AETE

Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

37ème COLLOQUE SCIENTIFIQUE

37th SCIENTIFIC MEETING

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"AETE Virtual"

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6th and 13th September, 2021

AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, and PHYSIOLOGY of REPRODUCTION

President´s letter

Dear Friends, dear Colleagues,

Summer time and holidays are arriving after a tough period of changes, adaptations to online meetings, limited social contacts etc. It is incredible how a very small virus can dominate the world. Nevertheless, the Board of the AETE did its utmost to stay present and provide our members with new and attractive content. I hope you understand that the decision to cancel our physical meeting in Dublin again was very hard to make. I would like to take the opportunity here to thank Patrick Lonergan and the Local Organising Committee for all the work they did to prepare this meeting. I would like to express my appreciation for their flexibility and understanding.

However, we moved forward as a team and the Board rapidly decided to provide an attractive alternative for our members. Instead of organizing a full digital 2-day conference, we opted for an attractive digital alternative: our webinar series. Five webinars are now planned during the second half of 2021. The first one, co-hosted by the Association of Embryo Technologies of the German speaking countries (AET-d) was a real succes. More than 250 people from 42 different countries registered, clearly showing that the topic and the format were well chosen. The webinar was chaired by Christine Wrenzycki and a series of very good speakers covered topics related to the new European “Animal Health Law”, the consequences for animal breeding and for the import and export of semen and embryos.

The next webinar will be on **6th October, 18h**, followed by our General Assembly meeting. Here again Zoom will be used as our digital platform, all nicely masterd by Roger Sturmey. This next webinar will cover a very important topic both for practitioners and scientists: "When good things go bad: failures and errors in the IVF lab"! We have Rita Vassena (Spain) already confirmed as our invited speaker. In the same webinar, other practitioners will provide examples of good practice when it comes to failing blastocyst formation, infections and working under Covid conditions. On **27th October, 18h**, the Italian Society of Embryo Technolgies (SIET) will co-host the next webinar covering an attractive subject: “Embryo transfer applied in dairy cow fertility management”. Here we have Daniela Demetrio as our confirmed speaker. She is the President of the AETA. We thank the SIET for this new and promising collaboration and we look forward to this very appealing webinar.

But what about our students? As you all know, students and young scientist are the cornerstone of our society. Approximately 50 scientific abstracts have been submitted this year. The best 10 abstracts were selected for oral presentation during our two “student webinars” on **September 6th and 13th, at 15h.** Each webinar will contain 5 presentations, with sufficient time for questions and discussion. Please be there and support our abstract presenters! A real live voting will make it possible to select the best oral presenter of each day. They will win a free registration for our meeting in Utrecht (2022) and a 250 euro prize.

Our next meeting ... what shall we say about it! Well, it has to be, and will be, a physical meeting. We are all looking forward to seeing each other again and to be able talk without the aid of a computer. We will meet in Utrecht, The Netherlands. The Local Organizing Committee is preparing the best venue ever ... So, please block your agenda on **September 15 th and 16 th, 2022**!

Of course, I like to thank our Sponsors as they all decided to stay on board during these challenging times. They are all looking forward to seeing you again in person next year in Utrecht.

Finally, I thank all members of the Board of our Society. Again, they have shown their commitment and loyalty to our society. We went through a difficult and challenging period demanding exceptional efforts from all of them. I thank my team for their continuous support and their hard work.

Enjoy your summer time!

Happy holidays,

Jo Leroy, President of the AETE

Commercial Embryo Transfer Activity

in Europe 2020

Collated by Hèlène Quinton

National data collectors

Country	Collector	Comment
Austria	Friedrich FÜHRER	
Belarus	Victor MADISON ; A. DESHKO	
Belgium (South)	Isabelle DONNAY	
Belgium (Flanders)	Peter VERCAUTEREN	No data for two years
Bosnia Herzegovina	Teodor MARCOVIC	No data for two years
Croatia	Mario MATKOVIC	No data for two years
Denmark	Henrik CALLESEN	
Estonia	Ants KAVAK	
Finland	Seija VAHTIALA	
France	Serge LACAZE	
Germany	Hubert CRAMER	
Greece	Foteini SAMARTZI	
Hungary	Szabolcs SIMAI	
Ireland	Patrick LONERGAN	No data this year
Israel	Amir SHIFMAN	No data this year
Italy	Giovanna LAZZARI	
Latvia	Ilga SEMATOVICA	
Lithuania	Rasa NAINIENE	
Macedonia	Toni DOVENSKI	No data for two years
Norway	Marja MIKKOLA	
Poland	Jedrzej JASKOWSKI	No data for two years
Portugal	Joao Nestor CHAGAS E SILVA	
Romania	Stefan CIORNEI	
Russian Federation	Victor MADISON ; Denis KNUROW	
Serbia	Aleksandar MILOVANOVIC	
Slovakia	Dalibor POLAK	
Slovenia	Aleksandar PLAVSIC ; Janko MRKUN	
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Sweden	Renee BÅGE	
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The Netherlands	Anna BEKER VAN WOUNDENBERG; Erik MULLART	
Turkey	Ebru EMSEN;Fatih DEMIRASLAN	No data for two years
Ukraine	Victor MADISON	No data this year
United Kingdom	Brian GRAHAM	No data for two years

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Greece	Foteini SAMARTZI	
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Turkey	Ebru EMSEN;Fatih DEMIRASLAN	No data for two years
Ukraine	Victor MADISON	No data this year
United Kingdom	Brian GRAHAM	No data for two years

Declared bovine *In vivo* embryo production

	Dairy				Beef				All		
Country	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions	% Coll. with sexed semen	Embryos & ova	Transferable embryos	Collec-tions total	Transferable embryos total	Embryos/ collection
Austria	522	4%	4 032	3 537	9	0%	123	63	531	3 600	6,8
Belarus	334	26%	1924	1069	35	0%	176	139	369	1 208	3,3
Belgium	58	0%	434	291	731	0	5155	3365	789	3 656	4,6
Denmark	794	0%	7 072	5 050	47	0%	677	505	841	5 555	6,6
Estonia					3	0%	32	31	3	31	10,3
Finland	433	8%	4 156	2 951	2	0%	28	20	435	2 971	6,8
France	5121	20%	49372	30332	1021	0,1263	13271	7173	6142	37 505	6,1
Germany	3143	0%	30 800	21 411	459	0	5095	3230	3602	24 641	6,8
Greece	12	0%	30	10	0		0	0	12	10	0,8
Hungary	346	0%	1 651	565	61	0%	402	347	407	912	2,2
Italy	2445	54%	25183	17359	131	0,084	1283	890	2576	18 249	7,1
Latvia	4	0%	18	15	0		0	0	4	15	3,8
Lithuania	4	0%	21	16	0		0	0	4	16	4,0
Netherlands	2246	3%	16 708	12 512	0		0	0	2246	12 512	5,6
Norway	223	2%	1 629	1249	5	0%	37	27	228	1276	5,6
Portugal	114	77%	112	536	5	0%	28	18	119	554	4,7
Romania	4	0%	21	21	0		0	0	4	21	5,3
Russian Federation	611	72%	7619	3155	410	0,0293	4041	2968	1021	6 123	6,0
Serbia	7	0%	29	25	0		0	0	7	25	3,6
Slovenia	28	57%	179	131	5	20%	45	27	33	158	4,8
Spain	169	59%	1674	945	116	12%	1583	1232	285	2 177	7,6
Sweden	91	14%	683	416	0		0	0	91	416	4,6
Switzerland	687	43%	4 996	4 860	0		0	0	687	4 860	7,1
Total	**17 396**	**20%**	**158 343**	**106 456**	**3 040**	**5%**	**31 976**	**20 035**	**20 436**	**126 491**	**6,2**

Declared bovine *In vitro* embryo production (OPU-IVP)

	Dairy				Beef				All		
Country	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	% OPU with sexed semen	Oocytes	Embryos	OPU	Embryos	Embryos /OPU
Finland	625	1%	5 697	1 569					625	1 569	2,5
France	725	6%	6 836	1962	63	0%	1 000	461	788	2 423	3,1
Germany	2 276	0%	31 858	6 487	219	0	3420	687	2 495	7 174	2,9
Italy	112	64%	1 169	331	70	0%	715	147	182	478	2,6
Netherlands	5 495	2%	58 691	12 657					5 495	12 657	2,3
Norway	137	0%	1062	297					137	297	2,2
Serbia	24	38%	115	32					24	32	1,3
Spain	75	67%	1 140	353	85	27%	898	423	160	776	4,9
Switzerland	83	35%	928	306	1	0%	19	5	84	311	3,7
Total	**9 552**	**3%**	**107 496**	**23 994**	**438**	**3%**	**6 052**	**1 723**	**9 990**	**25 717**	**2,6**

Declared bovine *In vitro* embryo production – abattoir

	Dairy			Beef		
Country	Donors	Oocytes	Embryos	Donors	Oocytes	Embryos
Greece	297	1 442	227			
Netherlands	0	5 181	656	0	0	0
Portugal	0	50	20	0	6 213	517
Romania	17	127	8	0	0	0
Serbia	0	0	0	5	53	12
Spain	64	1 944	894	15	6 219	3 018

Declared bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	In Vivo	In Vitro	In Vivo	In Vitro
France	1 599	0	1 455	0
Germany	5450	14	8	0
Netherlands	0	0	0	4 371
Spain	0	23	0	12
Total	**2 144**	**37**	**1 463**	**4 383**

Declared bovine embryo transfers and exports- *In vivo*

Country	Dairy				Beef				Total embryos transferred
	Fresh embryos transferred	Frozen embryos			Fresh embryos transferred	Frozen embryos			
		Domestic transferred	Foreign transferred	Exported		Domestic transferred	Foreign transferred	Exported	
Austria	1 469	803	564	41	10	0	30	40	2 876
Belarus	876	493	383	0	100	82	18	0	1 952
Belgium	31	99	20	9	519	2437	39	13	3 145
Denmark	2942	1 414	0	0	294	141	0	0	4 791
Finland	680	1 600	179	524	0	0	0	0	2 459
France	14241	11833	1048	261	1779	3656	119	240	32 676
Germany	9293	11945	0	95	924	1784	0	8	23 946
Hungary	485	269	98	0	14	75	116	0	1 057
Italy	7300	1912	0	0	0	0	0	0	9 212
Latvia	8	1	0	0	0	0	0	0	9
Netherlands	386	763	20	0	0	0	0	0	1 169
Norway	0	1093	7	0	0	11	31	0	1 142
Portugal	144	354	7	0	3	26	62	0	596
Romania	8	0	0	0	0	0	14	0	22
Russian Federation	1170	1368	57	0	128	174	0	0	2 897
Serbia	15	0	0	0	0	0	80	0	95
Slovenia	75	20	6	0	17	6	12	0	136
Spain	298	410	81	0	139	310	2	152	1 240
Sweden	220	1160	0	0	0	0	0	0	1 380
Switzerland	794	1217	0	48	0	0	0	0	2 011
Grand Total	**40 435**	**36 754**	**2 470**	**978**	**3 927**	**8 702**	**523**	**453**	**92 811**

Declared bovine embryo transfers and exports – *In vitro*

	OPU				Abattoir		
Country	Fresh embryos transferred	Domestic frozen embryos transferred	Foreign frozen embryos transferred	Embryos exported	Fresh embryos transferred	Domestic frozen embryos transferred	Total embryos transferred
Belgium	0	97	2	0	0	0	99
Finland	0	715	0	320	0	0	1 035
France	1 006	695	109	99	0	0	1 909
Germany	3 290	5 040	0	0	0	0	8 330
Italy	280	51	0	0	0	37	368
Netherlands	3 762	3 455	51	0	0	0	7 268
Norway	0	198	0	0	0	0	198
Portugal	0	0	19	0	20	0	39
Serbia	0	18	0	0	0	5	23
Spain	279	173	30	0	16	19	517
Grand total	**8 617**	**10 442**	**211**	**419**	**36**	**61**	**19 786**

Declared embryo production, transfer and export in other species – *In vivo*

		Embryo collection		Embryo transfer			
Species	Country	Collections	Viable embryos	Fresh embryos	Frozen domestic	Frozen foreign	Exported embryos
Sheep	Portugal	0	0	0	8	0	0
	Romania	4	27	27	0	0	27
	Russian Federation	0	0	0	0	2 683	0
	Serbia	0	0	12	0	0	12
	Spain	3	35	15	0	0	15
	Sweden	0	0	0	0	285	0
	Total	**7**	**62**	**54**	**8**	**2 968**	**54**
Goat	Spain	17	319	32	12	0	0
Horse	France	4 437*	2 219*	2 219*	0	0	0
	Russian Federation	4	3	3	0	0	0
	Spain	2	2	2	0	0	0
	Sweden	29	24	24	0	0	0
	Total	**4 472**	**2 248**	**2 248**	**0**	**0**	**0**

*Estimate from the number of officially declared pregnancies

Declared embryo production, transfer and export in other species – *In vitro*

		Oocyte collection			IVP embryo transfer			
Species	Country	OPU	Oocytes	Embryos	Fresh embryos	Frozen domestic	Frozen foreign	Exported embryos
Water Buffalo	Italy	5	216	70	0	8	0	0
Horse	Italy	2 825	28 614	5 345	25	527	0	2 689
	France	0	0	0	0	0	23	0
	Switzerland	41	254	14	0	0	0	0
	Total	**2 866**	**28 868**	**5 359**	**25**	**527**	**23**	**2 689**

SHORT COMMUNICATIONS

SHORT ORAL COMMUNICATION COMPETITION

Bisphenol S impaired oestradiol secretion during ovine basal folliculogenesis in vitro.

Claire Vignault[1,2], Véronique Cadoret[1,2], Peggy Jarrier-Gaillard[1], Pascal Papillier[1], Alice Desmarchais[1], Ophélie Téteau[1], Aurélien Binet[1,3], Fabrice Guérif[1,2], Sébastien Elis[1], Virginie Maillard[1]

[1]UMR 0085 PRC, INRAE, CNRS, Université de Tours, IFCE, Nouzilly, France; [2]Service de Médecine et Biologie de la Reproduction, CHRU Tours, France; [3]Service de Chirurgie pédiatrique viscérale, urologique, plastique et brûlés, CHRU Tours, France; claire.vignault@inrae.fr

Keywords: Ovary, Endocrine disruptor, hormonal secretions

Currently known as the principal substitute for bisphenol A, bisphenol S (BPS) has been shown to affect terminal folliculogenesis by impairing steroidogenesis in granulosa cells from different species (Teteau O, et al. Reproduction. 159:571-583; 2020. Berni M, et al. Domest Anim Endocrinol. 66:48-56; 2019. Campen KA, et al. Reprod Domest Anim. 53:450-457; 2018. Amar S, et al. Int. J. Mol. Sci.21:1821; 2020) and oocyte developmental competence (Desmarchais A, et al. Int J Mol Sci. 21:1238; 2020). Nevertheless, few data are available on its effects on basal folliculogenesis. Recently, a study showed that prenatal BPS exposition could decrease the number of primary follicles in neonatal mouse ovaries (Shi M, et al. Toxicol Sci. 172:303-315. 2019), but its impacts on mono-ovulatory mammals are still unknown. Our objectives were to assess in vitro the effects of a long-term BPS exposure on basal folliculogenesis, specifically during the transition of preantral to antral follicle, and the follicular hormonal secretions using an ovine model of follicular growth previously described (Cadoret V, et al. Reproduction 153:493-508; 2017). In this study, 168 peripubertal ovine preantral follicles (180-240µm diameter) were collected from ovarian cortex strips (slaughterhouse) and cultured with BPS (0.1µM, maximal concentration measured in human follicular fluid or 10µM, higher concentration), or with ethanol vehicle (control, diluent of BPS with the same adjusted concentration in all conditions, 1/10 000) over a 15-day (D15) period. No effect of ethanol (1/10 000) on follicular growth was verified comparing with ethanol-free control. Antrum appearance, follicular survival rate (viable follicle was structurally intact with internal clear oocyte and measurable follicular growth within one week) and follicular growth (determined by follicular diameter) were monitored for each follicle on D6, D13 and D15. Their hormonal secretions were assessed in medium at D13 for oestradiol (n=121) and at D15 for anti-Müllerian hormone (AMH) and progesterone (n=105). Hormonal secretions were measured in cultured medium from each viable follicle using ELISA. These parameters were statistically analysed using either Kruskal-Wallis test or non-parametric permutational ANOVA. We showed that oestradiol secretion from follicles treated with BPS (0.1 µM, and 10 µM) was decreased compared to control (-48,8%, $p<0,001$ and -9,2%, $p=0,0375$, respectively). However, BPS had no effect on antrum appearance, on follicular survival and growth, on AMH and progesterone secretions compared to control for the 15 days of treatment. In conclusion, this work shows that BPS could affect female steroidogenesis during the basal folliculogenesis. The involved steroidogenic pathways should be now investigated. At least, these findings underline the necessity to assess the risk of BPS exposure for reproduction of mono-ovulatory mammals. They also provide an additional argument to classify BPS as an endocrine disruptor, as BPA.

Acknowledgments: We thank Albert Arnould and Thierry Delpuech for the collection of ovine ovaries.

Fundings: INRAe and the French National Research Agency (project ANR-18-CE34-0011-01 MAMBO).

Effects of c-phycocyanin purified from Spirulina platensis on preserved ram semen quality and fertility rate

Abdellatif Rahim[1,2], Saad Salhi[1,2], Abdelkhalid Essamadi[2], Bouchra El Amiri[1]
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Keywords: C-phycocyanin, Skimmed milk, Ram semen quality, Liquid storage, artificial insemination

This study was undertaken to evaluate the effect of c-phycocyanin purified from *Spirulina platensis* on the quality and *in vivo* fertilization ability of ram semen. Samples were collected from four adult fertile Boujaâd rams and diluted to a final concentration of 0.8 x 10^9 spermatozoa/ml in skim milk (SM) without (control) or supplemented with pure c-phycocyanin at 1.2 µg/ml, 2.4 µg/ml, 3.6 µg/ml or 4.8 µg/ml. The fresh semen was then stored at 5 °C and several sperm parameters (motility, viability, abnormality, and lipid peroxidation) were assessed after 0, 4, 8, and 24h of storage. The best concentration of c-phycocyanin was then used to assess sperm fertilization ability by means of artificial insemination. Two groups of ewes were insemination after oestrus synchronization (sponges and ECG) and using semen extended in SM (control) or in SM including c-phycocyanin at a fixed concentration. Statistical analyzes were performed using the JMP SAS 11.0.0 (SAS Institute Inc. Cory, NC, USA) program. The results on fresh semen quality showed a significant improvement when c-phycocyanin was added to the skim milk at a concentration of 2.4 µg/ml, whatever the time point of storage was (4, 8, and 24h). The 2.4 µg/ml also showed a significant increase in progressive and total motilities and viability. Moreover, a significant decrease in sperm abnormalities and lipid peroxidation was observed. Based on heat detection, the results of artificial insemination showed that the 2.4 µg/ml concentration improved the fertility of sheep (80%) compared to the control group (60%). However, the right values will be calculated after lambing. In conclusion, the 2.4 µg / ml concentration of c-phycocyanins improved the quality of sperm during liquid storage and also increased the fertility rate. This ability is probably due to its antioxidant capacity to scavenge reactive oxygen species (ROS) and inhibit lipid peroxidation. The study is in progress to determine the exact fertility rates after lambing.

Acknowledgments: The authors would like to thank the OCP, OCP Foundation, University of Mohammed VI, and the CNRST, Ministry of Higher Education, Scientific Research Morocco (MESRSFC) for the financial support project SHS-ELM-01/2017.

Comparison of sugars as non-permeating cryoprotectant for immature equine oocyte vitrification.

Daniel Angel-Velez[1,2], Tine De Coster[1], Nima Azari-Dolatabad[1], Osvaldo Bogado-Pascottini[1,3], Ann Van Soom[1], Katrien Smits[1]

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Keywords: Equine oocyte, sugars, vitrification.

Oocyte cryopreservation in horses would be an important complement to the growing clinical ICSI programs, but the efficiency is not satisfactory yet. Sugars are non-permeating cryoprotectants that exert an osmotic effect during vitrification. However, the influence of different sugars during vitrification and warming of equine oocytes on subsequent embryo development has not been evaluated. Therefore, we aimed to determine the effects of three sugars on the developmental competence of equine oocytes. Cumulus-oocyte complexes (COCs) were obtained from slaughterhouse-derived ovaries. The cumulus cells were removed by pipetting until around 4 layers remained. Then, COCs were vitrified immediately in three groups: sucrose (S; n=155), trehalose (T; n=160) and galactose (G: n=153). Oocytes were equilibrated for 25 seconds in base solution (BS; TCM 199 with Hanks' salts and 0.4% (w/v) BSA) with 10% (v/v) ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO), and subsequently transferred to a vitrification solution (BS with 20% EG, 20% DMSO and 0.5 M sugar), loaded onto a custom-made minimal volume (<1 µL) cryo-device (Equine Vet J. 50(3). 391-397. 2018) and plunged into liquid nitrogen within 40s. For warming, the cryo-device was placed directly into a 0.5 M sugar solution and incubated for 5 min, then COCs were transferred and washed in BS. All procedures were performed on a thermal plate at 39˚C. Once warmed, COCs were matured in 500 µl M199 with Earls' salts and 10% FBS at 38.5˚C in 5% CO_2 in air for 28 hours. Oocytes with an extruded polar body were injected by piezo assisted ICSI and presumed zygotes were cultured in 20 µl droplets of DMEM-F12 with 10% FBS under oil for 7 – 10 days at 38.2˚C in 5% O_2, 5% CO_2 and 90% N_2. A control group (n=173) with non-vitrified oocytes was included in every replicate (5 replicates). The effects of the sugars on maturation, cleavage, and blastocyst rate were fitted in generalized and linear mixed-effects models and results are expressed as least square means with standard errors. Galactose tended to result in a lower maturation rate compared to trehalose (p = 0.060) and the control group (p=0.069), but there was no significant difference in maturation rate among sugars (S: 52.4±4.1%; T: 57.4±4.1%; G: 43.1±4.1%), and control (56.9±4.0%; P > 0.05). Cleavage rates were not different between treatments (S: 53.2±5.6%; T: 61.8±5.2%; G: 73.4±5.5%), but the cleavage rate after vitrification with sucrose was significantly lower than that of the control (75.3±4.8%; p=0.02) and tended to be lower than that of galactose (p=0.066). Finally, blastocyst rates for all vitrified groups (S: 5.0±2.5%; T: 4.3±2.2%; G: 7.6±3.4%) were significantly lower compared to the control group (26.5±5.7%). Nevertheless, galactose, a monosaccharide tested for the first time in equine oocyte vitrification, resulted in the highest blastocyst rates after vitrification, as well as in equal cleavage rates compared to the control. Therefore, galactose should be considered as an alternative sugar for future optimization of vitrification protocols for equine immature oocytes.

Acknowledgements: The authors thank Petra Van Damme for her excellent technical assistance.

High bovine embryo production in OPU-IVP commercial programs using heifer and cow donor oocytes and X-sorted and unsorted sperms

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Keywords: OPU-IVP, X-Sorted sperm

The OPU-IVP technology combined with X-sorted sperm is a promising alternative to produce more female embryos in a dairy cattle program. However, the use of sexed sperms results in variable blastocyst rates (Steele *et al.*, 2020). The aim of this study was to evaluate the efficiency in IVP of X-sorted sperm compare to unsorted semen on grade 1 OPU-*in vitro* embryo production in high genetic merit Holstein heifers and cows under commercial conditions performed at the Biotechnology AURIVA-ELEVAGE in Denguin, France. With heifer donors (n=107), 145 sessions were performed with unsorted semen (Groupe1, G1) and 29 with X-sorted semen (Group 2, G2). With cow donors (n=147), 108 sessions were performed with unsorted semen (Group 3, G3) and 334 with X-sorted semen (Group 4, G4). Donors were stimulated with decreasing pFSH doses (Stimufol; Reprobiol, Belgium) twice daily during 3 days (total dose: 300 µg for cows and 200 µg for heifers). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. COCs were fertilized with frozen–thawed unsorted or X-sorted sperm in modified Tyrode's bicarbonate buffered solution medium using different non pre-tested bulls. Presumptive zygotes were cultured in SOF medium up to Day 7 at 38.5 °C in 5% CO2 and 5% O2 with maximum humidity. Grade 1 expanded blastocysts (IETS classification) were recorded on days 6.5 and 7. Embryo production and quality was analyzed with ANOVA ($p<0.05$ was significant). No significant differences were detected in the total number of selected COCs for IVM between G1 and G2 groups (11.09±6.8 *vs.* 10.6±6.0 respectively, $p>0.05$) and between G3 and G4 groups (12.4±9.7 *vs.* 10.8±7.3 respectively, $p>0.05$). At D7, Grade 1 expanded blastocyst rates were not different between G1 and G2 groups: 42.5±21.6 *vs* 40.5±20.0 respectively ($p>0.05$) and between G3 and G4 groups: 59.0±20.5 *vs.* 52.7±25.0 respectively, ($p>0.05$). Moreover, grade 1 embryo development rate was higher in cows (G3+G4: 54.4±24.0) compared to heifers (G1+G2: 42.2±21.4; $p<0.001$). Reported per session, 4.7±3.6 *vs.* 4.3±3.7 grade 1 embryos were produced in G1 and G2 respectively ($p>0.05$) and 7.3 ± 5.4 *vs.* 5.6 ± 4.3 grade 1 embryos were produced in G3 and G4, respectively ($p<0.01$). Assuming a sex ratio of 50% in unsorted semen and 90% in X-sorted semen, differences were noted ($p<0.01$) in total number of presumptive female embryos produced per session in G3 group (3.7±2.7 female embryos per session) compared to G4 (5.1±3.9 female embryos per session). This difference has not been evidenced in heifers, indeed, 2.4±1.8 presumptive female embryos has been produced per session in G1 vs. 3.8±3.4 in G2 ($p>0.05$). In conclusion, our work confirms the efficiency of OPU-IVP technology to produce high number of grade 1 embryos using X-sorted under commercial conditions. Furthermore, OPU-IVF technology seems to be more efficient in donor cows than in donor heifers in terms of blastocyst rate.

Temporal changes in the oviduct following the introduction of an obesogenic diet in outbred mice: a focus on transcriptomic changes

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Keywords: in vivo mouse, oviduct, qPCR

Metabolic disorders associated with consumption of a western type diet (high fat/high sugar (HFHS) diet) are strongly linked with reduced fertility in women. Direct detrimental effects of such metabolic alterations on oocyte quality have been documented, however, the impact on the oviductal microenvironment where fertilization and early embryo development take place is less characterised. Furthermore, the onset and duration of changes after the start of a HFHS diet remain unclear. In this study, we aimed to test whether the introduction of a HFHS diet in mice can lead to cellular and oxidative stress (OS), and inflammation in the oviductal epithelial cells (OECs) at transcriptomic level. Five week old female outbred Swiss mice were fed with either a control (CTRL; 10% fat) or HFHS (60% fat in diet, 20% fructose in drinking water) diet. Mice (n=3 per treatment per timepoint) were sacrificed and OECs from the whole oviduct were collected at 3 days, 1 week (1w), 4w, 8w, 12w and 16w after the start of dietary treatment. Total RNA extracts were reverse transcribed and qPCR was used to study the expression of genes involved in response to OS (NRF1, NRF2, SOD2, PRDX1, PRDX3, PRDX6), endoplasmic reticulum (ER) and mitochondrial stress (BiP, ATF4, HSPA8, HSPD1 and HSPE1) and inflammation (IL1b). ACTB, B2M and H2AFZ were used as housekeeping genes. A Kruskal Wallis test was used to compare changes in gene expression over time within the CTRL group, showing no effects of age ($P>0.05$). Subsequently, the data were grouped into four phases: acute (3d), early (1w), mid (4 and 8w) and late (12 and 16w) phase. Independent sample t-tests on log-transformed data were used to compare gene expression of the HFHS and CTRL groups within each phase. Acute responses to HFHS diet (at 3d) indicated an increased level of OS in OECs, since both BiP (ER chaperon) and PRDX3 (mitochondrial antioxidant (AO)) were significantly upregulated ($P<0.05$), together with a tendency ($P=0.077$) for higher NRF2 expression (a transcription factor that regulates the expression of AO proteins such as mitochondrial SOD2). At the early phase (1w) this was indeed followed by an upregulated SOD2 ($P=0.044$) and a tendency for higher PRDX1 expression ($P=0.086$), whereas ER stress (BiP) was temporarily normalized ($P>0.1$). Extended exposure to a HFHS diet up to 4-8w (mid phase) was associated with a second wave of NRF2/SOD2 AO response ($P<0.07$) and ER stress (BiP, $P<0.05$). These mid phase responses remained obvious during the late phase and were accompanied by significant upregulation of inflammatory marker IL1b ($P=0.05$) as well as PRDX6 ($P=0.03$). PRDX6 is an AO that regulates TNF-induced apoptosis through IL1b production. In conclusion, exposure to a HFHS diet results in acute OS in the OECs evident just after 3 days, which then initiates a cascade of transcriptomic changes to control mitochondrial ROS production and ER stress. This, however, cannot avoid ultimate signs of inflammation. These results suggest that consuming a HFHS diet even for a short period might compromise the oviductal microenvironment and may thus compromise fertilization and/or early embryo development.

Impaired post-blastocyst ovine development in vitro by TGFß inhibition can be partially rescued by ROCK inhibitor

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Keywords: epiblast, ovine, TGFß signaling pathway

Most pregnancy losses in farm ungulates are reported during the second week of gestation. During this period, critical developmental processes take place in the embryo, such as trophoblast proliferation, hypoblast migration along the inner surface, and epiblast development into an embryonic disc (ED). The developmental defects leading to post-blastocyst mortality have been difficult to explore *in vivo*, but several evidences point to the epiblast as the most vulnerable lineage and TGFß is one of the main signaling pathways involved in epiblast development into an ED. Rho-associated protein kinase inhibitor (ROCKi) supplementation promotes epiblast survival *in vitro* (Ramos-Ibeas *et al.*, IETS, 2021). The objectives of this study have been to evaluate the effect of TGFß inhibition on embryo development after the blastocyst stage through a recently developed *in vitro* culture system (Ramos-Ibeas *et al.*, Reproduction 2020), and to assess a possible rescuing effect of ROCKi on embryo development. D6/7 *in vitro* produced ovine blastocysts were cultured over agarose gels in 1) N2B27 medium (N; n=91); 2) N2B27 supplemented with TGFß inhibitor (N+SB; 20 µM SB431542; n=98); 3) N2B27 supplemented with ROCKi (N+R; 8 µM Y27632; n=124); or 4) N2B27 supplemented with both inhibitors (N+SB+R; n=127). Embryos were sequentially imaged and fixed at days (D) 8, 10, 12 and 14. Embryo survival was recorded (not collapsed embryos showing growth), embryo area was measured with ImageJ and the development of specific lineages was assessed by immunostaining for SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (trophectoderm). Embryo survival was not affected by any inhibitor treatment, but embryo size was significantly reduced after TGFß inhibition at D14 (0.81 ± 0.15 mm^2 in N *vs.* 0.38 ± 0.07 mm^2 in N+SB; mean ± s.e.m.; One-way ANOVA; $p<0.05$). The surface of the embryo covered by hypoblast cells (SOX17+) was significantly reduced after TGFß inhibition at D14 (87.57 ± 5.76% in N *vs.* 30.75 ± 6.76% in N+SB), and this effect was partially rescued by ROCK inhibition (62.8 ± 4.9% in N+SB+R; One-way ANOVA; $p<0.05$). Epiblast (SOX2+) cell number was significantly lower after TGFß inhibition at D10 (57.71 ± 13.37 in N *vs.* 13.84 ± 3.81 in N+SB), 12 (60.63 ± 15.04 in N *vs.* 14.93 ± 4.19 in N+SB) and 14 (58.4 ± 19.4 in N *vs.* 10.69 ± 3.85 in N+SB). This effect could be counteracted by ROCKi, but only at D10 (42.46 ± 5.88 in N+SB+R; One-way ANOVA; $p<0.05$). Finally, the number of embryos that developed an ED was significantly reduced after TGFß inhibition at D12 (8/12 [~67%] in N vs. 0/12 [0%] in N+SB) and 14 (9/17 [~53%] in N vs. 2/16 [~12%] in N+SB), and this number was significantly increased by ROCK inhibition at 12 (7/28 [25%] in N+SB+R; Chi-square; $p<0.05$). In conclusion, TGFß inhibition from blastocyst formation until D14 negatively affected embryo size and the development of the hypoblast. The epiblast was the most affected lineage after TGFß inhibition, showing reduced SOX2+ cell number and ED formation rates from D10 onwards, although this effect could be partially rescued by ROCK inhibition until D12. Thus, TGFß signaling pathway is crucial for epiblast development and embryo survival after the blastocyst stage.

Support: AGL2017-84908-R and RYC2018-025666-I from MINECO, and StG-757886 from ERC.

Transcriptional, mitochondrial activity and viability of Egyptian buffalo's granulosa cells in vitro cultured under heat elevation

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Keywords: granulosa, heat shock, reactive oxygen species, mitochondrial activity, gene expression Heat stress negatively affects reproductive processes of dairy animals. However, few reports that have focused on the molecular and intracellular response of *in vitro* cultured buffalo granulosa cells exposed to heat shock. Therefore, the present study was designed to evaluate the effect of heat stress during *in vitro* culture of buffalo granulosa cells on viability, oxidative status, mitochondrial activity and gene expression patterns. Granulosa cells were harvested after aspiration of cumulus-oocytes complexes that were collected from abattoir ovaries. The granulosa cells were cultured *in vitro* in 6 replicates either at a normal physiological temperature suitable for oocyte maturation and embryo development (38.5°C) (G1) or exposed to elevated temperature considered as sub-lethal temperature for embryo development of 40.5°C for two hours at day 3 of culture (day of confluence) then continued at 38.5°C up to day 7 (G2) according to our previous study done in buffalo. The viability of granulosa cells per every group was measured using trypan blue and quality was estimated by measuring the level of intracellular reactive oxygen species (ROS) at day 7. Moreover, metabolic activity was performed by measuring the fluorescent intensity of mitochondria. Fluorescent intensity was analyzed by image J software. Moreover, transcriptional activity was done by profiling four selected candidate genes using quantitative real-time PCR. The data were analyzed by applying one-way ANOVA and were expressed as mean ± standard error of means. Comparisons were significantly different if $p < 0.05$. Statistical analysis of data was performed using the IBM SPSS Statistics 22 program (SPSS Inc., Chicago, Illinois, USA). The expression profiles of selected target genes were analyzed using the SAS (SAS 2004) using general linear model procedure. In addition, Duncan's multiple range test was used to detect differences among means. The results indicated that the granulosa cells viability rate significantly ($P \leq 0.05$) decreased in G2 (25.1 ± 3.7%), compared to the control group (36.6 ± 5.3%) at day 3. In addition, the viability rate at day 7 decreased in G2, compared to G1 (83.7 ± 4.5 and 97.4 ± 0.4%, respectively). Nonetheless, there was a non-significant difference in ROS profile between G1 (21.7± 1.3 arbitrary unit) and G2 (15.7 ± 0.7 arbitrary unit) at day 7. However, the mitochondrial activity was higher in G1 (21.9 ± 1.9) than G2 (15.4 ± 0.8) at day 7. The expression of cellular defense (HSF1) and apoptosis-inducing gene (P53) were significantly ($P \leq 0.05$) up-regulated in G2, compared to G1 but BCL2 gene showed non-significant expression between groups. On the other hand, the steroidogenesis regulating gene (StAR) was down-regulated in granulosa cells cultured in G2, compared to G1. In conclusion, Heat stress reduced the viability of granulosa cells by inducing the expression of an apoptosis-related gene (P53) and compromised expression of genes regulating the steroid biosynthesis, which resulted in up-regulation of cell defense gene (HSF1) in an attempt to ameliorate the deleterious effect of heat stress on the biological activity of the granulosa cells.

Early postpartum metabolic and anti-oxidant profile in dairy cows is associated with changes in the granulosa cell transcriptomic profile in the dominant follicle at the time of breeding

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Keywords: Negative energy balance, NEFAs, FFAs, antioxidants, granulosa cells, cow reproduction

In this study we hypothesized that fat mobilization and blood antioxidant (AO) concentrations (ß-Carotene; bC and Vitamin E; VitE) during the early postpartum period (2w pp) in high-producing dairy cows can be linked with granulosa cell (GC) functions of the dominant follicle (DF) at the time of breeding (8w pp). Ultrasound-guided transvaginal follicular aspiration of the DF (pre-LH surge) was performed from 16 estrous-synchronized healthy multiparous cows at 8w pp. Livestock management was the same for all cows.The transcriptomic profile of the GCs was analyzed by RNAseq and was compared between subgroups of cows classified according to the median (High vs. Low) or quartile (Q4 vs. Q1) values of blood NEFAs, bC and VitE at 2w pp. Differentially expressed genes (DEGs: P-adj <0.05, 5% FDR) were determined using DESeq2 and were functionally annotated using Bioconductor packages in R. Blood bC concentration at 2w pp was positively correlated with blood and FF bC concentrations at 8w (P<0.05). Blood NEFA concentrations at w2 were only correlated positively with FF Estrogen: Progesterone (E2:P4) ratio at 8w (P<0.05), but not with AOs. Comparisons based on w2 blood **bC** concentrations (median = 190 µg/dL, Q4 ≥267 µg/dL, Q1 ≤127 µg/dL) were associated with 131 DEGs (39↑ and 92↓) (n=7 high vs. 9 low) and 192 DEGs (9↑ and 183↓) (n=3 Q4 vs. 5 Q1). Blood **VitE** concentrations at w2 (median =2.1 mg/L, Q4 ≥2.85 mg/L, Q1 ≤1.75 mg/L) were linked with 23 DEGs (21↑ and 2↓) (n=7 high vs. 9 low) and 157 DEGs (118↑ and 39↓) (n= 4 Q4 vs. 5 Q1). Comparing cows with high concentrations of **both bC and VitE** (n= 3 Q4 vs. 3 Q1) at w2 was associated with the highest number of DEGS (341; 162↑ and 179↓). Only 60-70 of the DEGs detected in the bC and/or VitE comparisons at w2 were common with DEGs induced by FF bC at the moment of follicle aspiration. In contrast, no DEGs were detected when comparing cows with high (n=10) vs. low (n=6) blood **NEFAs** at w2 (median= 635 µM), and only 64 DEGS (16↑ and 48↓) were found when comparing NEFA Q4 (n=7, ≥773 µM) vs. Q1 (n=3 ≤420 µM). None of these genes were similar to those induced by the variation in FF E2:P4 ratio or AO concentrations at 8w. Top canonical pathways of the 2w bC upregulated DEGs (Q4 vs. Q1) are: protein folding chaperons and unfolded protein responses; while those of bC downregulated DEGs are: inflammatory responses (e.g. mast cell activation and leukocyte mediated immunity), autophagy, and catabolic processes. NEFA upregulated DEGs (Q4 vs. Q1) are involved in cellular response to stress, immune response (e.g. regulation of cytokine production), and response to lipid and ketones; while NEFA downregulated DEGs are related to lipid catabolic processes, carnitine and Co-enzyme A metabolic process and cellular nitrogen metabolic processes. In conclusion, these results provide evidence that the metabolic and anti-oxidant profile of dairy cows during the early postpartum period is linked with changes in the GC functions in dominant follicles at the time of breeding, which may suggest an impact on oocyte quality and fertility.

Impact of equilibration duration combined with temperature on bovine oocyte vitrification procedures

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Keywords: temperature, permeability, meiotic spindle, apoptosis, embryo

Oocyte cryopreservation certainly represents one of the most attractive developments in the field of reproductive technologies. Despite some successes, there still remain shortcomings with methods used to cryopreserve bovine oocytes. A wide variety of approaches have been used to try to improve and optimize methods of cryopreservation. However, these procedures employed are rarely designed to specifically take account of the osmotic response of the cells according to the temperature and time of cryoprotectant (CPA) addition. In this work, using in vitro osmotic observations, we propose shorter, dehydration-based protocols at different temperatures (25°C vs 38.5°C) as a first step toward developing an optimal cryopreservation method. First, we determined the duration of the shrink-swell response after exposure of IVM bovine oocytes to the equilibration solution (ES) at 25°C and 38.5°C. Subsequently, we tested the efficiency of the optimized cell-specific exposure times for each temperature prior to vitrification/warming on oocyte spindle configuration, DNA fragmentation, and further embryo development. In vitro observations of the oocytes' osmotic behavior indicate that the time required for the oocytes to reach the equilibrium cell volume upon exposure to standard ES (7.5% DMSO + 7.5% ethylene glycol in TCM199 medium + 20% fetal bovine serum) increases as the temperature decreases: original oocyte cell volume recovery is reached within 2 min 30 sec at 38.5°C and at 5 min 30 sec at 25°C. Then, IVM bovine oocytes were exposed to the aforementioned CPAs at 25°C for 5 min 30 sec min or at 38.5°C for 2 min 30 sec and vitrified/warmed as described previously (García-Martínez et al. Int. J. Mol. Sci. 2020, 21, 7547). Statistical differences among treatments were analyzed using ANOVA (p<0.05). No differences in percentages of oocytes exhibiting a normally configured spindle and DNA fragmentation were recorded in the fresh control group (66.69 ± 2.28% and 6.17 ± 2.57%, respectively) and oocytes vitrified at 38.5°C (53.49 ± 4.00% and 11.32 ± 3.21%, respectively). However, oocytes vitrified at 25°C exhibited a significantly higher apoptosis rate (32.37 ± 4.87%) as well as lower percentage of normal spindle configuration (38.38 ± 1.52%) due to a higher proportion of oocytes with decondensed or disorganized microtubules. Similar cleavage rate and blastocyst yield were observed between the 38.5°C vitrified and fresh control groups, while vitrification after 25°C CPA exposure produced lower percentages on cleavage and embryo development. Under this premise, we were able to successfully reduce the necessary time to prepare bovine oocytes for vitrification to 2 min 30 sec when vitrification was carried out at 38.5°C, obtaining similar results on spindle morphology, DNA fragmentation and embryo development to fresh control oocytes.

This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P and grant 2017-FI-00451) and the Generalitat de Catalunya (Project No. 2017 SGR 1229).

Kinetics of bovine embryonic lineages development during post-hatching embryo culture

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Keywords: Pregnancy signaling, prostaglandin, conceptus elongation.

Conventional embryo culture conditions allow bovine zygotes to develop up to blastocyst hatching, but further development requires a more complex culture medium termed N2B27. Following hatching, the ungulate embryo undergoes critical cell differentiation, proliferation and migration processes. The inner cell mass differentiates into hypoblast and epiblast, the later evolving into an embryonic disc. Hypoblast proliferates and migrates covering the inner surface of the trophectoderm, and both extra-embryonic lineages proliferate extensively leading to a substantial growth of the conceptus (embryo proper + extra-embryonic lineages). We have previously reported that key developmental landmarks including the formation of embryonic disc (ED)-like structures and complete hypoblast migration are achieved by about half of the Day 15 (D15) bovine embryos cultured in N2B27 medium, but whether these processes occur at earlier stages remains unclear. The objective of this study has been to determine when these developmental milestones are reached during *in vitro* culture. To that aim, bovine Day 7 embryos produced *in vitro* under conventional conditions (SOF medium) were cultured in N2B27 medium and fixed in 4 % paraformaldehyde at Days 10, 11 and 12 of development. Embryo size was determined in alive embryos by stereomicroscopy on Days 9, 10, 11 and 12 of development. Fixed specimens were analyzed by immunohistochemistry to detect trophectoderm (CDX2), hypoblast (SOX17) and epiblast (SOX2) markers. Embryo diameter increased gradually from D9 to D12 of culture (0.33±0.01 vs. 0.40±0.02 vs. 0.50±0.03 vs. 0.63±0.05 mm, mean±s.e.m. for D9, D10, D11 and D12, respectively; ANOVA $p<0.05$ for D12 vs. D9 and D10 and D11 vs. D9). The percentage of embryos showing complete hypoblast migration increased significantly at all time-points analyzed (2/35 vs. 13/46 vs. 24/45 for D10, D11 and D12, respectively, z-test $p<0.05$). The percentage of embryos showing an ED-like structure (a compact structure containing SOX2+ cells) increased significantly on D12 compared to D11 or D10 (6/35 vs. 10/46 vs. 22/45, mean±s.e.m. for D10, D11 and D12, respectively). However, the number of cells and SOX2+ cells allocated to the ED-like structures were similar at all time points analyzed (Total: 87.5±19.4 vs. 74.1±8.3 vs. 105.6±15.7; SOX2+: 52.8±16.9 vs. 44±8 vs. 47.2±9.8, mean±s.e.m. for D10, D11 and D12, respectively, ANOVA $p>0.05$). In conclusion, D12 embryos already show similar rates of ED-like formation than D15, but the stable number of SOX2+ cells evidences that optimal conditions for epiblast development are yet to be uncovered.

Support: StG-757886 from ERC, and AGL2017-84908-R and RYC2018-025666-I from MINECO

TAI/FTET/AI

Analysis by spectral doppler ultrasound of uterine arterial vascularity in breeding sows

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The process of insemination is not only a deposition of semen into the female genital tract. A new paradigm demonstrating the importance of semen transit modulating the female reproductive tract at the time of insemination is becoming evident (Bromfield. Animal. 12, 104-109. 2014). Actually, there is evidence showing that seminal plasma acts directly on female genital tissues favoring implantation, embryonic development, or subsequent offspring health. Infusion of seminal plasma into the porcine uterus induces cellular inflammation a few hours after deposition. It is still evident several days later, including an increase in uterus vascularity but only demonstrated by visual observation of the uterus (O'Leary. Reproduction. 128, 237-247. 2004). Then, this study aimed to evaluate the uterine arterial vascularity of sows by transabdominal Doppler ultrasound at different stages. A total of 9 sows (3-5 parities) were analyzed by Doppler ultrasound on 3 periods: 1) the day of estrus detection; 2) 2-3 h post-AI (artificial insemination) (24 h after the onset of estrus): sows were inseminated using 2×10^9 spermatozoa per dose in 60 ml; and 3) 96 h post-AI. The ejaculates of 3 different males (Pietrain) were used for the experiment. A transportable ultrasound scanner (MyLab25Gold, Esaote-Pie Medical, Barcelona, Spain) equipped with a 2.5-5 Mhz sectorial probe and software for the calculation of cardiac and vascular Doppler parameters was used for the analysis. Sows were allocated in pens to facilitate the scanning. Vessel located within uterine cross-sections were measured. The statistical analysis was performed using the free statistical software SAS University Edition (SAS, 2016). All the Doppler parameters were compared with the mixed model of SAS. The statistical analysis of the results did not reveal significant differences between the experimental groups ($p<0.05$) in any parameter studied: Vascular Flow Integral (ranged from 11.74 to 13.64 cm), Resistivity Index (0.54-0.71), Pulsatility Index (1.03-2.00), Mean Flow Velocity (12.34-19.29 cm/s), Peak Systolic Velocity (31.97-33.84 cm/s), TeleDiastolic Velocity (8.80-15.78 cm/s), Systolic Velocity/Diastolic Velocity ratio (2.52-3.15), Blood Flow (12.28-18.31 ml/min), Mean pressure gradient (0.09-0.20 mm Hg), Peak pressure gradient (0.43-0.48 mm Hg). In conclusion, the semen deposition by artificial insemination did not seem to modify the uterine vascularity of sows. Supported by Spanish Ministry of Science and Innovation (PID2019-106380RB-I00/AEI/10.13039/501100011033).

Keywords: echography, insemination, porcine, ultrasound

MODIFIED Ovsynch and progesterone supplementation of medium term duration reduced the reproductive performance in ewes during breeding season in tropics

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Keywords: AVIKESIL-S®, breeding season, ewes, fertility, Ovsynch, progesterone, sponge, synchronization

Economical yet efficacious hormonal interventions to augment fertility in ewes during the breeding season are still warranted to drive their utilization in India. Aim of the study was to evaluate the efficacy of modified synchronization protocols (Ovsynch; GnRH, 4.0 µg buserelin acetate–PGF2α, 125µg cloprostenol–GnRH, 4.0 µg buserelin acetate; MSD animal health, India); of the short (days 0–5, 7; group-I), medium (days 0–8, 9; group-II), or long term (days 0–12, 13; group-III) duration, along with progesterone (P4) therapy to enhance reproductive performance of crossbred (Nali×Rambuillet) ewes. AVIKESIL-S® intra-vaginal sponges (350 mg of natural P4/sponge, supplied by Central Sheep and Wool Research Institute, Avikanagar, India) were used for P4 therapy for either 5 days (0-5), 8 days (0-8), or 12 days (0-12) in group-I, -II and -III, respectively. The study was conducted during the breeding season (November to January months, autumn season) in India. Each group comprised of 50 randomly selected (parity-wise) ewes, and one group (group-IV) was kept untreated as a control group. Five proven breeding rams in each group were used for mating with the ewes exhibiting estrus. Data on estrus activity and fertility variables were analyzed using one-way ANOVA and Chi-square test. Results showed that estrus synchronization rate was higher ($p<0.01$) in ewes of the group-II (82%) as compared to in the group-III (56%). However, the duration of estrus in ewes of group-II was significantly ($p<0.05$) longer (35.5±3.2 versus 26.8±2 h) compared to the group-III. The pregnancy rate (70%, 66%, 66% and 32% in groups-II, -IV, -I and -III, respectively), lambing rate, and fecundity were significantly lower in ewes of the group-III compared to the than in the other three groups, which demonstrated similar rates. The prolificacy and twinning rate were similar ($p>0.05$) among all the groups. In conclusion, modified Ovsynch protocol (days; 0-12, 13) along with 12 days of progesterone therapy resulted in reduction of estrus induction and fertility outcomes, even compared to the untreated ewes (group-IV). Hence, further studies on follicular dynamics along with endocrinological inter-relationships in such protocols, and alternative approaches to augment fertility in ewes during the breeding season in India are warranted.

Efficiency of obtaining twins by using embryo transfer in artificially inseminated dairy cows

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Keywords: cattle, embryo, embryotransfer

The study herd was a livestock of cattle in the conditions of Limited Liability Company (LLC) "Agricultural enterprise Nikolaevskoye", the village Lugovets, Mglinsky district, Bryansk region, the Russian Federation. To increase the LLC's beef meat production a study was conducted to determine the efficiency of obtaining twins by using embryo transfer (ET) in artificially inseminated (AId) dairy cows. Recipient cows were divided into three groups depending on the breed of the animals. Group I consisted of 22 Swiss cows, group II of 13 Russian black-and-white cows, and group III of 15 cross breed cows. All selected cows were AId with female sex-sorted Belgian Blue semen. On the 7th day after insemination, frozen-thawed female embryos obtained from Hereford donor cows inseminated with sex-sorted Hereford X-semen were transferred into all recipients. The pregnancy diagnostics and determination of the number of developing fetuses in the uterus were carried out using ultrasound scanning on the 45th day after insemination of the cows. Calvings were supervised 24 h a day and special monitoring included the cows diagnosed with twins. There were no stillbirths nor calves born dead. The results were analyzed using the t test in the GraphPad Prism 7 program. Significance was considered at $P < 0.05$. Of the recipients in the groups I, II and III, 45.5 % (10/22), 15.4 % (2/13) and 20.0 % (3/15) had twins, respectively. An ET-derived calf was born to 18.2 % (4/22), 23.1 % (3/13) and 33.3 % (5/15) of the recipients in the groups I, II and III, respectively. An AI-derived calf was born to 31.8 % (7/22), 38.4 % (5/13) and 40.0 % (6/15) of the recipients in the groups I, II and III, respectively. The remaining cows in each group had no calf. The percentage of twin offspring was significantly higher ($P \leq 0.01$) in the group I compared to the groups II and III. In the groups I, II and III, total calf yield was 140.9 % (31/22), 92.3 % (12/13) and 113.3 % (17/15), respectively. In conclusion, the Russian black-and-white cows were inferior twin producers compared to Swiss and cross breed cows which were excellent and moderate, respectively, twin producers.

The abstract was written on the subject of state assignment No121052600344-8.

OPU/IVF and ET

Effect of roscovitine on the developmental competence of domestic cat embryos produced by parthenogenetic activation, in vitro fertilization and somatic cell nuclear transfer

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Roscovitine (RO) is a reversible inhibitor of MPF. Incubation of immature COCs with RO followed by IVM enhances oocyte competence. Additionally, it is know that SCNT is a complex procedure in which only a limited number of viable embryos can be generated. The reversible delay in oocyte maturation that generates RO might enhances the cytoplasmic maturation of domestic cat oocytes. This could be useful in the generation of embryos by SCNT, giving the possibility of reconstruct a higher number of embryos. However, it has been described that RO reduces the developmental competence of domestic cat embryos generated by IVF (Sananmuang *et al*. 2010). To prove the effectiveness of RO, the hypothesis of this research proposes that the incubation of domestic cat oocytes with RO allows the generation of embryos without affecting their developmental competence. The objective was to evaluate the effects of RO in the developmental competence of domestic cat embryos generated by different methods: parthenogenetic activation (PA), IVF and SCNT.

Six groups were done, in which COCs were subjected to IVM (IVM-IVF, IVM-PA, and IVM-SCNT) or incubation with RO followed by IVM (RO-IVF, RO-PA and RO-SCNT). IVM was done in TCM-199 with 0.3% BSA, 0.36 mM sodium pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 0.1 IU/mL FSH-LH, 1 µg/mL 17β-estradiol, 20 ng/mL EGF and 50 µg/mL gentamycin, in 5% CO_2 at 38.5°C for 24 h. RO incubation was done similarly, without hormones and adding 12.5 µM of RO, this for 24 h and then followed by IVM for 24 h. Matured oocytes were used for IVF, PA or SCNT. Embryos were cultured in SOF medium, in 5% O_2, 5% CO_2 and 90% N_2 at 38.5°C for 8 days. Cleavage and blastocyst rates were evaluated between IVM and RO groups using the Kruskal-Wallis non-parametric test ($P < 0.05$). Additionally, the blastocysts were stained with Hoechst, the total cell number of the blastocysts was estimated and a t-test was used to evaluate statistical differences ($P < 0.05$).

Three replicates were done in RO-IVF, RO-PA and RO-SCNT, two in IVM-SCNT and five in the IVM-IVF and IVM-PA. At day-2 of IVC, the cleavage rates were estimated in the RO groups (RO-PA: 43/62 [69.4%], RO-IVF: 63/141 [44.7%] and RO-SCNT: 43/54 [79.6%]) and the IVM groups (IVM-PA: 81/92 [88.0%], IVM-IVF: 76/183 [41.5%] and IVM-SCNT: 29/37 [78.4%]). At day-8 of IVC, the blastocyst rates were estimated in the RO groups (RO-PA: 22/43 [51.1%], RO-IVF: 19/63 [30.2%] and RO-SCNT: 11/43 [25.6%]) and in the IVM groups (IVM-PA: 33/81 [40.7%], IVM-IVF: 26/76 [34.2%] and IVM-SCNT: 6/29 [20.7%]). No statistical differences were observed in the cleavage and blastocyst rates between the RO groups in their respective counterparts from the IVM groups. Additionally, no differences were observed in the blastocyst total cell number (Mean ± SD) between groups: RO-PA (234.4 ± 110) and IVM-PA (210.6 ± 100.1); RO-IVF (270.1 ± 163.1) and IVM-IVF (329.4 ± 88.7); RO-SCNT (239.4 ± 115.8) and IVM-SCNT (367.0 ± 118.2). These are preliminary results and additional experiments are needed to validation. In conclusion, the incubation of domestic cat COCs with RO before IVM did not affect negatively the developmental competence of embryos.

Supported by ANID FONDECYT 3200352

Keywords: felid embryos, cloning, in vitro development

In vitro reconstitution of sheep demi-embryos in culture medium supplemented with vitamin C

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Keywords: bisection, in vitro culture, reconstitution.

Evaluation of *in vitro* reconstitution allows the selection of viable demi-embryos. Vitamin C has an antioxidant effect and improves *in vitro* embryo production. The study aimed to evaluate the reconstitution of demi-embryos with different quality, cultured in medium supplemented with vitamin C. Oocytes obtained from ovaries of abattoir were matured in TCM medium (In vitro, Mexico City, Mexico) supplemented with 10% FBS (Mayimex, Mexico City, Mexico) and hormones for 24 h, at 5% CO_2, 38.5°C, and humidity at saturation. The fertilization was in a commercial medium (In vitro, Mexico City, Mexico) with fresh semen (1×10^6 mL^{-1}). The zygotes were cultured in Cleavage (72 h) and Blastocyst medium (96 h) (Cook IVF, Brisbane, Australia), until the blastocyst stage. The blastocyst rate was 38.7%. The experimental design was completely randomized in a 2×3 factorial arrangement. The quality of the bisected embryo (1=excellent and 2=good, according to IETS, 2010), and the supplementation of the Blastocyst medium with vitamin C (Sigma Aldrich, Mexico City, Mexico) (0, 50, and 100 µg mL^{-1}). $T_{1(control)}$= 0, 1; T_2= 0, 2; T_3= 50, 1; T_4= 50, 2; T_5= 100, 1, and T_6 = 100, 2 (n=20 for each one). In total 120 blastocysts were divided using a microblade adapted to a micromanipulator (Leica, Wetzlar, Germany) and an inverted microscope (Nikon, Tokyo, Japan). The blastocysts were placed in drops of Biopsy medium (IVF Bioscience, Cornwall, England) and symmetrically split. The pairs of demi-embryos were cultured in drops of blastocyst medium. After 12 h, the rate and grade quality one of the reconstituted demi-embryos were determined based on morphological criteria. The diameter of the original embryo and the average diameter of the pair of demi-embryos was determined with a digital camera (AmScope, Irvine, United States). All the procedures were conducted by only one technician. The variables were analyzed with SAS 9.3. The rate and quality were analyzed with the GENMOD procedure. The mean diameter of demi-embryos was analyzed with the GLM procedure considering the diameter of the original embryo as a covariate and the TUKEY test for the multiple comparisons of least squares means. The rate of demi-embryos reconstitution was affected only by the quality (p<0.05), being lower in T_2*vs.* T_1, T_3, T_4, T_5, and T_6 (45.0±7.8% *vs.* 77.5±6.6, 70.0±7.2, 67.5±7.4, 75.0±6.8, and 67.5±7.4%, respectively). The percentage of quality one demi-embryos was affected only by the vitamin (p<0.05), thus, T_3, T_4, T_5, and T_6 were higher compared to T_1 and T_2 (82.1±7.2, 81.5±7.5, 93.3±4.6, and 88.9±6.0% *vs.* 74.2±7.9 and 61.1±3.9%, respectively). The diameter was also affected only by the inclusion of vitamin C (p<0.05), T_3 and T_6 showed greater diameter than T_1, T_2, T_4, and T_5 (201.8±8.0 and 200.7±8.2 µm *vs.* 159.8±7.9, 159.5±9.9, 188.3±8.6, and 187.4±8.2 µm, respectively). The reconstitution of semi-embryos was better when 50 µg mL^{-1} of vitamin C was included in the culture medium and it showed a greater positive effect in quality two embryos, under the conditions of this study.

In vivo ewe demi-embryo survival under temperate or warm climatic conditions

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Keywords: ewes, splitting, survival.

Ewe embryo survival following transfer can be affected by several factors associated with the embryo, the ewe, the ram and possibly the environmental conditions. The study evaluated the effect of two climatic conditions; temperate climate (CT) in one farm or warm climate (CC) in another farm on the survival of *in vivo* produced demi-embryos in ewes. Average ambient temperatures during the period of study were 14° and 33.6° C for CT and CC, respectively. The general management conditions in the two farms were similar. In total 57 hair type multiparous ewes in good body condition were included as recipients (38 in CT and 19 in CC) and pregnancy rate (PG), lambing rate (PP), fetal losses (PF), twining rate (PGEM) and demi-embryo survival rate (PDE) were evaluated. The ewes were synchronized with sponges containing 20 mg Fluorogestone acetate (FGA) (Chronogest, Intervet, Netherlands) for 12 days, and received 333 IU equine chorionic gonadotropin (eCG) (Novormon 5000, Zoetis, México) two days before sponge removal. The estrouses were detected every 6 h from 18 h to 48 h after sponge removal with a teaser ram equipped with a harness and crayon. Embryos were collected seven days after estrous by uterine surgical flushing from multiparous Charollais ewes. The morphological evaluation of the embryos was carried out with a stereoscopic microscope at 80x. The embryos were kept in conservation medium (Syngro Holding Media, Vetoquinol, Canada) while evaluation. Only expanded quality 1 blastocysts were selected. The embryos were split using an inverted microscope (TS100, Nikon, Japan), a mechanical micromanipulator (Leica, Leitz, Germany), a microblade (AB Technology, Pullman, WA) and a commercial splitting medium (ViGro Splitting Plus, Vetoquinol, Canada) utilizing the “scratched bottom” technique (Bredbacka, 1991. Reproduction in Domestic Animals, 26(2), 82-84). The demi-embryos obtained from the same entire embryo were transferred through laparoscopy in pairs into recipient ewes, which were selected on the basis of ovulation rate and morphological quality of the corpus luteum observed. The diagnosis of pregnancy was carried out 35 days later by ultrasonography using a transabdominal 3.5 MHz convex probe (Aloka, ProSound 2, Japan). The results were analyzed with GENMOD of SAS. Differences ($p<0.05$) were only found between CT and CC for PP (70.37 ± 0.88 *vs.* 38.46± 0.13), PF (29.63 ± 0.09 *vs.* 61.54 ± 0.13), PGEM (29.63 ± 0.09 *vs.* 7.69 ± 0.07) and PDE (35.53± 0.05 *vs.* 15.79 ± 0.06). In conclusion, CC conditions significantly reduced the survival of sheep demi-embryos produced *in vivo*.

Liquid marbles as a possible tool for three-dimensional in vitro maturation in bovine oocytes

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Keywords: liquid marble, culture system, oocyte maturation

In physiological conditions, the oocyte is immersed in a three-dimensional (3-D) structure which involves a complex communication between the oocyte and its surrounding somatic cells. Embryos resulting from routine 2-dimensional (2-D) *in vitro* matured oocytes are more prone to chromosomal abnormalities and epigenetic modifications than embryos derived from *in vivo* matured oocytes (Tsuiko *et al*. Human Reproduction, 2017). In the current study, we used liquid marbles (LM) to mimic the 3-D environment within the follicle during maturation. LM are droplets encapsulated with hydrophobic nanoparticles which prevent direct contact between the liquid inside and the surrounding environment while allowing gas exchange. These properties enable LM to be used as microbioreactors. To evaluate the efficiency of this technique, bovine cumulus-oocyte complexes (COCs) from slaughterhouse were cultured in three groups: A) five COCs encapsulated in 30 µL of maturation medium (TCM-199 with 20 ng/mL epidermal growth factor) using treated fumed silica particles (or LM), B) five COCs in 30 µL droplets under oil (2-D droplets), and C) 60 COCs in 500 µL without oil cover (2-D control). In experiment 1 (n = 182, 2 replicates), after 22 hours of maturation, oocytes were recovered from the LM, cumulus cells from all groups were removed by vortexing (8 min) in 2.5 mL Hepes-TALP, and oocytes were fixed and stained with Hoechst for nuclear maturation assessment. In experiment 2 (n = 502, 4 replicates), after *in vitro* maturation, LM were dissolved in maturation medium and oocytes from all treatments were fertilized in groups of 60 and cultured to the blastocyst stage in groups of 25 under oil, as previously described Wydooghe, E. *et al*. Reproduction, 2014. Data were fitted in logistic and linear regression models, and the replicates were set as a random effect. In experiment 1, all oocytes resumed meiosis (i.e. no germinal vesicles were found). Metaphase II stage was reached in the majority of oocytes in all groups (LM: 88.3±4.1; 2-D droplets: 84.9±4.9; 2-D control: 87.0±4.1; $p > 0.05$). Similarly, there was no significant difference in the proportion of oocytes that reached germinal vesicle breakdown (0–5%), metaphase I (3–13%) or were degenerated (0-3%). In experiment 2, maturation conditions did not affect the cleavage rate (80–88%; $p > 0.05$), however, oocytes matured in LM showed reduced day 7 (14.4±3.8%) and 8 (24.6±4.4) blastocyst rates compared to droplets (26.7±5.5% and 39.9±5.2%, respectively; $p < 0.05$) and control (31.9±7.6% and 41.7±6.7%, respectively; $p = 0.007$). Total cell number (TCN) was higher in control (116.3±8.57) than LM (86.4±5.1; $p = 0.009$) and droplets (74.9±5.1; $p < 0.001$) while inner cell mass (ICM)/TCN (32–34%) and apoptotic cells/TCN (2–4%) showed no differences among groups. In conclusion, LM did not affect nuclear maturation but its application is technically too complex for routine bovine oocyte maturation, and lower embryo development was achieved. Alternative 3-D approaches for oocyte *in vitro* maturation must be explored.

Effects of cytokines IGF-1, LIF and FGF-2 on in vitro bovine embryo development.

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Keywords: embryo production, cytokines, bovine

The current efficiency of the bovine IVP system barely reaches 40%. Therefore, there is still room for improvement and so contribute to reducing the high costs of in vitro embryo production (IVP). The addition of three cytokines: insulin-like growth factor-1 (IGF-1), leukemia inhibitory factor (LIF), and fibroblast growth factor (FGF-2), altogether termed FLI, during cattle in vitro maturation (IVM), increased the proportion of oocytes reaching the metaphase II stage of meiosis. Additionally, FLI during the embryo culture (EC) period increased development to the blastocyst stage, cytoskeleton integrity, and survival following slow freezing (Stoecklein *et al.*, PLoS One, 16(2):e0243727, 2021). The effect of such cytokines, when added both during IVM and EC, has not been tested yet. Since these cytokines can be considered embryokines, we hypothesize that the supplementation of IVM and EC media with FLI, at the concentration reported by Stoecklein *et al.*, would improve the development and quality of embryos produced in vitro. To test our hypothesis, we added (FLI group) or not (Control group) 2.61 nM IGF-1, 1.01 nM LIF, and 2.22 nM FGF-2 into the IVM and EC media of abattoir-derived oocytes and evaluated the yield, developmental kinetics, and morphological quality of the blastocysts generated. Briefly, IVM was performed in TCM-199 medium supplemented with sodium bicarbonate, sodium pyruvate, glutamine, gentamycin, eCG, hCG, and 10% fetal bovine serum (FBS). IVF was performed in Fert-TALP medium supplemented with heparin and gentamycin with two frozen-thawed straws per replicate from the same proven fertility bull. EC media consisted of Holms synthetic oviduct fluid (supplemented with 5% FBS. (Sena-Lopes *et al.*, Theriogenology (126), 222-229, 2019). The experiment was replicated 4 times with a total oocyte number of 355 and 352 in control and FLI groups, respectively. The variables studied were percentage of cleavage, percentage of blastocysts at day 7 and 8 post-fertilization, developmental kinetics (percentage of early blastocysts, blastocyst, expanded blastocyst, and hatched blastocysts at day 8) and number of blastomeres per blastocyst. Embryos were fixed in glutaraldehyde (0.5% in DPBS) for 30 min at RT and stained with Hoechst (33342, 1 mg/mL) for 30 min in the dark. Data were analyzed by T-Student for independent samples and one-way ANOVA using the IBM SPSS Statistics package. P values <0.05 were considered significant. The results showed a similar embryonic cleavage (78.6±2.2 and 79.0±2.2 %) and blastocyst yield on day 8 (41.6±3.0 and 37.1±2.9 %) for control and FLI, respectively. There were also no differences between groups for the developmental kinetics of the embryos or the mean number of cells per blastocyst (112.8±3.7 and 104.3±3,7 blastomeres/blastocyst, for control and FLI respectively). The high rates of maturation and embryonic division in our control group make it difficult to observe substantial improvements in basic parameters of embryonic quality, although beneficial effects of the addition of FLI might be observed in other development parameters such as gene expression, cryotolerance, pregnancy rates after ET, and the number of healthy born individuals. In conclusion, the addition of the IFG-1, LIF, and FGF-2 at the concentration used did not improve bovine embryonic quality, so further studies would be advisable before routinely incorporating the FLI cocktail into the bovine culture media.

Possibilities of oocyte recovery rate at the Carpathian Indigenous Buffalo, and their classification - A case report

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Keywords: Carpathian Indigenous Buffalo, COCs, Oocyte.The quality of the oocyte has an impact on fertilization, embryonic development, establishment and maintenance of pregnancy and fetal development. Oocytes obtained from slaughtered animals are the easiest accessible source of female gametes. There are many attempts on the implementation of embryo transfer to the buffalo, but sometimes with limited results. Although the procedures are similar, there are few studies on the Carpathian Indigenous Buffalo (CIB).

The aim of this study was to clarify the possibility of oocyte recovery in CIB and classify COCs, for future full IVF sessions.

Cattle and buffaloes are considered similar species, but there are some differences. It is known that in buffaloes the ovaries are smaller in size and have an incomparably smaller number of primordial follicles than cows, so recovery of female gametes can be difficult and impossible. On average, the ovaries of the CIB have been recorded the following morphometric landmarks: length 24.3 mm, width 18.25 mm, thickness 13.15 mm and a weight of 4.53 g (Liliana Ciornei 2021).

Research has recently been conducted on the ovaries of three buffaloes in the N-E area of Romania. After buffalo slaughter, the ovaries were collected in a thermally insulated container with 0.9% NaCl at 37°C. These were transported to the laboratory within 4 hours. By puncture and aspiration as recovery techniques, follicular fluids and oocytes (COCs) were collected from large follicles. After examination of the aspiration fluid, the identified follicle/oocyte ratio was 11/5, the recovery rate being 45.45%. By slicing the ovaries, as a technique (according Hufana-Duran D., Duran P., 2015), a much higher number of oocytes were obtained (51 COCs) compared to the previous method. The ovary was sliced after aspiration of follicles larger than 5 mm (Mircu C 2020, Gordon I 2003).Thus, we estimate that the average recovery rate of CIB oocytes is 8.5/ovary/slicing. The total number of oocytes collected from CIB ovaries after slaughter was 56, and the recovery rate was 9.3 oocytes/ovary. As a percentage 8.93% were obtained by follicular puncture and 91.07% by ovarian slicing. Morphological evaluation according to the quality criteria of COCs was performed at 40x magnification (after Wood TC and Wildt DE 1997), in grades I-IV, of which only the first two categories are accepted. 46.42% (26/56) represented culturable oocytes (I and II), they have a uniform appearance with a similar structure and a similar degree of development (described by IETS/Embryo Transfer Forms/2/A1). These represent a population of oocytes that can successfully continue to the next stages of IVF. Slightly more than half of the CIB oocytes collected and evaluated were classified as unculturable 53.57% (30/56).

Due to intermittent follicular waves and the evolution of a small number of developing follicles, doubled by the influence of seasonality, only few antral follicles that could be punctured and aspirated were identified on the surface of the ovaries in CIB.

In conclusion, compared to cattle (20-40 oocytes/ovary), in buffalo (CIB) the number of oocytes collected is low (9.3 oocytes/ovary), and represents less than 50% of good quality oocytes.

Does the way of retrieving embryos from donor cows affect embryo yield?

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Keywords: beef cattle, embryo, uterine flushing

The aim was to assess the influence of embryo retrieval ways from superovulated donor cows on embryo yield. The study was carried out on beef cattle of the Hereford breed (n=120) in Bryansk region of the Russian Federation. To induce estrus, the cows were injected intramuscularly with prostaglandin F2α (Enzaprost T, CEVA, France) at a dose of 5 ml per animal. The superovulation was induced with Pluset (Laboratorios Calier, S.A., Spain) intramuscularly at a dose of 1000 IU per animal on the 9th-12th day of the induced estrous cycle every 12 hours with decreasing doses (3, 3, 2.5, 2.5, 2, 2, 1.5, 1.5 ml) and on the 12th day the estrus was induced with prostaglandin F2α (Enzaprost T, CEVA, France). Donor cows were inseminated 3 times with an interval of 12 hours using 5 sperm doses (2, 2, 1). Embryos were flushed non-surgically on the 7th day after the first insemination, previously counting the corpus luteums (CL) per rectum by palpation/ultrasound scanning. 87 donor cows (72.5%) of all treated animals responded positively, i.e. had 3 or more CL's. They were selected for the uterine flushing and were divided into 4 groups: in group I (n = 22) the embryos were removed by gravity using Y-shaped hoses with clamps, in group II (n = 22) by injecting (Luer syringe), in group III (n = 21) by a combined way using Y-shaped hoses with clamps and a Luer syringe, in group IV (n = 22) by an electric pump method (EM). The EM is based on the operation of a design with an electric pump which automatically supplies flushing fluid with an adjustable pressure gradient from the container to the uterus and aspirates the fluid along with embryos from the uterus to filters for collecting embryos (patent of the Russian Federation for utility model №156768, 2015, Center of Experimental Embryology and Reproductive Biotechnologies, Moscow, Russia). All groups used two-channel catheters for non-surgical embryo retrieval (Minitube, Germany), Em Safe filters for collecting embryos (Minitube, Germany), and flushing fluid DPBS (Ltd Pan Eco, Russia). The total number of retrieved embryos/ova was assessed (transferable quality and degenerated embryos, unfertilized ova). Data was analyzed using ANOVA followed by Tukey test ($p<0.05$). The highest recovery rate was obtained in group IV. In average, the number of the CL per animal in group IV was 10.2 ± 0.538 and 9.2 ± 0.502 embryos were extracted (90.2%). The number of CL's and the number and percentage of embryos recovered, respectively, were in group III 10.1 ± 0.618 and 7,6 ± 0.512 (75.0%), in group I 9.9 ± 0.524 and 6.8 ± 0.411 (68.4%), in group II 9.7 ± 0.594 and 6.4 ± 0.52 (66.2%). There were no significant differences between the groups in the number of CL. The proportion of embryos/CL's in group IV significantly ($P<0.001$) exceeded those in group I by 21.8 %-points, by 24.0 %-points in group II, and by 15.2 %-points in group III. In conclusion, it should be noted that the efficiency of retrieving of embryos from the uterus of a donor depends on the way of non-surgical retrieving used. The article was written on the subject of state assignment No. №121052600344-8.

Folliculogenesis, oogenesis and superovulation

Characterization and miRNA profiles of small extracellular vesicles originated from porcine follicular fluids and their association with oocyte developmental competence

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Keywords: follicular fluid, extracellular vesicles, miRNA

Follicular fluid (FF) provides the essential microenvironment needed for oocyte development and subsequently influences its competence (ability to mature and sustain embryo development). Extracellular vesicles (EVs) have been detected in the ovarian FFs and reported to play essential roles in regulating follicular and oocyte development through their cargo molecules including microRNAs (miRNAs). The objective of this study was to characterize and identify miRNA expression profiles of small EVs from porcine FFs in association with oocyte competence. Approximately 600 antral follicles (2-6 mm; from prepubertal gilts) were aspirated individually and cumulus-oocyte complexes (COCs) were stained with 0.5% Lissamine Green B stain (LB), a vital stain for determining oocyte competence (*Batrkova et al. Anim. Reprod. 17, 2020*). Each COC was classified separately according to the stain into LB+ (stained; low-competence) and LB- (unstained; high-competence). FFs corresponding to each oocyte group were pooled together into FFL+ (low-competence) and FFL- (high-competence). EVs were isolated from FFL groups (3 biological replicates/group; 0.5 mL FF/replicate) using the Exo-spin kit based on precipitation and Size-exclusion chromatography separation technology. Isolated EVs were characterized by western blot (WB), transmission electron microscopy (TEM), and nanoparticle tracking analysis (NTA). Total RNA was isolated from EV samples using miRNeasy Micro Kit. Small-RNA libraries were prepared using QIAseq miRNA Library Kit and sequenced on an Illumina NovaSeq 6000. WB analysis proved the presence of specific EV-associated tetraspanin and protein markers (CD63 and ALIX) and the absence of cellular-specific protein markers (CytC and ATP5A). Morphological evaluation by TEM showed EVs as circular bilayer enclosed vesicles. The median size of EVs was 132.6 *vs.* 135.7 nm and the concentration was 9 $x10^9$ *vs.* $8.8x10^9$ particles/mL in the FFL+ *vs.* FFL- group respectively, with no significant differences ($P > 0.05$). Sequencing analysis revealed that a total of 295 known miRNAs were commonly detected in the EVs of both FFL groups. MiR-27b-3p, miR-140-3p, miR-29a-3p, miR-202-5p, and miR-16 were the top highly abundant miRNAs in both groups representing around 45% of the total reads. Differentially expression (DE) analysis using DESeq2 package exhibited that 22 miRNAs (including miR-9, miR-6516, and miR-206) were up- while 19 (including miR-193a-5p, miR-125b, and miR-320) were down-regulated in FFL+ compared to FFL- group (FC > 2; FDR < 0.05). DE-miRNAs target gene analysis uncovered pathways associated with oocyte development including oocyte meiosis, ubiquitin-mediated proteolysis, and signaling pathways (MAPK, PI3K-Akt, FoxO, and AMPK). Our findings indicated that FF-EVs contain different miRNA cargo in association with oocyte competence. These miRNAs could be used as potential non-invasive biomarkers for oocyte selection.

Supported by IAPG-Matoušek Award 2020 and Ministry of Education, Youth and Sports of the Czech Republic (CZ.02.1.01/0.0/0.0/15_003/0000460).

Superovulatory response of donor ewes under temperate and warm climatic conditions

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Keywords: superovulation, embryo, ewes.

The variation of response to superovulatory treatments in ewes can be affected by many environmental factors, e.g. heat stress. The study evaluated the effect of two climatic conditions recorded in two different farms but in the same season; temperate climate (TC) or warm climate (WC) on the ovulatory response to a superovulatory treatment in ewes. The average ambient temperature during the period of study was 14.0 °C and 33.6 °C for TC and WC, respectively. The general management conditions in the two farms were similar. In total, 25 multiparous Charollais donors in good body condition were superovulated (17 in TC and 8 in WC) and ovulation rate (OR) was determined as number of corpåora lutea on the flushing day, embryo recovery rate (ERR) defined as the ratio among the total number structures recovered and OR, percentage of transferable embryos (TE) which included only of quality one and two embryos from the total amount of embryos recovered, and rate of unfertilised ova (UFO) were recorded. The ewes were synchronized with two intravaginal sponges containing 20 mg of Fluorogestone acetate (FGA) (Chronogest, Intervet, Netherlands) for 12 days, remaining inserted six days each. For superovulation, 240 mg of FSH (Folltropin, Vetoquinol, Canada) were administered for four days every 12 h, being in total eight injections given following a descending protocol (40, 40, 40, 40, 30, 30, 10 and 10 mg), starting 60 h before sponge removal. After sponge withdrawal, the oestruses were detected every 6 h from 18 to 48 h with the help of a teaser ram equipped with an apron. In addition, 200 µg of Gonadorelin (GnRH, Sanfer, Mexico) were given at oestrus onset. The ewes were inseminated by laparoscopy with three doses of 100 x 10^6 spermatozoa as fresh semen from a single Charollais ram of known fertility. All injections were administered intramuscularly. The ewes were flushed seven days after oestrus by mid-ventral laparotomy in an attempt to obtain the embryos, using a phosphate-based buffer solution (ViGro Complete Flush Solution, Vetoquinol, Canada). Embryos were recovered and morphologically evaluated using a stereo microscope at 40x and 80x, respectively. The embryos were evaluated in holding medium (Syngro Holding Media, Vetoquinol, Canada). The results are presented as averages ± SEMs and were statistically analyzed with GENMOD of SAS. In total, 108 and 19 embryos were obtained from the ewes maintained in TC and WC, respectively, from which 102 and 19 were quality one and two (transferable embryos) and only 6 from the TC group were quality three or degenerated. No statistical differences ($p>0.05$) were found between ewes kept in TC or WC for OR (12.59±0.86 vs. 11.5±1.20) and ERR (0.57±0.03 vs. 0.63±0.05). However, TE was significantly higher ($p<0.05$) (0.83±0.03 vs. 0.33±0.06) and UFO significantly lower ($p<0.05$) (0.12±0.03 vs. 0.67±0.06) for ewes kept in TC and WC, respectively. In conclusion, ewes superovulated under WC conditions produced a higher percentage of low quality embryos, possibly due to heat stress, negatively affecting the TE and consequently the efficiency of an embryo transfer program.

Morphometric and ultrastructural analysis of follicles in a prepubertal caracal (Caracal caracal)

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Keywords: caracal, folliculogenesis, electron microscopy

The caracal (*Caracal caracal*) is widely known to exist in the semi-desert region of the Arabian Peninsula. Despite the IUCN reported occurrence of the Caracal in Oman in 2016 and ranked it under least concern with a fluctuating trend, the last domestically reported citation was made at least ten years ago, with as low as 5 animals housed in captivity currently. The objective of this study was to investigate the presence of functional and morphologically sound follicles in an 8-month old prepubertal female. In this threatened species, the known sexual maturity age is 15 months. The caracal was found stuck on the fence and strangled to death overnight. Necropsy gross examination revealed the absence of overt signs of illness. Ovaries were transported on ice to the lab from the reserve site in 30 min. The ovary was cut into 1mm fragments. Toluidine blue (Sigma, Germany) staining (1%) was carried out by sectioning (0.5µm) and were examined under a light microscope (Olympus, Japan). Similar ovary fragments of 1mm were used for scanning electron microscopy (SEM) analysis by immersion into a fixative (Kornovsky) for 4 h. Followed by several washes with post fixative media, dehydrated, infiltrated, embedded in capsules, polymerized prior to ultra-thin microtome sectioning (60-90 nm) and SEM imaging with JEM-1230 (JEOL, Japan). Morphometrically, primordial and secondary follicles stained with toluidine blue exhibited singular or multilayer surrounding of cuboidal granulosa cells averaging 6 and 16 cells, respectively. The spherical structure of secondary follicles manifested a noticeable asymmetrical arrangement and detachment of granulosa cells with a small gap between granulosa and theca cells. While a number of oocytes displayed an eccentrical nucleus, the absence of the entire oocyte was recorded in secondary follicles. Vacuolation and deformed oocytes were unnoticeable. SEM micrographs depicted a microvascular architecture of follicles embedded in a grid of meshes. Specifically, spherical shape with intense short microvilli casing the surface and the existence of a number of blebs. Metrically, primordial and secondary follicular diameter ranged between 7.9 and 20.7 µm with an average of 16 µm. In summary, this study reveals the existence of primordial and secondary follicles in the ovary of a prepubertal caracal female. The elaborate description provides evidence of morphometric inconsistencies in primordial follicular architect.

Physiology of male reproduction and semen technology

Effects of Phoenix Dactylifera date palm pollen on ram semen quality parameters during chilled storage

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Keywords: Sardi ram semen, Liquid storage, Phoenix Dactylifera

The date palm pollen (DPPs Phoenix dactylifera L.) is widely used in the traditional pharmacopeia of Moroccan oasis population. In fact, pure or mixed to medicinal plants, the pollen is used to treat metabolic diseases or to stimulate lactation in humans and cattle. DPPs and male palm flowers were traditionally claimed to be aphrodisiacs and fertility enhancers. In this context, the present study aimed firstly to compare the phenolic composition of the methanol, ethyl acetate, water and ethanol extracts from *Phoenix Dactylifera* date palm pollen. Secondly, this study aimed to assess the effect of the best extract derived from the first step on Sardi ram semen variables, lipid peroxidation during liquid storage at 5 °C for up to 24 hours in skim milk extender. Pure Date Palm Pollen (25 g) was macerated in solvents of increasing polarity at 80%, filtered through Wattman paper before going through a rotary evaporator. The extracts were then analysed for their DPPH and ABTS radical scavenging ability and polyphenol and flavonoid contents. 50 semen samples were collected from five rams at a rythm of 5 ejaculates per week, pooled and extended with skim milk (SM) extender containing Methanolic Date Palm Pollen extract at 0 µg/ml (control), 1 µg/ml, 2 µg/ml, 3 µg/ml and 4 µg/ml (0%, 1%, 2%, 3% and 4% respectively). The extended semen was made at a final concentration of 0.8 × 10^9 sperm/ml and stored for up to 24 h at 5 °C. The sperm variables were evaluated at different time periods (0, 4, 8 and 24 h) using a computer-assisted sperm motility analysis program (CASA; ISAS, version 1.0.17, Proiser, Valencia, Spain). Statistical analysis was performed using JMP SAS 11.0.0 (SAS Institute Inc., Cory, NC, USA) program. The METHEX extract revealed the highest values in term of flavonoid and polyphenolic contents (90.09±0.26 mg gallic acid equivalent/g and 11.71±0.05 mg quercetin equivalent/g). It also showed both the highest ABTS and DPPH free radical scavenging activity. Sperm viability, total and progressive motility showed a significant increase when conserved with METHEX extract supplemented skim milk at a concentration of 3µg/ml while a decrease in total sperm abnormality (7.12±0.21, 10.35±0.2, 12.58±0.17, and 14.94±0.19) and lipid peroxidation (0.54±0.04, 0.63±0.03, 0.71±0.04, and 0.86±0.04) and increase in viability (86.85±0.19, 84.98±0.13, 82.35±0.21, and 79.95±0.1) in T0, T4, T8 and T24 respectively were observed ($P< 0.05$). In conclusion, skim milk supplemented with 3% of METHEX *Phoenix Dactylifera* date palm pollen extract proved its ability to improve the quality of ram semen which makes it a viable solution for liquid storage. Further studies are in progress to verify the effects of this quality improvement on the fertility rate of Sardi ewes during artificial insemination.

Acknowledgments: Thanks to the Academy of Research and High Education (ARES) for its financial support for this study.

The oxygen consumption of boar sperm is increased after the incubation with uterine fluid and an in vitro selection

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Keywords: porcine, metabolism, biofluids, fertility

Following ejaculation, sperm selection takes place across the female reproductive tract, to the fertilization site. At first, sperm are not able to fertilize, but they acquire this ability along their way to encounter the oocyte. This involves many factors, such as the interaction with male (seminal plasma, SP) and female (uterine fluid, UF) reproductive fluids. Both fluids lead to sperm changes, through the adhesion of components (proteins, lipids, metabolites) which help to preserve functions like motility and acrosome integrity (Perez-Patiño, J Proteomics, 142:15-23, 2016; Luongo, Int J Mol Sci, 21, 17:6060, 2020). Sustaining such metabolic events requires energy (Magdanz, PLoS One, 14(10):e0223576, 2019), which sperm obtain via two pathways: glycolysis and/or oxidative phosphorylation. It is known that boar sperm preferably use glycolysis, but recently, a connection between oxygen consumption rate (OCR) and ATP synthesis was demonstrated (Nesci, Theriogenology, 144:82-88, 2020). Thus, the aim of this study was to investigate how the sperm (S) OCR changes after exposure to SP and/or UF. Four ejaculates were collected from 4 boars with proven fertility, and centrifuged (500 *g*, 5 min) to remove the SP. The following experimental groups were prepared at $50x10^6$ sperm/ml: 1) Control: S without reproductive fluids; 2) SP: S with 20% SP; 3) UF: S with 20%UF; 4) SP+UF: S with 20% SP and 20% UF. All the groups were incubated for 3h at 38.5°C. Then, an aliquot of each sample was prepared to carry out the *in vitro* sperm selection by Percoll 45/90% (P). For all samples [with or without *in vitro* selection; (+)/(-) P], $1x10^6$sperm in XFe Assay medium were loaded into a 96-wells cell culture plate coated with polylysine. Thus, the plate was centrifuged (300 *g*, 5 min). Finally, the OCR (pmol/min) was calculated by the Oligomicin A injection by the Seahorse XFe device (Agilent). Data were analyzed by SAS (2016) and ANOVA for repeated measurements test was performed. Results were expressed as mean±SEM and considered statistically different when $p<0.05$. Before *in vitro* selection, sperm did not show a significant difference between the groups, except SP+UF (75.4±6.1) which showed a higher OCR than control (39.7±5.8) ($p=0.01$). However, each (-)P group showed significantly lower values of OCR than the correspondence (+)P groups ($p<0.04$). Interestingly, after Percoll treatment, the groups previously incubated with UF (UF= 134.4±11.4, and SP+UF=136.5±7.1) showed higher OCR than control (68.8±4.8) ($p<0.0001$) and SP (88.3±9.9) ($p=0.002$). These results showed how boar sperm OCR increases after *in vitro* selection, and, particularly after exposure to UF. In conclusion, this study provides new knowledge about sperm metabolism before fertilization, suggesting how sperm functionality may be improved by *in vitro* selection depending on the fluid with which they enter in contact. Acknowledgments: Supported by the Spanish Ministry of Science and Innovation (PID2019-106380RB-I00/AEI/ 10.13039/501100011033).

Resolving adhesion and aggregation issues in motility analysis of boar spermatozoa submitted to media removal by centrifugation

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Keywords: pig, semen, aggregation

Assisted reproductive techniques (ART) often require sperm washing and analogous procedures, which can be stressful (centrifugation, extension, pelleting), modify plasma membrane undergoes modifications and proteins coating the sperm surface. One consequence is the increase in spermatozoa adhesivity, resulting in cells sticking to glass (slides, hemocytometers) or aggregating during microscopic examination. Many labs have tried to avoid these effects, but the solution remains elusive. We report an attempt to ameliorate this effect in boar spermatozoa processed by single-layer colloid centrifugation (SLC) and stored. Whereas SLC is an excellent technique for improving sperm quality or removing bacteria, it could increase sperm stickiness. Six ejaculates (six boars, Large White breed, 2 years old, AIM AI centre, León) were extended after collection and processed by SLC (15 ml of sample on 15 ml of 20% Porcicoll colloid, 600×g 20 min). The samples were resuspended at a concentration of 30×10^6 ml^{-1}, stored at 17 °C in 15-ml volumes, and examined by CASA after 7 days (most noticeable stickiness). Before evaluation, 50-µl aliquots were pipetted from each tube and mixed with 100 µl of PBS supplemented with either 0.75%, 1.5%, 3% and 6% of BSA, PVA [Poly(vinyl alcohol)], PVP (polyvinylpyrrolidone), or PVA/PVP. The occurrence of spermatozoa sticking to the glass or aggregation was recorded and analysed by x2. The effects of treatments on total and progressive motility were estimated by linear mixed-effects models. SLC did not increase stickiness (overall occurrence in 35.4% of the samples), but showed lower aggregation (41.7% of control samples vs 4.2% in SLC, $P<0.001$). The effect of the additives in stickiness and aggregation was significant ($P<0.016$ and $P<0.001$, respectively). PBS with no supplements yielded 75% of samples presenting stickiness, whereas its incidence was reduced by or below 25% by BSA and PVA (except at 0.75%), PVP (except at 3%), and PVA-PVP (at 0.75% and 3%). Aggregation was prevented (<10% of samples showing it) by all BSA concentrations, PVA (at 0.75% and 1.5%), and PVP and PVA-PVP (except at 6%). Considering the CASA analysis, PVA at 3% showed the highest effect (increasing total motility by 17.6±4.9 points, $P<0.001$, and progressivity by 7.4±2.5, $P<0.01$), followed by BSA 1.5%, BSA 3%, and PVA 0.75%. CASA results were not entirely associated with our visual observations on the overall incidence of stickiness and aggregation, possibly because these determinations were qualitative instead of quantitative. These molecules could interact with the sperm surface, maybe compensating for the modifications caused by manipulation and storage and preventing interactions with glass and other cells. Since this is a long-standing problem for sperm analysis, we expect these results to improve sperm assessment. Nevertheless, some additives could affect sperm motility or elicit capacitation, and therefore the lowest concentrations could be preferable.

Funded by grants RTI2018-095183-B-I00 (MCI/AEI/FEDER, EU) and LE023P20, (Junta de Castilla y León/FEDER, EU). We thank Topigs-Norsvin España, AIM (León).

Embryology, developmental biology and physiology of reproduction

Pregnancy rate in shortened natural and stimulated estrous cycles in mares

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Keywords: hormones, natural estrous cycle, pregnancy, mare, PGF2a treatment

Various hormonal treatment regimens are used for artificial insemination and embryo transfer in mares. However, the effect of hormones on pregnancy rate in stimulated oestrous cycles depend on many factors and do not always coincide with the expected. The aim of the work was to analyze data on the pregnancy rate of mares in natural and stimulated oestrus cycles of different duration in two horse breeding farms (central region of Russia). Mares with either signs of endometritis or prolonged (> 24 days) oestrous cycles, or old mares with reduced fertility or mares at the foaling heat were excluded from the analysis. Mares were divided into 2 groups: 1) natural oestrus cycles (n =299) and 2) PGF2a-stimulated oestrus cycles (n=89), in which 150-250 μg (0.6-1.0 ml) of cloprostenol (Magestrophan, "Mosagrogen", Moscow) were used to reduce diestrus period and stimulate new heat. The beginning of estrus was determined by the appearance of ultrasound signs of oedema in the uterus, presence of 1-2 follicles at least 30-32 mm in diameter and simultaneous absence of clear corpus luteum (>15 mm) in the ovaries. Ovulation was taken as the end of the diestrus phase (day 0 of the next oestrus cycle). In both groups fertility was analyzed in normal (19-24 days) and shortened (9-18 days) oestrus cycles with either normal (4-8 days) or short (2-3 days) heat. Mares were inseminated with fresh diluted semen of fertile stallions in 24-36 hours interval until ovulation. The data were statistically analyzed by Student-Fisher test. The results showed that in natural cycles (n=37) the decrease in the interovulatory interval occurred mostly due to reduction of the length of heat (78.4%, n=29). In stimulated shortened cycles (n=65) the numbers of short and normal heats were 43.1% (n=28) and 56.9% (n=37), respectively. It was established that the pregnancy rate of mares in short natural cycles (n=37) was significantly ($p<0.01$) lower (51.3%, n=19) than in normal natural (n=262) cycles (79.4%, n=208). In normal stimulated (n=24) and short stimulated (n=65) cycles the pregnancy rate was 79.0% (n=19) and 73.8 % (n=48), respectively. Pregnancy rate in shortened stimulated cycles with short heat (n=28) was significantly lower (53.6%, n=15, $p<0.001$) than in shortened stimulated cycles with normal (n=37) heat (89.2%, n=33). In natural short cycles (n=37), the difference in fertility of mares with short (n=29) and normal (n=8) heat was 44.8% (n=8) and 75.0% (n=6), respectively, $p<0.10$. It was concluded that 1) in shortened natural oestrus cycles pregnancy rate of mares significantly decreased; 2) the shortening of natural oestrous cycles occurred mostly due to reducing of the heat duration; 3) in shortened normal and stimulated oestrus cycles with short (2-3 days) heat, the pregnancy rate significantly decreased, probably due to lack of time to create appropriate conditions for sperm viability and fertilization, in the reproduction tract. It should be considered especially in the case of artificial insemination of mares with expensive frozen semen.

In vitro fertilization and embryo development applying two 'progressively motile sperm to oocyte' ratios with cryopreserved Duroc and Landrace sperm

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Keywords: Porcine, in vitro fertilization, progressive motility

Using cryopreserved sperm during in vitro embryo production (IVP) allow for increased repeatability. However, survival and motility of cryopreserved sperm vary more between boars and straws and are often lower compared to fresh sperm. Fertilization is usually performed with a concentration based on total sperm cells per ml, which includes both live and dead sperm, but in this study concentration was corrected for progressive motility (PROG). The aim of this study was to assess the effect of two different 'progressively motile sperm to oocyte' ratios on fertilization, polyspermy and embryo development with cryopreserved sperm from three Norwegian Duroc and three Norwegian Landrace boars. Random sow ovaries were collected after slaughter and follicles with a diameter of 3-8 mm were aspirated. Cumulus oocyte complexes were cultured for 44 h in porcine oocyte medium (POM) (6% CO_2, 38.8°C). Cryopreserved sperm was centrifugated through a 45/90% Percoll® density gradient and sperm motility was analysed by computer- assisted sperm analysis (CASA) to determine sperm concentration and PROG after centrifugation. Oocytes were fertilized with 250 or 500 progressively motile sperm cells per oocyte in porcine gamete medium (PGM). After 4 hours fertilization, presumptive zygotes were denuded by vortexing and cultured in porcine zygote medium (PZM-5) under mineral oil (6% CO_2, 7% O_2, 38.8°C). To analyse fertilization and polyspermy rates, some presumptive zygotes were assessed 10 - 12 hours after start of fertilization. Furthermore, cleavage rate at day 2 and blastocyst rate at day 6 of culture were assessed, defined as the number of cleaved zygotes or blastocysts divided by the total number of oocytes cultured. The effect of ratio and boar on fertilization, polyspermy, cleavage and blastocyst rates were analysed using two-way ANOVA and values were compared by post hoc Tukey test. Fertilization with 500 progressively motile sperm cells per oocyte resulted in a significantly higher blastocyst yield on day 6 ($P = 0.03$), while no effect of ratio on fertilization, polyspermy or cleavage rates was observed. Blastocyst percentages for the 500:1 and 250:1 ratios were 26.9% vs. 20.3%, respectively (n = 703 and 734 zygotes analysed). Individual differences between boars were observed for fertilization ($P = 0.0025$), cleavage ($P = 0.0007$) and blastocyst rates ($P < 0.005$). The boar with a significant lower fertilization rate (14.4% vs. 52.5%) also had a significant lower cleavage (35.2% vs. 60.3%) and blastocyst rate (8.9% vs. 31.0%) compared to the boar with the highest blastocyst rate. The lowest blastocyst rate of 4.3 ± 4.2% was observed for a Duroc boar with the 250:1 ratio while the highest blastocyst rate of 40.4 ± 4.3% was observed for a Landrace boar with the 500:1 ratio. In conclusion, fertilization with 500 progressively motile sperm cells per oocyte resulted in the highest blastocysts yield on day 6. Differences in IVP outcomes were observed between the individual boars and high blastocyst percentages could be obtained with both Duroc and Landrace sperm. Although corrected for progressive motility, differences in IVP outcomes were still observed between the boars.

Expression of Sex Dimorphism in bovine in vivo derived or in vitro produced preimplantation embryos.

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Keywords: transcriptome, sex-effect, cattle

Early mammalian embryo is very sensitive to environmental perturbations, which affect phenotypic traits in a sex-specific manner at adulthood. This assumes that sex-specific differences exist even before hormonal impregnation. BoSeX-Dim project aims to identify early sex-dimorphisms in bovine embryo and analyse how these dimorphisms are modified in *in vitro* produced embryos. We first compared gene expression in *in vivo* derived and *in vitro* produced embryos at two key developmental stages: Day7 blastocysts with recent literature demonstrating a certain degree of X chromosome inactivation in female embryos, and Day 18 conceptus.

In vivo derived embryos were recovered from superovulated females at Day7 post insemination (pIA) and from synchronized females at Day18 pIA. *In vitro* Day7 embryos were produced from slaughterhouse ovaries. After oocyte *in vitro* maturation and fertilization, they were cultured in SOF medium supplemented with 1% oestrus cow serum. Some of them were transferred to synchronized recipient females and recovered 11 days later to produce Day18 *in vitro* conceptuses. At Day7, inner cell masses (ICM) were separated from trophectoderms (TE) using a mild immunosurgery protocol which makes both ICM and TE recovery possible (Charpigny et al., Front. Cell Dev. Biol., vol 9, June 2021). At Day18 embryonic disks (Disc) and distal trophectoderms (TD) were manually dissected. Each embryo was sexed by PCR. Transcriptome analyses were performed by RNAseq using SMART-seq v4 kit (Clontech) on 5 pools of 10 ICM or 10 corresponding TE at Day7, and on 5 single Disc or TD at Day 18 for each sex, totalizing 80 samples. Libraries were prepared from 0.15 ng cDNA using the Nextera XT Illumina library preparation kit and sequenced (Paired-end 50–34 pb) on an Illumina NextSeq500 instrument. Reads were mapped to the ARS-UCD1 reference genome to which the Genbank accession CM001061.2 (Y chromosome) was concatenated, using STAR software. FeatureCounts was used to establish gene count table. Data normalization and differential expression were performed using the DESeq2. Genes with a significant sex or condition (*vivo* vs *vitro*) effect were identified with an adjusted p-value<0.05.

At Day7, 5376 and 3843 genes displayed a condition effect in the ICM and TE compartments respectively, when 1621 and 1153 genes had a sex effect. At Day18, 10 and 88 genes had a condition effect in the Disc and TD respectively, when 608 and 147 genes displayed a sex effect. In the extraembryonic part, 63 genes had a sex effect at both stages, while 76 genes had a sex effect in embryonic compartments at both stages. Only 17 genes had a sex effect in both tissues at both stages, all being located on the X or Y chromosomes. Very interestingly genes having both a sex and a condition effect were identified at Day7, evidencing that condition may affect the expression of sex-dimorphic genes. Work is going on analysing gene expression in fetal gonads, chorion, brain and liver at Day40 when gonad differentiation is underway, but hormonal impregnation by sexual steroids did not already start.

BoSex-Dim project has been funded by ANR.

Expression of early cell lineages markers in ovine and murine embryos

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Keywords: early cell lineages, sheep, mouse

Early embryonic losses constitute a major burden for reproductive efficiency of farm animals. *In vitro* produced embryos show high mortality rates during preimplantation development, when complex cell differentiation processes happen. The critical differences in embryo development between ungulates and mice, the most studied mammalian model, have precluded the identification of the mechanisms governing early lineages differentiation in livestock species. Better understanding of these processes and identification of suitable lineage markers are necessary to assess embryo quality. The aim of this study was to characterize the expression of early cell lineage markers in mouse and sheep preimplantation embryos. *In vitro* produced sheep embryos (n=374) were cultured in SOF medium until the blastocyst stage. D6/D7 blastocysts were transferred to a post-hatching *in vitro* culture system based on N2B27 medium with activin A and Rho-associated protein kinase inhibitor (n=99). *In vivo* derived mouse zygotes (n=102) were collected from superovulated CBAXC57BL6 F1 hybrid females and cultivated in KSOM medium. Ovine embryos were sequentially fixed at days (D) 1.5, 3, 4, 5, 6, 7, 8, 10 and 12, and murine embryos at D1.5, 2, 2.5, 3.5 and 4.5. Embryos were analyzed by immunostaining for SOX2 (epiblast); SOX17 or GATA6 (hypoblast) and CDX2 or GATA3 (trophectoderm), and counterstained with DAPI. Z-stack imaging was performed and cells expressing each marker and total cells were counted. To compare markers expression between species, embryos were grouped according to total cell number in: 2c, 3-4c, 5-8c, 9-16c, 17-32c, early (33-64c in sheep and 33-48c in mouse) and late (> 64c in sheep and > 48c in mouse) blastocysts. SOX2 expression started at 3-4c in sheep (8/12 embryos) and was observed in some 2c mouse embryos (6/14), later disappearing until the 9-16c stage (6/10). In both species, SOX2 was restricted to the epiblast from the blastocyst stage onwards (n=171 in sheep and 18 in mouse). SOX17 was detected from the 3-4c stage in sheep (2/4) and from 17-32c in mouse (2/11), and was restricted to hypoblast cells from the blastocyst stage in both species (n=89 in sheep and 6 in mouse). However, GATA6 expression started at 5-8c in both species (11/19 in sheep and 10/11 in mouse) but it was specific to hypoblast cells only in mouse blastocysts (n=12), while in sheep it was ubiquitously expressed until D10 (n=42). Finally, trophectoderm markers were detected earlier in mouse (GATA3 from 5-8c [7/11] and CDX2 from 9-16c [2/4]) than in sheep embryos (GATA3 from 9-16c [6/32] and CDX2 from 17-32c [2/14]) and the % of GATA3+ and CDX2+/total cells were significantly higher in mouse than in sheep at all stages (Mann Whitney test, $p<0.05$), suggesting that trophectoderm specification occurs later in ungulates than in mice. In conclusion, we provide an expression timeline of well-known lineage markers in mouse and sheep embryos. While all analyzed markers can be used to assess proper lineage commitment and embryo quality in mice, SOX2, SOX17, CDX2 and GATA3, but not GATA6, are lineage-specific in sheep blastocysts.

Support: AGL2017-84908-R and RYC2018-025666-I from MINECO, and StG-757886 from ERC.

Characterization of in vitro ovine embryos cultured up to gastrulating stages

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Keywords: conceptus, in vitro, ovine

Pregnancy losses in farm ungulates entail a significant economic burden. Most of these reproductive losses occur during the second week of pregnancy due to defective embryo development. After blastocyst hatching, the hypoblast migrates to cover the inner embryo surface, and together with the trophoblast, they proliferate to exponentially increase embryo size. Concomitantly, the epiblast forms an embryonic disc (ED) which initiates gastrulation. Most of these processes could only be observed *in vivo*, limiting our understanding of post-hatching embryo development. We have recently developed an *in vitro* post-hatching culture system that supports ovine embryo development *in vitro* up to gastrulating stages at day (D) 14. The aim of this study was to compare the developmental landmarks achieved by *in vitro* produced (IVP) embryos with *in vivo* embryos. D6/7 IVP blastocysts were cultured over agarose gels in N2B27 medium with Activin A and Rho-associated protein kinase (ROCK) inhibitor, and fixed at D8 (n=23), D10 (n=40), D12 (n=53) and D14 (n=107). *In vivo* embryos were collected from super ovulated ewes and fixed at days E9 (n=11), E11 (n=16), E12.5 (n=11) and E14 (n=3) post-mating. Embryos were imaged and their length was measured with ImageJ. The development of specific lineages was assessed by immunostaining for SOX2 (epiblast), SOX17 or FOXA2 (hypoblast), CDX2 or GATA3 (trophectoderm) and T (mesoderm). Although D8 *in vitro* embryos were significantly larger than E9 *in vivo* embryos, *in vitro* embryos remained spherical and their size was significantly restricted from D12 compared with their *in vivo* counterparts (t-test; $p<0.05$). However, when D10 *in vitro* embryos were transferred to synchronized ewes and recovered at D14, 3/8 (~ 38%) had initiated conceptus elongation, indicating that uterine secretions are required for proper proliferation of the extraembryonic membranes. Hypoblast migration gradually increased *in vitro*, reaching 79.94 ± 2.6 % of the embryo surface at D14. However, the % of the embryo covered by hypoblast was significantly reduced in *in vitro* embryos compared with their *in vivo* counterparts at all stages (t test; $p<0.05$). No significant differences were found in the number of embryos exhibiting SOX2+ epiblast cells *in vitro* and *in vivo* (Fisher exact test; $p>0.05$). However, SOX2+ cell number was significantly lower in D10 (28.76±5.57) and D14 (67.41±14.88) *in vitro* embryos compared to E11 (132.8± 21.04) and E14 (6714±1225), respectively (t-test; $p<0.05$). The % of embryos developing an ED was also lower *in vitro* (8/29 [~28%] D10 *vs.* 14/14 [100%] E11; 14/36 [~39%] D12 *vs.* 7/8 [~87%] E12.5 and 34/55 [~62%] D14 *vs.* 3/3 [100%] E14). Mesoderm differentiation was observed from E12.5 *in vivo* in all EDs analyzed (7/7 E12.5 and 3/3 E14), and in 5/21 (~24%) D14 *in vitro* EDs. In summary, although uterine secretions are still required for conceptus elongation and *in vitro* embryos are delayed in development compared to their *in vivo* counterparts, our post-blastocyst culture system supports hypoblast migration, epiblast proliferation, ED formation and initiation of gastrulation *in vitro*, an unprecedented advance in an ungulate species. Our system may help to elucidate the causes for the reduced rates of post-hatching development of IVP embryos without the need of experimental animals.

Support: AGL2017-84908-R and RYC2018-025666-I from MINECO, and StG-757886 from ERC

Boar semen samples inoculated with bacteria show improved chromatin status after single layer centrifugation with a low density Porcicoll colloid

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Keywords: pig, semen, bacteria

Artificial insemination (AI) is routinely performed in the pig industry. Quality control requires antibiotics for limiting bacterial growth but a need for eliminating their use. Thus, alternative methods have appeared, such as single-layer centrifugation (SLC) with a low-density colloid, enabling the recovery of spermatozoa while removing bacteria. We performed a test with the Porcicoll colloid, spiking boar semen doses with a 104 CFU/ml inoculum prepared from in-situ collected samples (AIM León, Topigs-Norsvin; Pseudomonas aeruginosa, Burkholderia ambifaria, and Staphylococcus simulans). Seven ejaculates were extended and split: CTL (control), CTLS (control spiked), P20 (spiked and SLC with Porcicoll 20%), P30 (Porcicoll 30%). The SLC was performed by centrifuging 15 ml of the sample through 15 ml of Porcicoll, 600×g 20 min, then resuspending the pellet in a laminar-flow bench. After 0, 3, and 7 days of storage (17 °C), samples were analyzed for bacterial growth and sperm chromatin by flow cytometry: SCSA for DNA fragmentation (%DFI) and chromatin maturity (%HDS); and monobromobimane (mBBr) for levels of disulfide bridges. Data were analyzed by linear mixed-effects models. Bacterial growth was evident through storage, detecting other bacteria despite hygienic protocols during collection (environmental bacteria e.g. Aeromonas and Micrococcus, and some coliforms). Bacteria were efficiently reduced by P20 and P30 ($P<0.001$ with CTLS and P30 vs CTL; $P<0.1$ P20 vs CTL), effectively reducing growth for the duration of the experiment by one order magnitude for P30 compared to CTL. In parallel, SLC resulted in significantly less %DFI by day 7 (CTL: $P<0.001$; CTLS: $P<0.05$) and less %HDS ($P<0.001$ vs. both controls). In any case, %DFI was low throughout the experiment, at 1.4%±1.0 for CTLS. The number of disulfide bridges from the mBBr stain was not affected by SLC or the spiking, although it decreased with storage time. Overall, bacterial presence significantly and positively correlated (Pearson r, $P<0.001$) with some chromatin alterations: %DFI with total CFU/ml (0.40), S. simulans (0.41), and E. coli (0.61); %HDS with S. simulans (0.41), and E. coli (0.44); and disulfide bridges with total CFU/ml (0.44). In conclusion, SLC with low-density Porcicoll is efficient in reducing bacteria and also in preserving sperm chromatin integrity. We hypothesize a double effect to explain its positive effects on sperm chromatin: indirectly by reducing noxious bacteria, and directly possibly by removing some damaged spermatozoa. Then, low-density SLC could enable the development of produces for allowing antibiotic-free, extended storage of boar semen.

Funded by grants RTI2018-095183-B-I00 (MCI/AEI/FEDER, EU) and LE023P20, (Junta de Castilla y León/FEDER, EU). We thank Topigs-Norsvin España, AIM (León).

Maternal high-fat/high-sugar diet in outbred mice induces a change in offspring oocyte's global DNA methylation level that is prevented by preconception diet normalisation

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Keywords: obesity, preconception care intervention, epigenetics

Maternal obesity has been linked with subfertility and increased susceptibility to metabolic diseases in the offspring (F1). Maternal obesity causes oocyte mitochondrial dysfunction and transmission of aberrant mitochondria through the female germline in inbred C57BL/6 mice (Saben et al., Cell Reports 16(1), 1-8, 2016). DNA methylation in the oocyte is dependent on mitochondrial functions and is crucial for epigenetic programming during embryo development. Nowadays, obese patients are advised to lose weight before pregnancy as this might improve maternal fertility. However, it is not known if the preconception weight loss can alleviate possible detrimental effects on F1 oocyte quality. We hypothesised that a maternal Western-type diet in outbred Swiss mice causes transmission of defective mitochondria to F1 oocytes and alters their global DNA methylation. We also hypothesised that these effects can be prevented by maternal diet normalisation or caloric restriction (CR) in the preconception period. In this study, 5 week old female Swiss mice were fed a control (CTRL; 10% fat) or high-fat high-sugar (HF; 60% fat in diet, 20% fructose in drinking water) diet for 7 weeks. Afterwards, HF-fed mice were maintained on the HF diet or switched to a CTRL or a 30% CR diet for 4 weeks, resulting in 4 treatment groups: CTRL_CTRL, HF_HF, HF_CTRL, and HF_CR. All mice were then mated with CTRL-fed males and maintained on their corresponding diets during pregnancy and lactation, except for HF_CR which were switched to CTRL diet. After weaning, F1 mice were kept on a standard chow diet. F1 female mice (n= 5/litter, 4 litters/treatment) were sacrificed at 11w after hormonal stimulation for oocyte collection. 30 cumulus-oocyte complexes (COCs) were fixed in glutaraldehyde for electron microscopy to evaluate the oocyte mitochondrial ultrastructure. Other COCs were denuded and 5 oocytes/mouse were fixed in 4% paraformaldehyde for 5-methylcytosine (5mC) immunostaining (n=220). Images were acquired with a Leica SP8 confocal microscope and quantified using Image-J. Data were analysed using ANOVA or Kruskal-Wallis followed by LSD for pairwise comparisons. When compared with the CTRL_CTRL group, F1 oocytes from HF_HF-fed mothers had a significantly increased intensity of 5mC immunostaining (1,46x fold-change, P=0.026) indicating a rise in the global DNA methylation level. This increase was persistent in the HF_CR group, but was prevented in the HF_CTRL group (P=0.001) where the 5mC intensities were similar to the CTRL_CTRL group. Analysis of TEM images did not show any significant changes in mitochondrial ultrastructure or autophagosome count between CTRL_CTRL and HF_HF oocytes. We conclude that maternal diet-induced obesity alters DNA methylation patterns in the offspring oocytes compared with controls. This effect was prevented by preconceptional diet normalisation but not by caloric restriction. Contrary to what we expected, mitochondrial aberrations were not persistent in the mature oocytes of the F1 from HF_HF-fed mothers at the time of oocyte collection. This might be due to a higher efficiency of mitophagy and mitochondrial rejuvenation mechanisms in outbred Swiss mice compared to inbred C57BL/6 mice.

Effect of cytokines on metaphase-II chromosomes and apoptosis resistance of bovine oocytes matured in vitro

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Keywords: in vitro maturation, bovine oocytes, cytokine

Fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF), and insulin-like growth factor 1 (IGF1) have been shown to play a positive role in maintaining the quality of mammalian oocytes maturing in vitro. In the present work, effects of these cytokines, when added in combination to the in vitro maturation (IVM) medium, on the nuclear status and apoptosis resistance of bovine oocytes were studied. Slaughterhouse-derived cumulus-oocyte complexes (COC) were cultured for 24 h in either maturation medium (TCM-199 supplemented with 10 % fetal calf serum (FCS), 0.2 mM sodium pyruvate, 10 μg mL−1porcine FSH, and 10 μg mL−1 ovine LH) (Control) or maturation medium supplemented with FGF2, LIF and IGF1. The eight combinations of cytokines (LIF/IGF1/FGF2) were: (1) 20/10/10 ng mL^{-1} (Group 1), (2) 20/10/40 ng mL^{-1} (Group 2), (3) 20/20/10 ng mL^{-1} (Group 3), (4) 20/20/40 ng mL^{-1} (Group 4), (5) 20/10 /10 ng mL^{-1} (Group 5), (6) 5/10/40 ng mL^{-1} (Group 6), (7) 5/20/10 ng mL^{-1} (Group 7), (8) 5/20/40 ng mL^{-1} (Group 8). After IVM, the state of the oocyte nuclear material was evaluated by the Tarkowski's method (N=529). Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI (N=454). The data (3 replicates, 45-62 oocytes per treatment) were analysed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). No effects of the systems on the nuclear maturation of oocytes were observed, with the maturation rate after culture was: 86.6±4.9% (Control), 84.0±2.4% (Group 1), 88.6±2.8% (Group 2), 74.7±2.9% (Group 3), 83.4±3.8 (Group 4), 83.0±4.5% (Group 5), 83.0±4.5% (Group 6), 83.7±5.7% (Group 7) and 77.7±5.8% (Group 8). The rate of M-II oocytes with degenerative changes of chromosomes (decondensation, adherence, clumping) was lower in the Group 1 (27.7±0.6%, $P<0.05$), Group 6 (23.7±2.2%, $P<0.05$) and Group 7 (27.5±1.0, $P<0.05$) compared with Control (36.4±1.0%), Group 2 (35.8±1.6%), Group 4 (37.1±2.1%) and Group 5 (35.5±1.2%), but not compared with Group 3 (33.9±0.6%) and Group 8 (33.5±2.0%). Also was found out that after culture of COC in Group 1 (20 ng mL−1LIF/10 ng mL−1IGF1/10 ng mL−1FGF2) the rate of matured oocytes with apoptotic signs was the lowest one. This rate did not significant differ as compared to Control (13.3±4.2% vs 21.4±1.9%) and cytokine-treated groups 2-7 (18.8±1.7, 21.4±2.7, 27.9±3.7, 22.1±1.5, 20.0±2.0 and 26.8±4.6% respectively) but was lower than Group 8 (33.8±5.2%, $P<0.05$). In conclusion, LIF, FGF2 IGF1 in optimal combination are able to maintain quality of bovine COC during their maturation in vitro by reducing the frequency of M-II chromosome abnormalities. This research was supported by RFBR (projects No. 18-29-07089) and the Ministry of Science and Higher Education of Russia.

The developmental potential of embryos is affected by dithiothreitol during the prolonged culture and in vitro fertilization of bovine cumulus-enclosed oocytes

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Keywords: dithiothreitol, bovine oocytes, developmental capacity

A better understanding of different aspects of culture of in vitro matured oocyte is of great importance for successful in vitro embryo reproduction (IVP).The aim of the present research was to study the effect of dithiothreitol (DTT) on the embryo development of bovine oocytes matured in vitro using two different systems. Slaughterhouse-derived cumulus-oocyte complexes (COC) were matured for 24 h in TCM199 supplemented with 10% fetal calf serum (FCS), 0.2 mM sodium pyruvate, 10 μg mL^{-1} porcine FSH, and 10 μg mL^{-1} ovine LH. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). In system 1, matured COC (n=459) were transferred to the fresh medium (TCM199 supplemented with 10% FCS) and cultured for 10 h in the absence (Control) or in the presence of DTT (2.5, 5, and 10 μM). At the end of culture, aged oocytes were activated by sequential treatment with ionomycin (5μM for 5min) immediately followed by 6-dimethylaminopurine (2mM for 4h). In system 2, IVM oocytes (n=650) underwent in vitro fertilization (IVF) as previously described (Singina et al., Reprod Fert Dev, 26:154, 2014) using Fert-TALP medium modified by addition of 10 μg mL^{-1}heparin, 20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine. In this case, DTT (at the above listed concentrations) were added directly to the IVF medium. Activated and fertilized oocytes were cultured in CRaa medium (Rosenkrans, First, J Anim Sci 1994, 72:434-7) until Day 5 and then were transferred to the same medium supplemented with 5 % FCS and cultured up to Day 7. All the cultures were performed at 38.5°C and 5% CO^2 in humidified air. At Days 2 and 7 after artificial activation (PA) and IVF, the cleavage and blastocyst rates were determined. In addition, obtained blastocysts were fixed with 4% paraformaldehyde, and the total cell number was determined by DAPI staining. The data from 4-7 replicates (111-168 oocytes per treatment) were analyzed by ANOVA. The addition of DTT (5 μM) to the aging medium raised the blastocyst rate from 9.8±1.2 % (Control for PA) to 18.8±2.5 % ($p<0.05$). Cleavage rate of aging oocyte after artificial activation and total cell number in parthenogenetic blastocyst were unaffected by DTT (except DTT 10 μM group). In the case of DTT 10 μM, the blastocyst rate and blastocyst cell number (9.6±1.3 and 35.4±1.1, respectively) was lower than in the DTT 5 μM group ($P<0.05$). By contrast when added to the IVF medium, DTT raised the blastocyst rate at a concentration of 2.5 μM (from 21.6 ± 2.2% (Control for IVF) to 33.3 ± 2.3%, $P < 0.05$). No effects (similar PA) of DTT on the cleavage rate and total cell number in IVP embryos at the blastocyst stage were found. Our findings indicated that DTT supplements during in vitro fertilization and prolonged culture of matured bovine oocytes may improve their capacity for the subsequent embryo development. This effect was probably due to the inhibitory influence of DTT on aging of ova.

This research was supported by the Ministry of Science and Higher Education of Russia.

Pre-hatching exposure to N2B27 medium improves post-hatching development of bovine embryos in vitro

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Keywords: In vitro culture, embryo quality, conceptus elongation

The developmental competence of *in vitro* produced (IVP) embryos still remains a matter of concern, as pregnancy rates are often reported to be lower for IVP embryos compared to their *in vivo* derived counterparts. Following embryo transfer and prior to implantation, the hypoblast must proliferate and migrate to cover the inner surface of the trophoblast, whereas the epiblast should form the embryonic disc from which the fetus will be developed. Several *in vivo* and *in vitro* evidences suggest that epiblast development is the most critical process to ensure embryo viability shortly after hatching, and we have previously observed that, in contrast to conventional embryo culture media, N2B27 medium supports epiblast development in Day 9 IVP bovine blastocysts (Ramos-Ibeas et al. Reproduction 2020). The objective of this study has been to determine if earlier exposure to N2B27 enhances subsequent development. As N2B27 does not support bovine embryo development from the zygote stage, all the IVP presumptive zygotes used in this study (1626) were initially cultured in SOF medium and then moved to N2B27 medium on days 5 (group N5), 6 (group N6), 7 (group N7) or 9 (group N9) post-fertilization. The number of total (DAPI), trophectoderm (CDX2+), hypoblast (SOX17+) and epiblast (SOX2+) cells were analyzed by immunohistochemistry (IHC) on 64, 30 and 38 Day 8 (D8) blastocysts from N5, N6 and N9 groups, respectively. Post-hatching development was assessed on Day 12 (D12) embryos by IHC using the same lineage markers than on D8 in 61, 35, 32 and 84 embryos from N5, N6, N7 and N9 groups, respectively. Blastocyst rates on D8 were similar in all groups (22.7±2.4 vs. 20.9±1.4 vs. 22.3±3.4 vs. 25.1±11.3 %; mean±s.e.m., for N5, N6, N7 and N9, respectively, ANOVA $p>0.05$). Total cell number on D8 was significantly higher in N5 vs. N9 (Total: 158.4±14.7 vs. 136.9±13.8 vs. 109.7±7 for N5, N6 and N9, respectively, ANOVA $p<0.05$), whereas SOX2+ and SOX17+ cell number was significantly higher in N5 and N6 vs. N9 (SOX2+: 53.9±6.4 vs. 41.5±6.6 vs. 25.5±2.3; SOX17+: 41±6.6 vs. 33±6.7 vs. 16.8±2, for N5, N6 and N9, respectively, ANOVA $p<0.05$). All groups displayed a similar number of CDX2+ cells (89.6±12.3 vs. 83.8±9.9 vs. 64.9±7.1, for N5, N6 and N9, respectively, ANOVA $p>0.05$). Survival rate (not-collapsed structures) from presumptive zygotes to D12 was significantly higher in N5 group vs. N9 (29.8±4.2 vs. 18±2.5 vs. 21.3±3.1 vs. 20.2±1.5, for N5, N6, N7 and N9 groups, respectively, ANOVA $p<0.05$). Finally, Complete hypoblast migration rate at D12 was significantly higher in N5, N6 and N7 groups compared to N9 (30/61 vs. 16/35 vs. 17/32 vs. 20/84, for N5, N6, N7 and N9, respectively, z-test<0.05). Similarly, significantly more embryos developed an embryonic disc structure in N5, N6 and N7 groups compared to N9 (25/61 vs. 16/35 vs. 15/32 vs. 16/84, for N5, N6, N7 and N9, respectively, z-test<0.05). In conclusion, culture of bovine embryos in N2B27 from Day 5 onwards enhance the development of hypoblast and epiblast lineages, whose survival is hampered by prolonged culture in SOF medium.

Support: StG-757886 from ERC, and AGL2017-84908-R and RYC2018-025666-I from MINECO

Arachidonic acid supplementation during post-hatching bovine in vitro development regulates the transcription of genes related to prostaglandin synthesis and signaling

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Keywords: Pregnancy signaling, prostaglandin, conceptus elongation.

Prostaglandins (PGs) are lipid signaling molecules that play critical roles during pregnancy. Descriptive studies reporting the expression of PGs receptors and synthesizing enzymes in the conceptus have suggested that conceptus-derived PGs may act as signaling molecules to the uterus and even play autocrine roles inside the conceptus during elongation. PGs and all other prostanoids derive from arachidonic acid (AA) by the cyclooxygenase pathway and AA is not present in a medium allowing post-hatching bovine development (N2B27), which contains linoleic and lipoic acids. Under such medium, bovine embryos develop beyond hatching as evidenced by the complete migration of SOX17+ hypoblast and the formation of a SOX2+ ED-like structure (Ramos-Ibeas et al. Reproduction 2020). The objective of this study has been to determine the effects of AA supplementation during *in vitro* bovine post-hatching development on the expression of candidate genes involved in PG synthesis and signaling. Day 7 blastocysts produced *in vitro* following conventional protocols were cultured in N2B27 in the presence or absence of AA at different concentrations, replacing half of the media every second day. Initial tests observed that AA supplementation to 100 µM abolished completely post-hatching development, whereas 20 µM impaired blastocyst growth. Consequently, D7 blastocyst were cultured in N2B27 medium supplemented with 5 (AA5) or 10 (AA10) µM AA or without supplementation (AA0). By Day 12 of culture (i.e., 5 days of AA supplementation) 5 groups of 3 embryos per experimental group were snap frozen and stored at -80 ºC until analysis. mRNA was extracted by Dynabeads mRNA DIRECT Micro kit, treated with DNAse and retrotranscribed by qScript cDNA SuperMix (Quantabio). Relative abundance of specific transcripts were determined by qPCR using $2^{-\Delta\Delta Cq}$ method and *H2Afz* as housekeeping gene. The candidate genes analyzed encode for the rate limiting enzyme of cyclooxygenase pathway (*PTGS2*), two PG synthethases (*PTGES* and *PTGIS*), a PG receptor (*PTGFR*), a receptor involved in signaling of lipid molecules (*PPARG*), the protein mediating pregnancy recognition signal in ruminants (*IFNT2*), and rate limiting enzymes for the formation of monounsaturated fatty acids (*SCD*), Krebs cycle (*CS*) and anaerobic glycolysis (*LDHA*). *PTGIS* and *PPARG* were significantly upregulated in AA0 vs. AA5 (*PTGIS*: 2.3±0.9 vs. 1±0.2 vs. 1.7±0.2; *PPARG*: 2.2±0.4 vs. 1±0.2 vs. 1.6±0.3, mean±s.e.m. for AA0, AA5 and AA10 respectively, ANOVA $p<0.05$). *SCD* and *LDHA* were significantly upregulated in AA0 vs. AA10 (*SCD*: 2.6±0.4 vs. 1.9±0.5 vs. 1±0.2; *LDHA*: 1.7±0.3 vs. 1.4±0.2 vs. 1±0.1, mean±s.e.m. for AA0, AA5 and AA10, respectively, ANOVA $p<0.05$). These results evidence that *in vitro* developed bovine post-hatching embryos are responsive to AA, but the negative regulation of specific genes in the presence of AA may suggest that AA requirement could be already fulfilled by the linoleic acid already present in N2B27 medium.

Support: StG-757886 from ERC, and AGL2017-84908-R and RYC2018-025666-I from MINECO.

Activity of the thyroid system after artificial insemination of dairy cows with different reproductive abilities

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Keywords: dairy cows, thyroid hormones, reproductive ability

Thyroid hormones are involved in the endocrine control of the intensity of metabolic processes and thereby can affect female fertility. In cattle, these hormones are present in follicular fluid and reproductive tract and the respective receptors are expressed in oocytes and cumulus cells, suggesting direct hormonal effects on the reproductive function (Ashkar et al., Exp Biol Med Maywood, 235:215, 2010; Costa et al., Theriogenology, 80:295, 2013). Furthermore, thyroid hormones at certain concentrations are able to exert beneficial effects in vitro on the development capacity of bovine oocytes or the quality of bovine embryos (Ashkar et al., Hum Reprod, 25:334, 2010). Therefore, the aim of the present research was to study thyroid profiles on the day of insemination and during the first month after insemination in dairy cows depending on the pregnancy success. Thirty lactating Russian Black Pied cows without clinical signs of postpartum metabolic or gynecological diseases, kept on one farm, were used. The animals were synchronized using the Ovsynch protocol and artificially inseminated (AI). Blood samples from the cows were collected on Days 0, 7, 14, 21, and 33 after AI. Hormonal levels in the serum were measured by ELISA. Pregnancy was confirmed by ultrasonography on Day 33 and progesterone concentrations throughout the studied period. The cows were divided into two groups: pregnant (n=18) and non-pregnant (n=12). Hormonal levels were compared by two-way ANOVA for repeated measurements followed by Tukey's HSD test. The serum concentration of thyroxine (T4) did not change considerably during the entire study period in pregnant cows and decreased between Days 7 and 14 after insemination (from 59.0±8.5 to 30.7±1.3 nmol/L, $p<0.05$) and then increased by Day 33 (to 83.7±12.3 nmol/L, $p<0.001$) in non-pregnant cows. After fruitful insemination, the total triiodothyronine (T3) content in the blood of cows was relatively constant for 1 month, varying between 0.96 and 1.24 nmol/L. In animals that remained infertile, this content, on the contrary, was high before insemination (1.41±0.14 nmol/L), reached a maximum value on Day 7 (1.70±0.22 nmol/L) and then decreased 1.8-fold by Day 14 (to 0.93±0.06 nmol/L, $p<0.001$). Meanwhile, on Day 7, the concentration of total T3 in non-pregnant cows was 1.4 times higher than in pregnant cows ($p<0.01$), while the concentration of total T4 did not differ. At the same time, the content of reverse T3 in the blood was the highest on the day of insemination (0.458 nmol/L) and decreased 1.2-fold ($p<0.01$) on Day 14 in animals of both groups. The concentration of free T3, which characterizes the bioavailability of the hormone for body tissues, did not depend on the pregnancy success and did not change during the entire observation period. Thus, during the first month after insemination, thyroid profiles were dependent on the pregnancy status of cows. In non-pregnant animals, the revealed changes in total T4 and total T3 levels may be due to an association with the estrous cycle, which suggests their possible use as additional markers for the pregnancy success. The study was supported by Russian Ministry of Science and Higher Education (0445-2021-0004).

Support Biotechnologies: Cryogenesis and Cryobiology, Diagnosis through Imaging, Molecular Biology and "Omics"

Comparison of sugars as non-permeating cryoprotectant for immature equine oocyte vitrification.

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Keywords: Equine oocyte, sugars, vitrification.

Oocyte cryopreservation in horses would be an important complement to the growing clinical ICSI programs, but the efficiency is not satisfactory yet. Sugars are non-permeating cryoprotectants that exert an osmotic effect during vitrification. However, the influence of different sugars during vitrification and warming of equine oocytes on subsequent embryo development has not been evaluated. Therefore, we aimed to determine the effects of three sugars on the developmental competence of equine oocytes. Cumulus-oocyte complexes (COCs) were obtained from slaughterhouse-derived ovaries. The cumulus cells were removed by pipetting until around 4 layers remained. Then, COCs were vitrified immediately in three groups: sucrose (S; n=155), trehalose (T; n=160) and galactose (G: n=153). Oocytes were equilibrated for 25 seconds in base solution (BS; TCM 199 with Hanks' salts and 0.4% (w/v) BSA) with 10% (v/v) ethylene glycol (EG) and 10% dimethyl sulfoxide (DMSO), and subsequently transferred to a vitrification solution (BS with 20% EG, 20% DMSO and 0.5 M sugar), loaded onto a custom-made minimal volume (<1 µL) cryo-device (Equine Vet J. 50(3). 391-397. 2018) and plunged into liquid nitrogen within 40s. For warming, the cryo-device was placed directly into a 0.5 M sugar solution and incubated for 5 min, then COCs were transferred and washed in BS. All procedures were performed on a thermal plate at 39˚C. Once warmed, COCs were matured in 500 µl M199 with Earls' salts and 10% FBS at 38.5˚C in 5% CO_2 in air for 28 hours. Oocytes with an extruded polar body were injected by piezo assisted ICSI and presumed zygotes were cultured in 20 µl droplets of DMEM-F12 with 10% FBS under oil for 7 – 10 days at 38.2˚C in 5% O_2, 5% CO_2 and 90% N_2. A control group (n=173) with non-vitrified oocytes was included in every replicate (5 replicates). The effects of the sugars on maturation, cleavage, and blastocyst rate were fitted in generalized and linear mixed-effects models and results are expressed as least square means with standard errors. Galactose tended to result in a lower maturation rate compared to trehalose (p = 0.060) and the control group (p=0.069), but there was no significant difference in maturation rate among sugars (S: 52.4±4.1%; T: 57.4±4.1%; G: 43.1±4.1%), and control (56.9±4.0%; P > 0.05). Cleavage rates were not different between treatments (S: 53.2±5.6%; T: 61.8±5.2%; G: 73.4±5.5%), but the cleavage rate after vitrification with sucrose was significantly lower than that of the control (75.3±4.8%; p=0.02) and tended to be lower than that of galactose (p=0.066). Finally, blastocyst rates for all vitrified groups (S: 5.0±2.5%; T: 4.3±2.2%; G: 7.6±3.4%) were significantly lower compared to the control group (26.5±5.7%). Nevertheless, galactose, a monosaccharide tested for the first time in equine oocyte vitrification, resulted in the highest blastocyst rates after vitrification, as well as in equal cleavage rates compared to the control. Therefore, galactose should be considered as an alternative sugar for future optimization of vitrification protocols for equine immature oocytes.

Acknowledgements: The authors thank Petra Van Damme for her excellent technical assistance.

Relative mtDNA amount in human cumulus cells does not correlate with oocyte´s developmental potential

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Keywords: Cumulus cells, oocyte quality, embryo transfer.

The developmental competence of the embryo constitutes a major factor for pregnancy success following embryo transfer and, thereby, the implementations of methods for embryo selection is crucial to enhance reproductive success following Artificial Reproductive Techniques. Morphological evaluation is the most widely used method for embryo selection, but pregnancy rates in humans remain around 35 %, suggesting that there is still room for improvement. Molecular analyses performed on cumulus cells may help to complement morphological evaluation in embryo selection. Cumulus cells are closely connected to the oocyte through folliculogenesis and oocyte maturation and are discarded prior to fertilization, constituting an interesting biological material on which to perform non-invasive analyses. Oocyte´s mtDNA content has been positively associated to the embryo developmental potential in both animal models and humans. However, as the analysis of mtDNA in oocytes is not compatible with subsequent development, relative mtDNA abundance in cumulus cells provide an alluring alternative. The objective of this study has been to determine whether relative mtDNA content in human cumulus cells correlates to oocyte´s developmental potential. To this aim, cumulus cells were obtained from IVF cycles performed at IVF Spain, Madrid. Inclusion criteria were 1) absence of uterine abnormalities, 2) donor age ≤37 years, 3) recipient age ≤50 years, and 4) normal male sperm count. To further minimize variability, the samples used were obtained only from ICSI cycles, using unfrozen oocytes and embryos not submitted to preimplantation genetic testing. Following individual oocyte denudation, cumulus cells were pelleted at 1500 g for 10 min, snap frozen in liquid nitrogen and stored at -80 ºC until analysis. Development to blastocyst was recorded on day 6 post-ICSI and pregnancy was assessed at 4 to 5 weeks post-ICSI by fetal heart rate detection by ultrasound echography. Samples were allocated into three groups according to their developmental potential: 1) oocytes not developing to blastocysts (Bl-), 2) oocytes developing to blastocyst but failing to establish a pregnancy (P-), and 3) oocytes developing to blastocyst able to establish pregnancy (P+). Cumulus cells were digested by PicoPure and qPCR was performed to determine relative mtDNA by comparing Cq values for a mtDNA (*COX1*) and autosomal (*PPIA*) sequence using $2^{-\Delta\Delta Cq}$ method in 109 samples (37, 38 and 34 for Bl-, P- and P+ groups, respectively). Relative mtDNA abundance was remarkably similar between groups (1.1±0.07 vs. 1±0.05 vs. 1.06±0.06 for Bl-, P- and P+, respectively, ANOVA $p>0.05$), suggesting that this parameter provides a poor predictive value of the oocyte´s developmental potential both *in vitro* and following embryo transfer.

Support: IND2017/BIO-7748 from Madrid Region Government.

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