AETE

Association Européenne des Technologies de l' Embryon

Association of Embryo Technology in Europe

36ème COLLOQUE SCIENTIFIQUE

36th SCIENTIFIC MEETING

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"Virtual AETE-Lite"

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10th and 11th September, 2020

AETE

Association Européenne de Transfert Embryonnaire

European Embryo Transfer Association

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President´s letter

Dear Friends, dear Colleagues,

It is with great pleasure that I invite you to this Annual Scientific Meeting of the Association of Embryo Technologies in Europe. This year, however, we go nowhere, we stay home. The corona virus obliged us to adapt our annual habits and forced us to look for a valuable alternative. It was not an easy decision taken by the board, to cancel our annual meeting. The local organizing committee in Dublin, chaired by Patrick Lonergan was very well prepared and ready to welcome us in a wonderful conference venue. However, these efforts will not be lost as we postponed our annual meeting with one year. So, our next (physical) AETE meeting will take place in Dublin at September 8-9 (Wednesday and Thursday), 2021. Please block your agendas and inform your network.

The board of the AETE unanimously decided to provide an online alternative for 2020; we call it AETE-lite. We want to maximize the opportunity for our young researchers to submit an abstract and to give an online presentation. We do realize that this is important for their scientific career. About 60 abstracts were submitted and accepted for publication in Animal Reproduction and for presentation during our AETE-lite meeting. 35 of these abstracts were submitted as "oral presentation" showing the high need for the authors to interactively present their work. Our AETE-lite meeting tries to cover that need and will take place on Thursday 10 and Friday 11 September. The first day is a rather "silent" day where all registered participants can virtually walk in their own time along the uploaded posters and materials submitted by the sponsors. Friday will be the interactive day, in real time. In short, there will be 9 real-time oral presentations and time for interaction both with the scientist and the sponsors. The best oral presentation will win an AETE-lite award! Do not forget that if you would like to participate in our AETE-lite meeting (be it as a presenter or as a delegate), that you will have to register through our website. We very much look forward to meeting you!

And of course, we have the keynote speakers. It was decided to postpone our main program together with the pioneer award winner with one extra year. Their interesting papers however, will be published in this summer volume of Animal Reproduction but will not be published in this proceedings book. I sincerely thank all speakers for making their agenda available in September 2021.

Also during this very difficult year, the intense collaboration between AETE and SBTE is very well appreciated by the board and all members. As you know, this year we jointly publish already for the sixth time our abstract proceedings and invited lectures in Animal Reproduction. At the Affiliated Society meeting at IETS 2020 (New York), this collaboration and joint publication efforts between AETE and SBTE were mentioned and really appreciated.

Finally, we cannot underestimate the great importance of our Sponsors in our Society. We are very grateful that following sponsors stayed on board this year, even without any physical meeting in September: (9th June 2020):

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Enjoy reading the special issue of our Annual Proceedings, covering the AETE-lite meeting and stay up to date at www.aete.eu.

Jo Leroy, President of the AETE
Summer 2020

Commercial Embryo Transfer Activity in Europe 2019

Collated by Hèlène Quinton

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*Countries without 2019 declared embryo transfer activity at the time of the publication

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	406	3%	3 884	2 984	19	0%	132	106	425	3 090	7,3
Belgium	48	0%	271	184	706	0%	4 857	3 412	754	3 596	4,8
Denmark	787	0%	0	5 011	0		0	0	787	5 011	6,4
Finland	408	12%	3 665	2 352	1	0%	18	15	409	2 367	5,8
France	4 691	8%	49 100	29 408	1 338	5%	14 263	7 415	6 029	36 823	6,1
Germany	2 078	9%	28 583	19 560	247	24%	3 624	2 223	2 325	21 783	9,4
Greece	5	0%		21	0		0	0	5	21	4,2
Ireland	610	0%	7 381	3 321	0		0	0	610	3 321	5,4
Israel	26	4%	115	94	0		0	0	26	94	5,4
Italy	2 590	56%	26 936	18 389	145	0%	1 522	1 015	2 735	19 404	7,1
Latvia	14	0%	97	59	0		0	0	14	59	4,2
Lithuania	4	0%	32	20	6	0%	39	27	10	47	4,7
Netherlands	186	6%	0	1 024	0		0	0	186	1 024	5,5
Norway	174	1%	1 421	1 021	16	0%	175	138	190	1 159	6,1
Portugal	139	35%	1 226	611	18	0%	235	127	157	738	4,7
Romania	15	0%	107	80	1	0%	4	4	16	84	5,3
Russian Federation	326	58%	3 668	1 912	1 869	14%	12 706	9 943	2 195	11 855	5,4
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	270	53%	2 455	1 397	202	9%	2 031	1 181	472	2 578	5,5
Sweden	56	5%	524	378	5	0%	0	0	61	378	6,2
Switzerland	327	50%	3 058	1 720	14	0%	133	60	341	1 780	5,2
Grand Total	**13 164**	**20%**	**132 731**	**89 702**	**4 592**	**9%**	**39 784**	**25 693**	**17 787**	**115 395**	**6,5**

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	Oocytes	Embryos	Embryos /OPU
Finland	714	5%	6 349	1 365	0		0	0	714	6 349	1 365	1,9
France	325	34%	3 967	960	14	0%	200	106	339	4 167	1 066	3,1
Germany	1 809	0%	24 647	6 570	58	0%	1 185	275	1 867	25 832	6 845	3,7
Italy	126	67%	1 849	473	0		0	0	126	1 849	473	3,8
Netherlands	513	10%	7 013	1 297	0		0	0	513	7 013	1 297	2,5
Romania	5	0%	31	13	0		0	0	5	31	13	2,6
Russian Federation	595	85%	5 735	547	0		0	0	595	5 735	547	0,9
Serbia	24	38%	115	32	0		0	0	24	115	32	1,3
Spain	182	84%	2 976	850	47	13%	711	335	229	3 687	1 185	5,2
Switzerland	94	67%	572	139	3	0%	12	0	97	584	139	1,4
Grand Total	**4 387**	**23%**	**53 254**	**12 246**	**122**	**5%**	**2 108**	**716**	**4 509**	**55 362**	**12 962**	**2,9**

Declared bovine *In vitro* embryo production – abattoir

	Dairy			Beef		
Country	Donors	Oocytes	Embryos	Donors	Oocytes	Embryos
Italy	2	123	30	3	139	53
Serbia	0	0	0	5	53	12
Spain	48	968	502	196	1825	584

Declared bovine embryo technologies – embryo genotyping

	Sexed embryos		Genotyped embryos	
Country	In Vivo	In Vitro	In Vivo	In Vitro
Finland	92	77	92	77
France	2595	0	2526	0
Germany	0	0	206	154
Spain	0	92	0	92
Total	**2687**	**169**	**2824**	**323**

Declared bovine embryo transfers and exports- *In vivo*

	Dairy				Beef				Total embryos transferred
	Fresh embryos transferred	Frozen embryos			Fresh embryos transferred	Frozen embryos			
Country		Domestic transferred	Foreign transferred	Exported		Domestic transferred	Foreign transferred	Exported	
Austria	1 161	1 316	38	58	31	54	23	0	2 623
Belgium	59	118	18	6	491	2 157	22	76	2 865
Denmark									4 411
Finland	491	1 121	176	611	0	0	0	0	1 788
France	13 740	12 405	1 192	208	2 194	4 215	182	219	33 928
Germany	8 136	12 300	0	132	493	1 152	0	0	22 081
Greece	0	0	9	0	0	0	0		9
Ireland									1 340
Israel	42	2							44
Italy									7 450
Latvia	44	25	0	0	0	0	0	0	69
Lithuania	0	0	0	0	0	7	0	0	7
Netherlands	302	557	63	0	0	0	0	0	922
Norway	37	553	47	0	23	7	33	41	700
Portugal	152	370	37	0	17	57	62	0	695
Romania	58	12	0	0	0	4	55	0	129
Russian Federation	451	1 635	0	0	881	10 150	381	0	13 498
Serbia	15	0	0	0	0	0	60	0	75
Slovenia	75	20	6	0	17	6	12	0	136
Spain	605	474	17	0	455	277	57	70	1 885
Sweden	200	770	0	0	0	0	0	0	970
Switzerland	625	958	492	51	7	21	10	0	2 113
Grand Total	**39 394**	**32 6364**	**2 095**	**1 066**	**4 609**	**18 107**	**897**	**406**	**97 738**

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	56	7	0	0	0	63
Finland	2	360	0	353	0	0	362
France	368	299	78	126	0	0	745
Germany	3 873	3 238	0	0	0	0	7 111
Italy	156	0	0	0	0	12	168
Netherlands	792	281	210	0	0	0	1 283
Portugal	0	0	17	0	0	0	17
Russian Federation	101	281	0	0	0	0	382
Serbia	0	18	0	0	0	5	23
Spain	474	56	10	0	12	12	564
Grand total	**5 766**	**4 589**	**322**	**479**	**12**	**29**	**10 718**

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

Species	Country	Embryo collection: Collections	Embryo collection: Viable embryos	Embryo transfer: Fresh embryos	Embryo transfer: Frozen domestic	Embryo transfer: Frozen foreign	Embryo transfer: Exported embryos
Buffalo	Italy	0	0	0	19	0	0
	Romania	5	6	6	0	0	0
	Total	**5**	**6**	**6**	**19**	**0**	**0**
Sheep	Greece	12	33	0	0	0	0
	Portugal	3	18	10	0	0	0
	Romania	2	11	8	0	0	0
	Russian Federation	0	0	0	0	681	0
	Serbia	0	0	12	0	0	0
	Spain	12	111	53	12	0	350
	Sweden	0	0	0	0	225	0
	Total	**29**	**173**	**83**	**12**	**906**	**350**
Horse	France	1543	783	939	0	0	0
	Italy	298	212	212	0	0	0
	Russian Federation	33	22	5	0	0	0
	Spain	9	7	7	0	0	0
	Sweden	34	20	20	0	0	0
	Total	**1917**	**1044**	**1183**	**0**	**0**	**1**

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

Species	Country	Oocyte collection: OPU	Oocyte collection: Oocytes	Oocyte collection: Embryos	IVP embryo transfer: Fresh embryos	IVP embryo transfer: Frozen domestic	IVP embryo transfer: Frozen foreign	Exported embryos
Sheep	Spain	15	340	142	142	0	0	0
Goat	Spain	31	1631	748	21	12	0	0
Horse	Italy	2045	25917	3711	48	501	0	0
	France	0	0	0	0	0	0	0
	Switzerland	49	236	11	0	0	0	0
	Total	**2045**	**25917**	**3711**	**48**	**501**	**0**	**0**

SHORT COMMUNICATIONS

TAI/FTET/AI

Cervical transcriptomic profiling of high and low fertility sheep breeds

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Keywords: RNAseq, cervix, ovine
In sheep, cervical artificial insemination (AI) involves depositing semen at the cervical opening, as it is not possible to traverse the sheep cervix due to its complex anatomy. However, this method yields low pregnancy rates of less than 30% worldwide when frozen-thawed semen is used. The only exception to this is in Norway, where vaginal (shot-in-the-dark) insemination with frozen-thawed semen to a natural oestrus is performed by farmers and yields pregnancy rates of approximately 70%. Research in Ireland has demonstrated this is due to the ewe breed, since sperm can traverse the cervix in greater numbers in some ewe breeds (Belclare) than in other breeds (Suffolk). However, the molecular mechanisms underlying differences in sperm transport through the cervix and its secretions remain unknown. The aim of this study was to profile the transcriptome of the ovine cervix in four ewe breeds with known differences in pregnancy rates following cervical AI using frozen-thawed semen. These were Belclare and Suffolk in Ireland (high and low fertility, respectively) as well as Fur and Norwegian White Sheep (NWS) in Norway (both with high fertility compared to the Irish ewe breeds). Cervical post mortem tissue samples were collected from the four ewe breeds (all the ewes were parity 3-5) at the follicular and luteal phases of the oestrus cycle (n=8-10 ewes per breed at the follicular phase of a natural and synchronised cycle and at the luteal phase of a synchronised cycle). Following euthanasia, the ovaries were assessed for the presence of an active corpus luteum (luteal phase = Day 9) or dominant follicles (follicular phase = 12 h post detection of standing oestrus, Day 0). The reproductive tracts were then longitudinally opened and two sections were taken from the mid region of the cervix. High-quality RNA extracted from the cervical tissue samples was analysed by RNA-seq and differential gene expression was assessed. We identified 7232, 7716 and 510 differentially expressed genes (DEGs) in NWS, Fur and Belclare ewes (respectively) compared to the Suffolk breed (reference level) at the follicular phase of the oestrus cycle. At the luteal phase, 1661, 4984 and 2087 genes were differentially expressed in NWS, Fur and Belclare, respectively (FDR < 0.01). Gene ontology analysis identified enriched pathways for transmembrane transport (CA5A, PLN, MT -COX1), inflammatory response (KLKB1, MLKL, TSPAN2) and cervical remodelling (TGFB1I1, NEXN, TAGLN, TPM1, COL9A2, TES). Although, there was a considerable overlap in the differential expressed genes between ewe breeds, with 262 and 202 genes in common for the four ewe breeds at the follicular and luteal phases, respectively (P < 0.05). In conclusion, this study has shown that there are breed- and phase-specific differences in cervical gene expression between ewe breeds known to differ in sperm transport across the cervix. This novel study provides the first transcriptional analysis of cervical tissue in four economically important European ewe breeds and aids our understanding on why frozen-thawed sperm can traverse the cervix in some ewe breeds but not in others.

Effects of environmental heat stress on ram's seminal plasma oxidative stress and proteome in INRA180 sheep

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Keywords: heat stress, seminal plasma, oxydative stress, proteome, ram

The testis temperature must range from 2 to 8°C below body temperature to ensure successful spermatogenesis and disruption of this condition causes varied degrees of damages to the gonadal parenchyma and sperm production. Thus, exposure of animals to high temperatures potentially affects their reproductive efficiency. Seminal plasma (SP) is a component within the semen having a great role in maintaining sperm motility in many species. Previous studies focus on heat stress scrotal insulation on semen quality but there is a lack of studies based on environmental heat stress on oxidative stress status and seminal plasma proteome. In Morocco, animals are suffering from such effects mainly during summer when animals are grazing far away from their sheepfold. Thus, the present work aimed to study the effects of heat stress on rams's seminal plasma proteome and oxidative stress in INRA180 sheep. From mid Jun to the end of September 2019, semen samples were collected by artificial vagina (AV) from 12 INRA180 rams and then centrifuged to obtain seminal plasma (SP). The animals were randomly assigned to 3 groups. The control group (G0) was housed under sub humid conditions and was exposed to the sun during the grazing time from 7 to 11 am and from 3 to 6 pm. In the remaining time, animals were kept in a ventilated shed. The experimental groups were housed under sub humid (G1) and semi-arid (G2) conditions and were exposed to the sun during the whole day. From 15th to 30th of June, SP were collected once a week and used as a control. During the two months (July-August) of heat exposure no sample was collected. Then during the whole September, the samples were collected once a week. Total proteins, SOD activity and the level of GSH were evaluated in seminal plasma. Seminal proteins were analyzed by mass spectrometry. Statistical analysis to estimate the animal group effect were performed using SAS, ANOVA program (SAS Institute Inc., Cory, NC, USA). To compare the estimated means, the Dunnett test were used. After heat exposure, the total proteins (mg/ml) were lower in G1 (24.24 ± 0.35) than in G0 (25.49 ± 0.02) and G2 (26.84 ± 0.06). The SOD (UI/mg prot) activity was significantly ($P < 0.05$) higher in G0 (78.39 ± 0.55) than G1 (76.33 ± 0.62) and G2 (73.22 ± 0.55). The level of GSH (mg/dL) is highly affected by the heat stress exposure. The lowest value was recorded in G0 (18.68 ± 0.13) and G1 (18.83 ± 0.14) while the highest level was obtained in G2 (19.64 ± 0.16). The preliminary results of the proteomics analysis showed that 444 proteins were identified, and their appearance depended on the experimental group. Label free protein quantification showed that heat shock protein alpha was higher in G1 (4.7 times) and G2 (5.5 times) than in G0. Some proteins (Serpin domain-containing protein for instance) were only present in G0. Others were present in G1 and G2 while they were absent in G0. To conclude, the results in this study suggest that the environmental heat stress affects the oxidative stress indicator levels and the proteome and might be associated with semen quality.
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Increasing the genetic potential in a nucleus of Romanian Buffaloes, by artificial insemination with sexed semen, after stimulation with OvSynch protocol - A Case Report

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Keywords: Indigenous Romanian Buffalo (IRB), artificial insemination (AI), sexed semen, genomically tested bulls.

Although artificial insemination (AI) may mean a significant improvement in buffalo genetic improvement, its practical application has been difficult due to the low visibility of estrus and its poor human detection, the variable duration of estrus and the difficulty of predicts the time of ovulation. More recently, the development of protocols for synchronizing ovulation and planned of AI in buffaloes has been used to overcome these constraints and to be able to use AI on a large scale. Genetic improvement is a dynamic process that must evolve over time, supporting and responding to the needs of breeders, the market and the local context.

As the number of buffaloes in Romania is decreasing in recent years, the application of current reproductive biotechnologies to these breeds is limited. However, these AI is used occasionally in private smallf arms. In Italy, the country consecrated with tradition, in the production of milk buffaloes, AI has the most extensive use, but a large part of farms still using mount bull.

The farm ″ Terra di Buffala ″ has buffaloes of the Mediterranean breed, the Indigenous Romanian Buffalo (IRB) variety, and through this study we wanted to form a nucleus of females with high genetic potential. Thus, by implementing AI, an attempt was made to obtain female fetuses using sexed and genomically tested semen. The experiment was performed on a number of 20 multiparous buffaloes at over 60 days postpartum and lactation. Two batches of 10 were created for A.I. separated by two bulls, Oro and Aton, whit 2 Millions female sperm straws. (S.C. Genomix, importer of the National Association of Young Buffalo Breeders from Italy). The females were at the beginning of the breeding season, February - March. The groups was compiled after a thorough gynecological and general examination, and subsequently the Ovsynch therapeutic protocol (Gn-RH, PGF, Gn-RH) was started. According to the protocol, the females received on day zero and nine, 0.01mg buserelin acetate (Receptal®, MDS-Intervet, Holland) and PGF received on the fifth day, cloprostenol 500 µg.IM (Estrumate®, MDS, Holland). The average body score was 3, the females had a completely involved and healthy uterus. The ovaries showed no signs of pathology, and their average size was 2 cm. Also, no corpus luteum was present at the beginning of the protocol. To prevent waste, they were AI only buffaloes that were at least interested in the bull, and that had a dominant follicle (DF) on the ovary (at least .9 mm).The AI method was tactile recto cervical, females were inseminated once at 18 hours after the second Gn-RH injection. The ovarian response was good, due to the selection of females, bull stimulation and the beginning of the breeding season. Therefore, 75% buffaloes (15/20) were diagnosed in estrus and inseminated. The conception rate was 60% (9/15), being diagnosed pregnant at 45 days (transrectal ultrasound white Honda HS-1600V®, Japan; 7,5 MHz). By categories of bulls the percentages were 50% in Oro (4/8) and 70% the Aton (5/7). 9 calves were born, the sex ratio was 88.8%, only one male was produced by Aton. We state that the goal of increasing the genetic potential of IRB by using AI with sexed sperm becomes achievable.

OPU/IVF and ET

The impact of progesterone releasing device on ovarian response and IVP success in stimulated cycles of transvaginal follicular aspiration (OPU) in cattle

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Keywords: progesterone, ovum pick-up, IVP

Two intravaginal progesterone (P4)-releasing devices are currently available for use in cattle in the UK. We previously established that the PRID Delta 1.55g (Ceva Animal Health Ltd, Amersham, UK) was suitable for use in peri-pubertal OPU donors, but could not confirm any IVP benefits relative to the CIDR 1.38g (Zoetis UK Ltd, Leatherhead, UK) (Black & Sinclair, *Cattle Practice* **25:** 276, 2017). The current study, therefore, sought to compare the ovarian response and IVP outcome for stimulated cycles of OPU (Nivet et al. *Reprod* **143:** 165, 2012) that used either a PRID or a CIDR to provide P4 support.

Following establishment of a reference oestrus (Day 0), eight sexually mature Holstein heifers underwent five stimulated cycles of OPU-IVP using established protocols (Nivet et al. *Reprod* **143:** 165, 2012). Briefly, each cycle consisted ablating all ovarian follicles ≥ 5 mm (dominant follicle removal; DFR) and insertion of a PRID or CIDR (Day 3), and FSH stimulation (6 x 70IU Folltropin (Vetoquinol UK Ltd, Towcester, UK) i.m. at 12 h intervals) commenced 48 h later.

Cumulus-oocyte complexes (COCs) were aspirated 38-42 h following final FSH injection (Day 9). A replacement P4 implant was inserted at OPU and the process repeated. Donor animals used the same type of device (i.e. PRID or CIDR; n=4) throughout. Blood samples were collected at DFR and OPU for P4 analysis by ELISA. All proportions were analysed using generalized linear mixed models that assumed binomial errors and used logit-link functions. Follicles aspirated and oocytes retrieved assumed Poison errors and used log-link functions. P4 concentrations were analysed by repeated-measures ANOVA.

The first two cycles of OPU were undertaken in the presence of a visible (by ultrasound) *corpus luteum* (CL) (P4 = 7.26 and 9.25 ng/mL (SED=2.13) for CIDR and PRID treatment groups respectively). The final three cycles of OPU were undertaken in the absence of a visible CL (P4 = 2.34 and 3.18 ng/mL (SED=0.537)). Number of follicles aspirated were 18.5±1.83 and 18.6±1.96 for CL present (CIDR vs PRID), and 16.7±1.42 and 22.3±1.64 (P=0.068) for CL absent (CIDR v PRID) respectively. Mean number of COCs retrieved were 12.1±1.69, 11.9±1.78, 9.8±1.23 and 14.4±1.50 (P=0.052) for the same respective combinations. Proportions of transferrable quality (IETS stages 7-9) blastocysts of matured were 0.443±0.0504, 0.410±0.0540, 0.171±0.0348 and 0.422±0.0376 (P=0.018) also for the same respective combinations.

These observations indicate that a PRID rather than a CIDR increases transferable embryo yields when a visible CL is absent. This may be due to increased P4 in PRID v CIDR devices, although the timing of P4 sampling (at P4 device changeover) prohibited us from confirming this. It will be necessary, therefore, to corroborate these findings to confirm elevated plasma P4 concentrations in the presence of a PRID than a CIDR with more frequent sampling.

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Effect of exogenous progesterone on the follicular dynamics, recovery, quality, and in-vitro developmental competence of embryos in Sahiwal cattle undergoing repeated ovum pick-up (OPU) sessions

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Keywords: Sahiwal cattle, Progesterone, In-vitro embryo production

The objective of present study was to evaluate the effect of exogenous progesterone (P4) on ovarian follicular population, oocyte recovery, oocyte quality and *in-vitro* developmental competence in Sahiwal cows (*Bos taurus indicus*). After follicle ablation (day 0), twenty (n=20) wave synchronized Sahiwal cows were randomly divided into high-progesterone (Treatment) and low-progesterone (Control) groups. The animals in the treatment group received a progesterone device (CIDR) inserted into the vagina for four days on day 0 while the animals in the control group received no treatment at all. In both groups, animals were subjected to transvaginal ultrasound guided oocyte aspiration on day 4 following dominant follicle ablation and after every 96 hours, seven consecutive OPU's were performed in both groups. Transrectal ultrasonic scanning for follicular dynamics was performed after every 12 hours between the OPU intervals. At the time of each OPU, blood sampling for serum progesterone was performed. The OPU aspirates were searched in the laboratory for the COCs and, under optimized culture conditions, viable oocytes (Grade A, B and C) were processed for IVC (4 replicates) following IVM and IVF until day 7. The COCs (3 replicates) were denuded and treated with Hoechst (Sigma 33342) to estimate oocyte nuclear maturation after 24 hours of IVM at 38.5ºC, 5% CO_2 and 95% humidity. The data were analyzed by independent t-test and chi-square test using SPSS. The results revealed that the mean growth (mm / day) of F1 (1.47 ± 0.11 vs. 1.71 ± 0.09) and F2 (0.96 ± 0.07 vs. 1.05 ± 0.09) was lower ($P > 0.05$) in the treatment group compared with control group, respectively. The mean concentration of serum P4 in the treatment group (2.31 ± 0.15 ng / ml) increased significantly ($P < 0.05$) by exogenous progesterone (CIDR) compared to control group (0.315 ± 0.03ng / ml). The mean number of medium-sized follicles (0.89 ± 0.13 vs. 1.58 ± 0.19) was significantly lower ($P < 0.05$) in the treatment group compared with control group, respectively. While the mean number of small-sized follicles (91.48 vs. 83.38 %) was significantly higher ($P < 0.05$) in the treatment group as compared to control group. Similarly, the oocyte recovery rate (54.22% compared with 42.53%; $P < 0.05$) and grade I and II oocytes per session (3.37 ± 0.49 compared with 2.21 ± 0.33; $P < 0.05$) were also higher in the treatment group compared with control group, respectively. However, the nuclear maturation rate (71.43 vs. 68%), cleavage rate (52.87 vs. 53.06%) and blastocyst rate (27.54 vs. 25%) did not differ ($P > 0.05$) between the groups. Taken together, exogenous progesterone (CIDR) has improved oocyte recovery and quality, but in both groups the *in-vitro* developmental competence of oocytes in terms of nuclear maturation, cleavage rate, and blastocyst rate remained the same.

Lycopene improves blastocyst development and quality in a bovine in vitro model

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Keywords: antioxidant, embryo production, embryo quality

Oxidative stress associated with excessive production and accumulation of reactive oxygen species (ROS) reduces embryo viability by interfering with essential cellular processes. Antioxidant supplementation may guard embryonic cells against ROS detrimental effects. Among antioxidants, lycopene is a carotenoid that has the ability to quench singlet oxygen and scavenge free radicals. This study aims to evaluate the effects of supplementation of lycopene (antioxidant), menadione (prooxidant), and their combination during *in vitro* oocyte maturation on subsequent embryo development and quality in a bovine model. Cumulus oocyte complexes, collected from slaughterhouse (n = 806), were matured in 4 groups of 60 in 500 µl of maturation medium (TCM199 medium + 50 mg/ml gentamycin (Life Technologies, Ghent, Belgium) + 20 ng/ml of epidermal growth factor (Sigma-Aldrich, Diegem, Belgium)) and supplemented with 0.2 µM lycopene, 5 µM menadione (Sigma-Aldrich, Diegem, Belgium), 0.2 µM lycopene + 5 µM menadione (L+M), or were not supplemented (control). Maturation and Fertilization were standardly performed in 5%CO2 in air, and embryos were cultured in serum-free medium with 5%CO2 and 5%O2 in all the groups. The effects of pro and antioxidant supplementation on *cleavage*, day 8 blastocyst, and embryo quality parameters were fitted in generalized and linear mixed-effects models, and results are expressed as least squares means and standard errors. Lesser cleavage rates ($P < 0.05$) were found in menadione supplemented oocytes (74 ± 3.7) than in lycopene (92 ± 1.9), L+M (83 ± 3.0), and control (87 ± 2.5). Lycopene supplementation resulted in greater ($P < 0.01$) day 8 blastocyst rates (56 ± 3.4) in comparison to the other groups (33 ± 3.4 for menadione, 40 ± 3.5 for L+M, and 43 ± 3.3 for control). In the lycopene group, total cell number (TCN), inner cell mass (ICM), and TCN/ICM ratio were higher, and numbers of apoptotic cells (AC) and AC/TCN ratio were lower than in menadione, L+M, and control groups ($P < 0.05$). Apoptotic cells and AC/TCN ratio were similar between L+M and control groups ($P > 0.05$). However, blastocysts from menadione supplemented oocytes presented greater AC and AC/TCN ratio than in all the other groups ($P > 0.05$). In conclusion, lycopene supplementation during *in vitro* oocyte maturation improves embryo development and quality and could have protective effects against oxidative stress (menadione supplementation). Further experiments should be conducted to study the molecular basis underlying the effects of lycopene supplementation on subsequent embryo development and quality.

Follicular fluid supplementation during bovine oocyte maturation in vitro improves blastocyst development and quality in an individual culture system

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Keywords: cow, embryo, IVM

Follicular fluid (FF) provides the natural environment for oocyte maturation. Therefore, we hypothesized that supplementation of FF during *in vitro* maturation (IVM) would enhance the bovine oocytes' developmental capacity. In this study, the effects of FF supplementation during IVM on embryo development and quality were assessed in a group and individual culture system. Follicular fluid was collected from slaughterhouse ovaries (follicles between 12 to 20 mm in diameter) and pooled in heparinized tubes (BD Vacutainer Precision Glide, Becton Dickinson, Franklin Lakes, NJ), centrifuged, and stored at – 80°C until usage. *In vitro* maturation medium with 50 µg/ml gentamycin (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and 20 ng/ml epidermal growth factor was supplemented with 0 (control), 1, 5, or 10% of FF. In Experiment 1, IVM, fertilization (IVF) and culture (IVC) were performed in groups (n = 1,056 oocytes in 5 replicates). In Experiment 2, oocytes and embryos were subjected to individual IVM, IVF and IVC (n = 567 oocytes in 7 replicates). After 22 h of maturation oocytes were co-incubated with 1×10^6 spermatozoa/mL for 21 h at 38.5°C in 5% CO_2 in humidified air in 500 µL IVF-TALP (Tyrode's Albumin Lactate Pyruvate) supplemented with bovine serum albumin (BSA) (6 mg/ml; Sigma A8806) and heparin (25 mg/ml) for group culture and in droplets of 20 µL for individual culture. After fertilization, presumed zygotes were transferred in groups of 25 to 50 µL droplets (group culture) and individually to 20 µL droplets (individual culture) of synthetic oviduct fluid (SOF), 0.4% BSA, and ITS (5 µg/ml Insulin + 5 µg/ml transferrin + 5 ng/ml selenium). Day 8 blastocysts were fixed and differentially stained as described by Wydooghe *et al.* (2011). Generalized mixed-effects models were used to test the effects of treatment on day 8 blastocyst rates and mixed linear regression models were used to test the treatment effects on differential and apoptotic staining parameters (cell numbers and apoptotic cell index). For both models, the replicate was set as a random effect and results are expressed as least square means and standard errors. In group culture, supplementation of FF did not affect the day 8 blastocyst rate (46.3 ± 3, 35.5 ± 2.9, 44.3 ± 3, and 45.5 ± 2.9 for control, 1, 5, and 10% FF supplementation, respectively) nor quality ($P > 0.05$). In the individual culture system, 5% FF supplementation increased day 8 blastocyst rate (37 ± 4.1%) in comparison to control (18.7 ± 3.3%; $P = 0.003$) and 1% FF supplementation (18.4 ± 3.2%; $P = 0.003$) but was not different from 10 % FF supplementation (28.8 ± 3.9%; $P = 0.4$). Moreover, 5% FF supplementation during individual culture resulted in (>10%) greater total cells number (110.9 ± 2.7) and (>20%) a higher proportion of inner cell mass cells (51 ± 1.9) than in all the other groups ($P < 0.05$), but apoptotic cell index was not affected ($P > 0.05$). Supplementation of IVM medium with 5% FF significantly increased blastocyst rate and embryo quality in a bovine individual embryo production system. However, the characteristics of slaughterhouse-derived FF are plausibly variable. Several FF batches should be tested to draft definitive conclusions.

Crossbreeding effect of double-muscled cattle on in vitro embryo development and quality

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Keywords: double-muscled cattle, embryo production, embryo quality

In the past years, several developing countries have started to breed double-muscled cattle to their native cattle to improve beef quality. However, the developmental competence of the resultant crossbreeding embryos is unknown. The objective of this study was to evaluate the effect of crossbreeding double-muscled (Belgian Blue; BB) semen with beef (Limousin; LIM) and dairy (Holstein-Friesian; HF) derived oocytes on embryo development and quality. As purebred BB control, BB oocytes fertilized with BB sperm was used. A single ejaculate of a single BB bull located in the breeding center of AWE in Ciney (Belgium; 50˚29 N, 5˚11 E) was used for all the experiments. Motility parameters of frozen-thawed sperm samples were evaluated using computer-assisted sperm analysis before used for *in vitro* fertilization. Ovaries were collected at the local slaughterhouse from each breed and transferred to the lab allocated in different bags without medium transport and all placed in a safety closed box. *In vitro* maturation and fertilization were performed (as described by Wydooghe E, Reprod Fertil Dev 26:717, 2014) and embryos were cultured in serum-free medium in three replicates (n = 1,720 oocytes). Basic Eagle's Medium amino acids, minimal essential medium non-essential amino acids (100 x), TCM-199-medium, kanamycin, and gentamycin were purchased from Life Technologies Europe (Ghent, Belgium). All other components were obtained from Sigma (Schnelldorf, Germany) unless otherwise stated. Cleavage was evaluated at 48 h post insemination and blastocyst development at day 8 post insemination. Embryo quality was evaluated via differential-apoptotic staining of day 8 blastocysts (as performed by Wydooghe E, Anal Biochem 416: 228–230, 2011). The effects of breed on developmental and differential-apoptotic staining parameters were fitted in mixed effects and mixed linear effects models, respectively. The replicate was set as a random effect for all the models and results are expressed as least square means with standard errors. Cleavage and day 8 blastocyst rates were greater ($P < 0.05$) for LIM (82.9 ± 6 and 27 ± 4.3%, respectively) than for BB (69.8 ± 8.5 and 19.6 ± 3.1%, respectively) and HF (45.1 ± 10 and 12.3 ± 2.2%, respectively). Holstein-Friesian presented lower cleavage and day 8 blastocyst rates than BB ($P < 0.05$). Limousin blastocysts presented a higher number ($P < 0.05$) of inner cell mass cells (ICM; 68 ± 7.8) than HF (40.4 ± 8.2). No other differential-apoptotic staining parameter differed among breeds ($P > 0.05$). In conclusion, crossbreeding double-muscled cattle by *in vitro* fertilization with LIM oocytes yields better embryo development and quality (ICM number) compared with the purebred combination, while the combination with HF oocytes produced the lowest rate of blastocysts. This finding might be due to one of the facts that culled HF cows are typically older than BB or LIM or are culled for infertility. This experiment consisted of a preliminary study to mimic what maybe happens in aspects of potential fertility in a BB breeding program. However, more studies need to be conducted to draw definitive conclusions.

Subjectivity in the morphological selection of bovine immature cumulus-oocyte complexes

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Keywords: COC morphology, visual examination, kappa statistics

Successful *in vitro* production (IVP) strongly depends upon the quality of the cumulus-oocyte complex (COC). The COC's morphological characteristics are often used to predict developmental competence. Whenever antral follicles are punctured for embryo production, COCs of different morphological characteristics are harvested and selection is performed by visual examination. This method is simple and non-invasive but it is also highly subjective. This study aims to determine the agreement of morphological evaluation of bovine immature COCs among IVP researchers. A non-random set of 29 pictures of immature bovine COCs was presented in duplicates to eight bovine IVP researchers, with different institutional backgrounds. Pictures were selected to balance for oocyte category (assessed by an experienced researcher who served here as referent) and were presented in random order. The observers were asked to categorize the oocytes to one out of four categories: A) compact cumulus of > 5 layers of granulosa cells that are completely surrounding the oocyte with homogenous ooplasm, B) cumulus is less compact and darker than A and ooplasm is dark and slightly granular, C) cumulus consists of ≤ 5 layers of granulosa cells and/or is not completely surrounding the oocyte with homogenous ooplasm, and D) cumulus cells are expanded, and the ooplasm is granular. The categorization of the most experienced observer was set as the reference value. Responses among and within observers were assessed using kappa statistics and sensitivity (se) and specificity (sp) tests. The referent observer classified 8 COCs in category B and 7 COCs in categories A, C, or D. Kappa values (κ) for inter- and intra-observer agreement were κ = 0.27 and 0.25 respectively, with κ = 1 referring to 100 % agreement. True positive rates (se) for categories A, B, C, and D were 52, 34, 50, and 45%, respectively. True negative rate (sp) was 71% for category A, 81% for category B, and 87% for categories C and D. The inter- and intra-observer agreements were poor. Evaluation of immature bovine oocytes based on their morphological characteristics is highly subjective with weak repeatability among observers. The moderate sp and low se suggest that it is easier to discriminate than to concur in the same oocyte category. There is a need to develop a simplified rating model to determine oocyte quality without losing practical feasibility. The implementation of automatized methods using artificial intelligence could significantly objectify this task.

Trypsin treatment of porcine oocytes impairs in vitro fertilization output.

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Keywords: zona pellucida, fertilization, porcine

Polyspermy is the main limitation of porcine *in vitro* fertilization (IVF) success. The extracellular matrix or Zona Pellucida (ZP) of oocytes have a fundamental implication on sperm penetration. Since ZP is sensitive to protease digestion a soft treatment with trypsin was evaluated in order to evaluate porcine IVF output. *In vitro* matured oocytes-cumulus complexes were mechanically decumulated by soft pipetting until all cumulus cells were removed and washed twice in Tyrode's albumin-lactate-pyruvate (TALP) medium previously equilibrated at 38.5°C under 5% CO_2. DO (decumulated-oocytes) were then divided into two groups: trypsin and control. Both groups were transferred to a new 4-well Nunc plate with 500 µl TALP for 30 minutes. Trypsin group was supplemented with 0.5% trypsin. Photographs of DO from both groups were taken at time 30 min for ZP thickness measurement using image-J software. Afterwards, DO were incubated in TALP with fresh boar spermatozoa selected by a Percoll® density gradient. At 18h after IVF, putative zygotes were fixed and stained for penetration rate, monospermy and efficiency (percentage of monospermic oocytes from total inseminated) evaluation. Five replicates with a total of 289 DO were used for the IVF assessment (143 control; 146 trypsin) and 45 DO for ZP thickness evaluation (22 control; 23 trypsin). The statistical analyses were performed using the software IBM SPSS statistics *vs*.24. Normal distribution (Shapiro-Wilks) of data and equality of variances (Levene`s test) were evaluated. IVF parameters were assessed by chi-square analysis and the ZP thickness was analyzed by Student`s t-test. It was observed that the penetration rate and the efficiency were significantly higher in the control group than in the trypsin group (80.4% *vs*. 6.2%, and 20.3% *vs*. 2.7%, respectively, $P<0.0001$). No differences were observed on monospermy (44.4% and 25.2%, respectively, $P=0.209$). Moreover, no differences were observed on ZP thickness on DO from control and trypsin group after 30 minutes (19.05±1.77 µm and 19.94±1.54 µm respectively, $P=0.282$). In conclusion, a soft trypsin digestion of the ZP impaired IVF performance without altering ZP thickness.

Inhibiting Diacylglycerol Acyltransferase-1 enzyme in bovine embryos produced in vitro reduces their lipid content and improves cryotolerance

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Keywords: triacylglycerol, cryopreservation, cattle

Diacylglycerol acyltransferase-1 (DGAT1) is an enzyme that catalyzes the final step in triglyceride synthesis, which is a major component of the lipid droplets in embryos. Intracellular lipids accumulated in embryos produced *in vitro* have been associated with reduced cryotolerance and quality. We have evidenced that inhibiting DGAT1 synthesis in culture media with 10 or 50 µM DGAT1 inhibitor (A922500® Sigma-Aldrich) improves bovine embryo quality in terms of mitochondrial activity and total cell number (Giraldo-Giraldo J. et al., Reprod Dom Anim, 54:122 (2019)). Thus, in the present study we assessed if DGAT1 inhibition in *in vitro* culture of bovine embryos reduces lipid content and improves post-vitrification survival. Zygotes were cultured in groups of 25 in 25µL drops of synthetic oviduct fluid (SOF) supplemented with 5% fetal calf serum (FCS) alone (control) or with 10 or 50 µM DGAT1 inhibitor (T10 and T50, respectively) or 0.1% dimethyl sulfoxide (T_{DMSO}: vehicle for DGAT1 dilution), from 54 hours post-insemination (hpi) until Day 8 at 38.5ºC, 5% CO_2, 5% O_2 and 90% N_2. A representative number of blastocysts on day 7-8 (grade 1 and 2 according to IETS manual) from each group was used for quality evaluation through (i) lipid content (n≈30/group) stained with Bodipy (lipid droplet area in µm^2) and (ii) survival after vitrification/warming (n≈70/group). Survival was defined as re-expansion of the blastocoel and its maintenance for 72 h after warming. Data obtained were analyzed using one-way ANOVA. No differences were found in cleavage rate at 54 hpi (control: 90.5 ± 1.1%, TDMSO: 88.28 ± 1.0%, T10: 88.8 ± 1.2% and T50: 89.8 ± 1.2%) or in blastocyst yield on Days 7 and 8 (control: 29.1 ± 1.3% - 33.3 ± 1.1%, T_{DMSO}: 26.1 ± 1.2% - 31.6 ± 1.2%, T10: 29.2 ± 1.3% - 35.7 ± 1.2% and T50: 29.2 ± 1.0% - 34.7 ± 1.0% Day 7-8, respectively). Lipid droplets area was significantly reduced ($P<0.05$) in T10 (0.08 ± 0.0 µm^2) and T50 (0.09 ± 0.0 µm^2) groups compared with control (0.39 ± 0.02 µm^2) and T_{DMSO} (0.36 ± 0.02 µm^2) groups. In terms of blastocyst cryotolerance, during the first 24 h after warming, there were no differences in survival between the groups, which ranged from 82.9 ± 1.4% to 88.6 ± 2.1%. However, 48 h after warming, the survival rates of blastocysts obtained from T10 (83.3 ± 1.9%) was significantly higher ($P<0.001$) than those of the T50 (75.1 ± 1.3%), T_{DMSO} (72.5 ± 1.2%) and control group (75.5 ± 2.0%). At 72 h after warming, those differences were even more marked (T10: 73.8 ± 0.8% vs T50: 56.1 ± 1.2%, T_{DMSO}: 55.9 ± 1.6% and control: 57.1 ± 2.0%; $P<0.001$). Hatching rate was also higher in T10: 57.2 ± 2.8% vs T50: 39.6 ± 2.0%, T_{DMSO}: 38.4 ± 3.5% and control: 40.7 ± 2.1% ($P<0.001$). In conclusion, inhibition of DGAT1-synthesis in bovine embryos produced *in vitro* contributes to reverse the negative effect of serum by decreasing their lipid content and the lowest dose improve embryo cryotolerance.

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Effect of prolactin on developmental competence of bovine OPU-oocytes matured in vitro

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Keywords: OPU-oocytes, maturation, prolactin

In vitro maturation (IVM) of the oocytes recovered through ultrasound-guided transvaginal follicular aspiration (Ovum Pick-Up, OPU) is an important step for in vitro embryo production (IVP) in cattle. In vitro culture during maturation decreases oocyte quality and therefore, IVM conditions need to be improved. The goal of the present research was to study effects of pituitary hormone, prolactin (PRL) on the nuclear maturation of OPU-derived bovine oocytes and their development competence in vitro. Cumulus-oocyte complexes (COC) were selected from non-stimulated Simmental heifers at the age of 17 to 23 months (n=4) twice a week (11 OPU-sessions per animal, 5.3±0.4 COC per session) and classified immediately after OPU. The viable COC (compact cumulus and homogeneous cytoplasm, n=171) were cultured in standard maturation medium (TCM-199 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) for 24 h in the absence (Control) or in the presence of 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia). Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). After IVM, a subpopulation of oocytes was fixed with 4% paraformaldehyde, and the nuclear state was determined by DAPI staining. The remaining oocytes (62 Control and 52 PRL-treated group) underwent in vitro fertilization (IVF) and in vitro culture (IVC). Frozen/thawed sperm of the same Simmental bull were prepared in Sperm-TALP medium by swim-up procedure. In vitro matured OPU-oocytes were co-incubated for 18 h with prepared sperm in the modified Fert-TALP medium containing 10 µg mL–1 heparin, PHE (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine), and 0.1% MEM nonessential amino acids. Fertilized oocytes were cultured in CR1aa medium until Day 5, transferred to the same medium supplemented with 5 % FCS and cultured up to Day 7. At Days 2 and 7 after fertilization, the cleavage and blastocyst rates were determined. All the cultures were performed in 100 µl droplets of medium covered with mineral oil at 38.5°C and 5% CO2 in humidified air. The data for nuclear state (5 replicates per treatment) and IVF/IVC (10 replicates per treatment) were analyzed by ANOVA. After 24 h of maturation, the rate of M-II oocytes did not differ between non-treated and treated groups and were 83.8±4.6 and 88.3±5.6 respectively. However, after IVF/IVC, the cleavage rates of oocytes matured in the medium supplemented with PRL was significantly higher compared with control medium (82.4±5.4% vs. 69.4±2.6%, ($p<0.05$)). Furthermore, a significant increase in blastocyst rate was observed in the PRL-containing medium (20.5±1.9%; 0.91 blastocysts per OPU-session) compared with the control group (12.3±1.6%, $p<0.01$; 0.56 blastocysts per OPU-session). The findings indicated that PRL supplements during IVM of bovine oocytes recovered from live animal through ultrasound-guided transvaginal follicular aspiration may improve their capacity for the subsequent embryo development in vitro.

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Poliovulatory response and embryo recovery rate in beef sheep in Romania, as a possibility for genetic development - A case report

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Keywords: MOET, poliovulation, ET, sheep
Embryo transfer (ET) technology gained commercial prominence in the international movement of bovine genetics. The development of this reproductive biotechnology in sheep has in the past had a similar development especially in important breeds of sheep and goats (like the Suffolk breed). Small ruminant ET is a well described and yet underexploited animal breeding technology. The size of sheep and goats, aspects of their anatomy and seasonal reproductive behavior, present challenges not common to cattle. Those considerations have not deterred serious breeders and ET practitioners in sheep and goat producing countries. The success of an ET protocol in sheep depends on many factors, but in the end, what matters is the number of embryos obtained, Recovery rates is an essential step in ET. The aim of our experiment was to observe the ovarian reaction to the treatment of Suffolk (UK) sheep polyovulation, and the recovery rate of embryos produced in vivo. A number of 6 Suffolk sheep were poliovulated at the beginning of the natural breeding season, using the P4-FSH-PGF protocol. The poliovulate (POV) method was based on the administration of intravaginal sponges containing 20 mg of flugestone acetate (Chronogest®, Intervet, Holland) followed by 500 IU FSH:LH (Pluset®, Calier, Spain) in decreasing doses in the last 4 days, and a cloprostenol (125 µg.IM), (Estrumate®, MDS, Holland) on day 11. The poliovulatory ovarian response was monitored by transrectal ultrasound (Honda HS-1600V®, Japan; 5 MHz) before estrus was detected, and on the day of embryo recovery. When estrus was detected, 3 mounts were performed at intervals of 12 hours, and 7 days later, the embryos were recovered by laparascopic surgical technique. Uterine flushings were made using Vigro complete flush™ (Vetoquinol, USA) a two-way catheter (Vortech 14Ch) and a filter (EmSafe Filter). Examination of the recovered flush fluid is routinely performed under magnification of 20-80X using a microscope. The size, morphology and developmental stages of small ruminant embryos are similar to those of bovine embryos. All POV sheep responded to stimulation and new follicular waves were identified on the ovaries. On the first day after POV, all ovaries had more than 5 dominant follicles, no differences were observed between sheep and between ovaries. But 7 days after estrus, corpus lutea (CL) was observed in only 83.3% (5/6) sheep. At the time of abdominal laparotomy, an average of 9.1 (0,11,12,15,9,8) CL/sheep were identified. The total number of CL observed was 55, 29 on the right ovary, 26 on the left. The distribution right/left of CL was 0/0, 6/5, 7/5, 7/8, 4/5, 5/3. The total number of embryos obtained was 48, and then the recovery rate (number of embryo/number of CL) was 83.3%. Its distribution was 10/11, 8/12, 13/15, 9/9, 8/8. 35 embryos (73%) were transferable. They all were excellent or good early blastocysts (stage 5 - quality 1, acording to the IETS recommended codification). The unviable embryos were degenerated or unfertilized. In conclusion, the POV protocol and the harvesting method applied have a positive effect in the production of in vivo embryos in Suffolk sheep and can garantee the success of ET activity of this breed in Romania”

Folliculogenesis, oogenesis and superovulation

Long-term changes in granulosa cells and oocytes following bacterial infection of the uterus in Holstein dairy cattle

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Keywords: Oocyte, endometritis, cattle

Postpartum uterine disease reduces fertility in dairy cows, even after the resolution of the bacterial infection. However, it is not clear how fertility continues to be impaired after the bacterial infection has resolved. We hypothesised that bacterial infection in the uterus compromises oocyte competence. To test our hypothesis, without the potential confounding effects of lactation or negative energy balance, we induced endometritis in virgin Holstein heifers (n = 4) and non-lactating Holstein dairy cows (n = 12). Animals were infused intrauterine with endometrial pathogenic bacteria, *Escherichia coli* and *Trueperella pyogenes* in 30 ml Luria-Bertani broth (Sigma, USA), and the animals developed endometritis; control heifers and cows (n = 6 and 11, respectively) received an intrauterine infusion of 30 ml sterile Luria-Bertani broth. In the heifers, we collected oocytes on day 60 after infusion by ovum pick-up, using an oocyte pick-up instrument with an 18-guage needle and a 7.5 MHz convex ultrasound probe, as described in detail previously (*Biology of Reproduction*, *https://doi.org/10.1093/biolre/ioaa069*), and granulosa cells were collected by aspiration from dominant follicles at the time of slaughter, as described in detail previously (*Reproduction*, *https://doi.org/10.1530/REP-19-0564*). In the cows we collected a total of 933 oocytes by ovum pick-up on days 2, 24, 45 and 66 after intrauterine infusion. In the heifers, we used RNAseq profiling and Ingenuity Pathway Analysis to compare the transcriptomes of oocytes and granulosa cells between bacteria-infused and control animals. We found that amongst the > 11,700 expressed genes, uterine bacterial infusion led to 539 differentially expressed genes (log2 fold change > 2) in oocytes collected 60 days after infusion, and 89 differentially expressed genes in the granulosa cells collected 94 days after infusion. Predicted upstream regulators of differentially expressed genes in the oocytes and granulosa cells included innate immunity (LPS, TLR4) and cytokines (IL-1, IL-6 and TNF). Oocytes collected from the cows were subjected to in vitro fertilization in BO-IVF media overlaid with mineral oil, and then embryo culture in BO-IVC embryo culture medium (all IVF Bioscience), as described in detail previously (*Biology of Reproduction*, *https://doi.org/10.1093/biolre/ioaa069*). Uterine bacterial infusion reduced the proportion of cleaved oocytes developing to morula compared with control (30.7% vs 45.0% for all oocytes collected 2, 24, 45 and 66 days after intrauterine infusion), with the greatest reduction for oocytes collected 24 days after intrauterine infusion (21.4% vs 45.6%). In conclusion, independent of lactation and negative energy balance, bacterial infection of the uterus in dairy cattle altered the transcriptome of oocytes and granulosa cells months later, and compromised the developmental capacity of oocytes. Our findings imply that bacterial infections of the uterus have long-term effects on oocyte competence, and that cows that have a history of postpartum uterine disease may not be optimal oocyte donors.

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Plasma extracellular vesicle miRNAs as potential biomarkers for ovarian superstimulatory response in cattle

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Keywords: EV-miRNAs, superovulation, bovine

Ovarian superstimulation (OS) in cattle is utilized to stimulate the growth and ovulation of multiple ovarian follicles to o btain a high number of viable embryos from elite donor cows. However, the high individual variability in response to OS is one of the disadvantages of this technology. Prediction of superstimulatory response could be a beneficial tool in assisted reproduction. The objective of this study was to analyse the extracellular vesicle microRNA (EV-miRNA) expression profiles in the blood plasma of heifers with variable response to OS. Oestrous cycles of crossbred beef heifers (n=25) were synchronized using an 8-d intravaginal progesterone device with GnRH at insertion and PGF2α 24 h before removal. On D10 after standing oestrus (=D0), OS was induced by the administration of decreasing doses of FSH twice a day for 4 d with PGF2α administered with the 6th FSH injection followed by AI 24 and 36 h after the last FSH injection. Blood samples were collected on D7 of the unstimulated (U) and superstimulated (S) cycle from each heifer. All heifers were slaughtered on D7 of the S cycle. Corpus luteum (CL) measurements, as well as the total number of recovered/transferable embryos, were recorded for each heifer. A subset of High (H, n=3) and Low (L, n=3) responders was selected depending on their response to OS and EV-miRNAs profiles were analysed in each. Total weight of luteal tissue (33.3±13.9 *vs.* 107±13.9 g) and the total number of recovered (6±6.2 *vs.* 16.3±4) and transferable (2.6±2.5 *vs.* 11.3±1.5) embryos were lower in SL *vs.* SH heifers, respectively. Total vesicular RNA, was isolated from blood plasma using exoRNeasy Serum/Plasma Kit (Qiagen). MiRNA expression profile was analysed in individual plasma samples using small RNA-seq technology (NextSeq500; Illumina). Approximately 200 known miRNAs were detected in each sample with 144 commonly detected in all samples. MiR-16, miR-125, miR-126, and members of let7 family were among the most highly abundant miRNAs in all samples. Differential expression (DE) analysis revealed that 12 miRNAs (including miR-1, miR-133a, miR-206, and miR-6517) and 14 (including miR-17-5p, miR-181a, miR-199c, miR-206, and miR-6517) were dysregulated in UH *vs.* UL and in SH *vs.* SL heifers, respectively. Interestingly, miR-206 and miR-6517 exhibited the same expression pattern in H compared to L heifers both before and after OS. KEGG pathway analysis for the DE miRNA-target genes revealed that estrogen, MAPK, and Wnt signaling were among the top pathways targeted by the downregulated miRNAs while FOXO, PI3K-Akt and RAP1 signaling pathways were targeted by the upregulated miRNAs in H compared to L heifers. In conclusion, heifers with divergent ovarian responses exhibited differential expression of plasma EV-miRNAs which may be used as a potential biomarker to predict individual animal response to OS.

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Different ovarian stimulation protocols used prior laparoscopic ovum pick-up in Saanen goats: preliminary results

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Keywords: goat, ovarian stimulation, laparoscopy, oocyte

Laparoscopic ovum pick-up (LOPU) is a convenient way to retrieve oocytes for in vitro embryo production from healthy efficient goats. This process requires ovarian hyperstimulation to obtain many big follicles (≥ 5 mm) that can be easily visualized by laparoscopic optics and conveniently aspirated with a high recovery rate. The objective of this study was to compare the effect of different ovarian stimulation protocols on the number of aspirated follicles and oocyte recovery rate. The study was conducted using 9 sexually mature Saanen goats with an average age 2.8 ± 0.2 years and average weight 75.6 ± 4.6 kg that were divided into 3 groups. Estrus synchronization was performed using intravaginal sponges with 45 mg flugestone acetate (Chronogest CR®, MSD, Walton, UK) for 14 days. Ovarian stimulation was induced by injecting PMSG (Sergon 500® Bioveta, Ivanovice na Hane, Czech Republic) intramuscularly with treatment regimens, as follows: group 1 - three doses (375, 250, and 125 IU) at 24 h intervals; group 2 - five doses (300, 300, 300, 250 and 1000 IU) at 24 h intervals; group 3 - six doses (500, 500, 500, 500, 250 and 1000) at 24 h intervals. The last dose in all protocols was applied 36 h before LOPU that was performed once for each goat. Although group 2 received almost 3 times more total dosage of hormone than group 1, there was no significant difference ($P > 0.05$, Mann–Whitney U test) between the two groups in the number of aspirated follicles (14.3 ± 2.5, 13.0 ± 2.0; mean ± SD), retrieved oocytes (9.3 ± 0.6, 8.7 ± 0.6) and recovery rate (66.0% ± 8.2%, 67.3% ± 6.6%). Group 3 showed significantly more aspirated follicles (22.0 ± 3.6; $P \leq 0.05$) and retrieved oocytes (16.0 ± 2.0; $P \leq 0.05$) than groups 1 and 2, however, the recovery rate was not influenced by the protocol (73.1% ± 4.2%; $P > 0.05$). To conclude, these preliminary results showed that ovarian stimulation protocol with six doses of PMSG provided higher number of follicles and retrieved oocytes in Saanen goats.

Changes in acetyl-CoA metabolism alter histone acetylation profile and global gene expression in bovine cumulus cells

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Keywords: oocyte maturation; acetylation; metabolic pathways

The cumulus cells (CC) are somatic cells that are closely attached to the oocytes. Among other functions, CC support maturation by allowing the bi-directional transfer of essential molecules involved with cell signaling and metabolism. Besides, in mammalian cells, most of the cytosolic acetyl-CoA, the major source of acetyl groups for histone acetylation, is derived from citrate produced in the tricarboxylic acid cycle (TCA). Thus, alterations in mitochondrial function could impact histone acetylation, with consequences to chromatin permissiveness and gene expression, possibly impacting the maturation process and the oocyte quality. In this work, we hypothesized that the modulation of mitochondrial function in bovine CC correlates with changes in histone acetylation profile and global gene expression. Bovine COCs (grade I from 3-5mm follicles) were collected from slaughterhouse ovaries and in vitro matured in the presence or absence (control) of 1.5 mM of dicholoracetate (DCA), an inhibitor of pyruvate dehydrogenase kinases (25 per group in 3 replicates – culture conditions: 90ul drops of tissue culture medium 199 (TCM-199), 10% fetal bovine serum (FBS), 0.2 mM pyruvate, 0.5 mg/mL FSH, 100 IU/mL human chorionic gonadotrophin and 1.25 mg/mL gentamicin 38°C, 5% CO2 and high humidity). COCs were collected at different time points (immature, 4, 8, 16 and 24 hours) and assessed for H3K9 acetylation levels (immunostaining) and global synthesis of new transcripts (Click-iT® RNA imaging kit). At 24h, mitochondrial activity was also assessed (MitotrackerTM Red CMXRos). At each time point, images were acquired by fluorescence microscopy (LAS X Life Science Software) under the same conditions and parameters. Fluorescence intensity of CCs was calculated considering a round area including the oocyte and approximately 10 CC layers (Image J software). Then, oocyte area was subtracted, and the resulting values were submitted to statistical analysis. Results were compared by t-student (treatment vs. control) with $p<0.05$. CC from DCA group had an increase in mitochondrial activity suggesting, albeit indirectly, the greater activity of the TCA cycle. Associated with that, CC from treated group showed higher H3K9 acetylation levels at all analyzed timepoints. Moreover, at 8 and 16h after the onset of IVM, we also observed a significant increase in the synthesis of new transcripts compared to control group. At 24h, however, levels of new transcripts did not differ between groups, suggesting the action of additional epigenetic regulation at the end of maturation. In conclusion, results corroborate our hypothesis and clearly demonstrate the close relationship between energy metabolism and epigenetic control in bovine CC, suggesting that a higher mitochondrial activity modulates the generation of substrates for histone acetylation, and leads to changes in the global transcription levels of CC.

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Pharmacokinetics of a long-acting progesterone formulation in female camels

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Keywords: camelids, embryo, progesterone

Progesterone administration is used extensively in camel embryo transfer programs for synchronization of recipients and donors. Daily intramuscular administration (IM) of 100 to 150 mg of progesterone in oil for 14 days is recommended in order to achieve the desired effect. Daily IM injections to a large group of animals present several difficulties associated with frequent animal handling and compliance with timing and dose of injection. In addition, frequent injections may render some animals less tractable. Other techniques of delivery of progesterone for several days include the use of CIDRs. However, these devices are not always well tolerated by camels, may be lost, and are associated with development of vaginitis. Similar problems have been encountered in other species such as horses. Studies in mares have shown that administration of Biorelease progesterone formulations results in serum progesterone levels comparable to those observed with normal luteal function, for a period of 10 to 12 days.

The present experiment was designed to evaluate progesterone pharmacodynamics following a single standard dose administration of compounded proprietary long-acting progesterone that was formulated for mares. Fourteen (n=14) nulliparous female camels of 3.5 years of age and of similar weights were included in the study. Each female was given an intramuscular injection of 5 mL of a proprietary progesterone formulation (BioRelease P4 LA300, 300 mg of progesterone per mL). All females were examined by transrectal ultrasonography and only females with no corpora lutea present on the ovaries were included in the study. Blood samples were collected daily starting one day prior (Day 0) and continuing for 14 days after injection. Serum was isolated and stored at -20°C until assayed for progesterone using radio-immunoassay. Change in daily progesterone level following treatment was examined using a repeated measurement ANOVA.

As expected progesterone level was low (Mean ± SEM = 0.2 ± 0.07 ng/mL) prior to injection and increased significantly (36.76 ± 3.8 ng/mL, $P<0.05$) within 24 hours of treatment. Serum progesterone level remained above 2 ng/mL in all animals for 10 days. By 12 days after injection only 50% of the females had progesterone levels below 2 ng/ml. By 14 days after treatment, five females (36%) had serum progesterone between 1 and 2 ng/mL while all the other has less than 1 ng/ml.

In conclusion, this study demonstrated that administration of 5 ml of BioRelease P4 LA300 to female camels provides elevated serum progesterone levels that are comparable to those expected during the luteal phase for at least 10 days. This treatment may be useful to eliminate the need for repeated daily administration for at least that period of time. Studies are underway to determine the effect of this compounded long-acting progesterone on ovarian function. Sources of variation in individual response need further examination.

Physiology of male reproduction and semen technology

Effects of environmental heat stress on ram's scrotal circumference and semen quality of the INRA180 sheep

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Keywords: ram, heat stress, scrotal circumference, semen quality

Due to its location between a temperate climate in the North to a tropical climate in the south, Morocco is a country suffering from extreme impact and sensitivity due to global climate changes. Recent scenario predicts a decrease in precipitations (11%) and an increase of temperatures (1.3°C) by 2050. In this context, it is well documented that ram's exposition to high environmental temperatures causes a seminal degeneration (Cárdenas-Gallegos, Archivos de Medicina Veterinaria, 47, 39-44, 2015) with intensity varying between breeds. Moroccan sheep raised in harsh conditions during summer suffer from such effects. However, there is a lack of studies regarding this issue. Thus, the present study aims to determine the effect of different environmental heat stress condition on the INRA180 ram semen quality. During summer period (July - October), 12 INRA180 rams were randomly assigned to 3 groups. The control group (G0) was housed under sub humid conditions, and was exposed to the sun during the grazing time: from 7 to 11 am and from 3 to 6 pm. The remaining time, animals were kept in a ventilated shed. The experimental groups were housed under sub humid (G1) and semi-arid (G2) conditions, and were exposed to the sun during whole day. The evaluation of fresh semen quality, mass motility (MM), individual motility (IM), concentration (C), volume (V) and scrotal circumference (SC) were recorded 15 days (at 10 am) before starting the experiment to determine the initial parameters. After 2 months of animal's exposure, the same measurements were recorded. During the experiment, the mean temperature was 27.30±1.48 and 31.55±3.4 in sub humid and semi-arid respectively. Statistical analysis were performed using SAS, ANOVA program (SAS Institute Inc., Cory, NC, USA). The results showed that the MM decreased significantly in the two groups (G1= 3.08±0.2; G2 = 2.43±0.4) compared to G0 (4.4±0.18). Individual motility decreased significantly in the two groups (G1=68.04±1.3%, G2= 32.33±1.75%) when compared to G0 (IM= 97.98±1.27%). For G2, C (2.04±0.68 x 10^9 spz/ml) decreased significantly ($P<0.05$) and not for G1 (3±0.77 x 10^9 spz/ml) ($P > 0.05$) when compared to G0 (C= 3.41±0.87 x 10^9 spz/ml). However, the volume and SC were not affected by the heat stress exposure, whatever the ram group was ($P > 0.05$). The present study shows that the exposure of INRA180 rams to environmental heat stress causes a considerable decrease for MM, IM and C. These results are in agreement with those of previous studies under environmental as well as direct testis insulation heat stress (Soleilhavoup, Journal of Proteomics, 109, 245–260, 2014). The increase in temperature stress disturbs or fails thermoregulation and consequently increases the testicular temperature (Moule, Aust. J. Agr. Res. 1, 456, 1950). It will lead to local hypoxia and deleterious effects on the tissue inducing an alteration of the spermatogenesis process and reducing the quality of ram sperm (Marai, Ann. Arid Zone, 39, 449-460, 2008). To conclude, the exposure of INRA180 rams to thermal stress negatively affects the quality of the ram's sperm in semi-arid and sub-humid conditions.

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Characterisation of the sire contribution to fertilisation failure and early embryo survival in cattle

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Keywords: bull fertility, superovulation, embryo

Reproductive efficiency is a major driver of profitability in cattle production systems, particularly in seasonal systems where high 6-week in-calf rates are critical. The preponderance of research effort in the field of bovine reproductive physiology has focused on ways to improve cow fertility, while the contribution of the sire to poor herd fertility has received less attention. However, bull fertility is a major contributor to overall reproductive performance, particularly in herds where natural service is predominately used. Even in highly selected bulls in AI centres, where sperm quality is scrutinized, significant variation exists in field fertility. It is not clear where along the developmental axis such differences originate. The objective of this study was to determine whether subfertility in AI bulls is due to issues of sperm transport to the site of fertilisation (based on accessory sperm number), fertilisation failure or early embryo development.

Holstein Friesian bulls (3 High, HF and 3 Low, LF, fertility) were selected from the national population of AI bulls based on adjusted fertility scores from at least 500 inseminations (n=840 bulls; HF: +4.34% and LF: -12.7%). Synchronized beef heifers (n=19, 3-4 per bull) were superovulated using follicle stimulating hormone. Heifers were blocked based on estimated number of follicles at the time of AI and inseminated with semen from HF or LF bulls. Following slaughter 7 days later, number of corpora lutea (CL) were counted and the uteri were flushed. Recovered structures (oocytes/embryos) were classified according to developmental stage and stained with DAPI to assess number of cells and accessory sperm.

Mean number of CL per superovulated donor was not different between groups (HF: 17.4 ± 8.2, LF: 17 ± 8.4). Overall recovery rate (total structures/total CL) was 52.6% (HF:49.6% v LF:55.3%; P>0.05). Mean number of embryos recovered per recipient was 8.6 ± 5.2 and 9.4 ± 5.5 for HF and LF, respectively (P>0.05). Overall fertilisation rate was 95.9% and was not different between groups. The percentage of morula (14.9 v 36.2%), blastocysts (40.5 v 23.1%) and expanded blastocysts (44.6 v 40.7%) was not different between HF and LF bulls. Mean embryo cell number was greater for HF (91.5±3.42) v LF (77.2±3.21) bulls (P<0.05). Overall, 17.0% (29/171) of structures had at least one accessory sperm. Number of accessory sperm was highly variable (range HF:0 to 45; LF:0 to 8; P<0.01). For those structures with accessory sperm, the mean number of sperm per structure was 12.7±3.66 v 2.9±0.75 (P<0.05) for HF and LF bulls, respectively.

In conclusion, while fertilisation rate did not differ between HF and LF bulls, the number of accessory sperm, a proxy for the number of sperm reaching the site of fertilisation, was lower in LF bulls, although highly variable. Despite differences in embryo cell number at Day 7, differences seen in field fertility between HF and LF bulls used in tis study are unlikely to be due to differences in fertilisation rate or early embryo development. It is likely that differences occur later in development, perhaps associated with maternal recognition of pregnancy or implantation.

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Bisphenol-A concentration affects ram and boar sperm motility

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There is growing concern about the effects of bisphenol-A (BPA) on fertility. There are reports on decreasing sperm fertility due to plastic quality, potentially associated with leaching (Porcine Health Manag 3:15, 2017). Therefore, we have tested the effect of BPA on boar and ram sperm motility. We used frozen ram semen doses from our cryobank (extended in TALP-HEPES post-thawing) and boar commercial doses (refrigerated) from AIM Ibérica, 3 males per species (triplicated by male). BPA (Merck, Darmstadt, Germany) was prepared in DMSO and added to sperm aliquots (30×10^6 ml^{-1}) at 500, 200, 100, and 10 µM, adding DMSO to the control. The tubes were incubated at 37 °C for 2 h. Videos of motile spermatozoa were acquired each 30 min (20-µm chamber, ×10 negative phase, 200 fps) and processed with the OpenCASA software (PLoS Comput Biol 15:e1006691, 2019). Data were analyzed by linear mixed-effect models. Total motility decreased as the concentration of BPA increased, with different dynamics for each species. Ram spermatozoa showed significant effects of incubation time and the concentration (main effects, no interaction). However, only 500 µM caused a significant decrease. Progressive motility showed similar results, with $P<0.05$ for 200 µM vs 10 and control. Boar data showed a significant interaction concentration×time, with 0 and 10 µM behaving similarly and decreasing with time, whereas 10 to 500 µM abolished motility from time 0 (most samples below 10%), remaining similar for the rest of the experiment. Sperm velocities (VCL: curvilinear; VAP: average-path; VSL: straight-line) showed significant interactions for ram, overall being lower in 500 (all times) and 200 µM (decreasing with time). For boar, the dynamics for each variable varied, in general showing small changes with BPA and incubation. Linearity parameters (LIN: linearity; STR: straightness) were affected by BPA in ram ($P<0.001$), decreasing with concentration but not as dramatically as for other variables, whereas WOB (wobble) was not affected. However, boar data showed interactions for LIN and STR ($P<0.01$), increasing with time for 100 to 500 µM, and time and dose-dependent decreases for WOB ($P<0.05$). The ALHmax (lateral head movement, maximum) showed a significant interaction in ram, with a decreasing trend for 100 and 200 µM, whereas 500 µM showed a lower value for the duration of the experiment. For boar, there was no interaction, with ALHmax decreasing with time and BPA concentration ($P<0.01$). In conclusion, BPA affected motility in both kind of samples. However, only boar refrigerated spermatozoa were clearly affected at concentrations below 500 µM. These results are relevant for quality control, and help to explain dramatic prolificacy changes reported in boar doses involving faulty plastic material. Furthermore, these results highlight the usefulness of boar sperm as biosensors (Basic Clin Pharmacol Toxicol 123:3, 2018).

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In vitro fertilization as an assessment of cryopreserved boar semen

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Keywords: porcine, oocyte, frozen semen.

Currently, artificial insemination in pigs uses fresh semen almost exclusively due to poor results using cryopreserved boar semen. The option to use frozen semen would be an advantage, *e.g.* due to long storage time and transport possibilities. The aim of this study was to investigate the usefulness of *in vitro* fertilization (IVF) as a fast method to assess the fertilizing ability of frozen semen from seemingly similar boars.

Semen from two Hampshire boars (A and B) cryopreserved using a slow cooling method, with similar post-thawing progressive motility as assessed by computer assisted sperm analysis, CASA (43% and 44% respectively) was compared. The semen was frozen in 500 µl straws with lactose and egg yolk solution with the addition of 3% glycerol and thereafter kept in liquid nitrogen until use. After thawing, the semen underwent colloid centrifugation (PorciColl) to select the best spermatozoa. *In vitro* matured pig oocytes (n = 1024) derived from slaughterhouse material, were randomly divided into groups of 30 for fertilization by boar A or B. The time of fertilization was 4 h (short) or 24 hours (long) and the number of spermatozoa/ml for fertilization was either 0.6 x10^6 (low) or 1.20 x10^6 (high).The presumed zygotes were observed for cleavage rate, blastocyst production and number of spermatozoa attached to the zona pellucida on day 6 after fertilization (DAPI staining).

The low concentrations of spermatozoa as well as the very short incubation time resulted in very few or zero fertilized oocytes for both boars. This contributed to a very large variation in the data which limited the possibility to use robust statistical analyses. The results are therefore only presented descriptively as means ±SD. Subjectively assessed sperm movement after thawing was similar between the boars (A: 28%±9, and B: 33%±9). Cleavage after low or short fertilization was similar between boar A and B (12%±8 and 16%±8, respectively). Cleavage after high and long fertilization was similar between boar A and B (56%±10 and 59%±12, respectively). Blastocyst development day 6 after low or short fertilization was lower in A than in B (0.6%±1 and 7%±5, respectively). Blastocyst development day 6 after high and long fertilization was lower in A than in B (3%±5and 14%±8, respectively). Mean number of spermatozoa attached to the zona pellucida for boar A was lower than for boar B (0.9±1.3 and 2.53±3.4). In all parameters measured in this small study, boar B consistently had the best results even though both boars were subjected to colloid centrifugation to select the best spermatozoa. It is no surprise that there are boar differences in IVF-systems but the IVF-outcomes still may reflect fertility after *in vivo* insemination using frozen boar sperm. However, more studies are needed to confirm these results.

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Application of complementary tests for the evaluation of chromatin structure in semen from AI boars

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Keywords: DNA fragmentation, spermatozoa, chromatin, SCSA

Producing a sire is costly due to breeding and innovation expenses. Moreover, AI centres can incur in compensations if they fail to detect subfertile boars. Thus, the pig industry has an interest on procedures for early detection of these boars. Sperm DNA fragmentation (SDF) affects fertility, but it is only one of many parameters in sperm chromatin. We tested SDF together with abnormal histone retention and disulphide bridges between protamines, as a proposal for routinely testing boars entering production. We used ejaculates from 11 boars of known fertility in an AI centre (Topigs-Norsvin). Samples (two ejaculates per boar in different weeks) were analysed on the day of collection and after 11 days of storage at 17 °C. Flow cytometry analyses (in duplicates) performed SCSA (DNA fragmentation, %DFI, and chromatin immaturity, %HDS), determination of disulphide bridge levels (DSF) and % of spermatozoa with histone retention (HR). We compared the techniques (linear mixed-effects models, male and ejaculate as random factors), assessed correlations and clustered the boars according to the chromatin parameters (R statistical environment). Results are shown as mean±SD. SCSA showed a low incidence of chromatin alterations. Storage increased DNA damage (%DFI; 1.1%±1.2 to 3.2%±2.9), and decreased HR (22.3%±3.3 to 9.1%±6.6) and DSF (36.4±5.6 to 25.2±4.7), all $P<0.001$. %HDS was not affected (7.6%±3.2 to 8.7%±2.9, $P>0.05$). %DFI negatively correlated with HR (-0.54, $P<0.001$) and DSF (-0,32, $P<0.05$), other correlations not being significant. A hierarchical cluster analysis yielded 4 groups of boars for each technique. Two boars with high initial %DFI or %HDS clustered individually and the other were grouped in two clusters of 2 and 7 boars respectively. The average DBE (Direct Boar Effect, a relative index) was higher for the first cluster (0.37 vs 0.04). The first cluster grouped boars with lower chromatin alterations or less detrimental changes with refrigeration, whereas the second cluster grouped boars with worse chromatin status and lower storage resilience. We were able to assess a small number of boars estimating chromatin status and resilience to storage. SCSA and DSF could be more discriminant, but all the parameters showed some association with fertility variables. The scope of this study is limited by the sample size, but our results suggest that chromatin analysis have a good potential for being applied in the AI centers. However, equipment cost is an important issue for considering routine use, since these techniques require flow cytometry.

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The Porcicoll colloid selects a population with improved chromatin status in fresh and refrigerated boar semen

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Keywords: Porcicoll, spermatozoa, colloid, centrifugation, chromatin

The pig industry undergoes increasing pressure for innovating while keeping costs low. Selecting an optimized sperm population could increase the useful life and efficiency of artificial insemination (AI) doses, while minimising problems due to low-quality ejaculates. The Porcicoll colloid allows easily selecting high-quality spermatozoa by single-layer centrifugation (SLC). Sperm DNA fragmentation reportedly decreases after selection, but few studies have explored other chromatin parameters. We aimed at testing if Porcicoll SLC was capable of modifying the chromatin structure in boar semen. Ejaculates from 10 boars (Topigs-Norsvin, León, Spain) were collected in 3 consecutive weeks. They were submitted to SLC (S1: 1 ml and S4: 4 ml Porcicoll) or analysed directly (C: control), on the day of collection and after 3 days of storage at 17 °C. Pellets resuspended in BTS were analysed by flow cytometry in duplicates by: SCSA (DNA fragmentation, %DFI; chromatin immaturity, %HDS), disulphide bridges levels (DSF, monobromobimane, mBBr) and histone retention (HR, CMA3). The effects of the SLC and processing day were tested by linear mixed-effects models (R software, results as mean±SEM). Treatment×day interactions were not significant, therefore both factors were analysed as main effects. %DFI was low in all cases and not affected by day. SLC decreased this important parameter, showing an effect removing spermatozoa with damaged DNA (C 0.83 vs S1 0.41, S4 0.36, SEM 0.05, $P<0.001$). The treatment had no effect on %HDS. DSF fell on day 3 respect to day 0 ($P<0.001$), not being affected by SLC. However, the medium-mBBr population (a measurement of free thiols) increased with SLC and decreased at day 3 ($P<0.001$) and high-mBBr decreased with SLC ($P<0.001$). HR as CMA3 mean fluorescence or moderate-CMA3 population decreased at day 3 ($P<0.001$), no effect of SLC. However, the high-CMA3 increased at day 3 ($P<0.001$) and decreased with S1 ($P<0.001$ vs C and $P<0.05$ vs S4). Porcicoll SLC modifies the chromatin status of boar semen, both fresh and refrigerated. Our study disclosed that changes not only affect DNA integrity but also other chromatin parameters rarely considered, nevertheless important. The Porcicoll variants S1 and S4 yielded similar effects. Therefore they could be used in different contexts according to practical requirements.

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Extended equilibration time and glutathione increase viability and chromatin compaction in post-thawed bull semen

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Keywords: semen, chromatin, cattle

Holding bull semen overnight before freezing is often used for samples that cannot be frozen on the day. However, there are contradictory reports regarding the effect on sperm quality. Also, supplementation of antioxidants to the semen extender could reduce the detrimental impact of reactive oxygen species (ROS). Therefore, this study aimed to assess the effects of extending the equilibration time to 24 h and the addition of glutathione in the post-thawing quality of bull semen. Semen from 8 Holstein and 4 Asturiana de los Valles bulls (an autochthonous breed in Asturias, Northern Spain) was collected by artificial vagina (3 ejaculates per bull) and frozen (BioXCell, IMV) after 4 (4E) or 24-h equilibration (24E) at 5 °C and with reduced glutathione (2 mM GSH, G2) or without GSH (G0). The cryopreserved doses were assessed immediately post-thawing (37 °C 30 s) and after a 5-h incubation at 37 °C (as a stress test). We analysed sperm viability, acrosomal status and apoptosis (YO-PRO-1/propidium iodide/PNA Alexa 647), and chromatin status (SCSA, acridine orange staining) by flow cytometry. Data were analysed by linear mixed-effects models in the R statistical environment (results as mean±SEM of %; no significant interactions detected). E24 improved viability (62.3%±1.0 vs 57.4%±1.2, P<0.001), and apoptotic ratio (9.7%±1.1 vs 12.4%±1.5, P<0.001) post-thawing, but reduced viability (40.2%±1.4 vs 45.7%±1.2, P<0.001) and acrosomal status (45.5±1.3 vs 52.0±1.1, P<0.001) after the incubation. G2 slightly improved viability (60.8±1.2 vs 59.0±1.8, P=0.013) post-thawing, and acrosomal status overall (63.4%±0.9 vs 62.1%±0.9, P=0.041). DNA fragmentation (%DFI) was not affected (1.9%±0.8 overall). Chromatin immaturity (%HDS) post-thawing was smaller in E24 (4.2%±0.4 vs 5.4%±0.3, P=0.005) and G2 (4.5%±0.2 vs 5.2%±0.3, P=0.038), with no significant differences after the incubation. Extending the equilibration time up to 24 h could be advantageous for work planning in breeding centres. E24 could even improve sperm quality in some cases. However, post-incubation results suggest the presence of sublethal damage not evident post-thawing. G2 yielded small effects, but it could be considered for freezing sensitive samples. Future work as insemination trials could elucidate if the lower resilience of samples submitted to E24 represents a threat. Nevertheless, differences were small and therefore increasing the equilibration time could still be considered if deemed convenient. In any case, individual bulls on high genetic value should be tested, since the sensitivity of the spermatozoa to extension times could vary among males.

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Functional characterisation of sperm from high and low fertility bulls

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Keywords: fertility, bull, sperm physiology

Traditionally, bovine artificial insemination (AI) centres have relied solely upon microscopy-based assessments, such as motility and morphology, as post-thaw measures of semen quality. However, amongst bulls deemed as acceptable based on these criteria, there is still considerable variation in fertility. The aim of this study was to investigate a range of sperm functional parameters in bulls of varying fertility to identify reliable discriminatory biomarkers. Holstein Friesian bulls classified as having either high (HF) or low (LF) fertility (n = 10 per phenotype) were used and had adjusted pregnancy rates of +4.1±0.15% or -7.6±1.50% (with a mean of 0%), respectively. For each bull, frozen-thawed spermatozoa were washed (300 x g, 5 min) and then incubated in modified TALP media and assessed at 0, 3 and 6 h (3 ejaculates assessed separately per bull). Motility and kinematic parameters were assessed using computer assisted sperm analysis (CASA) whilst nigrosin-eosin smears were prepared for morphology assessments at 0 h only (200 spermatozoa assessed per slide). For flow cytometric assessments, spermatozoa were incubated with two different staining combinations, 1) Alexa 647-PNA, merocyanine 540 and DAPI for acrosome integrity, lipid packing and viability and 2) MitoSOX red and Sytox Green for superoxide production and viability. Spermatozoa were stained with each combination for 15 or 20 min, respectively, at each time point prior to analysis. Potential differences between the fertility phenotypes were statistically analysed for each functional assessment using linear mixed model regression (REML; R version 3.4.1). Pairwise comparisons were determined using the predictmeans function with a Tukey adjustment. There were no differences between HF and LF bulls in relation to motility, kinematic parameters, morphology or superoxide production in viable cells, irrespective of incubation time ($P>0.05$). However, the percentage of viable cells with an intact acrosome as well as with high membrane lipid packing was greater overall in HF bulls (HF: 42.6±3.21% vs LF: 31.2±5.53%, $P<0.05$). Based on these findings, the tentative markers for fertility relate to cell viability and acrosome integrity. Since the flow cytometric assessment of viability is based on the integrity of the plasma membrane, further research is required to identify factors contributing to structural defects in the membrane and acrosome in bulls with low fertility.

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Comparison of the uterine inflammatory response to sperm from high and low field fertility bulls

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Keywords: AI, fertility, inflammatory response

Despite stringent quality control checks in animal breeding centres, some bulls with apparently normal semen quality yield unacceptably low pregnancy rates. We hypothesised that this was due, at least in part, to a differential uterine immunological response to sperm from high and low fertility bulls. The aims of this study therefore were to (i) optimise an *in vitro* protocol to assess the endometrial inflammatory response to frozen-thawed bovine sperm (ii) establish if sperm from high and low fertility bulls elicit a differential uterine inflammatory response and (iii) identify if the source of the uterine inflammatory response from frozen-thawed sperm is of sperm or seminal plasma origin. The experimental model used was heifer follicular phase uterine explants (8 mm biopsy punch) stimulated with washed frozen-thawed sperm *in vitro* (3-5 replicates per experiment). Experiment 1 investigated three sperm concentrations (5, 10 or 15 $x10^6$sperm/ml) and three incubation time points (1, 3 and 6 hours). Experiment 2 assessed sperm from 3 high (fertility rate of + 4.27% ± 0.35, mean ± s.e.m,) and 3 low fertility (-12.2% ± 1.81, mean ± s.e.m) bulls (average fertility = 0%) where fertility was based on an animal adjusted model (AAM) which is based on calving rate while adjusting for a wide range of factors. Experiment 3 included explant co-incubation with pooled caudal epididymal sperm (CES) from mature bulls of unknown fertility with and without seminal plasma. Each experiment included a control explant (no sperm added). In all experiments, a panel of inflammatory mediators namely, *IL1A, IL1B, IL6, TNFA* and *CXCL8* were analysed by qPCR. Quantification of IL1-ß and IL-8 in explant supernatants for experiments 2 and 3 were analysed by ELISA. For statistical analysis, repeated measures ANOVA was performed for experiment 1 and one-way ANOVA for experiments 2 and 3. In experiment 1, there was no effect of sperm concentration ($P>0.05$) but there was an effect of time for *IL6, IL1B* and *IL1A* with maximum expression at 6h ($P<0.05$). There was no sperm concentration by time interaction. There was no difference in the inflammatory response at the gene or protein level between high and low fertility bulls (Experiment 2) but a significant up-regulation of *ILB, TNFA* and *IL1A* gene expression from frozen-thawed sperm (irrespective of bull fertility status) was detected compared to the control. An up-regulation of IL-1ß and IL-8 protein concentrations compared to the control ($P<0.05$) was also detected. There was no difference between the uterine inflammatory response of CES or CES and seminal plasma and no up-regulation of cytokine expression or protein concentration compared to the control ($P>0.05$). Overall, this study demonstrated an up-regulation of inflammation in the uterus in response to frozen-thawed sperm *in vitro* but CES or CES with seminal plasma do not mirror these effects indicating inflammation could be coming from another component of the frozen-thawed semen. Based on this *in vitro* model there does not appear to be any difference in the uterine immunological response of sperm from high and low fertility bulls.

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Photo-stimulation with red-led light of Duroc pig seminal doses was not effective to improve fertility of Iberian sows

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Keywords: porcine AI, spermatozoa, reproductive outcomes

Different authors have pointed out the sensibility of the sperm cells from different species to be exposed to light (Shahar et al., Hum Reprod 26: 2274, 2011). In pigs, it has been reported an increase in the farrowing rate and litter size when the seminal doses for artificial insemination were photo-stimulated by red-led light (Yeste et al., Scientific Reports 6: 22569, 2016; Blanco Prieto et al., Rep Dom Anim 54: 1145, 2019). The former studies were developed with commercial breeds, mainly based in Large White and Landrace breeds. The reproductive parameters and outcomes from the Iberian breed pigs are different from the commercial breeds (Gonzalez-Anover et al., Theriogenology 75: 34, 2011). The aim of this study was to evaluate the possible effect of illumination of seminal doses with red-led light from Duroc boars and Iberian females in their specific production system. Semen samples were obtained from 33 fertile Duroc boars. Semen AI-doses were prepared from ejaculates that fulfilled the standard of sperm quality thresholds, using MR-A extender and stored at 16ºC up to 48 hours before application. Photo stimulation of the AI seminal doses were carried out thought a commercial system (Maxipig, IUL SA, Barcelona, Spain) that illuminated the samples with red led using the program of 10 min of light, followed by 10 min of darkness and finally 10 min additionally of light. The fertility study was conducted on 2 commercial farms at Murcia (Spain) using multiparous Iberian sows (farm A n=824; B n= 2137), that was randomly assigned to Led (L) or Control (C) groups. Post cervical insemination took place on 0 and 24 hours after the diagnosis of oestrus with seminal doses from the same ejaculate and same treatment. Categorical parameters as pregnancy and farrowing rate were analysed by Chi square test, continuous parameters as parity, pregnancy length and litter size were analysed by ANOVA. No differences were found between L and C groups in both farms ($p>0.05$) for parity, pregnancy rate, duration of pregnancy, farrowing rate and litter size (total, alive and died born piglets). Farrowing rates in farm A were 88.8% (n=383) for control and 89.6% (n=441, $p=0.67$) for led group. In farm B were C:90.5% (n=1035) and L: 90.1% (n=1102, $p=0.48$). In farm A total born piglets were 8.69 ± 0.11 for C and 8.71 ± 0.11 for L ($p=0.87$). In farm B 8.72 ± 0.7 for C and 8.70 ± 0.06 ($p=0.82$) for L.. Under the productive conditions of Iberian breed the photo-stimulation with red-led light of Duroc pig seminal doses was not effective to improve fertility of Iberian sows. According to the data reported by Blanco Prieto et al., 2019, and analysed by Chi square test the increase in farrowing rate after photo stimulation was significant ($p<0.05$) only in 6 from 31 farms evaluated. Specific characteristic of the Duroc spermatozoa, Iberian sows or the productive conditions of these farms could be related to the inefficiency of the photo stimulation system. The influence of these factors must be analysed in further studies.

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Embryology, developmental biology and physiology of reproduction

Antimicrobial resistance of Corynebacterium spp. in the vaginal flora of gilts and sows in Sweden

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Keywords: antimicrobial resistance, pigs, corynebacterium

Semen extenders for fresh boar semen include antibiotics, according to official guidelines, to control the growth of bacteria contaminating the semen during collection. However, it is not known if this use of antibiotics leads to the development of antimicrobial resistance in the vaginal flora of inseminated pigs or whether this resistance could spread to other animals (including human beings) or the environment.

Objective: The antimicrobial resistance pattern of *bacteria* isolated from the vagina from non-inseminated gilts and from sows that had already had three litters of piglets following artificial insemination.

Methods: Vaginal swabs were taken from 30 sows and 30 gilts on three farms in the middle of Sweden during the autumn of 2018. The swabs were directly cultured on blood agar, lactose purple agar, mannitol salt agar, Colistin-Oxolinic Acid-Blood Agar (COBA) and Man, Rogosa and Sharpe agar (MRS-agar).

In total, 280 bacterial isolates were identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry: 73 % of them consisted of *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. Fifty-two isolates of *Corynebacterium* spp. were tested for susceptibility against 16 selected antimicrobial substances, assessed with VetMIC™ Lact-1 and VetMIC™ Lact-2 (SVA, Uppsala Sweden), by determining the antimicrobial minimum inhibitory concentrations (MIC). Epidemiological cut-off (ECOFF) values for determining susceptibility were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The difference in antibiotic resistance for *Corynebacterium* spp. between gilts and sows was analysed by Chi-squared or Fisher's exact test.

Results: The *Corynebacterium* isolated were identified as *C. aurimucosum, C. casei, C. confusum, C. freneyi, C. glucuronolyticum, C. glutamicum, C. stationis,* and *C. xerosis*. Most (>80%) *Corynebacterium* spp. were resistant to clindamycin, but the difference between gilts (85.71%) and sows (95.83%) was not significant ($p = 0.45$). A few (<20%) *Corynebacterium* spp. were resistant to gentamicin (3.57%:4.17%), penicillin (10.71%:12.5%), vancomycin (3.57%:4.17%), ciprofloxacin (3.57%:4.17%) and rifampicin (0%:4.17%), but no significant differences were found between the gilts and sows ($p > 0.05$). None of the *Corynebacterium* showed any resistance to linezolid. *Corynebacterium* isolated from gilts were more often resistant to tetracycline compared with *Corynebacterium* from sows (15%:4.17%) ($p = 0.04$). However, since only a few farms were included in the study, with few individuals per farm, this significance should be interpreted with caution.

Conclusion: *Corynebacterium* from both gilts and sows showed low resistance to most of the antibiotics tested, with the exception of clindamycin. More isolates from gilts were resistant to tetracycline compared with isolates from sows, which may be due to contact with this antibiotic during the early life of the gilts, followed by the waning of resistance with time. Unfortunately, it is not known which antibiotics were contained in the semen extender used for the inseminations since it was not obligatory at the time for the manufacturer of the extender to provide such information.

Follicular size plays a critical role on durations of in-vitro maturation (IVM) in Bos indicus cattle oocytes

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Keywords:

Folliculogenesis involves a series of pivotal steps which lead to meiotic oocyte resumption. Oocytes harvested from various stages of follicular growth may vary accordingly. The research was designed to evaluate the effect of IVM period (21, 24, 27 h) for oocytes from different follicular sizes (small; ≤ 6 mm vs. medium; > 6 mm) on the nuclear maturation and early *in-vitro* embryo production. Ovaries were collected from a nearby abattoir, and follicles of small and medium size were aspired separately and poured into two separate 15 ml falcon tubes. After searching and washing of cumulus oocytes complexes (COCs), the good quality COCs (Grade I & II) were selected for further processing for IVM and were divided into three different groups: (1) 21 hours group, (2) 24 hours group and (3) 27 hours group.The COCs were incubated in the IVM media at 38.5ºC, 5% CO2 and 95% humidity. In Experiment 1, a total of 335 COCs, over six replicates, were processed for 21, 24, and 27h of IVM durations. The COCs were stripped off of the cumulus cell by gentle pipetting once the specific time window for each group was completed. The denuded oocytes were stained with Hoechst (Sigma 33342) and examined under inverted microscope equipped with fluorescence filter to estimate the nuclear maturation stages. Furthermore, Experiment 2 was performed to examine the effect of IVM period on the early embryonic development of oocytes. In Experiment 2, a total of 565 COCs, over 13 replicates, were processed for 21, 24, and 27h of IVM durations. The COCs were processed for IVF *after 24* h of incubation in the IVM media with the same bull's capacitated frozen thawed semen for each group. The presumptive zygotes were denuded and cultured for 7 days at 38.5ºC, 5% CO2, 5% O2 and 95% humidity after 16 hours of sperm-COCs incubation. On day 2 after IVF the cleavage rates were assessed. The data were analyzed using SPSS, using the Chi square method. For small-sized follicles, the maturation stage (MII) was highest at 27 h (48.1%) compared with 24 h (37.8%) and 21 h (32.1%) groups ($P > 0.05$), while the MII stage was the highest in 21 h (68.6%) group ($P > 0.05$) for medium-sized follicles. To conclude, oocytes harvested from small-sized follicles required 24-27h duration to achieve nuclear maturation while 21h duration is enough for oocytes from medium-sized follicles. Cleavage rates were highest at 21 h compared to 24 h and 27 h of IVM duration for oocytes aspirated either from small or medium-sized follicles ($P < 0.05$). 4-cell stage embryos were also significantly higher for oocytes aspired from small-sized follicles at 21 h (54.5%) compared to 27h (31.9%). The highest values for 4 cell stage embryos were observed at 24h (71.4%) of the IVM duration for oocytes aspired from medium sized follicles. It was concluded that the aging process begins after 24 h of IVM duration due to delayed nuclear maturation for oocytes aspired from either small or medium follicles, and that the developmental competence of these oocytes starts to decline after 24 h of IVM duration. Therefore, it is suggested that the oocytes should be processed for IVF between 21 to 24 h of IVM duration in *Bos indicus* cows for better developmental competence.

Effect of season on follicular population, oocyte quality, in-vitro maturation and fertilization in Nili-Ravi buffalo

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Keywords: Season, Nuclear maturation, Nili-Ravi buffalo

Unfavorable environmental conditions may influence the quality of COCs causing premature aging, and abnormal nuclear maturation leading to decrease in the reproductive efficiency of buffalo. The aim of the present study was to evaluate the effect of season on follicular population, COCs quality, nuclear maturation and *in-vitro* fertilization in *Nili-Ravi* buffalo under sub-tropical conditions. The season were classified as Autumn (Sep-Nov), Winter (Dec-Feb), Spring (Mar-May) and Summer (Jun-Aug). Ovaries (n=1836) were collected year-round from a local abattoir near Lahore and were categorized into cyclic and acyclic based on the presence or absence of the corpus luteum. To confirm the animals' cyclic or acyclic status, the animals were blood-sampled for plasma progesterone estimation. In the 1st Experiment, the cumulus oocyte complexes (COCs) were aspirated with an 18-G needle attached to a 10ml syringe. For further processing to IVM, the COCs with grade A and B (484 over six replicates) were selected and incubated for 24 hours at 38.5ºC, 5% CO2 and 95% humidity. The COCs were removed from the incubator after 24 hours of IVM incubation, and completely denuded by gentle pipetting and stained with fluorescent dye (Hoechst, Sigma 33343). The frozen semen from the same elite bull was thawed and utilized for IVF in the 2nd Experiment. A total of 904 COCs, over 8 replicates, were processed for IVF. The presumptive zygotes were processed for IVC at 38.5ºC, 5% CO2, 5% O2 and 95% humidity for 7 days after 6 hours of sperm-COCs co-incubation. The data were analyzed by One-way ANOVA and LSD test was used to test further differences between the groups. The proportional data were analyzed by Chi square test using SPSS. The meteorological data revealed that the temperature humidity index (THI) were (72 ± 4.0 vs. 56 ± 1.0 vs. 73 ± 4.1 vs. 81 ± 0.2) in Autumn, Winter, Spring and Summer, respectively.The results manifested that the follicle population per ovary were significantly higher for Autumn (3.35 ± 0.3) and Winter (2.94 ± 0.2) months compared to Spring (2.59 ± 0.3) and Summer (2.39 ± 0.2; P< 0.05) months while no significant difference was observed between Summer and Spring months. Moreover, it was observed that the follicular growth pattern started to improve after Summer months (medium and large-sized follicles improved in Autumn (0.29 ± 0.07, 0.13 ± 0.04) months compared with Summer (0.15 ± 0.03, 0.07 ± 0.03) months. Based on nuclear staining, the percentage of COCs, reaching the MII stage, improved during Autumn and Winter (69%, 73% Autumn and Winter seasons vs. 47%, 40% in Spring and Summer seasons respectively; P < 0.05). Furthermore, the Summer season also deteriorated the quality of COCs (only 25% A & B grade COCs during Summer months). However, the cleavage rate (54 vs. 48 vs. 52 vs. 39%) was not affected by season (P > 0.05). In conclusion, the ovarian dynamics, oocyte quality and maturation rate are affected by Summer season in buffalo under sub-tropical conditions as compared to other three seasons. It is therefore suggested that genetic harvesting for the production of embryos should be preferred in the Autumn and Winter when the heat stress is minimum.

Embryonic disc formation in extended in vitro culture of ovine embryos

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Keywords: embryonic disc, in vitro, ovine

The highest pregnancy losses in farm animals occur during the second week of pregnancy, when many critical events of embryo development take place: the inner cell mass differentiates into hypoblast, which covers the inner surface of the embryo, and epiblast, which will form the embryonic disc (ED). Unfortunately, the lack of an *in vitro* system able to support such events limits our understanding of those pregnancy losses. The aim of this study was to develop an *in vitro* culture system to achieve sheep embryo development after the blastocyst stage and ED formation. *In vitro*-produced blastocysts were cultured over agarose gels to prevent attachment from day (D)6/7 until D14. Blastocysts were randomly allocated to 5 different culture media: 1) SOF supplemented with 10% FBS (SOF-FBS n=16), 2) an *in vitro* culture medium (hIVC n=35) supporting ED formation in human embryos (Xiang et al., Nature, 2019), 3) chemically-defined N2B27 medium alone (N2B27 n=38), 4) supplemented with activin A (N2B27+A n=47) or 5) with activin A and Rho-associated protein kinase (ROCK) inhibitor (N2B27+A+R n=33). At E14, survival was recorded and embryo diameter, area and volume were measured with ImageJ. Cell apoptosis was analysed by TUNEL and development of specific lineages was assessed by immunostaining for SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (trophectoderm). Embryo survival (Chi-square test; $p<0.01$) and size (One-way Anova; $p<0.05$) were significantly reduced in embryos cultured in SOF-FBS. The percentage of apoptotic cells was significantly higher in surviving embryos cultured in N2B27+A+R (7.96 ± 1.19) than in SOF-FBS (3.87 ± 1.05) or hIVC (2.10 ± 0.25) (One-way ANOVA; $p<0.01$). Complete hypoblast migration was observed in most of the surviving embryos cultured in N2B27 (20/26 ~77%), N2B27+A (21/22 ~95%) and N2B27+A+R (13/16 ~81%), but only in 3/6 (50%) embryos cultured in SOF-FBS and in 8/28 (~28%) in hIVC. No epiblast cells were detected in any embryo developed in SOF-FBS (0/6), and only 1/22 (~4%) in hIVC showed 3 SOX2+ cells. In contrast, 11/28 (~39%), 24/36 (~66%) and 9/17 (~53%) embryos developed in N2B27, N2B27+A and N2B27+A+R, respectively, exhibited SOX2+ cells. SOX2+ cell number was significantly higher in N2B27+A+R (222.44 ± 65.20) than in N2B27 (52.45 ± 13.5) or N2B27+A (57.75 ± 14.75) (One-way ANOVA; $p<0.01$). ED formation, evidenced by a round compact structure formed by more than 50 SOX2+ cells, was observed in 5/11 (~45%) embryos cultured in N2B27, 12/22 (~54%) in N2B27+A and 8/9 (~89%) in N2B27+A+R. In conclusion, neither SOF-FBS nor hIVC medium supported epiblast survival *in vitro* in ovine embryos. On the contrary, N2B27 medium, although inducing a higher percentage of apoptotic cells, supported complete hypoblast migration and epiblast development. Activin A supplementation enhanced epiblast survival and ROCK inhibitor promoted epiblast proliferation and embryonic disc formation *in vitro*. This system could provide a significant advance to understand early embryo mortality in livestock without the need of experimental animals.

Role of granulosa cells as a monolayer on protecting in vitro buffalo embryo production under heat stress conditions

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Keywords: In vitro embryo production, Heat stress, Co-culture, Buffalo.

Buffaloes experience low conception rate during hot months. Therefore, the impact of co-culture system using granulosa cells (GCs) to alleviate the adverse effect of physiological heat shock, at the most sensitive preimplantation stage (zygote), on *in vitro* embryo development and related molecular response was the aim of the present study. Cumulus-oocyte complexes (COCs) were collected from ovaries (n= 448) of cyclic slaughtered buffalo cows. Good quality immature oocytes (n= 1512) were subjected to *in vitro* maturation and fertilization. Nuclear maturation was detected for oocyte chromosomal configuration using 1% aceto-orcein stain after fixed in 3:1, methanol: glacial acetic acid for 24 hour. Post *in vitro* fertilization (18-22 h), presumptive zygotes were randomly assigned into four groups: (G1) No heat shock (38.5ºC), (G2) Heat shock (40.5°C) and (G3) Co-culture with GCs monolayer and heat shock and (G4) Co-culture with GCs monolayer and no heat shock. Heat-shocked groups were exposed to temperature of 40.5°C for the first two hours of culture (as well established and published protocol in this species) then continued *in vitro* culture at 38.5°C up to day 8 (day of fertilization = day zero). Embryo development (cleavage rate at D3 and blastocyst rate at D8) was monitored throughout pre-implantation period. Expression profile of 7 candidate genes (CPT 2, GLUT 1, SOD 2, HSF1, HSP 90, NANOG and NFE2L2) was analyzed in blastocysts of all experimental groups using quantitative Real-time PCR after RNA isolation and cDNA synthesis. The embryo development data were analyzed by General Linear Univariate model using SPSS while gene expression data was analyzed using SAS statistical analysis package.

The results indicated that COCs expansion rate was 90.8±1.1% and nuclear maturation rate (telophase + metaphase II) was 73.8%. Cleavage rate as recorded at day 3 was significantly higher ($p \leq 0.05$) for G1 (71.1±10.5%), G3 (80.2±7.0%) and G4 (70.5±7.9%) than G2 (43.7±7.0%). In addition, embryos of G3 showed approximately the same rate of developed embryos (Morula and blastocyst stages at D 8 of culture) as of G1 (50.9±5.3 and 51.7±7.9, respectively). The expression profile of genes regulating metabolic activity (CPT2 and GLUT1) was increased ($p \leq 0.05$) in G1 and G3 compared to G2 and G4 groups. In addition, relative abundance of antioxidant gene (SOD2) showed comparable results between G1 and G3 being however higher than G2 group. Two members of heat shock protein family (HSF1 and HSP90) were significantly up-regulated in G2 and G3 compared to G1 group. While no statistical differences were observed for pluripotent regulating gene, NANOG and stress resistance transcript NFE2L2 among the study groups. In conclusion, embryos cultured in the presence of GCs as a monolayer has a beneficial impact on alleviating heat stress through the regulatory mechanism of genes involved in metabolic activity, defense system and heat shock response highlighting crucial role of these mechanisms for embryo viability when buffaloes exposed to severe heat stress.

APAF1-deficient bovine embryos develop normally through elongation

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Keywords: Elongation, haplotype, holstein, HH1, APAF1

Genomics-assisted genetic selection in cattle has uncovered several deleterious haplotypes. These haplotypes are never found on homozygosity and thereby must induce pre-term mortality. Holstein Haplotype 1 (HH1) produces a non-sense mutation in the gene *APAF1*, truncating the protein to approximately one-third. However the developmental timing when HH1 double-carriers (i.e., *APAF1* knock-out, KO) die remains unknown. The objective of this study has been to determine whether *APAF1* KO embryos display developmental defects before maternal recognition of pregnancy. *In vivo* produced embryos were recovered at Days 9 (E9, expanded blastocysts), 11 (E11, ovoid conceptuses) or 14 (E14, elongated conceptuses) post-fertilization from superovulated cows heterozygous for HH1 mutation (Hz, 2 cows/stage) inseminated with semen from an Hz bull. Embryos were fixed in 4 % paraformaldehyde for 10 min and kept at 4 ºC until analysis. E9 embryos were subjected to immunostaining with anti-CDX2 to determine trophectoderm (CDX2+) and inner cell mass (CDX2-) cell number. Total and epiblast cell numbers were determined on E11 conceptuses by immunostaining for SOX2. Conceptus and embryonic disc length were measured on E11 and E14 conceptuses. Finally, Sanger sequencing was performed to determine the genotype of each embryo. E9 embryos showed Mendelian distribution of alleles (5:8:4 for wild-type(WT):Hz:KO). At that stage, genotype did not determine blastocyst cell counts (TE: 98±7 vs. 106±4 vs. 105±5; ICM: 20±1 vs. 21±1 vs. 22±2; for WT, Hz and KO respectively, ANOVA $p>0.05$). E11 conceptuses also showed Mendelian distribution of alleles (4:12:5 for WT:Hz:KO). Conceptus or embryonic disc size was also similar across genotypes (conceptus length 535±84 vs. 546±89 vs. 446±68 μm, disc length 106±13 vs. 107±12 vs. 105±7 μm, for WT, Hz and KO, respectively, ANOVA $p>0.05$) and no differences were noted on total or SOX2+ (epiblast) cells (total cells 1262±209 vs. 1291±352 vs. 951±282; SOX2+ cells 50±4 vs. 54±10 vs. 50±7; for WT, Hz and KO, respectively, ANOVA $p>0.05$). Finally, Mendelian distribution was also unaltered in E14 conceptuses (2:3:3 for WT:Hz:KO) indicating that KO embryos are able to develop to elongated conceptuses. A significant cow effect was noted on conceptus and embryonic disc length at E14, but genotype did not influence any of these parameters (conceptus length 7.9±6.1 vs. 5.6±5 vs. 5.7±2.7 cm; disc length 0.52±0.11 vs. 0.44±0.16 vs. 0.57±0.13 cm, for WT, Hz and KO, respectively, two-way ANOVA $p<0.05$). In conclusion, APAF1 KO embryos develop normally to elongated conceptuses, suggesting that the developmental arrest induced by the causative mutation occur after maternal recognition of pregnancy.

Mitochondrial DNA replicates during mouse preimplantation development

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Keywords: Mitochondria, mtDNA, CRISPR, POLG, oocyte quality, embryo quality

Mitochondrial DNA (mtDNA) is believed to do not replicate during preimplantation development, as mtDNA copy number remains relatively stable up to the blastocyst stage. However, POLG, the nuclear-encoded polymerase responsible for mtDNA, is expressed in preimplantation embryos. The existence of mitochondrial replication during embryo development would imply that suboptimal culture conditions may alter mtDNA copy number contributing to embryonic damage. The objective of this study was to determine whether mtDNA replication occurs before reaching the blastocyst stage. For this aim, we have analyzed the mtDNA copy number in *Polg*-deficient mouse embryos (KO, generated by CRISPR/Cas9) compared with wild type (WT) embryos. C57CBAF1 female mice (7-8 weeks old) were superovulated by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG) and an equivalent dose of human chorionic gonadotropin (hCG) at a 48-h interval. Superovulated female mice were mated with C57CBAF1 stud males. Mouse zygotes, collected ~20 h after hCG injection, were injected with mRNA encoding for Cas9 alone (WT) or combined with sgRNA against *Polg* (POLG group). Microinjected embryos developed *in vitro* to blastocyst in KSOM medium or to egg cylinder stage (sequential system, Bedzhov et al. Nature 2014). Clonal sequencing (10 clones/embryo) was performed to determine which embryos were KO within POLG group. Embryos were deemed KO when all alleles disrupted the open reading frame of the gene. Cell count was performed at the blastocyst stage by immunostaining for the trophectoderm marker CDX2, CDX2+ cells were deemed TE cells, whereas CDX2- DAPI+ cells were deemed ICM cells. Relative mtDNA content was analysed by qPCR. Development to blastocyst was similar (~75 %) between WT and POLG groups, and no differences were noted on total, TE or ICM cells between WT and KO blastocysts (total: 103±6 vs. 100±7; TE 77±7 vs. 84±6; ICM 25±5 vs. 15±3; WT vs. KO, ANOVA $p>0.05$). Relative mtDNA content at the blastocyst stage was significantly reduced following *Polg* ablation (2.6±0.3 vs. 1±0.1 for WT and KO, respectively, ANOVA $p<0.05$). Embryo development to egg cylinder was significantly lower for embryos of POLG group (37.5±3.5 vs. 14.7±2.5, for WT and POLG groups, ANOVA $p<0.05$) and the differences in mtDNA between KO and WT embryos were more evident than at the blastocyst stage (192.6±42.8 vs. 1±0.6, for WT and POLG groups, ANOVA $p<0.05$). In conclusion, *Polg* ablation does not alter blastocyst formation, but reduces mtDNA content, indicating that mtDNA replication occurs already before reaching the blastocyst stage in mouse embryos.

Ovarian characteristics, and in vitro nuclear and cytoplasmic oocyte maturation in Duroc and Landrace pigs.

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Keywords: porcine, breed differences, oocyte maturation

Differences in reproduction traits are observed between pig breeds; on average 9.2 total number piglets born (TNB) per litter are reported in the Norwegian Duroc (ND) sire line compared to 13.8 TNB in the Norwegian Landrace (NL) dam line (Norsvin, 2019). Breed differences in this trait could be due to ovarian characteristics, which might also affect *in vitro* embryo production (IVP) outcomes. Therefore, the aim of this study was to assess ovarian characteristics and *in vitro* nuclear and cytoplasmic oocyte maturation in the ND and NL line. One day after weaning, follicular phase ovaries were collected from 37 ND and 20 NL sows. Ovary length and weight were measured, and the number of follicles (<3 mm and 3-8 mm) were counted. Cumulus oocyte complexes (COCs) were collected and cultured for 48 h in Porcine Oocyte Medium (POM) supplemented with 0.4% BSA (6% CO_2, 38.8°C). To assess individual COC area and to determine a cumulus expansion ratio per well, images were taken at 0 h and 20 h using a stereomicroscope. Oocytes were stained at 20 and 48 h with 8 µg/ml Hoechst-33342 and 100 µg/ml Lectin PNA-Alexa Fluor 568 to evaluate nuclear maturation and cortical granule (CG) distribution. Additionally, total glutathione (GSH) was measured at 48 h by a GSH/GSSG-Glo assay to further elucidate cytoplasmic maturation. Data was obtained from 3 replicates and mean values for ovarian characteristics, COC area, cumulus expansion and GSH content between the breeds were analysed by Student's t-test. Proportion of oocytes in the different nuclear stages and the six CG distribution classes were analysed between the breeds by Fisher's exact test. The data from parity one sows only (ND, n=11; NL, n=10) was used for analysis of ovarian characteristics as different parities were not equally represented across breeds. A larger average ovary length (3.0±0.3 cm vs. 3.2±0.3 cm, P=0.01) and a greater number of 3-8 mm follicles (13.6±5.4 vs. 21.6±7.9, P<0.001) were observed for NL ovaries compared to ND. For all sows (ND, n=37; NL, n=20), ND COCs had on average a significantly smaller area at 0 h (P<0.0001), but a higher cumulus expansion ratio was observed after 20 h compared to NL (364±46% vs. 278±27%, P=0.001). In addition, more ND oocytes exhibited advanced stages of nuclear maturation based on chromatin configuration at 20 h than NL oocytes. Significantly more ND oocytes were in the GV2 and MI stage compared to NL, while more NL oocytes were present in the GV1 stage. Contrary, the proportion of CG distribution groups of ≥ 4 showed more NL oocytes in the more advanced CG distribution groups compared to ND at the same timepoint (34% vs. 56%, P=0.0016). Maturation to MII stage at 48 h did not differ between ND and NL, 136/151 (90.1%) and 142/162 (87.7%), respectively. No differences were observed for GSH content or CG distribution at 48 h. In conclusion, differences with regard to ovarian characteristics as well as nuclear and cytoplasmic maturation at 20 h, but not at 48 h, were observed between breeds which could affect IVP outcomes. Further experiments are required to understand differences in fertilization and embryo development between the breeds.

Alginate-based encapsulation of bovine cumulus-oocytes complexes during in vitro maturation

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Keywords: alginate, oocyte maturation, cattle

Ovarian architecture prearranges oocyte quality. For example, the interaction between the stroma and the ovarian follicle has implications in polycystic ovary syndrome in humans (Fournier et al., 2017 Gynecol Obstet Fertil Senol 45) or culturing isolated preantral follicles in domestic species (Brito et al., 2016, Reprod Domest Anim 51). *In vitro* maturation (IVM) is a process in which cumulus-oocyte complexes (COCs) are removed from the ovary, and the final stages of oocyte development occur *in vitro*. Thus, IVM occurs in an altered physical environment, since cumulus-oocyte complexes (COCs) are deprived of the extracellular support that exists within the ovarian follicle. Full *in vitro* recapitulation of the events associated with successful oocyte maturation is not always achieved during IVM with only 30% of immature oocytes developing to the blastocyst stage (Lonergan and Fair, 2016 Annu Rev Anim Biosci 4). Here we tested the hypothesis that maintaining COCs within a three-dimensional structure during IVM for a better recapitulation of the physiological environment could improve nuclear and cytoplasmic maturation of the gamete. Inert biomaterials, such as alginate, have been used in numerous biomedical engineering applications due to its biocompatibility and ease of gelation (Shea et al., 2014 Annu Rev Biomed Eng 16). Thus, we examined the effect of encapsulating bovine COCs in alginate hydrogels on nuclear maturation and cortical granules (CG) migration. For IVM in alginate (ALG), 10 COCs were transferred to 0.5% ALG, aspirated with an automatic pipette in 10 µl volume, and transferred to the cross-linking solution (50 mM $CaCl_2$, 140 mM NaCl) to allow ALG gelification. ALG beads containing COCs were then washed and transferred to 500 µl BO-IVM medium (four beads/well). As a control, groups of 40 unencapsulated COCs were placed in IVM wells. Both groups were cultured for 22 h (38.5ºC, 5% CO_2 in air) and then COCs were released from the ALG beads following a brief treatment with 10 IU/ml alginate lyase. COCs in both treatment groups were denuded, and the oocytes were recovered. To assess CG distribution and nuclear status in the oocytes, the zona pellucidae were digested (0.1% pronase) and cells were fixed (4% paraformaldehyde) and stained (10 µg/ml *Lens Culinaris-Agglutinin*-FITC and DAPI-Vectashield). Confocal microscopy revealed no difference in the percentage of oocytes that reached metaphase II between groups (69.9±5.4%, N = 73 *vs.* 82.1±5.2% N = 56 for control and ALG, respectively). The proportion of oocytes that showed type III CG (CG fully migrated, arranged under oolemma and ready to be exocytosed) was 76.7±5.0% (control) and 82.1±5.2% (ALG). In conclusion, while there was a tendency of encapsulation to improve meiotic maturation and favorable CG distribution, these results were not significant. Additional fertilization and embryo development studies are warranted to examine whether encapsulation during IVM improves the developmental competence of the gamete.

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Embryonic disc formation following post-hatching bovine embryo development in vitro

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Keywords: bovine, embryonic disc, in vitro

Embryonic mortality during the second week of gestation accounts for the greatest pregnancy losses in bovine, exerting a relevant economic impact on farming. By day 9, epiblast and hypoblast have emerged from the inner cell mass. The hypoblast then migrates to cover the entire inner surface of the embryo, and the epiblast forms a flat embryonic disc. These developmental events are poorly understood, mainly because they cannot be recapitulated *in vitro*. Previous studies have established an *in vitro* post-hatching development system that supports bovine embryo development beyond the blastocyst stage (Brand*ão* et al., Biol Reprod, 2004; Vajta et al., Theriogenology, 2004). This system, based on agarose gel tunnels and glucose-enriched (PHD) medium, achieves trophoblast expansion and proliferation of hypoblast cells, but embryonic disc formation is impaired. The aim of this study has been to develop an *in vitro* system able to support embryonic disc formation. *In vitro*-produced D9 blastocysts were allocated to SOF:PHD (1:1) or N2B27 and at D11 they were measured and cultured individually in PHD or N2B27, respectively, in different culture substrates: 1) inside agarose tunnels (n=69) or free-floating over an agarose-coated dish (n=48) in PHD medium (Synthetic Oviduct Fluid supplemented with 27.7 mM glucose and 10% FCS); 2) in PHD medium (n=62) or in a chemically-defined enriched medium (N2B27, n=47) over an agarose-coated dish and 3) with (n=32) or without (n=47) agarose coating in N2B27 medium. At D15, survival was recorded (dead embryos were clearly distinguishable as they collapsed and degenerated), embryo length, area and volume were calculated using Fiji, the abundance of transcripts encoding interferon Tau (*IFNT2*) and metabolic enzymes was analysed by RT-qPCR, and the development of specific lineages was assessed by immunostaining for SOX2 (epiblast), SOX17 (hypoblast), and CDX2 (trophectoderm). No differences were found on embryo survival until D15 and the main factor determining survival was the initial embryo size at D11 ($p<0.05$, Chi-square test). Culture inside agarose tunnels shaped embryo morphology by physical constriction, but it reduced embryo area and volume (1.92 ± 0.31 mm^2 and 1.52 ± 0.24 mm^3 inside tunnel *vs.* 3.98 ± 0.92 mm^2 and 7.50 ± 2.33 mm^3 free-floating, $p<0.05$, t-test) and did not provide any significant advantage in terms of development of hypoblast and epiblast lineages. *IFNT2* expression was higher in PHD medium and anaerobic glycolysis-related genes were upregulated in D15 vs. D9 embryos irrespective of the media used. In contrast to PHD, N2B27 medium supported complete hypoblast migration and epiblast survival *in vitro*, even in the absence of agarose coating: ~56 % of D15 embryos developed in N2B27 showed SOX2+ cells (6/11 over agarose and 9/16 without agarose) and ~22 % developed embryonic disc-like structures formed by SOX2+ cells (2/11 over agarose and 4/16 without agarose). In summary, we provide a culture system supporting trophectoderm proliferation, hypoblast migration and epiblast survival beyond the blastocyst stage.

Characterization and analysis of miRNA content of bovine oviduct and uterine extracellular vesicles across estrous cycle

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Keywords: oviduct, uterus, extracellular vesicles

Recently, extracellular vesicles (EVs) found in various biological fluids and particularly in reproductive fluids, have gained a considerable attention for their possible role in cell- to- cell communication. Among, the different bioactive molecules cargos of EVs, MicroRNAs (miRNAs) are emerging as promising diagnostic biomarkers with high clinical potential. Therefore, aiming to understand the roles of EVs in bovine reproductive tract, we intended to characterize and profile the EVs of oviduct and uterine fluids (OF, UF) and their miRNA across the estrous cycle. For this, 5 reproductive tracts for each stage of estrous cycle (S1: days 1 to 4; S2: days 5-10; S3: days 11-17; S4: days 18-20) were selected according to their corpus luteum morphology from slaughtered heifers and transported to the laboratory on ice. EVs were isolated by size exclusion chromatography from a flushing of 1ml and 3ml of OF and UF, respectively, and concentrated by ultracentrifugation. The obtained EVs pellet was suspended in 100 µL of PBS. One part was used for EVs characterization by Nanotracking analysis (NTA), Transmission electron microscopy (TEM) and western blot. The other part was used for RNA extraction and miRNA expression profiling by primer based real-time PCR of 383 mature miRNA sequences. Statistical differences in miRNA level were assessed by ANOVA. Both NTA and TEM observations confirmed the existence of EVs in OF and UF at all stages, with a mean size ranging between 135-180nm. The NTA quantification showed an average concentration of 3.4 x 10^{10} EVs/ml and 6,0 x 10^{10} EVs/ml for OF and UF respectively, whatever the cycle stage. Moreover, western blot analysis evidenced the EVs expression of some classical markers described for exosomes: tetraspanin cell surface proteins (CD67 and CD9); heat shock protein 70 (HSP70). The miRNA analysis revealed the abundance of 232 and 332 miRNAs in OF and UF, respectively. 67% and 82% of these miRNAs are common to all stages of estrous cycle. 9 miRNAs were differentially abundant in OF between stages of cycle: 8 of them displayed a progressive increase from S1 to S4 ($P<0.05$) and one miRNA showed a reduction ($P <0.05$). In UF a total of 14 miRNA were differentially abundant between stages. Greater differences were observed between S1 and S3, with 11 miRNAs enriched in S3 compared to S1 ($P <0.05$). S2 showed enrichment of 4 miRNAs in relation to S1 ($P <0.05$). Reduction of 4 miRNAs was also observed in S4 compared to other stages ($P <0.05$). In conclusion, these preliminary results indicate a possible hormonal regulatory effect of the estrous cycle on miRNA content in EVs of bovine OF and UF. Ongoing bioinformatics analysis are aiming to predict the genes targeted by these miRNAs, their signaling pathways and functional annotation clusters associated with their biological processes.

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Porcine sperm attracted by chemoattractants, showed the highest curling tail when incubated in adverse osmolarity condition

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Keywords: Boar spermatozoa, chemotaxis, osmolarity condition

The hypo-osmotic swelling (HOS) test enables the identification of sperm with functionally intact membranes and is one of a range of tests commonly used to determine sperm viability. The osmotic stress caused by the hypo-osmotic medium is enough to induce an influx of water into the cell, which results in an increase in volume and hence curling of the tail. The aim of the study was to evaluate the reaction ability of porcine sperm in hypo-osmotic solution previously attracted by follicular fluid (FF), oviductal fluid (OF), conditioned medium (CM) and progesterone (P4). The chemotaxis system consisted of two wells (A and B) connected by a capillary. Five wells (A) were filled with fresh sperm washed (20x106/mL diluted in 500 µL of TALP) from different fertile boars. The opposite wells (B) were filled with TALP and TALP supplemented with corresponding chemoattractant: FF: 0.25%, OF: 0.25%, CM: 0.13%, and P4:10 pM. After 20 min of chemotaxis, the sperm (wells B) were incubated with a final hypo-osmotic solution of 100 mOsm/L for 20 min at 5% CO_2, 38.5 °C, with saturated humidity. A total of 6000 sperms were evaluated in five replicates. ANOVA was used for the statistical analysis, and the means were separated using the Tukey test at $P < 0.05$. Results show that a higher curled tail concentration (%) was detected with FF (87.4±3.2a), OF (89.9±2.2a), CM (87.8±2.9a) P4 (88.6±2.1a) than control (84.3±3.2b) ($p<0.05$). In conclusion, our chemotaxis system, in combination with different chemoattracts, selects a pool of viable spermatozoa, as evidenced by the hypoosmotic swelling test. Further studies should be done to analyze both the phenotype and genotype of these spermatozoa, in the attempt to improve sperm selection prior to in vitro fertilization in the porcine species. *Supported by Fundación Séneca, Saavedra Fajardo (20020/SF/16). MINECO-FEDER (AGL 2015-66341-R).*

Development of in vitro matured porcine oocytes after in vitro fertilization with ejaculated or epididymal spermatozoa

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Keywords: boar spermatozoa, in vitro fertilization, embryo

In vitro production of pig embryos (IVP) is still the object of multiple studies, because a number of issues related to this method have not been resolved yet. Among problems connected with IVP are the quality of sperm after capacitation and the quality of *in vitro* obtained embryos. The aim of this study was to determine the quality and fertilization ability of ejaculated and epididymal boar spermatozoa. Ejaculated (n=7) and epididymal (n=4) sperm before and after capacitation were evaluated under a microscope and with the computer-assisted analysis method. The following parameters were evaluated: motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL) and hyperactivity. Ejaculated and epididymal spermatozoa underwent *in vitro* capacitation and *in vitro* fertilization (2 and 3 sessions, respectively) with the matured *in vitro* oocytes. Potential zygotes were cultured *in vitro* up to the expanding blastocyst for 6-8 days. The total cells number (mean) and number of apoptotic nuclei (mean) per blastocyst were assessed. Before and after capacitation, statistically significant differences (p<0.05, t-Student test) were found when comparing progressive motility of ejaculated (60.77% and 68.8%, respectively) and epididymal spermatozoa (30.0% and 55.7%, respectively). Moreover, after capacitation significant differences (p<0.01, t- Student test) between these two types of semen were recorded in the following motility parameters: VCL (49.2% and 83.3%, respectively) and hyperactivity (50.5% and 13.2%, respectively). Significant differences (p<0.01, t- Student test) in terms of hyperactivity before and after capacitation of ejaculated spermatozoa (14.81% and 50.5%, respectively) were also observed. We showed a similar *in vitro* fertilization ability of ejaculated and epididymal boar spermatozoa. The developmental potential of porcine embryos obtained as a result of fertilization with ejaculated and epididymal spermatozoa was also similar (53.7% of cleaved embryos and 25.9% of blastocysts, n=16; and 55.1% of cleaved embryos and 30.5% of blastocysts; n=4, respectively). A higher total number of cells per blastocyst was obtained as a result of *in vitro* fertilization with ejaculated spermatozoa (mean 34.2±8.3, n=70) when compared to epididymal spermatozoa (mean 28.5±6.45, n=13) (p<0.05, Chi^2 test). However, the number of apoptotic cells in blastocysts was similar in both groups (differences statistically nonsignificant). In conclusion, boar spermatozoa irrespective of type (ejaculated or epididymal), displayed a similar susceptibility to *in vitro* capacitation and *in vitro* fertilization. Developmental competence of porcine embryos obtained after *in vitro* fertilization with ejaculated and epididymal spermatozoa was also similar. Blastocysts obtained after *in vitro* fertilization with ejaculated spermatozoa showed a higher total number of cells in comparison to the same procedure carried out with epididymal spermatozoa.

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Seminal plasma downregulates the Signal Transducer and Activator of Transcription 5A gene (STAT5A), a modulator of immune response, in the preovulatory porcine endometrium

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Keywords: seminal plasma, transcriptomics, pig

Seminal plasma (SP) is a complex secretory mix of proteins, cytokines, and hormones that acts as a carrier for spermatozoa as well as actively elicits molecular responses when females are inseminated. Thus, SP is playing a major role in male-female signaling and positively impacting early embryo development (*Martinez CA et al., Front Vet Sci, 6:465, 2019*; *Alvarez-Rodriguez M et al., Int J Mol Sci, 20(3):E513, 2019*). Alongside, glucocorticoid signaling, deemed essential for normal reproduction, seems to involve a wide range of molecules. Among them, there are the STAT-proteins, involved in JAK/STAT signaling, that modulate cytokine production during inflammatory processes, and heat shock 70kDa protein HSPA4L, implicated in osmotic stress adaptation and fertility. The present study evaluated the effects of infused boar SP on the expression of glucocorticoid related genes in the preovulatory reproductive tract of weaned fertile sows (n=8) on the 1st day of estrus. Samples of cervix, distal and proximal uterus, utero-tubal junction, isthmus, ampulla and infundibulum were surgically removed 24 h after cervical infusion with pooled sperm-free SP from the whole ejaculate (SP-Total; n=4) from mature fertile boars (n=5) or after infusion with the protein-free extender Beltsville Thawing Solution (control, n=4). RNA was isolated following a TRIzol-based protocol and analyzed for global transcripts using specific microarrays (PORGENE 1.0 ST GeneChip® array, Affymetrix) aiming to identify the specific expression for 22 glucocorticoid-related genes. The data was normalized (Robust Multiarray Average) and analyzed with the Transcriptome Analysis Console (RMA-method, -1>log fold change>1; $p<0.05$). Molecular functions and biological processes of all the analyzed genes were identified by PANTHER classification system. Functional pathways were described using the KEGG database. Significant changes in gene expression were triggered by SP in distal uterus (*HSPA4L* upregulation and *STAT5A* downregulation), and infundibulum (*PTGS2*, *STAT6*, and *NR3C1* downregulation) when compared to the control. The JAK-STAT (ssc04630), and also Th1, Th2 (ssc04658) and Th17 (ssc04659) cell differentiation pathways were enriched in the downregulated genes. Owing to the active role of *STATs* in inflammation, this SP-mediated *STAT5A* downregulation might modulate an increased uterine inflammatory to avoid a plausible harmful effect to spermatozoa during sperm transport towards the oviductal reservoir.

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Natural mating represses pro-inflammatory responses in the pre-ovulatory porcine endometrium and endosalpinx (ampulla) by down-regulation of caspase-1 (CASP1) and caspase-12 (CASP12)

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Natural mating and artificial insemination (AI) elicit molecular responses in the sow genital tract upon the entrance of semen (Alvarez-Rodriguez M et al., Sci Rep, 10, 5061, 2020). Alongside, cysteine proteases i.e. caspases, play important functions in innate immunity, activating cytokines, and apoptosis. The inflammatory caspase-1 (*CASP1* gene) is activated in response to pathogen-derived and endogenous mediators, inducing secretion of the pro-inflammatory cytokines interleukin-1ß and interleukin-18, both key modulatory factors in placenta development and attachment during early pregnancy in pigs. Besides, *CASP1* expression is upregulated by the presence of viable conceptuses in the porcine uterine lumen. Caspase-12 (*CASP12* gene), on the other hand, negatively regulates inflammatory processes, inhibiting the activation of caspase-1 and preventing the production of pro-inflammatory cytokines. This study investigated the effects of natural mating and AI on *CASP1* and *CASP12* gene expression along the sow reproductive tract during the pre-ovulatory stage. Samples of the reproductive tract (cervix, distal and proximal uterus, utero-tubal junction, isthmus, ampulla, and infundibulum) were surgically removed from sows 24 h after natural mating (NM, n=4) or cervical AI with 10 mL of the sperm-peak fraction (Semen-AI, n=4). Sows cervically infused with the protein-free extender Beltsville thawing solution were used as controls (n=4). RNA was isolated following a TRIzol-based protocol and analysed for global transcripts using specific microarrays (PORGENE 1.0 ST GeneChip® array, Affymetrix). Data was normalized (Robust Multiarray Average). Only *CASP1* and *CASP12* expression were analysed with the Transcriptome Analysis Console (-1>fold change>1, p<0.05). After NM the *CASP1* and *CASP12* expression were downregulated in uterus (distal: -1.71, -1.28; and proximal: -2.14, -1.73) and ampulla (-1.59, -1.32). Also, both NM and Semen-AI shared a downregulation of *CASP1* expression in the isthmus (-1.38, -1.42) and ampulla (-1.59, -1.53). The expression of *CASP12* in the ampulla was downregulated by NM (-1.32), whereas Semen-AI upregulated *CASP12* expression (1.44). Finally, Semen-AI downregulated *CASP1* expression in the utero-tubal junction (-1.47). Results indicate that both *CASP1* and *CASP12* were downregulated by the presence of spermatozoa in the female reproductive tract during the pre-ovulatory stage, although the verification of the results could be performed by PCR. Results confirm the presence of a mechanism of immune tolerance for spermatozoa and suggest that endometrial *CASP1* and *CASP12* expression is more strongly regulated by natural mating than for AI, probably induced by the act of mating itself regardless the presence of semen.

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In vitro bovine embryo production in high palmitic acid conditions decreases DNMT1 expression in bovine zygotes and lowers global DNA methylation in the produced morulas

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Maternal metabolic disorders are associated with subfertility. Upregulation of lipolysis causes a rise in non-esterified fatty acids in the blood, which is reflected in the follicular and oviductal micro-environment. This has a lipotoxic effect on oocyte and embryo development, mainly due to elevated palmitic acid (PA) concentrations. Such disturbances in this micro-environment can cause persistent defects in later life. This is possibly mediated through epigenetic changes, like DNA methylation at cytosine residues (5-methylcytosine (5-mC)) in the oocyte or embryo. DNA methyltransferase 1 (DNMT1) is an important enzyme that maintains the DNA methylation pattern during mitosis. The dynamic nature of the epigenetic landscape during oocyte maturation and early embryo development makes this a vulnerable window for epigenetic alterations. We hypothesized that exposure of bovine oocytes to PA during *in vitro* maturation (IVM) and culture (IVC) alters expression of DNMT1 in the resulting zygotes and modifies global DNA methylation levels in the resulting morulas. In this study, bovine oocytes were exposed to a pathophysiological concentration of PA (150µM) or Solvent Control (SCONT) media during IVM (24h). Oocytes were *in vitro* fertilized (for 20h) and presumptive zygotes were cultured in either PA (230µM) or SCONT media, respectively. Cleavage rates were recorded at 48h post insemination (p.i.) and blastocyst rates at day 7 (D7) and 8 (D8) p.i. (n=538 oocytes, 4 replicates). Zygotes were collected at 20h p.i. and snap-frozen in pools of 5-10/treatment (4 replicates) to evaluate mRNA expression of DNMT1 by qPCR. This was measured in duplicates and normalized based on the expression of validated reference genes YWHAZ and GAPDH. Relative fold change was calculated with the delta-delta Ct method. Also, morulas (D5.7; n=7/treatment) were fixed in 4% paraformaldehyde for 5-mC immunostaining. Images were acquired with a Leica SP8 confocal microscope and quantified using Image-J to measure integrated density of the nuclei in different z-stacks (SCONT: mean cell number=36.3±7.5; PA: mean cell number=32.3±3.7). Developmental competence data were analysed using logistic regression, other numerical data using an independent samples t-test. Exposure of bovine oocytes and embryos to PA during IVM and IVC resulted in significant reduction of cleavage rates (68.3%) compared to the SCONT group (81.9%) ($P<0.001$). Blastocyst rates were significantly lower in the PA group (D7: 15.6%; D8: 25.2%) compared to the SCONT group (D7: 32.8%; D8: 39.1%) (D7: $P<0.001$; D8: $P<0.001$). DNMT1 expression in PA-exposed zygotes was significantly decreased (38-fold downregulation, $P=0.02$) compared to SCONT zygotes. Also, staining intensity of 5-mC was significantly decreased in morulas exposed to PA (34,7% reduction compared to SCONT, $P=0.017$). We conclude that exposure of bovine oocytes and embryos to PA during IVM and IVC hampers development, and alters global DNA methylation levels which is detected at the morula stage.

Dietary caloric normalization or restriction as preconception care strategies: impact on oocyte developmental competence and blastocyst quality in high fat/high sugar-induced obese outbred mice

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Keywords: obesity, preconception care intervention, oocyte development

Maternal metabolic disorders like obesity are linked to decreased oocyte and embryo quality, and reproductive failure. Obese patients are advised to lose weight before conception to increase pregnancy chances. However, as human studies show no univocal guidelines, more fundamental research might provide additional answers. Furthermore, it is unclear if oocyte quality can be restored over time after starting a preconception care intervention (PCCI). Therefore, we aimed to test the effect of diet normalization or caloric restriction (CR) as PCCI in high fat/high sugar (HF)-fed obese mice and examined the impact on oocyte development and embryo quality. Five week old female outbred Swiss mice were fed a control (CTRL; 10% fat) or a HF (60% fat in diet, 20% fructose in drinking water) diet for 7 weeks. Afterwards, HF-mice were put on different PCCIs for 2 or 4 weeks, resulting in four treatment groups: 1) CTRL diet for 9 or 11w (CTRL_CTRL), 2) HF diet for 9 or 11w (HF_HF), 3) switch from a HF (7w) to an *ad libitum* CTRL diet for 2 or 4w (HF_CTRL) and 4) switch to a 30% CR diet for 2 or 4w (HF_CR). Change in body weight (BW) was recorded twice a week (n=192 mice). *In vivo* matured oocytes were collected after superovulation, *in vitro* fertilized and cultured (n=6 mice/group/timepoint). Oocyte developmental competence (n=722 oocytes) was examined by recording cleavage (24h p.i.) and blastocyst rates (5 days p.i.). Blastocyst quality (n=183) was determined by caspase-3 immunostaining and DAPI. Total cell count (TCC) and apoptotic cell indices (ACI) were calculated. Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni-corrected. In comparison with the CTRL group, HF diet increased BW after 7 weeks by 24.19% (*P=0.000*). After the start of the PCCI, both HF_CTRL and HF_CR mice progressively lost weight and reached values similar to control mice after two weeks. After **2 weeks** of PCCI, oocytes from HF_HF mice displayed lower cleavage rates than those from CTRL_CTRL mice (36.26% *vs.* 64.52%, *P=0.002*) but blastocyst rates (26.37% *vs.* 35.48%, *P*>0.1) were not different. HF_CR, but not HF_CTRL, oocytes showed higher cleavage rates (68.48%, *P=0.000*) compared with HF_HF oocytes. Moreover, both HF_CTRL (44.64%, *P*=0.033) and HF_CR (59.78%, *P*=0.000) oocytes showed improved blastocyst rates when compared to the HF_HF group (26.37%). After **4 weeks** of PCCI, HF_HF oocytes also displayed lower cleavage rates compared with CTRL_CTRL mice (42.17% *vs.* 62.11%, *P=0.040*) while blastocyst rate (34.94% *vs.* 45.26%, *P*>0.1) was not affected. HF_CR, but not HF_CTRL, oocytes showed higher cleavage (65.28%, *P=0.018*) but not blastocyst rates (52.78%, *P*>0.05) when compared to HF_HF. No significant differences in blastocyst TCC and ACI could be detected among relevant treatment groups at both time points (*P*>0.1). Based on this information, switching to a caloric restriction diet (HF_CR) seems to be more efficient in restoring oocyte quality in an obese mouse model than diet normalization (HF_CTRL).

Cloning, transgenesis and stem cells

Conventional CRISPR is unable to edit mtDNA in mouse zygotes

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Keywords: Mitochondria, mtDNA, embryo, CRISPR

Mitochondria contain their own genome (mtDNA) encoding for proteins essential for their function. Mutations in mtDNA can cause mitochondrial diseases and CRISPR may provide a way to correct these mutations or to eliminate mtDNA harbouring unwanted mutations. The objective of this study has been to analyse whether conventional CRISPR system, developed for nuclear DNA modification, is able to induce mtDNA mutation or mtDNA degradation. Mouse zygotes were injected with mRNA encoding for Cas9 alone (control group) or combined with sgRNA against one of two mitochondrial sequences: *Cytb* or *Nd4*. *In vitro* blastocysts produced from microinjected embryos were snap frozen. To determine whether mtDNA was edited by CRISPR, target sequences (*Cytb* or *Nd4*) were amplified by PCR and Sanger sequenced. Possible mtDNA degradation was determined by relative mtDNA content analysis by qPCR. Development to blastocyst was similar in all three groups (83±10 vs. 79±2 vs. 74±6 % for control, *Cytb* and *Nd4* groups, respectively, ANOVA $p<0.05$). In contrast to the >90 % edition rates we routinely obtain when targeting nuclear sequences, no mtDNA mutation was detected on embryos microinjected with CRISPR components against mitochondrial sequences (18 embryos analysed for *Cytb* and 20 embryos analysed for *Nd4*). Relative mtDNA content was also similar between groups (1.2±0.1 vs. 1±0.1 vs. 1±0.1 for control, *Cytb* and *Nd4* groups, respectively, ANOVA $p<0.05$). These results suggest that the CRISPR system conventionally used for nuclear genome modification, which includes a Cas9 protein tagged with a nuclear localization signal, is unable to access the mitochondrial matrix where mtDNA is located.

Timing of mRNA CRISPR/Cas9 microinjection with respect to in vitro fertilization affects embryo development and mutation efficiency in porcine embryos

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Keywords: CRISPR/Cas9, TPC2, pig

Current advances on *in vitro* fertilization (IVF) techniques in pig, allow the generation of embryos with improved quality, enabling the use of IVF as a means to produce genetically modified pigs. In contrast to *in vivo* production, IVF allows precise control of microinjection timing of gene editing tools with respect to fertilization and the beginning of DNA replication. The objective of this study was to determine the effect of microinjection timing on embryo developmental and genome edition rates. For this aim, Cas9 mRNA and single guide RNA (sgRNA) targeting the two-pore channel 2 gene (TPC2) were microinjected at three different times: oocyte microinjection before IVF (BIVF), zygote microinjection 5-6h after IVF (5IVF) and zygote microinjection 10-12h after IVF (10IVF). A non-injected group was used as a control.

Porcine cumulus-oocyte complexes matured in NCSU37 medium for 40-44h (38.5ºC, 5% CO_2) were inseminated in TALP medium with ejaculated frozen-thawed boar spermatozoa selected with NaturARTs-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). At 18h post insemination, putative zygotes were *in vitro* cultured in NCSU23 medium until day 6 post insemination. Blastocyst yield (blastocyst/oocyte) was recorded and mutation rates were analysed by fluorescent PCR-capillary gel electrophoresis.

A total of 869 oocytes were used for this experiment in four different replicates. Results showed that microinjection after IVF detrimentally affected the embryo development. Thus, blastocyst yield was significantly lower ($p<0.05$) in 5IVF (19.9±2.8 %) and 10IVF (17.9±2.7%) groups compared to the BIVF group (28.8±3.3%), but no group was significantly different to the control (21.6±2.8%). Mutation rates despite were numerically higher at the late microinjection times, being the highest edition rate obtained with 10IVF (14/33, 42.4%) followed by 5IVF (11/32, 34.4%) and BIVF (12/40, 30.0%), but these differences between groups were not statistically significant. Mosaicism incidence was low in all groups analysed (0, 9.1 and 21.4% for BIVF, 5IVF and 10IVF groups, respectively). Finally, the efficiency of the system, measured as the proportion of edited blastocysts without mosaicism from total oocytes injected, was 8.6% in BIVF, 6.2% in 5IVF and 6.0% in 10IVF. In conclusion, the efficiency of the system for obtaining pig embryos without mosaicism tended to be higher when oocytes were microinjected with Cas9 mRNA and sgRNA before IVF.

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First generation of two-pore channel 2 (TPC2) mutant pigs by ribonucleoprotein CRISPR/Cas9 microinjection before in vitro fertilization

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TPC2 is a lysosomal cation channel protein involved in NAADP-mediated Ca^{2+} signalling and endolysosomal trafficking in the cell. TPC2 has been shown to be involved in a variety of pathophysiological processes including smooth muscle and heart contraction, neo-angiogenesis and cancer, immune responses, autophagy, skeletal muscle development, diabetes, brown adipose thermogenesis (reviewed in Patel et al. 2018 Biochimica et Biophysica Acta. Molecular Cell Research), and SARS-CoV-2 (Ou et al., 2020 Nature Communications) and Ebola infection (Penny et al. 2019 Biochim Biophys Acta Mol Cell Res). Most insights into TPC2 function have come from studies of TPC2 knockout (KO) mice. The importance of TPC2 is such that further valuable insights might be gained by studying the consequences of loss of TPC2 expression in species physiologically closer to humans, and pig is a very suitable model in this respect (Perleberg et al., 2018 Disease Models & Mechanisms). Therefore, this study aimed to generate TPC2 KO pigs by using *in vitro* matured oocytes that were immediately microinjected with anti-TPC2 CRISPR/Cas9 ribonucleoprotein (RNP) before being subjected to *in vitro* fertilization (IVF). The goal of injecting before IVF was to see if this led to gene editing before the first DNA replication thus reducing mosaicism incidence.

Porcine cumulus-oocyte complexes matured in NCSU37 medium for 40-44h were inseminated in TALP medium with ejaculated frozen-thawed boar spermatozoa selected with NaturART-PIG sperm swim-up medium (EmbryoCloud, Murcia, Spain). At 18h post insemination (p.i.), putative zygotes were cultured *in vitro* in NCSU23 medium until day 2 p.i. Embryos at the 2-4 cell stage were surgically transferred (120±10 embryos/sow) to one oviduct of 6 sows 3-5 days after natural heat detection. After delivery, ear, tail, muscle and blood tissue were collected from born piglets and mutation rates were analysed by fluorescent PCR-capillary gel electrophoresis and Sanger DNA sequencing.

Embryo transfer resulted in 2 pregnancies (33.3%) with one litter of 5 piglets (4 males and 1 female) and another with 7 piglets (1 male and 6 females). The mean litter size was 6 piglets (41.7% males and 58.3% females). Regarding mutations, 2 females and 1 male were homozygous KO in all analysed tissues. One of the females and the male showed a double deletion of 11bp whereas the other female had a double insertion of 16 bp; both should result in a frameshift in the TPC2 open reading frame. From birth to day 28 of life, all mutant pigs had a phenotype and growth rate similar to that observed in wild-type animals. In conclusion, this is the first generation of KO TPC2 pigs generated by any means to our knowledge. The mutant animals obtained will be now used to generate a TPC2 KO pig colony to obtain more animals that will be available for different studies.

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

Cumulus cells transcript abundance as a proxy for bovine oocyte quality

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Keywords: Cumulus cells, gene expression, oocyte quality, RNAseq

The efficiency of *in vitro* embryo production protocols in cattle is hampered by the reduced developmental competence of *in vitro* matured oocytes. Oocytes are enclosed by cumulus cells which serve essential nourishing and signalling functions during folliculogenesis. These cells provide an attractive matrix on which to perform analyses aimed to understand the molecular roots of oocyte competence. The objective of this study was to analyze the transcriptional differences between cumulus cells from oocytes exhibiting different developmental potentials by RNA-seq. Cumulus-oocyte complexes were obtained from slaughtered cattle and individually matured *in vitro*. Following IVM, cumulus cells were removed by hyaluronidase treatment, pelleted, snap frozen in liquid nitrogen and stored at -80 ºC until analysis. Cumulus-free oocytes were fertilized using semen from a single bull and cultured *in vitro* individually. Cumulus cells were allocated into three groups according to the developmental potential of the oocyte: 1) oocytes developing to blastocysts following IVF (Bl+Cl+), 2) oocytes cleaving following IVF but arresting development prior to the blastocyst stage (Bl-Cl+), and 3) oocytes not cleaving following IVF (Bl-Cl-). RNAseq was performed on 4 (Bl-Cl-) or 5 samples (Bl+Cl+ and Bl-Cl+) per group, using the Illumina platform with >30 M reads/sample. Each sample contained cumulus cells from 10 cumulus-oocyte complexes (COCs). Differential expression was analysed by DESeq2 software. RNAseq analysis revealed 1609, 1466 and 1420 differentially expressed genes (DEGs) for the comparisons Bl+Cl+ vs. Bl-Cl+, Bl+Cl+ vs. Bl-Cl- and Bl-Cl+ vs. Bl-Cl, respectively, using a raw p value <0.05. These DEGs were narrowed down to 77, 80 and 32 DEGs for the comparisons Bl+Cl+ vs. Bl-Cl+, Bl+Cl+ vs. Bl-Cl- and Bl-Cl+ vs. Bl-Cl-, respectively, when an adjusted p value <0.05 was used. From these subsets of DEGs, 49, 50 and 18 DEGs, respectively, exhibited a fold change greater than 1.5. Focussing on DEGs in cumulus cells obtained from oocytes developing to blastocysts, 10 DEGs were common to both comparisons (10/49 from Bl+Cl+ vs. Bl-Cl+, 10/50 from Bl+Cl+ vs. Bl-Cl-). These DEGs correspond to 6 downregulated genes (*HBE1, ITGA1, PAPPA, AKAP12, ITGA5 and SLC1A4), and 4 genes upregulated (GSTA1, PSMB8, FMOD and SFRP4)* in Bl+Cl+ compared to the other groups. In conclusion, cumulus cells transcript abundance could be used as a predictor of the developmental potential of their enclosed oocyte.

Early embryo morphokinetic parameters to predict bovine in vitro blastocyst development

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Keywords: blastocyst, early cleavage, time-lapse

Monitoring morpho-kinetics using time-lapse technology at initial cleavage stages may assist embryologists in selecting embryos with the greatest developmental potential. The objective of this study was to establish the best cut-point on blastomere division times based on the probability of detection statistics for bovine *in vitro* blastocyst development using time-lapse technology. Cumulus oocyte complexes, collected from the slaughterhouse, were matured and fertilized in groups of 60 using routine methods. After 21 h of sperm-oocyte coincubation, presumed zygotes were randomly selected for culture in 9- or 16-microwells, and their development was monitored using time-lapse technology (Primo Vision; Vitrolife®, Göteborg Sweden). Remaining zygotes were conventionally cultured in groups (n = 25) as a control. Time-lapse technology allowed to take pictures of the zygotes every 10 min. Morpho-kinetic data included time (hours) to reach the first (1-2 cell), second (3-4 cell), third (5-8 cell), and fourth (9-16 cell), cell division. A receiving operator characteristic (ROC) and the area under the curve (AUC) were built in to find the cut-points with the highest sensitivity (Se) and specificity (Sp) to predict day 8 blastocyst development using the morpho-kinetic data of blastomere division times. Generalized mixed-effects models were used to test the effect of the cleavage time based on the best cut-point on day 8 blastocyst development. The cut-point for the first blastomere division was 31.6 h and had a better combination of Se (60.4%) and Sp (72.8%) and higher ($P < 0.05$) AUC (68.7%) than the second (44.2 h; Se = 88.7 and Sp = 34%; AUC = 55.2%), third (45.6 h; Se = 81.1 and Sp = 36.7%; AUC =50.9%), or fourth (77 h; Se = 86.5 and Sp = 40.6%; AUC = 53.9%) blastomere division. In 15 replicates, control (n = 366) resulted in a higher day 8 blastocyst development (43.8 ± 2.8%) than time-lapse (n = 294; 30.7 ± 2.8%; $P = 0.0006$). The cleavage rate was similar ($P > 0.05$) between control (77.4 ± 2.3%) and time-lapse (71.4 ± 2.6%). From cleaved zygotes, slow-cleaving embryos (based on the first blastomere division) presented lower ($P < 0.001$) day 8 blastocyst development (25.4 ± 3.8%) in comparison to fast-cleaving (58.3 ± 5.3%) and control embryos (55.7 ± 3.1%). The day 8 blastocyst development was similar ($P > 0.05$) for fast-cleaving and control embryos. Other studies have already shown that early cleavage is associated with higher blastocyst development. However, the categorization of cleavage speeds of previous studies was mainly based on arbitrary cut-points. This study, for the first time, implemented a ROC curve and the AUC to establish cut-points of blastomere division to predict blastocyst development. The ROC curve and the AUC are considered as the golden standard for determining how a predictor (blastomere division time) is capable of distinguishing between classes (slow vs. fast). Moreover, we also developed cut-points for further blastomere divisions (second, third, and fourth) and discovered that the speed of the first division is the best predictor for blastocyst development.

Differential expression of mRNAs and miRNAs in the sperm of bulls of contrasting fertility

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Keywords: bull, sperm, non-coding RNAs

Bulls used in artificial insemination with apparently normal semen, as assessed under the microscope, can vary significantly in their field fertility. A range of more advanced flow cytometric assessments of i*n vitro* sperm function have failed to reliably predict the field fertility of bulls. At a molecular level, it is known that the sperm transcriptome contains a rich population of messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs), such as microRNAs (miRNAs). Together, these various transcripts are fundamental for sperm function and for successful fertilisation and embryo development. The objective of this study was to characterize the transcriptomic profile of sperm from high (HF) and low fertility (LF) bulls at the mRNA and miRNA level, in order to identify differentially expressed transcripts with potential as novel markers of fertility. Holstein Friesian bulls were assigned to either the HF or LF group (n=10 per group) based on adjusted fertility scores calculated from a record of at least 500 inseminations (+4.1±0.15% for HF and -7.6±1.50% for LF; mean of 0%). Total RNA was extracted from a pool of frozen-thawed semen straws from 3 ejaculates per bull. Absence of contamination with gDNA and RNAs originating from somatic cells was confirmed by RT-PCR. Transcriptomic profiles were obtained by performing mRNA-seq and miRNA-seq, which generated data from an average of 67.4 and 14.8 million reads, respectively. Six mRNAs and 13 miRNAs, respectively, were found to be differentially expressed between HF and LF bulls. Notably, the highly abundant protamine 1 (PRM1) mRNA had a higher expression in LF than HF bulls ($P<0.05$). As PRM1 is involved in sperm chromatin condensation during spermatogenesis, this could suggest that LF bulls exhibit an abnormal sperm chromatin structure. The gene pathways targeted by the 13 differentially expressed miRNAs were related to embryonic development and gene expression regulation, suggesting that, collectively, these miRNAs may have an impact on early embryo development. This study has identified potential biomarkers that could be used for improving semen quality assessment and predicting bull fertility.

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JUNO coated beads to discriminate sperm from high or low fertility bulls

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Keywords: JUNO, sperm binding, cattle

The molecular mechanisms involved in gamete interaction should be explored to develop new consistent methods to predict sperm fertility. Our knowledge of this process at the molecular level is relatively poor, particularly in mammals. JUNO is the only oocyte plasma membrane receptor described in mice and humans to be involved in gamete binding. It is believed that it is conserved amongst mammals, since JUNO orthologues have been found in all mammalian genomes sequenced to date (Bianchi et al., Nature 508(7497):483-7, 2014). Thus, we hypothesized that the molecules involved in fertilization could be useful in the evaluation of sperm fertilizing capacity. Therefore, we propose that a sperm-binding assay using beads conjugated with recombinant bovine JUNO (rbJUNO) protein mimicking the egg's shape could discriminate sperm with different fertilizing capacity.

rbJUNO was expressed in Chinese hamster ovary cells and its successful conjugation to the beads was confirmed by SDS-PAGE and western blot probed with commercial antibodies. Two sperm-binding experiments were performed. In Experiment 1 (4 replicates), groups of 50-60 B_{JUNO} and B_{Ctrl} (beads without protein) were co-incubated with bull sperm. In Experiment 2 (3 replicates), sperm from bulls categorized as low (LF) or high (HF) fertility based on field fertility data were used. Moreover, the selection of the bulls was narrowed down based on cleavage rate following IVF, being >80% and <40% for the HF and LF bulls, respectively. 50-60 B_{JUNO} were co-incubated with thawed sperm from 2 HF and 2 LF bulls. Motile sperm in both experiments were selected through a 45/90% gradient (final concentration of 200,000 sperm/mL) and incubated in Fert-TALP for 2 h at 39 ºC, in 5% CO_2 and 20% O_2. Then, beads were fixed with glutaraldehyde (0.5 % v/v) and stained with Hoechst 33342 (0.01 mM). The number of sperm per bead was recorded and results analyzed by one-way ANOVA. A P value <0.05 was considered statistically significant.

rbJUNO exhibited the expected molecular weight (30 kDa) and its conjugation to the beads was time-stable. In Experiment 1, the mean number of sperm bound to beads (S/B) was higher (P<0.001) for B_{JUNO} (13.39 ± 0.71; n=277) than B_{Ctrl} (5.69 ± 0.32; n= 257), confirming that sperm mainly bind to beads coated with its specific receptor and suggesting that the JUNO-IZUMO1 interaction is conserved in cattle. In Experiment 2, (S/B) was higher (P<0.05) in the HF bull group (19.20 ± 0.90; N=310) compared to LF bulls (13.60 ± 0.80; N=288).

In conclusion, these preliminary data suggest that this sperm-binding assay is a valuable tool to investigate the role of proteins involved in gamete interaction and that it is able to discriminate bulls of different fertilizing capacity. Future studies using the proposed model, could help understand the molecular aspects of the sperm-egg binding process in depth.

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In vitro maturation with glutathione ethyl ester improves embryo development potential of vitrified bovine oocytes

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Keywords: oxidative stress, ROS, cryopreservation

Despite the increasing interest and applications of oocyte cryopreservation, fertilization rates and embryo developmental competence of vitrified bovine oocytes are still low. Vitrification may induce oxidative stress in mammalian oocytes, which result from unbalanced amounts of reactive oxygen species and the depletion of ooplasmic levels of glutathione (GSH). Glutathione ethyl ester (GSH-OEt), a cell-permeable GSH donor, has been proved to increase GSH content and improve oocyte maturation rate, spindle morphology and fertilization rate in bovine *in vitro* matured oocytes (Curnow *et al. Reprod. Fertil. Dev*, 2010, **22**, 597–605). We hypothesized that addition of GSH-OEt to the *in vitro* maturation (IVM) medium would increase the cryotolerance of bovine oocytes to vitrification by increasing embryo developmental competence. Viable COC's were *in vitro* matured in: 1) Control: IVM medium: TCM199 + 20% Fetal Calf Serum + 10 ng/mL epidermal growth factor and 50 µg/mL gentamycin (n=381); and 2) GSH-OEt: IVM medium supplemented with 5mM of GSH-OEt (n=307). After 22 h of IVM, half of the oocytes from each group were vitrified/warmed using the Cryotop method (Morató *et al*. Cryobiology, 2008, **57**, 137–141) and allowed to recover in their respective IVM media for 2 additional hours (VIT (n=136) and VIT GSH-OEt groups (n=134), respectively). After 24 h of IVM, oocytes were inseminated and *in vitro* cultured. Cleavage rate, 16-cell stage and blastocyst yield were assessed at 48 h, 96 h and day 7 (D7) and day 8 (D8) post-insemination, respectively. After checking normal distribution of the data by using Shapiro Wilk test, a lineal Mixed-Effect followed by a pairwise comparison test (Tukey-adjustment) was performed to analyze differences in embryo development ($P<0.05$). Cleavage rate and D7 blastocyst yield were higher ($P<0.05$) for fresh non-vitrified oocytes (Control: 73.1±3.8% and 14.7±1.7%; GSH-OEt: 74.8±3.3% and 20.2±6.5%, respectively) than for vitrified oocytes (VIT: 42.8±6.9% and 4.1±1.3%; VIT GSH-OEt: 45.5±10.2% and 6.6±0.8%, respectively), regardless of GSH-OEt treatment. However, embryos derived from non-vitrified groups or VIT GSH-OEt group exhibited significantly higher percentages of 16-cell stage embryos and D8 blastocysts (Control: 53.7±8.8% and 23.1±5.2%; GSH-OEt: 45.6±2% and 28.6±8.1%; VIT GSH-OEt: 37.5±2.4% and 13.8±3.2%, respectively) when compared to vitrified oocytes (25.5±4.1% and 5.3±0.6%, respectively). Similar D8 blastocyst hatching ability was observed among non-vitrified oocytes (27.1±14.7% and 22.7±5.4%) and oocytes vitrified after IVM with GSH-OEt (9.5±9.5%) while none of the D8 blastocysts derived from vitrified oocytes was able to hatch. In conclusion, supplementation with GSH-OEt during IVM improved embryo development of vitrified-warmed bovine oocytes, probably by protecting the oocytes from the oxidative stress induced by the vitrification.

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Effect of equilibration time on gene expression of vitrified/warmed in vitro produced bovine embryos using the VitTrans device

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Keywords: mRNA, apoptosis, oxidative stress

For the successful application of vitrification technology to field conditions, the procedures used for warming and transfer of the cryopreserved *in vitro* produced (IVP) bovine embryos should be as simple as possible. The device VitTrans, designed by our group, enables warming/dilution of IVP embryos and their transfer directly to recipient females in field conditions (Morató and Mogas, Cryobiology, 68, 288, 2014). During vitrification, the exposure time of the embryo to the vitrification solution is commonly considered to be critical, and should be strictly controlled within 1 min. However, the exposure time to the equilibration solution is flexible, ranged from 2 min to 15 min. This study aimed to examine the effect of different equilibration times on gene expression of bovine blastocysts vitrified/warmed using the VitTrans device. IVP day 7 expanded blastocysts were exposed to equilibration solution (7.5% ethylene glycol (EG) + 7.5% dimethyl-sulfoxide (DMSO) in TCM199 + 20% Foetal Calf Serum) for 3 min (Short equilibration: SE) (n=55) or 12 min (Long equilibration: LE) (n=51). Blastocysts were then transferred to vitrification solution (15% EG + 15% DMSO + 0.5M sucrose in TCM199 + 20% Foetal Calf Serum), loaded onto the VitTrans device and plunged into liquid nitrogen. The entire process from embryo immersion in the vitrification solution to plunging was completed within 1 min. Fresh non-vitrified blastocysts (n = 56) were set as a control group. Warming was performed by injecting 0,3 mL of warming solution (0.5 M sucrose in TCM199 + 20% Foetal Calf Serum) at 45°C into the inner channel of the device. Blastocysts were then cultured in SOF medium at 38.5° C in a 5% CO_2, 5% O_2 humidified atmosphere. The relative mRNA abundance for *BAX, BCL-2, SOD1, AQP3, CX43* and *IFNτ* genes of vitrified/warmed viable blastocysts was assessed at 24 h post-warming using RT-PCR. Gene expression was analysed by Kruskall-Wallis test and followed by two-way ANOVA. Significance was set at $P \leq 0.05$. Analysis for gene expression revealed no differences in *BAX, AQP3, CX43* and *IFNτ* genes between blastocysts vitrified after SE or LE, whereas significantly higher abundance of *BCL-2* and *SOD1* transcripts was observed in blastocysts vitrified after SE when compared to blastocysts exposed to LE. A clear trend (p=0.07) towards higher *CX43* expression was present in blastocysts exposed to SE when compared to LE group. To conclude, this study showed that embryos exposed to equilibration solution for 3 min previously to vitrification/warming using the VitTrans methodology regulated relative expression level of anti-apoptotic (*BCL-2*) and antioxidant enzyme (*SOD1*) genes, which may be indicative of a better embryo competence. Further studies are warranted to determine if these observations are related with a higher pregnancy rate.

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Health status and blood biochemistry of calves born from cryopreserved and fresh IVP embryos

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Keywords: Cryopreservation, calf health, blood biochemistry

Reproductive techniques can lead to developmental abnormality and phenotypic changes in the offspring (Guo XY et al, Fertil Steril, 2017). In cattle, changes in calf phenotype due to embryo vitrification/warming (V/W) and freezing/thawing (F/T) are not well known. In this study we compared basic blood parameters and health of calves born from embryos transferred after V/W and F/T vs. fresh.

Abattoir oocytes were matured and fertilized in vitro with N=4 Asturiana de los Valles (AV) and N=3 Holstein bulls. Embryos were cultured in SOFaaci-BSA (with or w/o 0.1% FCS) from Day-0 to Day-6, and then w/o protein. Expanded blastocysts (Day-7; N=112) were singly transferred to recipient heifers (Holstein, AV and their crosses). Calves born were clinically examined and blood samples were taken before and 1 to 4h after colostrum intake. Blood was analysed in situ in a Vetscan i-STAT One analyser (Scil Animal Care, Madrid, Spain; CG4+ and CHEM8+ cartridges). Data were analysed by parametric (GLM; $P<0.05$) and non-parametric (Krustal-Wallis) tests ($P<0.05$), with REGWQ as a post-hoc test. Calves born after F/T (N=22), V/W (N=14) and from fresh (N=12) embryos did not differ in birth weight (39.4±2.3, 39.8±2.4 and 40.9±2.5 kg, respectively from now on) and gestation length (283.1±1.6, 284.5±1.2 and 283.0±1.7 days). Within F/T, V/W and fresh embryos, respectively, capillary refill time (CRT; 3.63±0.16, 3.22±0.17 and 2.51±0.19 s), rectal temperature (39±0.11, 38.9±0.12 and 38.3°C±0.12) and creatinine (3.8±0.21, 3.94±0.23 and 2.7±0.26 mg/dL) were higher ($P<0.0004$) after V/W and F/T. Partial pressure of CO_2 (PCO_2; 50.29±1.38, 53.21±1.47 and 56.6±1.68 mmHg) decreased ($P<0.0151$) after F/T, and packed cell volume (PCV; 23.01±1.07, 28.71±1.25 and 25.62±1.33 %PCV) and hemoglobin (g/dL) were higher ($P<0.0002$) after V/W. Colostrum intake decreased CRT ($P<0.001$), heart rate (beats/min; $P<0.0282$), pCO_2 ($P<0.0001$), creatinine ($P<0.0125$), PCV ($P<0.0036$) and hemoglobin ($P<0.0034$), while pH ($P<0.0011$) and base excess (mmol/L; $P<0.0329$) increased. All differences were subtle, as all parameters analyzed were comprised within healthy ranges. Further metabolomics studies are in course, in parallel to identification of putative epigenetic changes due to cryopreservation.

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The transcriptomic landscape of the rabbit oviduct through the pre-implantation embryo development

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Keywords: oviduct, transcriptomics, rabbit

The mammalian oviduct is a specialized tubular structure surrounding the ovaries and connected with the uterus in which pre-implantational embryo development from fertilization up to the early blastocyst stage takes place. The understanding of the crosstalk between gametes or embryos and the oviduct is a challenge which we studied *in vivo* by the means of the omics in a polyovulatory and induced ovulator species such as the rabbit (*Oryctolagus cuniculus*). Twelve sexually mature New Zealand rabbit does received 0.02 mg Gonadorelin (im; Fertagyl, Merck., Kenilworth, USA) at insemination time (t=0 h) to induce ovulation. Isthmic tissue was collected after euthanasia at 10 h (pre-ovulatory; n=3), 20 h (post-ovulatory/zygote; n=3), 56 h (morula; n=3) and 88 h (blastocyst; n=3) post-insemination. RNA was extracted and samples with an RNA integrity number > 7 were analyzed by microarray GeneAtlas System (chip RabbitGene 1.1 ST, with 496321 probes covering 23282 genes, Affymetrix). Data analysis was done with R (v3.6), raw intensities were background corrected and RMA normalized. Differentially expressed genes (DEGs) were defined as those with a fold change > |2| and false discovery rate (FDR) < 0.05 obtained through a variance analysis and FDR adjustment with RankProd. All DEGs between the six possible comparisons were organized in clusters with SOTA (Self Organized Tree Algorithm). Biological meaning was assessed with Gene Ontology enrichment with DAVID (v6.8). The transition between the pre- and post-ovulatory stages involves 96 DEGs, the comparison between the post-ovulatory and the morula stage involves 122 DEGs, and the difference between the morula and blastocyst stages involves 48 DEGs. Interestingly, the transitions between the four stages involve meaningful changes in the expression of genes coding for secreted and exosomal proteins which could influence the local environment where embryos develop. Between the pre and post-ovulatory stages, 14 of these genes are downregulated and 10 upregulated. In the case of post-ovulatory to morula transition, 35 genes are downregulated and 24 upregulated. Furthermore, between the morula and blastocyst stages, 9 genes are downregulated. Genes involved in the extracellular matrix organization (e.g. collagens, and metalloproteases such as MMP7) account for most of these secreted protein coding genes downregulated between the pre-ovulatory to morula transition. Moreover, other important processes are associated with the zygote-morula transition: genes coding proteins involved in cell adhesion and lysil oxidases are downregulated, while chemoattractant protein coding genes are upregulated. To our knowledge, this is the first *in vivo* study of the transcriptomics of the oviduct in the presence of embryos in several stages of development. The findings reveal a series of changes in the transcriptomics of the oviduct which could be related to the communication of this organ with the pre-implantational embryo.

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