo.

A195E Embryology, developmental biology and physiology of reproduction

Long-term antiapoptotic action of progesterone on bovine oocytes during the second phase of IVM is not mediated through the Bax/Bcl2 pathway

Ekaterina Shedova, Galina Singina, Aleksandr Lopukhov, Irina Lebedeva

L.K. Ernst Federal Science Center for Animal Husbandry,Podolsk, Russian Federation.

Keywords*:* progesterone, oocyte apoptosis, Bax/Bcl2 pathway.

Endogenous progesterone (P4) secreted by cumulus cells exerts an antiapoptotic effect on bovine oocytes during IVM through cumulus-expressed proteins (O'Shea et al., Biol Reprod, 89:146, 2013). However, the action of exogenous P4 on the oocytes is not so obvious and may be dependent on the stage of the meiotic maturation. Therefore, the present work was aimed to study: (1) a pattern and duration of effects of P4 and luteotropic hormone prolactin (PRL) on bovine oocyte apoptosis during the second phase of IVM (from M-I to M-II) and (2) a role of the Bax/Bcl2 pathway in these effects. In one-step IVM, bovine cumulus-oocyte complexes (COCs) were matured for 24 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH (1st IVM medium). In two-step IVM, COCs were cultured in the 1st IVM medium for 12 h and then transferred to TCM 199 containing 10% FCS (2nd IVM medium) and cultured for next 12 h. The 2nd IVM medium was either free of additives (Control) or supplemented with 50 ng/ml P4 or 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia). After one-step and two-step IVM, a half of COCs was cultured for additional 24 h in TCM 199 containing 10% FCS to test long-term hormonal effects during aging. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). At the end of culture, oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI. The expression of apoptosis-related genes (*Bax* and *Bcl2*) in oocytes was analyzed by qPCR following RNA isolation by Trizol method and reverse transcription to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). Levels of the gene expression were normalized by the reference gene *GAPDH*. The data for apoptosis (n=5-6, 87-113 oocytes per treatment) and gene expression (n=3, 30 oocytes per each replicate for each treatment) were analyzed by ANOVA. The rate of M-II oocytes was similar in all the compared groups (83.3-94.3%). Following 24 and 48 h of culture, the apoptosis frequency and the expression of *Bax* and *Bcl2* genes in oocytes did not differ between one-step IVM and the control group of two-step IVM. During 24 h of oocyte aging, the apoptosis frequency increased 2-3 times ($P<0.001$) in all the groups, whereas the relative levels of the transcripts changed only slightly. The addition of P4 (but not PRL) to the 2nd IVM medium resulted in the reduction ($P<0.05$) in the rate of apoptotic oocytes from 11.7±1.2 to 5.9±1.7% after 24 h of maturation. Furthermore, this rate in the P4-treated group was lower than in one-step IVM (17.6±1.6 vs. 24.3±0.4%, $P<0.05$) after 24 h of aging. Meanwhile, P4 did not affect the expression of *Bax* and *Bcl2* genes in matured or aged oocytes. Thus, during the second phase of IVM, exogenous P4 can exert the long-term antiapoptotic effect on bovine oocytes that is not related to modulation of the Bax/Bcl2 pathway. The role of the AVEN-associated pathway is currently under consideration.

A196E Embryology, developmental biology and physiology of reproduction

Bovine oocyte quality when cultured in one-step and different two-step IVM systems

Galina Singina, Ekaterina Shedova, Irina Lebedeva, Evgeniya Tsyndrina

L.K. Ernst Federal Science Center for Animal Husbandry, Podolsk, Russian Federation.

Keywords*:* two-step IVM system, MII chromosomes, Bax/Bcl2 pathway of oocyte apoptosis.

In vitro matured oocytes are widely used for commercial and research purposes. The quality of oocytes acquired during in vitro maturation is the main limitative factor affecting their capacity for further development. The aim of the present research was to study effects of different conditions of IVM on the state of M-II chromosomes and apoptosis of bovine oocytes. Cumulus-enclosed oocytes (CEOs) were matured in either one-step or two-step IVM systems. In the case of the one-step protocol, CEOs were cultured for 24 h in TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml porcine FSH, and 10 μg/ml ovine LH (standard medium) at 38.5°C and 5% CO2. In the case of the two-step procedure, CEOs were first cultured for 16 h in the standard medium and then transferred to one of three experimental media and cultured for additional 8 h. The following media for the two-step IVM system were tested: (1) TCM 199 containing 10% FCS (Group 1), (2) TCM 199 containing 3 mg/ml BSA (Group 2), or (3) Fert-TALP medium supplemented with 6 mg/ml BSA (Group 3). Fert-TALP with traditional for IVF concentration of BSA was selected because it can potentially be used throughout maturation and fertilization. At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method (N=251). Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI (N=212). The expression of involved in apoptosis genes *Bax* and *Bcl-2* in oocytes was analyzed by real-time RT-PCR (N=332). The data (4 replicates, 69-114 oocytes per treatment) were analysed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rate of M-II oocytes was similar in all groups and reached 84.3-87.7%. No effects of the systems on the frequency of M-II chromosome abnormalities (decondensation, adherence, clumping) were observed, with the frequency after culture was: 29.5±3.0 (one-step IVM), 36.5±3.1 (Group 1), 31.7±2.7 (Group 2) and 33.6±1.9% (Group 3). In the one-step system, the rate of matured oocytes with apoptotic signs was 15.1±2.0%. Transfer of CEOs after 16 h of incubation in the standard medium to TCM 199 containing BSA (Group 2) caused a decrease in the rate of oocyte apoptosis to 6.9±1.3% ($p<0.05$). Moreover, the rate of apoptotic oocytes in Group 2 was lower than in Group 1 (19.4 ± 1.1%, $p<0.01$) and in Group 3 (14.5 ± 2.7%, $p<0.05$). The expression level of pro-apoptotic gene *Bax* after oocyte maturation did not differ between groups or systems. Meanwhile, oocyte culture in Group 2 (but not in Group 1 and 3) led to an increase in the transcript abundance for anti-apoptotic gene *Bcl-2* and a decrease in the ratio of *Bax* and *Bcl-2* transcript levels as compared to the one-step system ($p<0.05$). Our data indicate that bovine oocyte culture in the two-step IVM system including oocyte transfer to TCM 199 containing BSA can increase the apoptosis resistance of the oocytes by enhancing expression of anti-apoptotic gene *Bcl-2* and may be used as an alternative for the standard one-step IVM.

The study was supported by RFBR (No. 17-29-08035).

A197E Embryology, developmental biology and physiology of reproduction

Effect of different culture conditions on bovine embryos derived from metabolically compromised oocytes

Anouk Smits, Waleed F.A. Marei, Jessie De Bie, Peter E.J. Bols, Jo L.M.R. Leroy

Gamete Research Centre, University of Antwerp, Wilrijk, Belgium.

Keywords*:* FFA-exposed oocytes, in vitro culture conditions, supplementation.

Metabolic disorders e.g. obesity lead to elevated saturated (stearic; SA, palmitic; PA) and mono-unsaturated (oleic; OA) free fatty acids (FFAs) in serum and follicular fluid. Exposure of maturing oocytes to these FFAs, particularly to PA, hampers embryo development. Supplementation of embryo culture media with Insulin-Transferrin-Selenium (ITS) or serum is used to enhance embryo production; however the effect of such enrichment on development of metabolically compromised oocytes has not been investigated. Here, bovine oocytes (n=3737) were exposed to either 1) pathophysiological high PA, SA and OA concentrations (150, 75, 200 µM, respectively; **HFA**); or 2) high PA, basal SA and basal OA (150, 28 and 21 µM; **HPA**); compared to 3) basal PA, SA and OA (23, 28, 21 µM; **BASAL**) as a physiological control. Zygotes were cultured in SOF medium containing 1) BSA (2%) only or supplemented with 2) ITS (10 µg/mL insulin; 5,5 µg/mL transferrin and 6,7 ng/mL selenium) or with 3) serum (5%). Cleavage (48h) and blastocyst rates (day 7 (D7) and D8 post insemination) were recorded. D8 blastocysts were analyzed for apoptotic cell indexes (ACI) (caspase-3 immunostaining), embryo metabolism (glucose consumption and lactate production), or mRNA expression of genes involved in ER unfolded protein responses (UPR^{er}) (*Atf4, Atf6*), oxidative stress (*SOD2, GPx, CAT*) mitochondrial UPR (*HSP10, HSP60*) and mitochondrial biogenesis (*TFAM*). Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni corrected. Cleavage rate was significantly (P<0.05) reduced in HPA embryos compared with BASAL when cultured in BSA. However, ITS or Serum in culture alleviated this negative effect. Compared with BASAL, HPA exposed oocytes showed significant lower D7 and D8 blastocyst rates after culture in BSA and Serum, but not in ITS containing SOF medium. Within the PA-treated group, ITS significantly increased D7 and D8 blastocyst rates compared with BSA. HFA did not have significant effects on development under all IVC conditions. For embryo quality, ACI was not different among BASAL, HFA and HPA groups in BSA culture. Surprisingly, supplementation of ITS during IVC significantly increased ACI of HPA and HFA embryos compared to BASAL (P<0.05). Serum supplementation also increased ACI of HPA embryos compared with HFA and BASAL (P<0.05). Regardless of IVM treatment, embryos cultured in Serum showed increased lactate/2glucose ratio compared with BSA and ITS, confirming the reported preference for Warburg metabolism. In contrast, HPA-derived embryos cultured in ITS or Serum had significantly lower lactate/2glucose ratio compared to BASAL and HFA. At the blastocyst transcriptomic level, HPA increased *HSP60* expression compared to BASAL when cultured in BSA, indicating activation of mitochondrial stress responses. ITS and Serum alleviated this increase in *HSP60.* In conclusion, enrichment of embryo culture media with ITS or serum can improve developmental competence of oocytes after maturation in lipotoxic conditions. However, the surviving blastocysts exhibit higher apoptosis and altered metabolism indicating inferior quality.

A198E Embryology, developmental biology and physiology of reproduction

Relationship between nitric oxide in follicular fluid and ovarian response among oocyte donors

Florentin-Daniel Staicu[1,2], Analuce Canha-Gouveia[1,2], Juan Carlos Martinez-Soto[2,3], Jorge Chavarro[4], Carmen Matas[1,2]

[1]Department of Physiology, Veterinary Faculty, University of Murcia, International Excellence Campus for Higher Education and Research (Campus Mare Nostrum), Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB), Murcia, Spain; [3]IVI-RMA Global, Murcia, Spain; [4]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, USA; Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, USA.

Keywords*:* nitric oxide, follicular fluid, oocyte.

The identification of a family of enzymes catalyzing the synthesis of nitric oxide (NO) in the oviduct, oocytes and cumulus cells of several species [Rosselli M, Mol Hum Reprod. 2(8):607-12, 1996; Lapointe J, Endocrinology. 147(12):5600-10, 2006; Tao Y, Mol Cell Endocrinol. 222(1-2):93-103, 2004], suggested that NO is a key component of the oocyte microenvironment [Romero-Aguirregomezcorta J, PLoS One. 9(12):e115044, 2014]. Interestingly, it has been reported that NO modulates the granulosa cell function, follicular maturation and ovulation (Yalçınkaya E, J Turk Ger Gynecol Assoc. 14(3):136-41, 2013). The present work aimed to determine if the NO levels in human follicular fluid (FF) correlate with the number of total and MII oocytes retrieved from donors. Seventy-two women participating in the oocyte donation program at IVI-RMA Global Murcia (Spain) took part in this study. FF was obtained at oocyte retrieval in 93 donation cycles to measure the levels of stable-end products of NO oxidation, nitrite (NO_2) and nitrate (NO_3). For each donor, demographic, lifestyle and donation cycle-related data were also recorded. NO_2 and NO_3 were determined by HPLC-UV/VIS. Multivariate mixed Poisson and logistic regression models with random slopes to account for repeated observations within woman were used to compare total and MII oocyte yields for women across tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio while adjusting for age, body mass index, hours of sleep, coffee intake, smoking and physical activity. NO_2 levels ranged from 0.7 to 96.1 µM, NO_3 levels ranged from 4.9 to 39.7 µM, total NO levels ranged from 5.6 to 109.5 µM and NO_3/NO_2 ratio ranged from 0.1 to 31.5. NO_2 and NO_3 concentrations were unrelated to each other (r=-0.01). FF NO_2, NO_3, total NO or NO_3/NO_2 ratio were unrelated to total or mature oocyte yield. The multivariable-adjusted MII yield (95% CI) for women in the lowest and highest tertiles of NO_2 was 12.4 (10.2, 15.1) and 13.2 (10.9, 16.0) (p=0.53); 14.1 (11.7, 17.1) and 12.2 (9.9, 15.0) for NO_3 (p=0.21); 13.7 (11.4, 16.5) and 12.2 (10.1, 14.6) for total NO (p=0.26); and 14.1 (11.7, 16.9) and 12.2 (10.1, 14.8) for the NO_3/NO_2 ratio (p=0.15). When MII oocytes were considered as the proportion of total oocytes, however, the proportion of MII oocytes increased with increasing FF NO_2 levels but decreased with increasing NO_3 levels. The adjusted proportion (95%CI) of MII oocytes for women in the lowest and highest FF levels of NO_2 were 68% (58-77%) and 79% (70-85%) (p, linear trend=0.02); whereas the proportion of MII oocytes for women in extreme tertiles of FF NO_3 levels were 79% (70-85%) and 68% (57-77%) (p, linear trend=0.03). In conclusion, NO and its metabolites did not predict the number of mature oocytes retrieved from donors, but NO_2 and NO_3 correlated with the MII proportion. The fertilization rate, embryo quality and pregnancy rates should be analyzed in patients who received these oocytes to determine any correlations with NO levels in FF.
Supported by H2020 MSC-ITN-EJD 675526 REP-BIOTECH and MINECO-FEDER (AGL 2015-66341-R).

A199E Embryology, developmental biology and physiology of reproduction

Effect of month of birth on the development of Belgian Blue calves

Habib Syaiful Arif Tuska, Gretania Residiwati, Karel Verdru, Ann Van Soom, Geert Opsomer

Ghent University, Belgium.

Keywords*:* month of birth, Belgian Blue calves, organs.

Intra-uterine growth is important in beef cattle since it determines weight and morphometrics of neonatal calves and hence contributes to ease of calving. Earlier, we have shown in dairy cattle that environmental circumstances like for example season of birth and parity of the mother significantly influence birth weight of calves. The present study aimed to evaluate the effect of month of birth on the intra-uterine development of Belgian Blue calves based on the measurement of 10 body parts shortly after birth: body weight (BW), withers height (WH), oblique length (OL), heart girth (HG), width of the back (WB), shoulders width (SW), circumference of the head (CH), diameter of the head (DH), length of the metatarsus (LM), and length of the underarm at the front leg (LA). Furthermore, we also investigated the effect of month of birth within the sex of calves. The data include 73 records of calves born in the Clinic of Reproduction and Obstetrics at the Faculty of Veterinary Medicine, Ghent University (Belgium), collected between 2016 and 2017. The results show that the average BW was 52.46 kg, WH 70.08 cm, OL 64.78 cm, HG 80.08 cm, WB 25.95 cm, SW 24.45 cm, CH 50.49 cm, DH 13.39 cm, LM 30.57 cm, and LA 25.28 cm. There was a significant association between the month of birth and the length of the metatarsus as well as the length of the underarm at the front leg. In comparison to calves born in winter, calves that were born in autumn had both a longer metatarsus (31.13 versus 29.82 cm; $P<0.05$) and a longer underarm at the front leg (26.13 versus 24.14 cm; $P<0.05$). Furthermore, the data showed an effect of the gender of the calves, as the male calves had a significantly ($P= 0.026$) bigger HG (81.69 cm) than their female counterparts (78.67 cm). Based on these results, it appears that the season of birth and the gender of the calves both have a significant effect on some of the neonatal morphometrics in Belgian Blue calves, which is important to know in terms of calving ease.

A200E Embryology, developmental biology and physiology of reproduction

Dynamic transcriptome changes during embryonic diapause and reactivation in the embryo and endometrial epithelium of the European roe deer

Vera Anna van der Weijden[1], Jochen T. Bick[1], Stefan Bauersachs[2], Frank Goeritz[3], Katarina Jewgenow[3], Thomas Hildebrandt[3], Barbara Drews[1], Susanne E. Ulbrich[1]

[1]ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Switzerland; [2]Genetics and Functional Genomics, Vetsuisse Faculty Zurich, University of Zurich, Switzerland; [3]Leibniz Institute for Zoo- and Wildlife Research, IZW, Berlin, Germany.

Keywords*:* diapause, embryo, endometrium.

The European roe deer pre-implantation embryo development is characterized by a four-month period of embryonic diapause, after which the embryo rapidly elongates and implants. Pre-elongation developmental pace is 10 times slower than in cattle. In roe deer, endometrial secretions at implantation are 1.5-fold higher than during diapause, and morphological changes of the embryo coincide with changes of the uterine fluid composition. To identify the reactivation initiating mechanism, we investigated the embryonic and endometrial transcriptome changes during diapause and following reactivation. Samples were collected at regular huntings between September and January 2015-2017. A total of 360 animals was sampled and 537 pre-implantation embryos were collected (77% recovery rate). A group of six day 14 *ex vivo* flushed embryos from captive roe deer represent the early blastocysts. Embryonic DNA was extracted for DNA content determination and cell number estimation. Endometrial luminal epithelial was collected by laser-capture micro-dissection. Total embryonic RNA from 87 embryos and total endometrial RNA from 56 different females was subjected to RNA-sequencing. Raw sequence reads were analyzed using a customized Galaxy pipeline. A pseudotime analysis (CellTree) was performed to gain insight into the transcriptome dynamics of diapausing embryos, in which the number of embryonic cells was used as proxy for developmental progression. Differentially expressed transcripts (DET) were identified in a time-course dependent manner with the ImpulseDE2 algorithm with an FDR <1%. To elucidate dynamic gene expression changes, a self-organizing tree algorithm (SOTA) was used. Gene set enrichment analysis and gene ontology were used to identify enriched hallmarks between diapause and elongation. As determined by a rise in DNA content, embryonic cells divide every two weeks during diapause. With developmental progression, an overall increase in the number of embryonically expressed transcripts was observed. The pseudotime analysis of both embryos and luminal epithelium showed grouping of the early blastocysts on one end of the trajectory, the elongated embryos on the other end with diapausing embryos dispersed heterogeneously in between. Embryonic time-course analysis revealed 13,193 DET out of 29,575 transcripts. The DET grouped into 7 SOTA clusters. Gene set enrichment analysis and gene ontology revealed an enrichment of MYC targets, MTORC1 signaling, PI3K, AKT and MTOR signaling, unfolded protein response, peroxisome and the glycolytic pathway in elongated embryos. In the luminal epithelial cells, changes were less dynamic; 2,754 DET grouped into 2 clusters and lacked any enriched biological pathway. Taken together, roe deer embryos divide at a slow pace and are transcriptionally active during diapause. Enriched pathways indicate cell proliferation following reactivation. Targeted transcript analyses will emphasize on the identification of diapause-related regulatory pathways and aim at identifying conserved mechanisms of cell cycle control.

A201E Embryology, developmental biology and physiology of reproduction

Effect of the zona pellucida removal on the developmental competence of domestic cat embryos generated by *in vitro* fertilization

Daniel Veraguas, Soledad Saez, Constanza Aguilera, Darling Seaz-Ruiz, Fidel Ovidio Castro, Lleretny Rodriguez-Alvarez

Laboratorio de Biotecnología Animal, Departamento de Ciencia animal, Universidad de Concepción, Chillán.

Keywords*:* felids, in vitro embryo production, zona pellucida.

The domestic cat is a valuable model for the development of assisted reproductive techniques that might be used in the conservation of endangered felids. However, the efficiency of the *in vitro* embryo production in the domestic cat remains low compared to other species. In the bovine and equine, the zona pellucida removal enhance the developmental competence of the embryos generated *in vitro*, allowing the birth of live offspring (Gambini *et al*., 2012; Rodriguez *et al*., 2008). The objective of this research was to evaluate the effects of zona pellucida removal in domestic cat embryos generated by in vitro fertilization (IVF). To achieve this purpose, two experimental groups were made, 1) domestic cat embryos generated by IVF and *in vitro* cultured (Zona-included), 2) domestic cat embryos generated by IVF and cultured without zona pellucida (Zona-Free). To evaluate the effect of the zona removal, the developmental capacity and morphological quality of the embryos generated in the Zona-free group were compared against the Zona-included group. For this, the ovaries of domestic cats were collected by ovariohysterectomy and the cumulus-oocyte complexes (COCs) were recovered by slicing. The COCs were *in vitro* matured in supplemented TCM-199 Earle's salts medium for 26-28 hours, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC. The IVF was realized using epididymal refrigerated sperm. 1.5 – 2.5 x 10^6 spermatozoa /mL were incubated with 20-30 COCs in supplemented TALP medium for 18 hours, in a 5% CO_2 atmosphere, at 38.5ºC. After cumulus cell removal, the zona pellucida of the presumed zygotes was removed by 2-4 minutes incubation in 2 mg/mL of pronase (Sigma-Aldrich, P8811, USA). The presumed zygotes were cultured using the well of the well system (Vajta *et al*., 2000) in supplemented SOF medium, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC, for 8 days. The cleavage, morulae and blastocysts rates were estimated at day 5 and 8. The Zona-included and Zona-free groups were performed at different times, with nine and six replicates respectively. The diameter and total cell number of the blastocysts were evaluated. The Wilcoxon non-parametric test was used to analyze the developmental competence and the t-test was used to analyze the diameter and total cell number. Regarding to the results, no statistical differences were observed between the Zona-included and Zona-free groups in the cleavage rate: 155/239 (64.9%) and 116/177 (65.5%), morulae rate: 115/155 (74.2%) and 68/116 (58.6%), and blastocysts rate: 51/155 (32.9%) and 36/116 (31.0), respectively ($P > 0.05$). No differences were observed in the total cell number (mean ± SD) of the blastocysts generated in the Zona-included (279.9 ± 148.1) and Zona-free group (313.1 ± 164.9) ($P > 0.05$). Finally, the diameter (mean ± SD) of the blastocysts from the Zona-free group (253.4 ± 83.3 μm) was significantly higher than the diameter of the blastocysts from the Zona-included group (210.5 ± 78.5 μm). In conclusion, the zona pellucida removal did not affect negatively the morphological quality and developmental competence of domestic cat embryos in our cultured conditions. However, more studies are needed to evaluate the *in vivo* competence of these embryos.

A202E Embryology, developmental biology and physiology of reproduction

Effects of serum and serum substitutes on *in vitro* maturation (IVM) and embryo development of porcine oocytes

Lucy M. Vining[1], Giuseppe Silvestri[2], María Serrano-Albal[2], Lucas G. Kiazim[2], Louisa J. Zak[3], Grant A. Walling[4], Egbert F. Knol[3], Simon C. Harvey[1], Darren K. Griffin[2], Katie E. Fowler[1]

[1]School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, Kent, United Kingdom; [2]School of Biosciences, University of Kent, Canterbury, Kent, United Kingdom; [3]Topigs Norsvin Research Center, Beuningen, The Netherlands; [4]JSR Genetics, Southburn, Driffield, East Yorkshire, United Kingdom.

Keywords*:* Embryo development, blastocyst, pig.

Porcine *in vitro* embryo production (IVP) protocols have traditionally relied on the use of follicular fluid and serum, which results in undefined media with undetermined levels of growth (and other) factors. Media composition can alter the efficiency of embryo development, and more importantly gives rise to a potential biohazard. Moreover, the use of serum in IVP has been linked to alterations in embryo transcriptional activity (Oliveira *et al.* 2006, Reprod Domest Anim. 41:129-36). Hence, our aim was to establish whether a serum substitute (formed by a combination of cytokines currently under NDA) could be used efficiently in pig IVP. Here, we compared the use of this serum substitute during *in vitro* maturation (IVM) vs. sow follicular fluid (sFF) as stand alone treatments or in combination, and followed the development of the resulting zygotes. Oocytes collected from abattoir-derived ovaries were matured for 44 h in supplemented or non-supplemented Porcine Oocyte Medium (POM). There were four treatment groups: 1) Non-supplemented (control), 2), 10% sFF, 3) serum substitute, or 4) combination of 10% sFF + serum substitute (n=100 oocytes/group). Fertilisation of matured oocytes was carried out using extended boar semen (JSR, Driffield, UK). *In vitro* culture (IVC) of zygotes across treatment groups remained consistent, and occurred in defined Porcine Zygote Medium 5 (PZM5) supplemented with our serum substitute and with partial media changes at 48 h and 96 h post-IVF. Cleavage and blastocyst rates were assessed at 48 h and 144 h post-IVF, respectively. Differences between the groups were analysed using a comparative General Linear Model followed by Tukey's *post-hoc* test. Cleavage rates, as compared to the control group (36.7%), were significantly higher in both serum substitute (57.4%, p=0.02) and combination groups (70.4%, $p=6x10^{-6}$). There was no significant effect of using 10% sFF compared to controls (50.4%, p=0.25). Interestingly, there was no significant difference in the proportion of blastocysts per cleaved embryo between the control (30.3%), 10% sFF (26.8%), serum substitute (26.8%) and combination groups (30.9%)(F_3=1.85, p=0.14). The use of serum in combination with cytokines might have resulted in a higher proportion of growth factors, providing a possible explanation to the combinatory effect here described for cleavage rates. Moreover, the fact that cleavage but not blastocyst rates differed between these groups advocates that the effects of the supplementation in IVM did not extend to IVC. Certain IVM treatment groups produced a higher proportion of developmentally competent oocytes, as highlighted by the difference in cleavage rates. At the same time, the use of a standardised IVC protocol following IVM might have had a role in ensuring consistent development to the blastocyst stage following successful fertilisation. To further characterise this observation, future experiments will assess the effects of supplementation with sFF and serum substitutes on IVC, while IVM will be kept consistent. While our serum substitute appeared suitable for IVM and IVC, the combinatory effect observed suggests that the development of a more complex and efficient serum substitute should be possible.

A203E Embryology, developmental biology and physiology of reproduction

Using a time-lapse system to study the morphokinetics of blastocysts derived from heat-shocked oocytes

Shira Yaacobi-Artzi, Dorit Kalo, Zvi Roth

Department of Animal Sciences, Robert H.Smith Faculty of Agriculture, Food and Environment,The Hebrew University of Jerusalem, Rehovot.

Keywords*:* heat shock, embryo morphokinetics, time-lapse system.

Embryonic development involves multiple dynamic events that are remarkably sensitive to environmental changes. We used a non-invasive time-lapse system (TLS) to continuously monitor the kinetics of embryonic development. The objectives were to: (1) compare embryonic development in a conventional incubator and in an incubator equipped with a TLS; (2) characterize the precise cleavage time and duration (i.e., morphokinetics) of individual in-vitro-derived embryos; (3) examine whether exposing oocytes during maturation to heat shock affects the morphokinetics of the developing embryo. Ovaries were collected from a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated, matured (22 h) and fertilized (18 h) in a conventional incubator (humidified air, 5% CO_2 at 38.5°C). Statistical analysis was performed using JMP-13 software. In the first experiment, putative zygotes were cultured in a conventional incubator (n = 192, control) or an incubator equipped with a TLS (n = 126, TLS) for 8 days. The proportion of oocytes that cleaved to the 4-cell stage (74.3±7 vs 89.2±2.3%, respectively) and that of blastocysts (20.6±6.9 vs 25.9±5.8%, respectively) did not significantly differ between control and TLS groups. In the second experiment, the morphokinetics of embryos (n = 427) cultured in the TLS-equipped incubator were individually recorded. Findings revealed that the median of cleavage into 2-cells stage was 27.5 h post fertilization (pf): from 2- to 4-cell stage was 37.5 h pf, from 4- to 8-cell stage was 50.5 h pf and to the blastocyst stage was 127.5 h pf. In the third experiment, COCs (n = 421) were matured for 22 h in an incubator under normothermic conditions (5% CO_2, 38.5°C, control) or exposed to heat shock (6% CO_2, 41.5°C, HS) in a conventional incubator. Following fertilization (18 h), embryos were cultured for 7 days under normothermic conditions in the TLS-equipped incubator and embryo morphokinetics was recorded. In the control group, embryo cleavage was characterized by two waves of divisions; the first between 22 and 28 h pf and the second between 28 and 36 h pf. In the HS group, cleavage was characterized by one wave of divisions that occurred between 28 to 36 h pf. The median of the cleavage into 2-cell stage was 31.5h pf and 27.5h pf for HS- and control groups, indicating a delay in cleavage.Blastocysts from both control and HS groups were collected for real-time PCR assay to evaluate the expression of selected transcripts (*OCT4*, *NANOG*, *SOX2*, *DNMT1*, *PTGS2*, *GDF9*, *STAT3*). In summary, use of a TLS adds to our understanding of the mechanisms by which heat stress can impair oocyte developmental competence. We documented the precise morphokinetics of bovine embryo development from oocytes matured under normothermic or heat-shock conditions during maturation. These were associated with reduced developmental competence in the HS group. PCR findings will enable a comparison of gene expression in blastocysts developed from control and heat-shocked oocytes.

A209E Cloning, transgenesis, and stem cells

RS-1 increases CRISPR-mediated Knock-in rate in bovine embryos

Ismael Lamas-Toranzo[1], Álvaro Martínez-Moro[1,2], Elena O´Callaghan[3], José María Sánchez[3], Gema Millan[1], Pat Lonergan[3], Pablo Bermejo-Alvarez[1]

[1]1Animal Reproduction Department, INIA, Madrid, Spain; [2]Procreatec, Madrid, Spain; [3]School of Agriculture and Food science, University College Dublin, Belfield, Dublin 4, Ireland.

Keywords: CRISPR, bovine, homologous recombination.

The insertion of genomic sequences at specific loci (targeted Knock-in, KI) has been challenging due to the low efficiency of homologous recombination (HR). This efficiency has been boosted by the use of endonucleases, such as CRISPR, that generate a double-strand break (DSB) at the target locus. However, CRISPR-generated DSB can be repaired by one of two mechanisms: 1) HR, which can lead to the intended targeted KI if a donor DNA is provided or 2) non-homologous end Joining (NHEJ), which generates random mutations. The objective this study has been to test the effect of an enhancer of HR pathway, RS-1, on the KI rates following CRISPR injection in bovine zygotes. A preliminary study (3 replicates) was conducted to evaluate the highest concentration of RS-1 compatible with normal percentages of developmental. Bovine zygotes were incubated in SOF supplemented with 0, 7.5 or 15 µM RS-1 for 24 h and subsequently cultured in the absence of RS-1 for 8 days. RS-1 at 15 µM significantly reduced embryonic cleavage and blastocyst development, whereas 7.5 µM resulted in similar percentages of development to the control group (cleavage: 71.8±3.0; 83.6±1.8; 84.0±3.4 %; blastocysts 16.9±3; 30.3±3; 37.8±6.5 %, for 15, 7.5 and 0 µM groups, respectively; mean±s.e.m. logistic regression and ANOVA $P<0.05$). In a second experiment, zygotes where injected with CRISPR components (300 ng/µl Cas9 mRNA and 100 ng/µl sgRNA) and a single-stranded donor DNA (100 ng/µl) to mediate the insertion of an XbaI restriction site on a target non-coding region. Following microinjection, zygotes were transiently incubated for 24 h in SOF containing no RS-1 or RS-1 at 3.75 µM or 7.5 µM. As expected, the three groups displayed similar percentages of embryonic cleavage (77.8±3.3; 78.8±2.5; 73.7±4.0 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates) and development to blastocyst (25.8±1.7; 27.0±2.0; 23.2±1.8 %, for 0, 3.75 and 7.5 µM groups, respectively; mean±s.e.m., 5 replicates). Resulting blastocysts were genotyped to detect genome edition (Sanger sequencing of PCR product) and targeted KI (XbaI digestion of PCR products). A significantly higher incidence of targeted insertion was achieved in embryos exposed to 7.5 µM RS-1 following microinjection compared with other groups (53.1% -17/32- for 7.5 µM vs. 26.5% -9/34- and -9/39- 23.1 % for 0 and 3.75 µM, respectively; Fisher's exact test $P < 0.05$). In conclusion, transient exposure of bovine embryos to 7.5 µM RS-1 following CRISPR microinjection enhances targeted insertion of genomic sequences, highlighting its potential for animal research.

This study is supported by projects AGL2017-84908-R from the Spanish Ministry of Economy and Competitiveness, 757886-ELONGAN from the European Research Council, and 13/IA/1983 from Science Foundation Ireland. ILT is supported by a FPI contract and AMM by the project IND2017/BIO from Madrid region government.

A210E Cloning, transgenesis, and stem cells

Vital tagging of potential naïve pluripotency in a mouse model

Iqbal Hyder[1], Naresh L Selokar[1,3], Wiebke Garrels[2], Sabine Klein[1], Wilfried A. Kues[1]

[1]Friedrich Loeffler Institut - Institute of Farm Animal Genetics, Neustadt, Germany; [2]Institute of Laboratory Animal Science, Medical School Hannover, Germany; [3]Central Institute for Research on Buffaloes, ICAR, Hisar, India.

Keywords: pluripotency, naive cells, endogenous retrovirus.

Recent studies suggested that two distinct pluripotent states exist in mouse and human embryonic stem cells that are termed as naïve and primed. The naïve pluripotency constitutes a ground state with full differentiation potential, whereas the primed state has a restricted differentiation potential (Boroviak et al. Nat. Cell Biol. 16, 516–528). The preimplantation epiblast represents a naïve pluripotent state, whereas the post-implantation epiblast reaches a primed state. Recently, it was shown that the expression of particular classes of endogenous retrovirus (ERV) mirror the naïve state in murine and human pluripotent cells (Wang et al. Nat Protoc. 11(2):327-46, 2016). We hypothesized that the naïve pluripotency could be tagged in vivo by a fluorescent reporter construct driven by the long terminal repeat (LTR) promoter of the human ERV7. To achieve this, the LTR7-GFP construct in a Sleeping Beauty (SB) transposon together with the SB transposase vector were co-injected into the cytoplasm of murine zygotes. The zygotes were transferred to surrogate animals, and subsequently the newborn mice were genotyped. A total of four founder transgenic animals were identified and used to establish stable lines. Expression of the LTR7-GFP was restricted to a subpopulation of the inner cell mass of late murine blastocysts. Additionally, expression of the reporter could be reactivated by reprogramming of fetal fibroblasts to induced pluripotent stem (iPS) cells. In somatic cells, no reporter expression was detected by fluorescence microscopy and RT-PCR. Thus the vital reporter labels a subpopulation of pluripotent cells in vivo and in vitro. Currently, additional experiments are ongoing to characterize this subpopulation in detail. This mouse model can be used to understand the development of pluripotency during early ontogenesis, and may provide insights into the regulation of pluripotency especially by temporal divergence in expression patterns of various endogenous retroviruses.

A211E Cloning, transgenesis, and stem cells

Generation of vascular deficient porcine embryos

Marta Moya-Jódar[1], Giulia Coppiello[1], Gloria Abizanda[1], Juan Roberto Rodríguez[1], Francisco Alberto García-Vázquez[2], Felipe Prósper[1], Xabier L. Aranguren[1]

[1]Regenerative Medicine Program, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain; [2]Department of Physiology, University of Murcia, Murcia, Spain;

Keywords: organ generation, CRISPR/Cas9, porcine embryos.

Organ transplantation is, in many cases, the only life-saving treatment for end-stage organ failure, but the main problem is the shortage of these organs. Currently, organs donations are insufficient to cover demand, so other alternatives are needed. Generation of human organs from pluripotent stem cells (PSCs) in animal recipients would provide an endless source of organs for clinical use. Blastocyst complementation is an extraordinarily promising approach to fulfil this unmet medical need. This technique allows the development of an organ/tissue that a genetically modified embryo is unable to form. It consists in the microinjection of PSCs into the cell type/organ-deficient preimplantational embryo and the completion of the embryonic development in the uterus of a foster mother. In this environment, the microinjected PSCs colonize the empty developmental niche and contribute entirely to its formation. Using blastocyst complementation strategy, a cell type/organ deficient pig embryo could be used to generate a humanized/human organ, owing to pigs are physiologically similar to humans as well as the size of their organs. Therefore, as first step, organ/tissue deficient pig embryos need to be generated. Because endothelial cells play a very relevant role in organ rejection upon xenotransplantation, we are generating vascular deficient pig embryos using CRISPR/Cas9 technology to erase ETV2 gene, since it is a master regulator of hematoendothelial lineages. For a preliminary study, we designed 5 different guides (Benchling, USA) against pig's ETV2 gene and were tested *in vitro* on pig's fibroblast. Genome editing was analyzed by Surveyor Mutation Kit (IDT, Spain) and 4 out of 5 guides showed cleavage capacity. Subsequently, the 4 selected guides were individually microinjected with Cas9 protein complex (100 ng/µl Cas9 protein and 50 ng/µl sgRNA) (IDT, Spain) 6h post fertilization into 1 cell-stage pig embryos. The embryos were cultured until blastocyst stage in a humidified atmosphere at 38.5ºC, 5% O_2 and 5% CO_2. Next, DNA amplification by PCR were performed before the deep sequencing analysis. Mutant embryos were obtained with the four microinjected guides, but only two of them achieved biallelic ETV2 disruption, although at low efficiency (Guide 1; 5% KO, Guide 2; 5% heterozygous, Guide 3; 5% KO and 5% Mosaic, Guide 4; 10% Mosaic and 5% heterozygous). Based on the foregoing, future experiments are required in order to optimize the generation of vascular deficient embryos. In the future, efficient ETV2 KO pig embryos could be used as recipient for human chimera-competent cells, that one may generate human vascularized organs. Moreover, this strategy can be expanding to any other organ in a deficient specific model embryo thus permitting the generation of fully humanized organs in a livestock animal and one day, resolve the organ shortage in the clinic.

Acknowledgements: RETIC TerCel RD16/0011/0005, Ramón y Cajal State Program (MINECO/FSE RYC-2015-17233), "Retos de la Sociedad" State Program (MINECO/FEDER SAF2015-64224-R).

A212E Cloning, transgenesis, and stem cells

First production of Calpain3 KO pig embryo by CRISPR/Cas9 technology for human disease modelling: efficiency comparison between electroporation and intracytoplasmic microinjection

Sergio Navarro-Serna[1], Raquel Romar[1], Martxel Dehesa-Etxebeste[2], Jordana S. Lopes[1], Adolfo López de Munain[2], Joaquin Gadea[1]

[1]University of Murcia Dept. Physiology, Murcia, Spain. International Excellence Campus for Higher Education and Research "Campus Mare Nostrum" and Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Murcia, Spain; [2]IIS Biodonostia, Neuroscience, San Sebastián, Spain.

Keywords: transgenesis, CRISPR/Cas9, porcine.

Limb-Girdle Muscular Dystrophy Type2A is an autosomal recessive myopathy caused by mutations in the Calpain3 gene. Currently the disease has not treatment and lacks good animal models. Thus, the study of this disease in pig would offer a great valuable tool to understand the disease and its possible treatments. In porcine species, somatic cell nuclear transfer and CRISPR microinjection (MI) are the main techniques to produce genetically modified embryos (GME). Previous studies have demonstrated that electroporation (EP) allows the production of GME embryos and further offspring (Tanihara, Sci. Adv. 2, e1600803, 2016). In this work we compare the use of MI and EP to produce GME for Calpaine3 evaluating embryo quality and mutation rate. In vitro matured porcine oocytes were treated before insemination with 100ng/µl of CRISPR/Cas9 ribonucleoprotein (RNP) by using two Calpain3 RNAguides. Five groups were used to compare mutation efficiency and embryo development achieved by MI and EP: oocytes microinjected with RNP (MI group), oocytes microinjected without RNP (sham group); oocytes electroporated with 4 (EP4 group) or 6 (EP6 group) pulses (30mV, 1 ms); and non-treated oocytes (Control group). All treatments were performed before IVF. Oocytes were inseminated in TALP medium with frozen-thawed boar spermatozoa selected with NaturARTs-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). Eighteen hours after insemination, putative zygotes were cultured (NCSU-23 medium) for additional 156 h to evaluate the blastocyst yield, regarding the total number of oocytes, and the gene deletion by PCR previous digestion of zona pellucida to remove bound spermatozoa. Experiment was repeated 4 times with 50-55 embryos per group and variables were analyzed by one-way ANOVA. Results showed similar cleavage rate among groups (63.2-72.9%) except for EP6 group which was the lowest (42.9; $p<0.01$). Blastocyst yield decreased in all treatment compared to control group (32.9±3.2%), being similar between MI (22.5±2.9%), sham (21.1±2.8%) and EP4 groups but again the EP6 was the lowest (11.1±2.2%; $p<0.01$). Regarding mutation rate, 41.5% (17/41) of MI derived blastocysts had a large gene deletion whereas EP4 showed 20.7% (6/29) and EP6 was 17.65% (3/17). As for the biallelic KO, it was similar in all blastocysts independently of the treatment applied: 14.6% (6/41) for MI; 17.2% (5/29) for EP4 and 11.8% (2/17) for EP6. These results confirm that EP is a valuable technique to produce KO embryos using CRISPR/Cas9 technology. Despite the low efficiency, the easiness to produce a greater number of embryos in a shorter time as well as the requirement of less high-qualified personnel and high-value equipment than with MI increase the possibility of its use to generate KO embryos. This is the first report about production of Calpain3 KO pig embryos, opening the doors to generation of KO big animals and promising further advances in the relevant field of human disease study. Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

A213E Cloning, transgenesis, and stem cells

Generation of a polled phenotype in cattle using CRISPR/Cas

Felix Schuster[1], Patrick Aldag[1], Antje Frenzel[1], Petra Hassel[1], Maren Ziegler[1], Andrea Lucas-Hahn[1], Heiner Niemann[2], Björn Petersen[1]

[1]Institute of Farm Animal Genetics, Friedrich-Loeffler-Institute, Mariensee, Germany; [2]TWINCORE, Hannover Medical School, Hanover, Germany.

Keywords: genome editing, somatic cell nuclear transfer, cattle.

In modern livestock farming horned cattle pose an increased risk of injury for each other as well as for the farmers. Dehorning without anesthesia is associated with stress and pain for the calves and raises concerns regarding animal welfare. Naturally occurring mutations causing polledness are known for most beef cattle but are rarely distributed within dairy populations such as Holstein-Friesians and Brown Swiss. The propagation of polled Holsteins and Brown Swiss is limited due to the rather low genetic merit of the offered polled bulls which originate from a few founder bulls. In beef cattle, a mutation consisting of a 210 bp insertion and an 8 bp deletion (*Celtic mutation*) causes the polled phenotype while in Holsteins an 80 kbp duplication accompanied by several single point mutations is causative (Medugorac, Seichter et al. 2012). In this project, we used the CRISPR/Cpf1 system (Cas12a) to introgress the *Celtic mutation* into the *horned locus* of Holstein-Friesian and Brown Swiss fibroblasts derived from horned individuals with the aim of producing polled clones from originally horned bulls. The *Celtic mutation* was isolated from an Angus cow via PCR and cloned into a transfection vector as a knock-in template. Editing efficiencies in this locus were low, so multiple CRISPR/Cpf1 target sites were evaluated in order to improve knock-in efficiencies. Furthermore, we used the CRISPR/Cas9 system to create a novel knock-out mutation in the *horned locus* to examine whether also a deletion in this genomic area causes a polled phenotype. For this purpose, two target sites flanking a 300 bp sequence were used to create a large knock-out mutation. Cell clones carrying the desired mutation were propagated further to serve as donor cells for the somatic cell nuclear transfer (SCNT). In the fetus, horn buds are histologically detectable from day 90 of the gestation (Allais-Bonnet et al. 2013), hence the first pregnancy of each experiment will be aborted prematurely to examine the development of horn buds. All other pregnancies will be carried to term. Sequencing data and PCR results showed the desired integration for both the knock-in and knock-out experiment. First cloning experiments showed that development rates of edited embryos (blastocyst rate: 23,68 %; (9/38)) were comparable with those of wild type embryos (blastocyst rate: 20,29 %; (14/60)). The edited embryos were recently transferred into surrogate mothers. In conclusion, we successfully edited the genome of bovine fibroblasts by using different variants of the CRISPR system to introgress a complex mutation (*Celtic mutation*) and also create a novel knock-out mutation. Furthermore, we were able to reliably produce embryos from edited cell lines using SCNT. Once the embryo transfers result in pregnancies the fetuses and offspring, respectively, will be examined for polledness.

A214E Cloning, transgenesis, and stem cells

Establishment of several cloned pregnancies of buffalo breeding bulls

Naresh Selokar, Monika Saini, Rasika Rajendran, Seema Dua, Dharmendra Kumar, Rakesh Sharma, Prem Singh Yadav

ICAR-Central Institute for Research on Buffaloes, India.

Keywords: buffalo, cloning, breeding bull, embryo.

Buffalo cloning is a valuable tool to improve the genetic potential of buffalo. Despite reported births of cloned buffaloes worldwide, the birth of several clones (more than 5 clones) of an individual buffalo have not yet been reported. Thus, in the present study, we attempted to produce multiple clones of an individual buffalo. The skin-derived fibroblast cells of two buffalo breeding bulls, namely M-29 and NR-480, were used as nuclear donors. The cloned embryos were produced using optimized handmade cloning (HMC) of our laboratory. The blastocyst production rate ranges from 35-40% in each experiment for both bulls. We transferred one or two cloned blastocysts on day 7 and 8 post oestrus into recipient buffaloes. We used transrectal ultrasonography to confirm the pregnancies at Day 30 post embryo transfer, and reconfirmed it again at day 60. We established 13 pregnancies (13 pregnant/56 recipient buffaloes, represents 23% conception rate) of M-29 bull, of which three were aborted at the first trimester. For NR-480 bull, we established 3 pregnancies (3 pregnant/7 recipient buffaloes, represents 42% conception rate). These established pregnancies are continuing at six to two months of gestation. To establish more cloned pregnancies, the embryo production and embryo transfer experiments are on-going. In conclusion, we established multiple cloned pregnancies of an individual buffalo using HMC embryos. HMC can be used to multiply elite buffalos in short period.

A226E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Comparisons of lipid content and genes of lipid metabolism in follicular cells and fluid in follicles of different size in bovine

Priscila Silvana Bertevello[1], Anna-Paula Teixeira-Gomes[2], Valerie Labas[1,2], Luiz-Augusto Cordeiro[1], Marie-Claire Blache[1], Pascal Papillier[1], Svetlana Uzbekova[1,2]

[1]1 INRA, UMR Physiologie de la Reproduction et des Comportements F-37380 Nouzilly, France; [2]PAIB (Pôle d'Analyse et d'Imagerie des Biomolécules), Plate-forme CIRE (Chirurgie et Imagerie).

Keywords*:* Lipid metabolism, FA metabolism, follicle-size, mass spectrometry.

Ovaries of mammals have thousands of follicles intended for atresia and only a few become dominant and designated to ovulate. The energy cost for follicular growth is high and requires different substrates, including fatty acids (FA). Somatic follicular cells and oocyte have molecular machinery to metabolize FA into energy. Within bovine biotechnologies, oocytes of the large follicles are more competent for the in vitro embryo development compared to the small ones. The objective of our study was to elucidate the specificity of the lipid composition and the metabolism of FA in antral bovine follicles of different sizes. MALDI-TOF mass spectrometry (MS) imaging allowed the mapping of 281 lipid characteristics in ovarian compartments. Lipid analysis using Red Nile demonstrated differential size dependent distribution of neutral lipids in granulosa (GC) and theca (TH) cell layers. MALDI-TOF MS lipid fingerprints of isolated follicular cells and follicular fluid (FF) of small (SF, medium size 5 mm) and large follicles (LF, mean size 13 mm) acquired by MALDI-TOF MS revealed drastic changes in follicular fluid lipidome (more than 55% of the detected characteristics varied more than twice between LF and SF, Student's t-test, $P<0.05$). The size of the follicle significantly influenced the lipid composition of TH, GC and cumulus cells (ranged from 5%, 15%, and 10%, respectively) in contrast to oocytes that had less than 2% of lipid profile modulation between SF and LF. Identified differential lipids (in total 17%) revealed potential changes of membrane lipids in both somatic follicular cells and fluid along with follicular growth. Among them, the phospholipids containing long and very long chain FAs were preferentially found more abundant in the cells of the LF. In the oocytes from SF., two identified phosphatidylcholines (PC29:1, PC31:1) and 1 sphingomyelin (SM 32:1) were more abundant than in LF. Analysis of gene expression in TH and GC in LF compared to SF suggests a significant increase of FA beta-oxidation and oxidative stress, respectively (observed by expression of *ACADVL, HADHA,* and *GPX4*). Gene *ACOT9*, coding for a thioesterase catalyzing the hydrolysis of long-chain Acyl-CoAs, showed overexpression in TH of the SF. In summary, FA metabolism in follicular cells changes through follicular growth and significantly modulates lipid composition of FF. Differential distribution and abundance of lipids, including signaling molecules may, therefore, influence either follicular atresia or dominance; also, an increase in long-chain FA can provide substrates for post-ovulation body-luteal progesterone production.

Financial support**:** Bertevello is funded by University do Estado do Rio Grande do Norte (Brazil).

A227E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

A new contribution to the improvement of human embryo culture media: a comparative study of low-abundance proteins of reproductive fluids and plasma of fertile women

Analuce Canha-Gouveia[1], Antonio Ramos-Fernández[2], Alberto Paradela[2], Maria Teresa Prieto-Sánchez[3], María Luisa Sánchez-Ferrer[3], Fernando Corrales[2], Pilar Coy[1]

[1]Department of Physiology, Faculty of Veterinary, University of Murcia, Campus Mare Nostrum, IMIB-Arrixaca, Murcia, Spain; [2]Proteomics Laboratory, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), 28049 Madrid, Spain; [3]Department of Obstetrics & Gynecology, "Virgen de la Arrixaca" University Clinical Hospital, IMIB-Arrixaca, Murcia, Spain.

Keywords*:* low-abundance proteins, human reproductive fluids, salpingectomy.

The improvement of the embryo culture media is gaining relevance as demonstrated by the growing number of publications describing its influence on successful implantation rates, pregnancy, neonatal outcomes and potential effects in the adult life. The ideal conditions for embryo development are those naturally occurring in the female reproductive tract, i.e., the oviductal and uterine fluids. These fluids provide all the nutrients, hormonal and non-hormonal factors, electrolytes, macromolecules as well as precisely regulated volume, pH and osmolality required for the gametes, zygotes, and later, embryo development. In order to shed light on the differences between chemical and natural media, a detailed study of the composition of the female reproductive fluids is imperative. Here, we performed the first comparative study of the low abundance proteins in plasma, uterine and oviductal fluid collected from January 2016 until June 2018, simultaneously, from healthy and fertile women that underwent a salpingectomy. In order to select the most homogenous samples for this study, 3 women (out of 62 initially recruited) were selected based in the following criteria: similar age (31, 33 and 39 years old), evidence of healthy progeny and phase of their menstrual cycle (secretory phase). Samples were collected with a modified Mucat® device. The amount and quality of the collected samples allowed us to perform an efficient antibody-based depletion of the most-abundant serum proteins to facilitate the detection of the lower-abundance proteins of each fluid. The rationale for this design derives from the fact that high-abundant proteins in these fluids usually come from blood serum and usually mask the detection of low abundant proteins, which presumably could have a significant role in specific process related with the reproductive function. Differential regulation was measured using label-free quantitative shotgun proteomics, and statistical significance was measured using q-values (FDR). All analyses were conducted using software from Proteobotics (Madrid, Spain). The proteomic analysis by 1D-nano LC ESI-MSMS has shown a higher number of differentially expressed proteins in the oviductal fluid (131) than in the uterine fluid (22) when compared to plasma. From these 131 proteins, 92 were upregulated and 39 downregulated. Regarding the up-regulated proteins identified, they were predominantly involved in cellular catabolic processes, biosynthesis of aminoacids and organic substances, organic and aromatic compounds and catabolic acid signalling. The differentially expressed proteins of uterine fluid were mainly proteins implicated in immune response and granulocyte activation. In conclusion, this study presents a high-throughput analysis of female reproductive tract fluids, which constitutes a novel contribution to the knowledge of oviductal and uterine secretome.
Supported by MINECO-FEDER (AGL 2015-66341-R), 20040/GERM/16 and European Union, Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526.

A228E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Transcriptome of porcine blastocysts stored in liquid state for up to 48 h

Cristina Cuello[1,2], Josep Miquel Cambra[1,2], Maria Antonia Gil[1,2], Inmaculada Parrilla[1,2], Manuel Alvarez-Rodriguez[3], Heriberto Rodriguez-Martinez[3], Emilio Arsenio Martinez[1,2], Cristina Alicia Martinez[1,2,3]

[1]Department of Medicine and Animal Surgery, Faculty of Veterinary Medicine, University of Murcia, Murcia, Spain; [2]Institute for Biomedical Research of Murcia (IMIB-Arrixaca), Campus de Ciencias de la Salud, Murcia, Spain; [3]Department of Clinical & Experimental Medicine (IKE), Linköping University, Campus US, Linköping, Sweden.

Keywords*:* embryo, pig, liquid storage, transcriptome.

Recently, we have developed a method for the liquid storage of *in vivo*-derived porcine blastocysts. Storage is done at 25 ºC in NCSU-BSA medium without controlled CO_2 gassing for up to 48 h, thus facilitating the commercial application of embryo transfer (ET) in pigs. In a preliminary study with few ETs, 30% liquid stored blastocysts for 48 h were able to develop *in vivo* until day 38 of pregnancy. However, subsequent ET-studies with liquid-stored blastocysts, showed impaired farrowing rates compared to that of fresh or 24 h-stored blastocysts. Therefore, to elucidate the causes of these pregnancy losses, we evaluate hereby the transcriptional patterns of *in vivo*-derived blastocysts stored in liquid state for 24 and 48 h. Blastocysts were collected by laparotomy at Day 6 of the cycle (D0=onset of estrus) from weaned cross-bread sows (N=7). Some fresh blastocysts (control group) were frozen immediately after collection and stored at -80ºC until transcriptome analysis. The rest of the blastocysts were stored in 1 mL of NCSU-BSA in Eppendorf tubes at 25ºC for 24 or 48 h upon which they were morphologically evaluated under a stereomicroscope. A total of 30 viable blastocysts (three pools of 10 blastocysts) per group were transcriptomically analyzed. Transcripts (24,123) were evaluated in a microarray (GeneChip Porcine Genome Array, Thermo Fisher Scientific). A False Discovery Rate adjusted analysis p-adjusted <0.05 and a fold change cut-off of ±1 were set to identify differentially expressed genes. Data were analyzed using Partek Genomic Suite 7.0 software, which also identified altered KEGG pathways. None of the stored blastocysts had hatched by the end of storage. The blastocyst survival rates at 24 (97 %) and 48 h (94 %) of storage were similar to those achieved in control blastocysts (100%). However, the number of differentially expressed genes of stored blastocysts compared to controls dramatically increased during storage, from 127 genes by 24 h to 4,175 genes by 48 h). Blastocysts stored for 24 h displayed 70 down-regulated and 57 up-regulated genes. Only seven pathways (Axon guidance, PPAR signaling, Long-term potentiation) had an enrichment score >4, with less than 5% of their genes modified with respect to the control blastocysts. In contrast, in blastocysts stored for 48 h, 2,120 genes were down-expressed and 2,055 over-expressed. Thirty-six pathways had an enrichment score >4. In addition, 12 pathways showed more than 30% of their genes altered, related to pathways fundamental for embryonic development and pregnancy as: Protein processing in endoplasmic reticulum, Metabolic pathways, Cell cycle, Oxidative phosphorylation, Notch signaling pathways, Mismatch repair, Nucleotide repair and DNA replication. These results would not only certainly explain the very low pregnancy rates obtained with 48 h-stored blastocysts in our previous studies but also help designing novel target strategies to improve liquid storage systems for porcine embryos.
Supported by MINECO-FEDER (AGL2015-69735-R), Seneca Foundation (19892/GERM/15) and FORMAS, Stockholm (2017-00946).

A229E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Influence of different extenders on post-thaw quality of cryopreserved yak (Poephagus grunniens) semen

Sourabh Deori[1], Vijay Paul[2], Dinamani Medhi[2], Prithiviraj Chakravarty[2]

[1]ICAR Research Complex for NEH Region, Umaim, India; [2]ICAR-National Research Centre on Yak, Dirang, India.

Keywords*:* extender, post-thaw, semen, yak.

Yak is a unique livestock of high altitude surviving under hypoxic and extreme cold conditions above 3000 metre from mean sea level. They are considered as multipurpose animal as they provide milk, meat, fibre/wool, hide, fuel and the much needed transportation to the highlanders. Yak breeding face a lot of challenges under field conditions due to geographical isolation of the herds and repeated use of same breeding bull for generations. Cryopreservation of semen and use of Artificial Insemination (AI) can be one of the effective tools for overcoming the breeding problem in yaks. Yak semen has been successfully cryopreserved using Tris-citrate-fructose-egg yolk-glycerol (TFYG) extender (S. Deori, SAARC. J. Agric., 15, 215-218, 2017). Therefore, present study was designed with an objective to compare commercially available soybean based and liposome based extenders with TEYG extender on post-thaw quality of yak semen following cryopreservation. Semen was collected from 4 mature yak bulls (aged between 4 to 5 years) using artificial vagina. A total of twenty ejaculates (5/bull) having initial motility >70 percent were used for the study. Each ejaculate was splitted and diluted with BioXCell®, OPTIXCell® (IMV Technologies, France) and TFYG extenders to achieve a concentration of 30 x 10^6 per 0.5 mL straw. TFYG extender consists of 20 percent egg yolk and 6.4 percent glycerol. The straws were cooled to 5°C and equilibrated for 4 hours before freezing and cryopreserved in liquid nitrogen (BKD Borah et al., Int. J. Chem. Stud., 6, 509-511, 2018). The straws were thawed at 37°C for 10 seconds and evaluated for post-thaw sperm motility under phase contrast microscope (400x) and recorded from 0 to 100 based on the percentage of progressive motile sperm, sperm viability by Esoin-Nigrosine stain and acrosomal integrity by Giemsa stain. For sperm viability and acrosomal integrity a total of 200 sperm were counted and recorded in percentage. The mean values of the post-thaw sperm motility (%) in TFYG, BioXCell® and OPTIXCell® extenders were 53.50 ± 0.53, 54.50 ± 0.34 and 56.75 ± 0.55 respectively. The corresponding values for percent sperm viability and acrosomal integrity were 63.95 ± 0.53, 64.55 ± 0.51 and 66.35 ± 0.43, and 87.95 ± 0.67, 88.50 ± 0.66 and 91.10 ± 0.35 respectively. Analysis of variance indicated that post-thaw sperm motility, sperm viability and acrosomal integrity were significantly ($P<0.05$) higher in OPTIXCell® extender in comparison to BioXCell® and TFYG extenders. The values did not differ significantly between BioXcell and TEYG extenders. In conclusion, OPTIXCell® – a liposome based extender may serve as an alternative for successful cryopreservation of yak semen.

A230E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Combined embryo and recipient metabolomics improves pregnancy prediction in cattle

Enrique Gomez[1], Nuria Canela[2], Pol Herrero[2], Adria Cereto[2], Susana Carrocera[1], David Martín-Gonzalez[1], Antonio Murillo[1], Isabel Gimeno[1], Marta Muñoz[1]

[1]SERIDA, Gijon, Spain; [2]Centre for Omic Sciences, EURECAT, Reus, Spain.

Keywords*:* Embryo, recipient, metabolomics.

Pregnancy prediction within IVP embryos or recipients often fails, as competence of the embryo is not defined in recipient studies and vice-versa. We designed a multi-variate study with controlled factors (i.e. embryo breed, recipient breed, Day-6 embryonic stages) to identify pregnancy biomarkers in recipient plasma and embryo culture medium (CM; SOFaaci). Abattoir oocytes matured and fertilized with Asturiana de los Valles (AV) or Holstein sperm were first cultured in groups, and singly from Day-6 to Day-7 (12 µL CM). Expanded blastocysts were vitrified/warmed (V/W) and the CM was stored at -150°C until metabolomic analysis by GC-qTOF/MS. V/W embryos (N=24 [AV] and N=12 [Holstein]; N=6 bulls) were transferred to synchronized recipients (N=13 AV, N=17 Holstein and N=6 crossbred), and blood plasma was collected on Day-0 (PD0; N=35) and Day-7 (PD7; N=36). Independent Holstein embryos and recipients (N=13 ETs) were used for validation. Pregnancy was diagnosed on Day-62 and birth. Metabolites identified were N=36 (CM) and N=71 (plasma). Metabolite values in CM were subtracted from incubated blank controls. Data were transformed by Pareto scaling and weighed by embryo breed, bull and embryonic stage on Day-6 (embryos), and recipient breed. Pregnancy-regulated metabolite concentrations were identified by GLM ($P<0.05$ and FDR ($P<0.05$)). Biomarkers were obtained in two ways: 1) singly, by ROC-AUC>0.650 ($P<0.05$; FDR<0.05); and 2) by F1 score, as a Boolean product of 1 metabolite from CM and 1 metabolite from plasma. Thus, concentrations of metabolites in pregnant plasma and CM were paired (True (T)*T = T), and open samples outside the pregnant range considered T (one, another, or both, plasma and CM, outside the pregnant range) or false (F; both CM and plasma in the pregnant range). Biomarkers in CM at birth were capric acid (C, P=0.021) and monostearin (M; P=0.016); CM at Day-62: no metabolite. PD0 at birth: creatinine (CR, AUC: 0.690, P=0.024) and azelaic acid (AZ, AUC: 0.694, P=0.047). PD7 at Day-62: Leucine (L, AUC: 0.744, P=0.029). Fold changes in CM were >3.0, while in plasma were <2.0. Combining non-significant PD0 metabolites as glycine (G, 22 –correct- /35 –total- samples), hydrocinnamic acid (HY, 23/35) and hippuric acid (HI, 21/35) with the CM biomarker C improved sample classification (GxC: 28/35; HYxC: 30/35; HIxC: 28/35), and their respective F1 score (0.780, 0.872, 0.829; $P<0.003$) over C AUC (0.755; 23/36). Similar increases were observed with the CM biomarker M (not shown). In contrast, PD0 significant biomarkers as CR and AZ did not improve F1 accuracy as combined with C or M. Target metabolomics using pure analytical standards coinjection confirmed the identity of C, M, HY and HI in the analyzed samples. Validation of M in Holsteins led to AUC=0.769 (10/13), while C showed AUC=0.615 (8/13). Reliable birth predictions are feasible once: 1) biomarkers are identified in CM and plasma; and 2) embryo and recipient biomarkers are confronted. Acknowledgements: MINECO (AGL2016-78597-R and AGL2016-81890-REDT). GRUPIN 2018-2020 (IDI/2018/000178). FEDER. COST Action 16119 (Cellfit). ASEAVA. ASCOL.

A231E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Pregnancy after short exposure of cryopreserved porcine embryo to cryoprotective agents

Florence Guignot[1], Florine Dubuisson[1], Camille Voiry[1], Eric Royer[2], Anais Giovanetti[2], Jonathan Savoie[2], Christian Moussu[1], Frédéric Elleboudt[1], Henri Woelders[3], Pascal Mermillod[1], Elisabeth Blesbois[1]

[1]Inra, UMR85-PRC, F-37380 Nouzilly, France; [2]Inra, UEPAO, F-37380 Nouzilly, France; [3]Wageningen UR Livestock Research, Wageningen, the Netherlands.

Keywords*:* porcine embryo, vitrification, short exposure.

The pig industry has nowadays an increasing demand for a reliable and cost-effective porcine embryo cryopreservation allowing long-term conservation, transport and widespread dispersion of high-quality genetics resources. Progress in embryo vitrification process made it possible to use the method in pigs, but lower and variable pregnancy rates are achieved with frozen embryos compared to fresh one. High concentrations of cryoprotective agents (CPAs) used for vitrification are believed to negatively affect developmental competence (Woelders et al, Cryobiology, 2018). The aim of the present study was to test the viability of cryopreserved porcine embryo after short exposure to CPAs during vitrification process. Embryos were surgically recovered 6 days after ovulation from Large White sows. Only embryos at the blastocyst stage were selected and vitrified in superfine open pulled straw (SOPS). Embryos were firstly placed in equilibration solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% DMSO, and then in vitrification solution (VS), containing 16% EG, 16% DMSO and 0.4M sucrose. Embryos were incubated 2 min in ES and 30 sec in VS (short exposure to CPAs) or 3 min in ES and 1 min in VS (control, Cuello et al, RFD, 2010). Embryos were then loaded into straws and plunged into liquid nitrogen. After thawing, they were transferred to Talp-Hepes PVA with decreasing sucrose concentrations (0.13 and 0M) for 5 min each. In vitro and in vivo survival were tested. For in vitro survival, embryos were cultured for 3 days in 50µL of NCSU-23 + 10% FCS at 38.8°C in a humidified atmosphere of 5% CO_2 in air. For in vivo survival, embryos were surgically transferred in uterine horn of synchronised Meishan recipient (30 blastocysts per recipient). Three hundred and four embryos were used to test in vitro embryo survival after short and control exposure to CPAs. In the first experiment, the survival rate was better with shorter exposure to CPAs (66.2% vs 45.6%, p=0.008, n=145), but in the second experiment, it was identical (47.4% vs 60.6% ; p=0.101, n=159). New embryos were produced and collected (n=157) to test in vivo survival. Transfers were performed with embryos vitrified according to short exposure to CPAs. Among 4 recipients, one is pregnant. Farrowing is expected in next weeks. Our results show that a short exposure to CPAs is as efficient as the longer exposure usually employed for porcine embryo vitrification. As short exposure decreases the embryos contact with high level of toxic CPAs, reducing potential harmful and epigenetic modification of embryonic genome, this short exposure to CPAs should be chosen for porcine embryo vitrification.

This work was part of the IMAGE project which received funding from the European Union's Horizon 2020 Research and Innovation Programme under the grant agreement n° 677353.

A232E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Cumulus-oocyte complexes-like 3D models to analyze sperm binding

Julieta Gabriela Hamze[1], María Jiménez-Movilla[1], Raquel Romar[2]

[1]Departamento de Biología Celular e Histología, Facultad de Medicina, Universidad de Murcia. IMIB, Murcia, España; [2]Departamento de Fisiología, Facultad de Veterinaria, Campus Espinardo, Universidad de Murcia. IMIB, Murcia, España.

Keywords*:* zona pellucida, gamete interaction, magnetic beads.

The oocyte encapsulated by a glycoprotein matrix named zona pellucida (ZP) is surrounded by cumulus cells forming the cumulus oocyte complexes (COCs) that are ovulated in a plug in polytocous mammalian species. In recent years, a new 3D model to study gamete interaction in depth under *in vitro* conditions has been developed (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). The model, consists of porcine ZP recombinant proteins conjugated to magnetic Sepharose® beads (B_{ZP}), supports sperm binding and resembles oocyte's size and shape being a valuable tool to simulate gamete interaction studies. In this work, we have taken a step forward to improve the model by better imitating the shape and possible function of the native COCs by incubating the B_{ZP} with cells cumulus thus generating cumulus-oocyte complexes-like 3D models ($CB_{ZP}C$) to evaluate whether they support further sperm binding. In order to obtain the $CB_{ZP}C$ models, B_{ZP} were generated as previously described with recombinant porcine ZP2, ZP3 and ZP4 proteins (Hamze, Animal Reprod 13: 647, 2016; Hamze, Animal Reprod 14 (3): 974, 2017). B_{ZP} were incubated for 24 h with cumulus cells isolated from *in vitro* matured porcine COCs (2,500 cells/ B_{ZP}). Then, groups of 50-55 $CB_{ZP}C$ were incubated for 2 h with fresh ejaculated porcine sperm separated by double centrifugation method (200,000 sperm/mL) at 38.5 ºC, 20% O_2, 5% CO_2, and saturated humidity. After co-incubation period, $CB_{ZP}C$ were washed twice in PBS, fixed and stained with Hoechst 33342. The mean number of sperm bound per $CB_{ZP}C$ ($S/CB_{ZP}C$) was scored by epifluorescence microscopy. Data was analyzed using Systat v13.1 (Systat Software, Inc San Jose, CA, USA) by one-way ANOVA and the values compared by Tukey's test when P value <0.05. The preliminary results (3 replicates) show that $S/CB_{ZP}C$ was significantly higher for the $CB_{ZP}C$ conjugated to ZP2 (11.88 ± 0.72, N=176) than beads conjugated to ZP3 (5.82 ± 0.41, N=195) and ZP4 (8.83 ± 0.61, N=176). These results are consistent with the ones previously reported with the 3D models without cumulus cells (B_{ZP}) (Hamze, Animal Reprod 13: 647, 2016) as well as the ones described in human and mice (Avella, J Cell Biol. 205(6):801-9, 2014; Avella, Sci Transl Med. 27;8(336):336ra60, 2016) suggesting that ZP2 could act as a sperm receptor in porcine species as well. In conclusion, this study offers a more physiological 3D model offering data consistent with previous observations thus reinforcing the viability of these models as a valuable tool to study gamete interaction. Moreover, as recombinant technology it is easily transferable to other species.

Supported by MINECO and FEDER (AGL2015-70159-P), Fundación Seneca-Agencia de Ciencia y Tecnología de la Región de Murcia (20887/PI/18, Ayudas a proyectos para el desarrollo de investigación científica y técnica por grupos competitivos).

A233E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Effect of highly dispersed silica nanoparticles on the functional activity of actin cytoskeleton in native and devitrified bovine oocytes during IVM

Tatyana I. Kuzmina[1], Diana N. Tatarskaya[2], Tatyana I. Stanislavovich[1]

[1]Russian Research Institute of Farm Animal Genetics and Breeding - Branch of the L.K. Ernst Federal Science, Russian Federation; [2]EmbryLife Center of Reproductive Technologies, St.Petersburg, Russia.

Keywords*:* oocyte, cytoskeleton, vitrification.

Actin takes over various essential function during oocyte meiosis (UrajiJ, et al., J Cell Sci,131 22: 1-6, 2018). Nanoparticles are widely used in various fields including reproduction. The mechanisms of the influence of *highly dispersed silica nanoparticles* (HDSns) on the functioning of intracellular organelles are still not clear. The aim of the present study was to identify the effects of HDSns (Chuiko Institute of Surface Chemistry, Ukraine) on the functional activity of the actin cytoskeleton [the intensity of fluorescence of rhodamine-phalloidin (IFRF) conjugated with actin filaments] in dynamics of meiosis of native **(unfrozen)** and devitrified (DV) oocytes. IFRF was evaluated in: native oocytes; native oocytes were cultured with 0.001% of HDSns; DV oocytes; DV oocytes pre-treated with 0.001% of HDSns before vitrification (20 min) and were cultured with 0.001% of HDSns. Vitrification was performed by equilibration of cumulus oocyte complexes (COCs) **before IVM** in: CPA1:0.7 M dimethylsulphoxide (Me2SO) +0.9 M ethylene glycol (EG), 30 sec; CPA2:1.4 M Me2SO + 1.8 M EG, 30 sec; CPA3:2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose, 20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199. COCs were cultured 24 h in TCM 199 + 10% (v/v) FCS + 50 ng/ml PRL with 10*6granulosa cells /ml. For assessment of chromatin and IFRF fixed oocytes were incubated sequentially in rhodamine-phalloidin(RF, R415 Invitrogen, Moscow, Russia), 1 IU/ml, for 30 min to label actin. Then oocytes were incubated in 4',6-diamidino-2-phenylindole, 10 |μg/ml, for 10 min to label chromatin. Oocytes were examined using confocal laser scanning system Leica TCS SP5 with inverted fluorescent microscope. Diode 405 nm, argon 488 nm and helium-neon 543nm laser lines were used for fluorochrome excitation. IFRF were expressed in arbitrary units. All chemicals, except for RF, were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by ANOVA**.** Chromatin status and IFRF of 391 native and DV oocytes (in 3 replicates, 30-34 oocytes/group) were evaluated during IVM. There were no differences between the IFRF in native oocytes and native oocytes treated with HDSns before and in dynamic of culture (23±1.1 vs 21.1±1.08; 14 h of IVM - 55.8±5.6 vs 49.2±6,7; 24 h of IVM - 29.8±5,8 vs 21.3±7.3, respectively). The lowest level of IFRF were tested in DV oocytes before, after 14 h and 24 h of IVM (14.7±4.4, 16.1±3.8, 10.5±6.1, respectively). Treatment of DV oocyte with HDSns increased the IFRF after 14 h and 24 h of IVM (16.1±3.8 vs 37.8±5.9 and 10.5±6.1 vs 23.5±4.9, respectively, $P < 0.01$, $P < 0.05$). The data of study showed that the treatment of COCs with 0.001% of HDSn influences on actin cytoskeleton integrity of bovine oocytes during vitrification. The mechanisms of the realization of this effect are under the further investigation.

This study was funded by RFBR and Minobrnauka of RF (projects # 18-016-00147A and # 18-118021590132-9).

A234E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Quality of bovine in vitro produced embryos derived from frozen-thawed oocytes

Alexander Makarevich[1], Lucia Olexiková[1], Linda Bedeova[1], Elena Kubovicova[1], Peter Chrenek[1,2]

[1]NPPC- Research Institute for Animal Production Nitra, Slovak Republic; [2]Slovak University of Agriculture in Nitra, Slovak Republic.

Keywords*:* bovine, cryopreservation, in vitro fertilization.

Bovine oocyte cryopreservation has recently been a promising method of preserving genetic resources. A prerequisite for the proper development of preimplantation embryos after IVF is good quality of thawed oocytes. The aim of the study was to compare quality of IVP embryos derived from vitrified-warmed (V) oocytes with those obtained from fresh (C; control) oocytes. Embryo quality was evaluated basing on blastocyst cell number. Bovine cumulus-oocyte complexes were matured *in vitro* and then frozen by an ultra-rapid cooling vitrification technique in a minimum volume of vitrification solution (E-199 medium, 25 mM HEPES, 30 % ethylene glycol, 10% FBS) using 300 mesh electron microscopy nickel grids as a carrier. After warming the oocytes were fertilized in vitro and cultured in a Menezo B2 medium on the cumulus cell monolayer until the blastocyst stage. Embryo cleavage rate was counted on the Day 2 and blastocyst rate - on the Day 7 of embryo culture. For cell number counting, DAPI staining on nuclei observed under a Leica fluorescent microscope was used. For evaluation of differences between the experimental and control groups in embryo cleavage and blastocyst rate a Chi-square test, and in blastocyst cell number the t-test were used. Cleavage rate (Day 2) in V oocytes was lower (55.81%) compared to C (72.5%) group. Similarly, the blastocyst rate (D7) in V oocytes was different (11.82%) compared to C group (23%). In the V group, the higher incidence of asynchronous division at 3-cell stage, irregularly divided blastomeres at advanced embryo stages, and occurrence of blastocyst-like embryos (appearance of a blastocoel, but only a few nuclei) were observed in contrast to fresh oocytes. However, the cell number in truly D7 blastocysts in the V group (85 ± 10.16) was not statistically different from the control (97.3 ± 6.43) blastocysts. In conclusion, although the quality (the cell number) of V-derived blastocysts is comparable with those of fresh control, their development is affected by an asynchronous/irregular division resulting in a lower blastocyst rate. Further optimization of an oocyte cryopreservation regimen is required.
Grant support: the Agency for the support of Science and Technology, Slovak Republic (grant no. APVV-15-0196).

A235E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Proteomic analysis of ejaculated and epididymal sperm associated with freezability in Iberian ibex (Capra pyrenaica)

Lucía Martínez-Fresneda[1,2,5], Marc Sylvester[3], Farhad Shakeri[4], Andreas Buness[4], Juan C. Del Pozo[6], Francisco A. García-Vázquez[5], Christiane Neuhoff[1], Dawit Tesfaye[1], Karl Schellander[1], Julian Santiago-Moreno[2]

[1]Department of Animal Breeding and Husbandry, Institute of Animal Science, University of Bonn, Bonn, Germany.; [2]Department of Animal Reproduction, INIA, Madrid, Spain.; [3]Core Facility Mass Spectrometry, Institute of Biochemistry and Molecular Biology, University of Bonn, Bonn, Germany.; [4]Core Unit for Bioinformatics Analysis Universitätsklinikum Bonn, Bonn, Germany.; [5]Department of Physiology, Faculty of Veterinary Science, International Excellence Campus for Higher Education and Research "Campus Mare Nostrum", University of Murcia, Murcia, Spain.; [6]Center for Biotechnology and Plant Genomic, Polytechnic University of Madrid-National Institute for Agricultural and Food Research and Technology (UPM-INIA), Madrid, Spain.

Keywords*:* proteome, small ruminants, spermatozoa.

The sperm proteome is known to affect cell cryoresistance and is reported to differ between epididymal and ejaculated sperm in small ruminants (C.J. Li *et al*., Animal Reproduction Science, 173, 1–7, 2016; T. Pini *et al*., Journal of Proteome Research, 15, 3700-11, 2016). However, studies aiming at identifying proteins involved on sperm freezing-tolerance are scarce. The aim of this study was to investigate the association between the freezing capacity and the proteome of ejaculated and epididymal sperm of the Iberian ibex. Ejaculates were collected from anesthetized animals by transrectal ultrasound-guided massage of the accessory sex glands combined with electroejaculation (n = 6), whereas epididymal samples were collected post-mortem by flushing (n = 6). After seminal/epididymal fluid removal, sperm cells were conventionally cryopreserved by slow freezing. Sperm quality parameters were assessed in fresh and frozen-thawed sperm to evaluate sperm freezability. Motility parameters were assessed by computer-assisted sperm analysis system and membrane and acrosome integrity were assessed by fluorescence microscopy. Tandem mass tag-labeled peptides were analyzed by high performance liquid chromatography coupled to a mass spectrometer (MS; Orbitrap Fusion Lumos) in three technical replicates. A false discovery rate of 1% was applied as protein identification threshold. The MS raw data were processed in Proteome Discoverer 2.2.0.388 and the statistical analysis was done using the moderated t-test of the R package limma. Epididymal sperm showed higher post-thaw total motility (57.46±8.58% *vs* 23.19±3.05%), progressive motility (37.70±6.38% *vs* 8.65±1.83%), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) than ejaculated sperm ($P<0.0001$). Post-thaw acrosome (89.50±0.56% *vs* 61.95±3.48%; $P<0.001$) and membrane integrity (57.33±7.26% *vs* 35.73±3.39%; $P<0.05$) were also higher in epididymal sperm. A total of 1660 proteins were quantified in both epididymal and ejaculated samples among which 310 proteins (18.7% of the total) were differentially expressed between both types of sperm when using a cut-off for significance (adjusted p-value <0.05) and fold-change (abs(log2 (fold-change)>1). Out of those proteins, 212 were significantly more abundant in epididymal sperm and 98 were more abundant in ejaculated sperm. Peroxiredoxin-4 (PRDX4) and superoxide dismutase [Cu-Zn] (SOD1) are proteins involved in cell protection against oxidative stress and were more abundant in epididymal than ejaculated sperm. Heat shock protein HSP 90-alpha (HSP90AA1) is a chaperone involved in structural maintenance and cell cycle control that was also more abundant in epididymal sperm. Besides updating the sperm proteome of small ruminants, this study revealed differences of cryoresistance between epididymal and ejaculated sperm of the Iberian ibex contributing to identification of candidate markers of sperm freezability.

This work was supported by funding from European Union (Horizon 2020 Marie Sklodowska-Curie Action, REPBIOTECH 675526; AGL2017-85753-R).

A236E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Effect of different egg yolk extenders on in vitro fertility of thawed ram semen

Cristina D. Munuera, Roser Morato, María Jesús Palomo

Autonomous University of Barcelona, Spain.

Keywords*:* thawed semen, egg yolk extenders, in vitro penetration.

An attempt to optimize sperm freezing extenders in ram was made by the replacement of fresh egg yolk (FEY) by powdered egg yolk (PEY). Therefore, ejaculates from 8 rams (2 years old) were collected by artificial vagina and mixed immediately. Pooled semen was centrifuged twice and the pellet was split into two aliquots and resuspended in a Tris-citric acid-glucose solution with 5% glycerol and 15% of PEY or FEY for 4 h at 5ºC before freezing in liquid nitrogen vapours. Secondly, cumulus-oocyte complexes were obtained by slicing from prepubertal sheep ovaries, selected and matured in 100 µL drops of BO-IVM® medium (IVF Bioscience, UK) plus estrous sheep serum (ESS, 10%) for 24 h at 38.5ºC in 5% CO_2 atmosphere. Then, FEY and PEY semen samples were thawed and spermatozoa were selected by 2 mL density gradient (40%/80%), using BoviPure® and BoviDilute® (Nidacon, Mölndal, Sweden) for its preparation. Once the column was centrifuged at 1200g for 10 min, the supernatant was discarded, the pellet resuspended with 2 mL of BoviWash® and then centrifuged again at 1200g for 5 min. After discarding the supernatant, total and progressive motility was assessed from the selected sperm populations by a CASA system (ISAS® , PROISER SL, Valencia, Spain). Then, the selected sperm were co-cultured with the in vitro matured oocytes in 100 µL drops of BO-IVF® medium (IVF Bioscience, UK), supplemented with 2% ESS, at a final concentration of $2x10^6$ sperm/mL. After 17 h of co-culture, oocytes inseminated (n=126 for FEY and n=127 for PEY) were mechanical decumulated, washed and fixed in 4% paraformaldehyde (v/v) at room temperature for 1 h approximately. Afterwards, oocytes were stained with Hoechst 33342 for 15 min in the dark at 4ºC. Then, oocytes were mounted on slides and kept at 4ºC in darkness until analysis under an epifluorescence microscopy (ZEISS Axioskop 40, Oberkochen, Germany). The parameters assessed were the penetration rate (number of oocytes penetrated by at least one spermatozoon of the total number of the potential mature oocytes) and the monospermy rate (number of oocytes penetrated by a single spermatozoon of the total penetrated oocytes). Statistical analysis was performed using a General Lineal Model procedure (SPSS® 20, IBM® Corporation, Armonk, NY, USA). The results (mean ± SD) did not show significant differences between extenders on penetration rate (82.6 ± 6.7 and 88.2 ± 3.9, for FEY and PEY, respectively) neither on monospermy rate (58.5±13.6 and 60.4±13.4, respectively). As well, no significant differences were found between extenders on total sperm motility (41.6±13.1 and 37.1±12.1 for FEY and PEY, respectively) and progressive motility (14.9±5.3 and 9.2±5.6 for FEY and PEY, respectively). In conclusion, powdered egg yolk can substitute successfully the conventional fresh egg yolk in ram sperm freezing extenders, providing higher biosecurity, due to its pasteurization process, and greater homogeneity in the composition of the diluents.

Supported by INIA (RZP2014-00001-00-00) and PIVEV (AGL2016-81890-REDT).

A237E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Incubation with seminal plasma after thawing reduces immature sperm in Blanca de Rasquera goats

Linda U. Ohaneje, Uchebuchi I Osuagwuh, Manuel Álvarez-Rodríguez, María Jesús Palomo

Autonomous University of Barcelona, Spain.

Keywords*:* cryopreserved sperm, seminal plasma, male age.

Addition of seminal plasma (SP) to cryopreserved sperm has been suggested as a potential alternative for improving sperm quality after thawing. DNA status, measured by the Sperm Chromatin Structure Assay (SCSA) provides an accurate measurement of sperm with a high DNA stainability (HDS), which has been related to sperm immaturity. Based on this, a preliminary study was conducted to evaluate the addition of SP to post thawed goat sperm on HDS levels in cryopreserved sperm from 1 and 2 years old bucks, thus studying a potential male age effect. Briefly, ejaculates from 8 bucks were collected via artificial vagina twice a week in 2 consecutive breeding periods. Fresh ejaculates were immediately pooled, centrifuged twice and diluted in a Tris-based media containing 15% powdered egg yolk (NIVE, Nunspeet Holland Eiproducten, Ochten, The Netherlands). Then, diluted samples were refrigerated for 4 h at 5°C before freezing in liquid nitrogen vapours. After thawing at 37°C for 30 seconds, sperm samples were selected by a single layer centrifugation using BoviPure® (Nidacon, Mölndal, Sweden), according to the manufacture instructions. Then, selected sperm sample was split in 2 aliquots and incubated in different media consisting on: a) *in vitro* fertilization commercial media (BO-IVF, IVF Bioscience, UK) and b) BO-IVF media plus 20% SP, for 3 h at 38,5ºC in a 5% CO_2 atmosphere at a final concentration of $40x10^6$ sperm/mL. Then, HDS sperm proportion was determined using flow cytometry after acid-detergent treatment for 30 s and acridine orange staining according to SCSA methodology described by Evenson et al (Journal of Andrology, 2002; 23, 1, 25-43). Viability was also assessed by flow cytometry using SYBR14 and PI probes. Statistical analysis was performed using a General Lineal Model procedure (SPSS 19.0). Results showed no significant differences on HDS sperm proportion (mean % ± SE, n=6) between samples collected from 1 (4.4±0.8) and 2 years old males (3.7±0.6) after incubation for 3h in BO-IVF media. Likewise, no differences were found between sperm samples from 1 (3.0±0.6) and 2 years old males (1.6±0.2) after 3h incubation in BO-IVF media plus seminal plasma. However, this HDS population was significantly lower in sperm from 2 years old males incubated in the presence of seminal plasma compared to the sperm incubated only in BO-IVF media, while in 1 years old males no significant differences were observed. However, neither the male age nor the incubation media had a significant effect on thawed sperm viability, showing values (mean % ± SE) of 12.0±2.6 and 16.6±4.8 in 1 year old male samples and 15.3±3.6 and 14.8±1.8 in 2 years old male sperm incubated in BO-IVF or BO-IVF+SP for 3h, respectively. In conclusion, this study showed that the age of the donors had no identifiable beneficial effect on the HDS-parameters in goat sperm. Further studies are needed to elucidate the potential beneficial effect of seminal plasma on HDS levels in cryopreserved, especially in older male sperm. Supported by INIA (RZP2014-00001-00-00) and PIVEV (AGL2016-81890-REDT).

A238E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Intracytoplasmic sperm injection using sex-sorted sperm in the bovine

Larissa Richter, Franziska Kotarski, Bettina Zimmer, Christine Wrenzycki

Clinic for Veterinary Obstetrics, Gynecology and Andrology, Chair for Molecular Reproductive Medicine, Germany.

Keywords*:* ICSI, paternal contribution, cattle.

Intracytoplasmic sperm injection (ICSI) involves the fertilization of oocytes at the metaphase II stage (MII) by direct injection of a sperm cell. Despite the similar success rates between ICSI and conventional IVF in humans, the success rates in cattle are poor with low developmental rates compared to those obtained via IVF. One reason might be the need of an artificial activation of the oocyte after sperm injection. This activation could also lead to parthenogenetic embryonic development up to the blastocyst stage and beyond without paternal contribution. Checking the morphology of the obtained blastocysts is not sufficient to distinguish between parthenogenetic and fertilized embryos after ICSI. Therefore, we used Y-sorted sperm for ICSI. Sexing the subsequent blastocysts employing bovine and Y-chromosome specific primers will provide reliable results regarding the sex of the embryos. Bovine cumulus-oocyte-complexes collected from follicles out of slaughtherhouse ovaries were in vitro matured employing a standard protocol (Stinshoff et al. Reprod Fertil Dev. 2014;26(4):502-10). After denudation, only MII oocytes were used for ICSI. Bovine Y-sorted sperm from four (bull 1-4) different bulls (1.5-2 years of age) with proven fertility (ICSI Y1, Y2, Y3, Y4) was prepared via a SpermFilter™ centrifugation. After immobilization of the sperm cell, it was sucked into the ICSI pipette using Eppendorf manipulators mounted on an Olympus microscope and injected into the oocyte. Chemical activation was performed with 5 µM ionomycin for 5 minutes followed by a 3 h culture period and an additional incubation for 3 h in 1.9 mM 6-DMAP. After activation, oocytes were cultured in SOFaa for 8 days. Oocytes which have been fertilized conventionally by co-culturing oocytes and sperm (IVF), which have only been activated (CA) and those which have been injected with non-sorted (ICSI non) and X-sorted (ICSI X) sperm served as controls. Experiments were repeated at least four times with an average of 25 oocytes per run. Cleavage (day 3) and developmental rates at day 8 (mean±SD) ranged from 15.8±5.4 to 40.9±13.1 % and 0.0 to 11.0±3.6 % in the ICSI Y1-Y4 groups. Within the control groups of embryos the following data could be obtained: 66.7±9.7, 33.0±7.4 % (IVF); 44.0±11.9, 9.7±3.5 % (CA); 49.2±18.0, 11.6±10.6 % (ICSI non); 51.4± 7.7, 7.1±2.8 % (ICSI X). After sexing, only 4 of the tested 21 embryos stemming from the ICSI Y1-Y4 group were male. Only 1 male embryo could be obtained using non- sorted semen for ICSI (1/24 embryos tested). Embryos out of the CA (16/16) and ICSI X groups (3/3) were all female as expected. After IVF, 38 out of the total number of 59 tested embryos were female. Taken together, these data indicate that there is a bull-specific suitability of sperm to be used for ICSI. Furthermore, even when an advanced developmental stage has been reached, the paternal contribution needs to be verified.

We acknowledge Stephane Alkabes (Masterrind GmbH, Germany) providing the sorted semen.

A239E Support biotechnologies: Cryopreservation and cryobiology, imaging diagnosis, molecular biology and "omics"

Histological cut of a paraffin-embedded blastocyst: optimized protocol for murine blastocysts

Alejandra Usón Gracia[1], Marta López Morató[1], José Mijares Gordún[1], Soledad Sánchez-Mateos[1], Francisco Miguel Sánchez-Margallo[1], Ignacio Santiago Álvarez Miguel[2], Nuria Hernández Rollán[1]

[1]Jesús Usón Minimally Invasive Surgery Centre, Spain; [2]Cell Biology Department. Faculty of Medicine. University of Extremadura.

Keywords: blastocysts, Histological-cuts, paraffin-embedded.

Paraffin-embedded tissues have been used for research and therapeutic applications for decades, as they represent a valuable tool in histology and for molecular analysis, as well as being a way to preserve tissue samples for long periods at a low cost. The information currently available to embed blastocysts into paraffin blocks include: protocols using gelatin and paraffin, protocols to embed a piece of uterus containing the blastocyst already implanted, and a few protocols for *Xenopus* or bovine embryos, using specific equipment that might not be available in every laboratory. Nonetheless, little information is available on easy protocols to embed mouse blastocysts into a paraffin block to then make histological cuts. The purpose of this work was to create an optimized protocol to embed mouse blastocysts into paraffin blocks, without using gelatin, and to perform histological cuts of the sample with the morphology well preserved, which can then be used for subsequent analysis. For this protocol we used 3 in vivo mouse blastocysts and performed 20 cuts in total with the microtome. Each one of them was fixed with 4% PFA for 30 min, permeabilized with 0.2% of Triton X-100 for 30 min, stained with 2 % eosin for 15 seconds to facilitate its visualization, dehydrated using different concentrations of ethanol (96% and absolute) for 10 min each, immersed in xylene for 5 min and embedded into the paraffin block. The next day, the paraffin block was cooled down and cut into 6 μm sections with a microtome, and the sample was processed to remove the paraffin, stain the cellular structures and be visualized under a microscope. Out of the 20 histological cuts performed, 65% of the sections contained just some cells of the blastocyst or the blastocyst folded onto itself, but the protocol was optimized based on the results and problems encountered until good histological cuts of the blastocyst with the morphology well preserved were obtained. Sections were considered of good quality when the samples had a circular shape and the nucleus and cytoplasm of the cells could be identified. This optimized protocol can be used to obtain good quality histological sections of a blastocyst, which can be used for studies involving *in situ* hybridization, immunohistochemistry, enzyme histochemistry, DNA, RNA or protein extractions, analysis of biomarkers, characterization of surface markers of stem cells integrated into the embryo, to analyze the effect of potential compounds that could be used to improve the embryo culture media, to prepare histological material for educational purposes, etc. Some of these studies could represent a valuable source of new information for the field of reproductive biology.

Workshop I: The impact of heat stress on dairy cows fertility-The management point of view

Efficient cooling management and remedial hormonal treatment to alleviate the effect of heat stress on ovarian function in dairy cows

Zvi Roth

The Hebrew University of Jerusalem, Israel.

Keywords*:* Heat stress, cooling management, hormonal treatments, reproduction

The impact of elevated temperature on the reproductive performance of dairy cows is becoming a worldwide problem, as a result of climate change and intensive genetic selection for high milk production. Reduced fertility is no longer confined to subtropical and tropical climates; adverse effects of elevated temperature have been reported in dairy farms located in cooler regions, such as Europe, and in temperate and Mediterranean climate zones. Furthermore, the effects of heat stress are not limited to the hot months: they carry over to the following cooler months, resulting in long-term effects throughout the year. Various heat-abatement strategies have been developed in the last four decades; these include providing shade to block direct solar radiation, ventilation, and indirect and/or direct cooling with water. The direct cooling approach is based on short-term spraying of water followed by its evaporation from the skin) constituting one cycle). Efficient cooling requires several cooling windows per day, each consisting of several cycles and lasting about 30–50 min. The efficiency of cooling on commercial farms can be conveniently compared by calculating the ratios between summer and winter milk production and conception rate. Calculations demonstrate that by using efficient cooling management, it is possible to maintain 98% of the winter milk production in summer; however, summer conception rate reaches only 68% of that in winter. Findings indicate that the reproductive system is highly susceptible to thermal stress and additional means are required to improve conception in the summer. Understanding the mechanism by which thermal stress impairs ovarian function led to the development of supporting hormonal treatment. The long-term effects of seasonal heat stress on the hypothalamus–pituitary–ovarian axis involve various impairments. These include alteration of the ovarian pool of follicles and their enclosed oocytes and impaired function of the corpus luteum, expressed by reduced plasma progesterone concentration. Induction of three consecutive 9-day follicular waves during the summer and fall improved conception rate (37 vs. 53% for control and treated cows, respectively) in primiparous cows. Treatment was more effective for multiparous cows with a high body condition score (BCS) and low somatic cell count. Administration of a controlled intravaginal progesterone-releasing device on day 5 ± 1 post-AI for 13 days was found beneficial for a subgroup of cows with low BCS at peak lactation compared to their control counterparts (53 vs. 27%, respectively) and for cows exhibiting both low BCS and postpartum reproductive disorders, compared to their control counterparts (58 vs. 14%, respectively). In summary, the reproductive tract, and in particular the ovarian components (i.e., follicles, oocytes, corpus luteum), are highly sensitive to elevated temperatures. Using an efficient cooling system to maintain normothermia in cows is a prerequisite for any additional remedial approach. Given that the effect of heat stress on fertility is multifactorial in nature, a combination of several treatment approaches might be most effective.

Embryo transfer- a promising tool for improving fertility during heat stress

Peter James Hansen

University of Florida, United States of America.

Keywords: embryo transfer, heat stress, cattle.

The major cause of infertility caused by heat stress is damage to the oocyte and early embryo by direct effects of elevated temperature and the physiological changes in the cow caused by heat stress. The developing embryo develops increased resistance to maternal hyperthermia by Day 3 of pregnancy. It is this characteristic of embryonic development that makes embryo transfer, typically performed at Days 6 to 8 after estrus, an effective tool to increase fertility during heat stress. Pregnancy rates following embryo transfer in the summer can be twice as high as pregnancy rates after artificial insemination. Moreover, differences in pregnancy rates between summer and winter are much less for embryo transfer than for artificial insemination. Coupling embryo transfer with an ovulation synchronization scheme like Ovsynch can make it possible to bypass effects of heat stress on estrus detection. The major limitation to the economic use of embryo transfer in commercial dairy and beef system is the cost of the procedure, which must be kept low to make implementation of an embryo transfer program economical.

Using thermoprotective factors to alleviate the effects of heat stress on the ovarian pool of oocytes: lessons from the lab bench to the field

Fabíola Freitas de Paula-Lopes

Federal University of Sao Paulo, Brazil.

Keywords: antioxidant, anti-apoptotic, survival factors.

Growth and development of ovarian follicles and oocytes require a series of coordinated events leading to successful ovulation, oocyte maturation, fertilization and preimplantation embryonic development. In cattle, such events can be disrupted by high environmental temperature leading to heat stress. Adverse environmental temperatures observed during the hot months of the year in subtropical and tropical climates reduce fertility in lactating dairy cows. Heat stress compromises follicular development, hormonal secretion, endometrial and oviductal function, oocyte and preimplantation embryonic development. Oocyte susceptibility to heat stress has been demonstrated during the germinal vesicle (GV) and maturation periods. In vivo and in vitro studies indicated that exposure of bovine oocytes to elevated temperature affects the cellular and molecular machinery required for proper oocyte function. For example, heat stress increased mitochondrial production of reactive oxygen species (ROS) altering the oocyte balance between ROS accumulation and removal by intracellular antioxidants. Heat stress also compromised mitochondrial function, nuclear and cytoplasmic maturation, induced cytoskeleton disorganization and apoptosis. Many efforts have been employed to alleviate the low fertility associated with heat stress. Therefore, the objective of this work is to highlight basic and applied strategies to protect oocytes from heat stress. Recently, molecules such as insulin-like growth factor I, astaxanthin, melatonin, epigallocatechin gallate, caspase inhibitors and sphingosine-1-phosphate were identified as thermoprotective factors for bovine oocytes. These factors rescued several cellular functions damaged by heat stress enhancing the ability of the oocyte to be fertilized and reach the blastocyst stage. Moreover, in vivo administration of antioxidants improved reproductive performance in heat stressed animals. Therefore, manipulation of themoprotective molecules has the potential to mitigate the deleterious effects of heat stress on the bovine oocyte improving fertility during summer.

Adaptation to heat stress in sows: an unfinished business

Pol Llonch

School of Veterinary Science, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain.

Keywords*:* animal welfare, heat stress, pigs.

Animal welfare and production efficiency are impaired by stress. Exposure to heat (measured by the Temperature Humidity Index) can lead to physiological and behavioral changes indicative of stress. According to recent predictions on climate change, severe heat events are forecasted to increase in intensity and frequency throughout the next decades, likely affecting animal production. This is of special interest in pig production as according to FAO, more than 50% of pig production occurs in warm climates, with predicted faster temperature growth than temperate areas. Heat stress response is a multisystemic adaptive mechanism triggered to cope with high environmental temperature. When heat exceeds the adaptation capacity of pigs, the body temperature increases, unbalancing homeostasis, which has negative consequences for production and welfare. Intensive breeding have led to a reduction of the adaptation capacity that confer a reduction in resilience towards environmental challenges. Pigs are particularly sensitive to heat because they lack functional sweat glands and the presence of a thick layer of subcutaneous adipose tissue that prevents heat dissipation. Besides the previous limitations, pigs kept in intensive conditions are prevented to perform most of natural behavior regulating body temperature, such as wallowing, which exacerbates the diminished adaptation capacity. The effects of heat stress in pigs are diverse, perturbing several body systems. Respiration rate increases to optimize the transpiration capacity and reduce body temperature. Activity decreases in order to reduce the metabolic source of heat due to muscle contraction. An increase in satiety during heat events alter feeding behavior and reduce the feed intake. Reproduction performance is linked to this reduction in feed intake, as lower access to nutrients leads to a loss of body condition and a negative energy balance, provoking reproductive problems associated with inadequate ovarian function. These effects are readily apparent on-farm, including anestrus, reduced farrowing rates, increased abortion rates, and reduced litter size. Heat is the environmental stressor that produces the highest (negative) impact on pig production, and it is likely that reproduction plays a major role.Strategies of heat stress mitigation can either focus on reducing the environmental challenge to which pigs are submitted or alleviating the consequences of the stress response on the organism. In the first approach, the reduction of ambient temperature (5–7°C) inside swine barns using evaporative cooling systems is the most extended strategy in intensive pig production. Alleviating the consequences of heat stress, stimulating feed intake using dietary strategies (i.e. additives) and feeding management are, to date, the most efficient strategies. Out of housing and management strategies, improving pig resilience to heat stress through breeding and epigenetic strategies are promising approaches addressing the fundamental root, which is adaptation.

Supported by Juan de la Cierva – Incorporación fellowship (IJCI-2016-30928).

Workshop II: Social acceptance of reproductive technologies in livestock

Social acceptance of Reproductive Technology in livestock

Roger Sturmey

Centre for Cardiovascular and Metabolic Research, Hull York Medical School, York, United Kingdom.

The manipulation of animal fertility has been essential for the development of current agricultural practise. The practice of utilising reproductive performance of animals can be traced back thousands of years, and on the whole, there is widespread acceptance of the strategic use of animal fertility in modern agriculture, even if this acceptance is borne of a certain 'rational ignorance'. Reproductive technology is a rare example of scientific research that has made immediate translational impact into society. However, it's position in society is more broad than 'science' since it touches on an emotive subject – about which almost everyone has an opinion. Indeed, few other areas of biological research have captivated front page headlines in quite the same way as reproductive breakthroughs; from the birth of the first IVF baby in 1978, through to successful cloning in the mid 90s and to the current era of genomic editing. There are a number of studies that explore the societal and ethical aspects of reproductive technology in human clinical medicine, however its use in agriculture can present even more challenging topics for society. The concept of combining of reproductive technology with agricultural practices can stir evocative and powerful views in wider society. However, these views may arise from limited information, or sensationalist representation in the media and online. It therefore becomes an imperative to take a proactive approach to defining and describing the use of reproductive technologies in a responsible and considered manner in livestock. Terms that are familiar to practitioners and researchers may be unclear and, even alarming to individuals with less understanding. The AETE seeks to embark on a journey to critically reflect on the real and perceived impacts of modern assisted reproductive practices on animal welfare and to consider the potential perception by the wider public. Moreover, this workshop will seek to consider the representation and appearance of reproductive technologies as they are used in livestock management and will run a workshop at the upcoming annual meeting. The aspiration from this workshop is to improve the readiness of the AETE to become involved in playing a wider role in informing public opinion.

Authors index