Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Secretome of follicular fluid influences cytokine uptake by equine cumulus-oocyte complexes when added during in vitro maturation

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Keywords: cytokine, oocyte, equine

In vitro matured equine oocytes exhibit poor developmental competence compared to their *in vivo* counterparts, being in part related to a suboptimal composition of commercial maturation media (Fernández-Hernández, P.Animals; 10(5):883.2020). Cytokines present in follicular fluid (FF) play a role in ovulation and oocyte maturation (Liu, X. Front. Cell Dev. Biol; 8:578.2020). Our aim was to assess the presence of cytokines in the secretome obtained from mare preovulatory FF and its effect on equine oocyte *in vitro* maturation (IVM). When a preovulatory follicle was detected, 3000 IU of hCG were administered IV and 32 hours later FF was retrieved by flank aspiration (4 mares) as previously described (Hinrichs, K. Theriogenology; 34(1):107-112.1990). 3 ml of FF from each mare was diluted 1:1 individually in sterile PBS and centrifuged (4000 g,1 h at 4°C) using a 10K Amicon® Ultra-15 Centrifugal Filter Unit. The protein concentration of the retrieved secretome was measured and aliquots were kept at -80°C. Cumulus-oocyte complexes (COCs) were recovered from five mares in eight ovum pick-up sessions and matured in TCM-199 with 10% FBS, FSH (5 mIU/ml) in 5% CO_2/95% air atmosphere at 38.2°C and 100% humidity for 28 hours. COCs were matured (20 µl of IVM medium/COC) in the absence (CTR) or presence of secretome (pooled from two mares) at 40 µg/ml (S40) (Marinaro, F. Biology of Reproduction;100(5):1180–1192.2019). IVM medium was recovered before (pre-IVM) and after maturation (post-IVM) and kept at -80°C. Sixteen cytokines were measured using a MILLIPLEX MAP Equine Cytokine/Chemokine Magnetic Bead in the secretome from individual mares (n=4) and in IVM medium (n=5). Normal distribution was ensured using a Shapiro Wilk test and a repeated measures t-test was used to compare each cytokine in pre-IVM and post-IVM (*p < .05); data are presented as the mean ± standard error of the mean (pg/ml). Only FGF, Eotaxin, IP10 and RANTES were detected in secretome, the remaining cytokines laid below the detection range. Secretome cytokine concentrations (pg/ml) were: FGF (78.8±1.7); Eotaxin(49.6±25.2); IP-10(173.1±36.3) and RANTES(1.1± .3). The cytokine concentration pre-IVM vs. post-IVM was: CTR group, FGF (36.9±2.9 vs 37.4± 3.7); Eotaxin (37.4± 3.7 vs 54.2±12.3) and RANTES (1.4± .03 vs2.0± .9); S40 group, FGF (37.4± 3.7vs17.8± 5.6)*, Eotaxin (72.7± 6.3 vs41.9±10.8)*, RANTES (1.3± .06 vs 1.1± .06). FGF and Eotaxin concentrations dropped significantly in the S40 treatment after IVM compared to control (p<0.05). Different cytokines were detected in the secretome from preovulatory FF and the addition at 40 µg/ml during IVM seemed to induce uptake of FGF and Eotaxin by COCs. More studies are required to elucidate the role of these cytokines in oocyte maturation and if secretome addition enhances equine oocyte developmental competence.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Cumulus cell gene expression identifies key mechanisms influencing porcine oocyte developmental competence

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Keywords: cumulus cells, L-carnitine, FAO

Cumulus cells (CCs) can transfer metabolites and small molecules to the oocyte and have pivotal roles during oocyte growth and maturation through supporting e.g. metabolic processes and the capacity to regulate oxidative stress. The aim of this study was to identify key mechanisms within metabolism influencing porcine oocyte developmental competence and distinguish genes whose expression in CCs could predict oocyte quality. Oocytes collected from prepubertal gilts and cycling sows were used to compare oocytes of low and high developmental competence (Silva et al, Molecular Reproduction and Development., 90:323, 2023). Random gilt and sow ovaries were collected after slaughter and follicles with a diameter of 2-6 mm were aspirated. For both gilts and sows, immature cumulus-oocyte complexes (COCs) were randomly placed into the immature or *in vitro* maturation (IVM) groups, where total RNA from CCs was extracted either directly following aspiration or after 44 h IVM in porcine oocyte medium (POM) (6% CO_2, 38.8 °C). For all groups, CCs from triplicate pools of 50-60 COCs were used for RNA extraction and downstream analyses. RT-qPCR was performed employing TaqMan hydrolysis probes (Applied Biosystems, Foster City, California) and relative gene expression was calculated for in total 11 genes by the ΔCq method with efficiency correction after normalisation against *ACTB*, verified as the most stable reference gene by NormFinder. Normally distributed data were analysed using two sample t-test assuming unequal variance. Relative to CCs from sows, gilts showed a 14.8 fold increase (P=0.026) of *BBOX1* transcripts responsible for the last step of the L-carnitine biosynthesis pathway, while transcripts encoding CPT2, an indicator of the rate of fatty acid oxidation (FAO), exhibited a level of 0.48 (P=0.037) before IVM. After maturation there were no significant differences between gilts and sows in the genes involved in FAO, while transcripts encoding key enzymes of the pentose phosphate pathway (PPP) (G6PD), and glycolysis (ALDOA), were present in gilt CCs at levels of 0.64 (P=0.044) and 0.49 (P=0.070), respectively, relative to those in sows. The results suggest that FAO was downregulated in gilt CCs at the time of aspiration, caused at least in part by an insufficiency in L-carnitine as indicated by elevated levels of *BBOX1* transcripts. After IVM there appears to be sufficient L-carnitine to sustain a similar level of FAO in gilt CCs to that seen in sows. This study implies porcine COCs have the molecular machinery to modulate L-carnitine synthesis, and additional supplementation of the media with L-carnitine should be exercised with caution as excessive concentrations could yield adverse effects. Gilt COCs might not have adequate stores of ATP and be less competent in responding to oxidative stress compared to sow COCs at the end of maturation, as demonstrated through delayed FAO and downregulation of glycolysis and the PPP. Higher expression of *CPT2* in CCs before maturation and of both *G6PD* and *ALDOA* after maturation are potential markers of oocyte quality.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Supplementation of Mito-TEMPO during in vitro maturation and its effects on development and cryogenic viability of bovine embryos

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Keywords: Embryo, Antioxidants, Vitrification

Despite many efforts, bovine embryos produced in vitro are still characterised by lower development rates, lower pregnancy rates and reduced cryogenic viability compared to ex vivo embryos. It has already been shown that the addition of mitochondrially active antioxidants to the maturation medium has a positive effect on ROS levels, development rates and cryogenic fitness of bovine embryos. Therefore, the aim of the present study was to investigate a possible effect of supplementing the maturation medium with Mito-TEMPO (Sigma, St. Louis, USA) on early embryonic development and cryogenic viability of bovine blastocysts. For the study, cumulus-ocyte complexes (COC) were obtained from the ovaries of slaughtered cows by slicing. Subsequent maturation was performed in TCM199 medium supplemented with (treatment) or without (control) 1 µM Mito-TEMPO for 22 hours in 4-well plates (NUNC, 400 µl, no oil overlay) with 50-70 COC per well (39°C, 5% CO2, 20% O2). For fertilisation, frozen semen was purified with SpermFilters® (IVF Bioscience, Falmouth, UK) and added to mature oocytes at a concentration of 2×106 cells/ml (Fert.-TALP. medium, 400 µl, NUNC). At 19 hours post fertilisation, COC were denuded by vortexing and then cultured for 8 days in synthetic oviduct fluid (SOFaa + 0.3% BSA, 400 µl, NUNC, oil overlay, 39°C, 5% O2, 5% CO2). On day 7 of culture, intracellular reactive oxygen species (ROS) levels were quantified in blastocysts from both experimental groups using fluorescence staining with 5 µM DCFDA (Sigma, St. Louis, USA) and comparative analysis was performed using an image analysis tool (ImageJ). In addition, day 7 blastocysts from both experimental groups were individually vitrified using BO-VitriCool™ media (IVF Bioscience) and the Cryotop® vitrification system (Kitazato, Shizuoka, Japan). Warming (BO-VitriWarm™ media, IVF Bioscience) of the vitrified blastocysts was followed by another post-warming culture for 72 hours to determine viability, expansion and hatching rates. GraphPad Prism software (version 9.3.1., Boston, USA) was used for all analyses and ANOVA was used to highlight differences between the two groups. The level of statistical significance was set at $p < 0.05$. The results of our study showed no significant effect of Mito-TEMPO supplementation during maturation on early embryonic development measured as blastocyst and hatching rate during in vitro culture. The same was observed for the measured ROS levels of blastocysts in both experimental groups and for the viability and expansion rate after warming. In contrast, the Mito-TEMPO supplemented group had a significantly ($p < 0.05$) higher hatching rate at 24, 32, 48, 56 and 72 hours after warming than the control group. These results confirm our hypothesis that the antioxidant Mito-TEMPO has a beneficial effect on mitochondrial properties, resulting in a reduction of cryo-induced damage after vitrification.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Sex-specific contours of mitochondrial respiration characteristics at the pre-elongation stage in individual bovine embryos

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Keywords: embryo metabolism, extracellular FLUX-analyzer, embryo sex

Sexual differences related to metabolism are known in in vitro produced (IVP) bovine embryos (Gómez et al., Theriogenology, 114, 180-184, 2018). Long-term effects of IVP on early preimplantive embryos or advanced developmental stages out of culture are still unclear. In view of partielly occuring reduced female calf ratio after transfer of IVP-derived blastocysts to recipients, we asked whether sex-related differences in metabolism still exist in pre-elongation stage embryos (D13) after transfer to recipients at expanded blastocyst stage (D7). Therefore, the aim of the present study was to compare mitochondrial respiration characteristics of male and female IVP-derived bovine embryos at day 7 (D7) and 6 days after transfer to recipients at day 13 (D13). Therefore, male and female expanded bovine IVP-derived blastocysts were produced by routine IVP procedures (IVM: modified TCM (Sigma); IVF: modified Fert-TALP (Parrish,Theriogenology, 81, 67-73, 2014), IVC: SOFaa (Holm et al., Theriogenology, 52,683-700, 1999) + 0.6% fatty acid-free BSA, 5% CO2, 5% O2, 38.8° C) using male and female sex-sorted semen from the same bull, respectively, for in vitro fertilisation. A subset of these male and female IVP-derived D7 embryos were transferred to synchronised recipients followed by embryo flushing at day 13 of development. Energy metabolism analysis was performed on pools of D7 embryos (male vs. female embryos, pools of 10 embryos, 6 replicates) and individual D13 pre-elongation stage embryos comparing male (n=26) and female (n=28) embryos using an extracellular FLUX analyser (Seahorse XFp). This was supported by the use of a custom designed embryo cage system and the Cell-Mito Stress Test Kit (Agilent) containing three serial injections (Oligomycin 0.5µM; FCCP 4.0µM; Rotenone/AA 0.25µM), which allows the investigation of specific mitochondrial characteristics (Wave Software, Agilent). The results of our study showed that female embryos used a significantly higher (unpaired t-Test, $p<0.05$) proportion of the total oxygen consumed for mitochondrial respiration compared to their male counterparts at day 7 (87.5% vs. 70.5%) as well as at day 13 of development (54.3% vs. 35.71%). Consistently, female embryos show significantly higher ($p<0.05$) maximal respiration rates (1.3-fold at day 7 and 1.7-fold at day 13) compared to male embryos during the Cell-Mito stress test. The total amount of oxygen consumed for ATP-linked respiration was significantly higher in female embryos at both day 7 (1.3-fold) and day 13 (2.4-fold). This was accompanied by a higher mitochondrial coupling efficiency in male embryos compared to female embryos at day 7 (74.5% vs. 64.4%). In conclusion, our study revealed sex-specific implications for several mitochondrial respiration characteristics in IVP-derived bovine D7 embryos. Strikingly, both maximal respiration and ATP-linked respiration remain at significantly higher levels in female embryos for at least 6 days after transfer to recipients, even into the pre-elongation stage. As it is currently unclear how long these differences between male and female embryos persist and whether they are physiological or due to inadequate culture conditions, further studies are required.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Description of a new quantitative method for the evaluation of mitochondrial distribution pattern in equine mature oocytes

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Keywords: Mitochondria, oocyte, equine.

In immature equine oocytes mitochondria are homogeneously distributed throughout the oolema (Ambruosi, International Journal of Endocrinology, 6, 1-5, 2011). In equine oocytes undergoing maturation, mitochondria migrate and result in the so known "aggregated patten" in which mitochondria are not evenly distributed within the cytoplasm being associated with cytoplasmic maturation (Alm, J Reprod Fertil Suppl 56, 473–482, 2000). However, no quantitative methods have been optimized to evaluate MDP in equine oocytes and only qualitative methods are currently used (Torner, Reprod Dom Anim, 42,176-183, 2007). Our aim was to design a quantitative method to evaluate the MDP based on the ratio of peripheral to central fluorescence intensity of the oocyte using confocal fluorescence. Equine cumulus-oocytes complexes (COCs, n = 56) were retrieved by ovum pick up (OPU). The oocytes were subjected to *in vitro* maturation (IVM) in TCM-199 medium, with 10% FBS and 5 mU/ml FSH for 26-28 hours in 5% CO2/95% air atmosphere at 38.2 °C. Oocytes were denuded and stained with MitoTracker™ Red CMXRosat 50 nM in TCM-199 with Hank´s and 10% FBS (v/v) for 15 minutes at 38 °C in the dark. Then, the oocytes were fixed with 4% formaldehyde in PBS + 0.01% PVA (w/v) and kept in the dark at 4 °C. The oocytes were also counterstained with 2.5 µg/ml of Hoechst 33342 for 10 minutes at 37 °C, mounted on slides and visualized; only metaphase II (MII) oocytes were considered. Fluorescence measurements were run on an inverted epifluorescence microscope (Axio Observer 7, Carl Zeiss, Germany) using a LD LCI Plan-Apochromat 25×/0.8 multi- immersion objective at a zoom of 1.3× with image acquisition (Axiocam 712 mono, Carl Zeiss, Germany) and analysis system for video-microscopy (ZEN Blue 3.4, Zeiss). Image processing and qualitative and quantitative analyses were performed using Fiji-ImageJ software. Firstly, based on qualitative analysis, MII oocytes were divided into two groups according to the distribution of mitochondrial pattern: homogeneous (HoD, n = 17) and heterogenous (HeD, n = 39). For the quantitative analysis, the background was subtracted and a 1-pixel line along the diameter was drawn; the intensity profile was calculated, and a fitting curve was generated. Next, fluorescence intensities were normalized and ratios of peripheral to central fluorescence intensity was calculated. Statistical analysis was performed using the Shapiro-Wilk test combined with t- test; $p < 0.05$. MDP ratio was (mean (arbitrary units) ± standard error of the mean): 0.8 ± 0.02 for HoD and 0.3 ± 0.02 for HeD; significant differences were observed between groups ($p < 0.001$). We describe a new approach to quantify mitochondrial distribution pattern in mature equine oocytes.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

High concentrations of lipopolysaccharides are associated with decreased progesterone concentrations in equine follicular fluid

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Keywords: follicular fluid LPS, steroids and cytokines, mares

Accumulation of lipopolysaccharides (LPS) in follicular fluid (FF) impairs steroid production and oocyte developmental competence in cows and mice. This has not been investigated previously in mares. This study aimed to assess the FF concentrations of LPS and their association with steroid concentrations (E_2, estradiol and P_4, progesterone) and inflammatory response (IL-6, interleukin-6 and TNF-α, tumor necrosis factor-alpha) in FF of mares. At the slaughterhouse, FF and the follicle wall of the largest viable follicle (>30 mm in diameter) were collected from nonpregnant mares. To assess follicle viability, follicle walls were fixed (Bouin's solution) and stained with hematoxylin and eosin. Viable follicles (*n*= 16) with no degenerative changes were selected for further analysis. For LPS detection in FF, a colorimetric assay (Pierce™ Chromogenic Endotoxin Quant Kit, ThermoFisher Scientific, USA) was used. Immunoassays were used to measure the FF concentrations of E_2 (DRG, Germany) and P_4 (Progesterone III, Roche Diagnostics, Germany). Concentrations of IL-6 and TNF-α in FF were measured using ELISA Nori® kits (Genorise Scientific, USA). Spearman correlation coefficients between all measured variables in FF were calculated. Independent samples t-test was used to compare means of E_2 and P_4 concentrations in mares with high (≥LPS median concentration; 7.18 EU/mL, EU= endotoxin unit) and low (<LPS median concentration; 7.18 EU/mL) LPS values. A P value <0.05 was considered significant. The minimum and maximum concentrations were 5.21-12.08 EU/mL for LPS, 0.08-16.66 µg/mL for E_2, 28.10-79.50 ng/mL for P_4, 101.87-1080.71 pg/mL for IL-6, and 54.02-689.46 pg/mL for TNF-α. There were negative correlations between LPS concentrations and the concentrations of P_4 (r=-0.679, P=0.005), IL-6 (r=-0.556, P=0.025), and TNF-α (r=-0.637, P=0.008). Taken together, LPS is detectable in FF of mares and is negatively associated with progesterone concentrations in FF. There is no clear explanation regarding the negative associations between LPS concentrations and the concentrations of pro-inflammatory cytokines in FF. It is well-known that the IL-6 and TNF-α in FF are not only produced locally by follicular cells, but also transported from the systemic circulation. The crosstalk between maternal health related to Gram-negative bacterial infection and inflammation may alter the oocyte's microenvironment, which may affect oocyte quality. Further studies are ongoing to investigate the effect of LPS on the oocyte's developmental competence and subsequent embryo quality in mares.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Derived-proteome of extracellular vesicles from uterine fluid is modified depending on seminal plasma fraction during early pregnancy

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Keywords: extracellular vesicles, proteomics, seminal plasma, uterine fluid.

In porcine artificial insemination (AI) seminal doses are prepared using only the rich fraction of the ejaculate. However, this method is controversial, as seminal plasma (SP) promotes embryo development via specific signaling pathways within the female genital tract. Extracellular vesicles (EVs) participate in these pathways establishing intercellular communication by transferring their contents (RNA, proteins and lipids) to target cells. We hypothesized that SP from ejaculate fractions differentially affect the protein content of EVs in uterine fluid (UF). Therefore, this study aimed to characterize the uterine EVs (uEVs) proteome of sows, after being inseminated and prior to embryo implantation with different cumulative fractions of the boar ejaculate. A total of 6 fertile boars (Pietrain) were used and 3 different seminal doses were prepared ($30x10^6$ spermatozoa/60 ml): 1) F1=sperm rich fraction, 2) F2=F1+intermediate fraction and 3) F3=F2+poor fraction. Subsequently, 15 crossbred sows (Large-White x Danbred) with similar parity and body condition were inseminated with seminal doses (5 per group) using post-cervical AI method. Five non- inseminated sows, were used as a control group. After 6 days of AI, all sows were sacrificed, their genital tracts were dissected and UF was extracted by flushing the uterus with PBS. Embryos were isolated from the flushes under stereomicroscope to ensure pregnancy. Then, uEVs were isolated by ultracentrifugation and characterized by TEM. Size and protein concentration of uEVs were also analyzed by DLS (range from 142.40±30.53 to 202.92±18.20 nm) and Bradford (range from 0.57±0.27 to 3.35±0.97 µg/µl). Finally, 20 samples were analyzed by HPLC-MS/MS to study their proteome. Bioinformatic analysis was performed by GO analysis and most statistically significant (FDR<5%) GO terms were checked with REVIGO to discard redundancy. The classification of the proteins was assessed using DAVID. A total of 142 proteins were identified in uEVs, of which 16 were common to all groups. Thirty proteins were detected exclusively in uEVs from non-inseminated sows. Whereas 31 proteins were detected only in uEVs from sows inseminated by F1, 6 proteins were detected exclusively in F2 group and other 6 proteins were only detected in F3 group. These proteins exclusive to each group were shown to be involved in protection against oxidative stress, immune tolerance, embryogenesis, blastocyst attachment and angiogenesis during pregnancy. In conclusion, we revealed protein cargo of uEVs depending on the boar ejaculate fraction, however, we must consider that uEVs during pregnancy can be of embryonic and endometrial origin and the presence of embryos could influence uEVs proteome. Therefore, further validation is needed to ensure the presence of specific proteins in uEVs from different experimental groups.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Effects of a polycaprolactone and polyethylene glycol diacrylate, 3-D printed scaffolds, on bovine embryo development in vitro

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Keywords: polycaprolactone (PCL), polyethylene glycol diacrylate (PEGDA), bovine, embryo development, bovine embryo assay

Despite the improvements in Assisted Reproductive Technologies (ART), in vitro environment is still far from physiological, causing low blastocyst quality and impaired epigenetic reprogramming. In the last years, 3D printing has been implemented in several research areas and can be used to create an IVF device that mimics the oviduct, the organ where fertilization takes place. However, before creating the device, the feasibility of the materials to support IVF needs to be tested. We aimed to evaluate the suitability of scaffolds printed with polycaprolactone (PCL) (CELLINK, Gothenburg, Sweden) and polyethylene glycol diacrylate 500 (PEGDA500) (CELLINK, Gothenburg) to support IVF and further embryo development. We carried out a bovine embryo assay (N=3 replicates) and the following experimental groups were settled: Rinse PCL (N=148) and rinse PEGDA500 (N=143), where the IVF was performed in IVF medium conditioned by the scaffolds during 24h; Scaffold PCL (N=144) and scaffold PEGDA500 (N=131), where the IVF was performed in the different scaffolds, and control group (CTRL) (N=259), where no scaffold nor conditioned medium were used. Prior IVF, both materials were sterilized in 70% ethanol for 5 min, washed in PBS for 30 min and 24h in IVF medium. For IVF, in vitro matured oocytes were incubated during 22h in Fert-TALP medium (Parrish et al, Theriogenology, 25, 591-600, 1986) with frozen-thawed bull sperm ($1x10^6$spz/ml) selected by Bovipure gradient (Nicadon, Sweden). After IVF, zygotes were washed and cultured in SOF medium supplemented with 0.3% BSA (w/v) covered with paraffin oil (NidOil, Nicadon) on a normal petri dish. To assess IVF outcomes, blastocyst rate at day 7 (BR7) and 8 (BR8) were registered. At day 8, blastocysts were fixed and stained with Hoechst 33342 to assess the cell number per embryo under fluorescence microscopy. Data were analyzed by One-Way ANOVA. Differences were considered significant when $p<0.05$. We found significant differences in blastocyst rate (expressed as BR7±SD, BR8±SD) between CTRL (20±4%, 21±0%) and rinse PEGDA500 (4±3%, 7±5%), and between CTRL and scaffold PEGDA500 (7±6%,10±7%), while we did not find significant differences between CTRL and rinse PCL (18±4%,22±6%) or scaffold PCL (25±10%,29±8%). Results showed similar cell numbers, being 100.65±39.49 for CTRL, 116.59±37.14 for rinse PCL, 83.96±32.87 for scaffold PCL, 103.11±30.28 for rinse PEGDA500 and 77.09±23.08 for scaffold PEGDA500. These data suggest that PEGDA500 has a detrimental effect on bovine embryo development since it promotes a lower blastocyst rate at day 7 and 8. This is an unexpected effect since PEGDA500 has been proposed as a nice candidate to perform studies for embryogenesis and organogenesis (Hribar et al, Lab Chip, 15, 2412-2418, 2015), and could be due to the different cell types or to some leaked compound used to print and stabilize the scaffold. On the other hand, PCL shows great biocompatibility since embryo development was not impaired by the presence of the scaffold during IVF. In conclusion, the current data suggest that PCL could be used to construct an IVF device.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

The effect of iloprost on energy status of the in vitro produced bovine blastocysts

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Keywords: bovine, embryos

Oviduct fluid is composed of substances and co-factors that support cleavage and early development *in vivo*. Imitating the oviductal environment has been the challenge for making *in vitro* culture (IVC) successful, but there is still no general consensus defining IVC conditions for *in vitro*-produced (IVP) embryos. None of the attempts to improve this conditions have produced consistent bovine blastocyst production rates above 40-50%. Prostacyclin (PGI2) is a lipid molecule synthesised primarily in endothelial cells as well as oviductal endothelial cells. For this reason, several studies have tested the role of PGI2 in pre-preimplantation period by supplementing the IVC medium with iloprost, a PGI2 analogue. The results of the study performed on pigs demonstrate that the use of iloprost during culture improves the *in vitro* development of porcine embryos. The authors showed that an analogue of PGI2 plays an important role in meiotic progression in porcine oocytes through the regulation of cAMP/PKA activity (Kim et al., 2010). In cows, iloprost positively affected the development of IVP and somatic cell nuclear transfer (SCNT) embryos cultured *in vitro* by stimulating the cAMP response element-binding protein (CREB)-COX2 signalling pathway (Song et al., 2009). Although, in our previous study we found that, treating cumulus oocytes complexes (COCs) with iloprost improved oocyte quality and maintained their developmental capacity during *in vitro* maturation (IVM) the role of iloprost during IVC remains unknown. Therefore, in the present study, we examined the effects of iloprost on blastocyst developmental rates and quality, as well as mitochondrial function in the *in vitro* produced bovine embryos. Ovaries were collected from mature Holstein cows at a local abattoir and cumulus-oocyte complexes (COCs) were isolated by aspiration from ovarian follicles (n=1308). Following 24h of *in vitro* maturation (IVM; TCM 199 Maturation Medium, 19990/0010, Minitube) COCs were *in vitro* fertilized (IVF; TL sperm capacitation medium, 19990/0020, Minitube) and embryos were *in vitro* cultured (IVC; SOF synthetic oviduct fluid medium, 19990/0040, Minitube) for 7 days. Two groups were established to conduct the study: 1) control, and 2) experimental, embryos exposed to an iloprost (50μM). All methodology of IVP are described in Kowalczyk-Zieba et al., (2020). The analyses were performed using the statistical software GraphPad PRISM 6.0. Iloprost had no direct effect on blastocyst rates on Day 7 (control 134±20.4 vs. experimental 147±22.8, $P>0.05$). However the number of expanded (65±48.5 vs. 84±56.4, $P<0.05$) and hatched (9±6.7 vs. 15±10.1, $P<0.05$) blastocysts was higher in iloprost-treated groups. Embryos treatment with iloprost during *in vitro* production resulted in reduction in intracellular reactive oxygen species levels in blastocysts Day 7 ($P<0.05$). Iloprost impoved mitochondrial membrane potential and active mitochondria, using JC-1 fluorescent reaction and MitoTrckerRed CMXRox, respectively in blastocyst Day 7 ($P<0.05$). Furthermore, supplementation the IVC medium with iloprost influenced on mRNA expression of the genes involved in mitochondrial function (Clpp, GLu1, GPx4 and Polg2) and blastocyst quality markers (OCT4, SOX2, NANOG, PLAC8) using RT-qPCR. In the blastocyst produced with iloprost we found higher mitochondrial DNA copy number ($P<0.05$) analysed by qPCR. In conclusion, our results demonstrate that treating bovine embryos with iloprost may improve blastocyst quality and maintain their developmental capacity during IVC. We thus propose iloprost as a new agent for the prevention of developmental loss of bovine embryos in ARTs.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Stage- and quality grade-dependent expression of developmental competence gene markers in comparison with prostaglandin E2 synthesis in the early- and late-cleaved bovine blastocysts

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Keywords: cow, prostaglandin E2, blastocyst

Prostaglandin (PG) E2 plays a role in oviductal transport of gametes, hatching from the zona pellucida and early embryonic development. The expression profiles of 3 PGE2 synthases were differ between early- and late-cleaved bovine embryos, with higher PTGS2 expression and lower cPGES mRNA level in 16-cell embryos and with higher PTGS2 and cPGES expression in expanded grade C blastocyst from late-cleaved group. The aim of the study was to examine whether the stage- and quality grade-dependent (classified by morphological assessment) expression profile of embryo developmental competence markers is associated with expression of PGE2 synthases in bovine blastocyst from early- and late-cleaved embryos. Ovaries were collected from Holstein cows at a local abattoir and cumulus-oocyte complexes (COCs) were isolated. After 24h of IVM, COCs were fertilized, and according to Lonergan et al. (1999), early-cleaved embryos (good quality) were separated at 30 hpi whereas late-cleaved embryos (bad quality) were isolated at 36 hpi. All IVP methodology and blastocyst classification are described in Boruszewska et al. (2019). For RNA isolation, 5 repetition of 5 embryos/tube from each category of stage and quality within 2 analyzed group were used. The expression of mPGES1, mPGES2, cPGES, OCT4, SOX2, IGF1R, IGF2R, PLAC8 were examined by RT-qPCR. Statistical analyses were conducted using two-way ANOVA (fixed factor: experimental groups; random factor: developmental stage/quality of blastocysts) followed by Tukey's multiple comparison test or a correlation analysis using Pearson correlation coefficient (GraphPad PRISM). We found that PTGS2 mRNA level was higher in hatched and in grade C blastocysts; and cPGES level was higher in early blastocysts, blastocysts, and expanded blastocysts and in grade A, B and C blastocysts from late-cleaved group ($p < 0.05$). The mRNA level of SOX2, OCT4, IGF1R, IGF2R and PLAC8 was higher in early blastocysts in late-cleaved group ($p < 0.05$). The IGF1R and IGF2R mRNA levels were higher in grade A and B blastocysts, OCT4 and PLAC8 mRNA levels were higher in grade B blastocysts, and SOX2 mRNA level was higher in grade B and C blastocysts in late-cleaved group ($p < 0.05$). In early-cleaved embryos, PTGS2 mRNA level correlated positively with mRNA level of all markers in expanded blastocysts from late-cleaved group ($p < 0.05$). The cPGES mRNA level correlated positively with mRNA level of IGF1R, IGF2R, SOX2 and PLAC8 in expanded blastocysts from early-cleaved embryos; and with mRNA level of SOX2, OCT4 and PLAC8 from late-cleaved group ($p < 0.05$). In summary, the mRNA levels of PGE2 synthases and developmental competence genes were affected by the embryonic stage of development and quality classified by morphological assessment. Our research accounts for many correlations between mRNA level of gene markers and PGE2 syntheses (mainly PTGS2 and cPGES), that vary in bovine blastocysts depending on the time of first cleavage.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Sex-specific gene expressions of bovine elongated embryos triggered by the physiological conditions of the recipients

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Keywords: Male, female, elongated, embryo, gene

Sex-biased embryonic losses due to the differential secretion of signalling molecules by male and female embryos in response to the maternal microenvironment are believed to be one of the causes of skewed male to female ratio at birth in cattle and other animals. Therefore, this study was conducted to understand the gene expression patterns in bovine elongated male and female embryos developed in multiparous cows or heifers. For this, *in vitro* produced 4-8 cell stages of male and female embryos were transferred to multiparous cows and heifers. The elongated embryos were then recovered on day 13 of the gestation period and classified according to their origin and sex. Total RNA was isolated from each group of embryos using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). RNA sequencing libraries from 5 elongated embryos per group were prepared using the NEBNext Ultra II RNA library prep kit for Illumina (NEB, Ipswich, MA, USA) and sequenced using Illumina NovaSeq in a 2x150 bp configuration. Adapters were trimmed using Trim Galore (Babraham Bioinformatics) and clean reads were mapped to the bovine reference genome using the bowtie2 alignment tool. Quantitation was performed using the RNA-Seq quantitation pipeline of the Seqmonk tool (Babraham Bioinformatics). Differential expression analysis was done using the EdgeR package (Robinson et al. 2008, Bioinformatics, 26, 139-140) and genes which showed expression differences with p-value < 0.05 and false discovery rate < 0.1 were filtered. Functional enrichment analysis was performed using gprofiler (https://biit.cs.ut.ee/gprofiler/gost). The results indicated that 13948, 13775, 13972 and 13341 genes were expressed in both male and female elongated embryos developed in cows and heifers, respectively. Among these, including *ACTG1, COX1, COX2* and *COX3,* the expression level of 38 genes was highly expressed with > 9000 read counts in all sample groups. These are involved in energy production and metabolism. On the other hand, including *CYP39A1, CYP2R1, CYP27B1, CYBRD, PAG8* and *PAG12,* a total of 197 genes were differentially expressed between the male and female elongated embryos developed in cows. Some of these are involved in steroids and lipid biosynthetic processes. Similarly, including *GSTO1, PRKD1*, *POU2AF1, NOS2,* and *HSD3B1,* a differential expression of 293 genes was detected between the elongated male and female embryos developed in heifers. Some of these genes are involved in organ development, tissue morphogenesis, and female sex differentiation. Therefore, this study indicates that although genes associated with energy production and metabolism were highly expressed in all embryo groups, the cow's maternal environment could induce differential expression of genes that are potentially associated with steroid and lipid biosynthetic processes in male and female embryos, but the heifer's maternal environment could induce the expression of genes associated with sex differentiation and organogenesis or tissue morphogenesis in male and female embryos differently.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

The BMP15 added to IVM medium of prepubertal goat oocytes increases oocyte EGFR expression and cumulus-oocyte communication

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Keywords: goat, BMP15, IVEP

Oocyte competence for embryo development depends on bidirectional communication with cumulus cells (CCs; Gilchrist, Hum Reprod Update, 14(2):159-77, 2008). This is mediated by the transzonal projections (TZP), which are thin cytoplasmatic filaments that project from the CCs and penetrate through the zona pellucida to the oocyte. Oocyte-secreted growth factors (OSFs) such as bone morphogenetic protein 15 (BMP15) induce proliferation and differentiation of CCs and improve embryo development (Sudiman, J Assist Reprod Genet, 31(3):295-306, 2014). OSFs are suggested to promote the epidermal growth factor network, which is associated with oocyte competence (Richani, Hum Reprod Update, 24(1):1-14, 2018). Our aim was to study the effect of adding BMP15 to IVM medium on embryo competence of prepubertal goat oocytes (1-2 months old), the TZP and the epidermal growth factor receptor (EGFR) expression. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and matured in TCM-199 with FSH, LH, estradiol, EGF and cysteamine during 24h at 38.5ºC with 5% CO_2. IVM medium of the experimental group (BMP15 group) was supplemented with 100 ng/ml of BMP15 (R&D systems, USA) . The control group was IVM medium without BMP15. A total of 733 IVM-oocytes (7 replicates) were in vitro fertilized with $4x10^6$ sperm/ml frozen-thawed semen in BO-IVF medium (Bioscience, UK) for 19h. Presumptive zygotes were cultured for 8 days at 38.5ºC with 5% CO_2 and 5% O_2 in BO-IVC medium (Bioscience, UK). A sample of 10 COCs/replicate (3 replicates) were recovered at several time points during IVM (0h, 6h, 12h and 24h) for TZP density assessment. Actin filaments were stained with phalloidin-FITC and TZP density was quantified as phalloidin-FITC average fluorescence intensity in the zona pellucida area. The EGFR protein levels of MII-oocyte were assessed after 24h of IVM. A total of 40 MII-oocytes for BMP15 group and 26 for control group (4 replicates) were quantified by immunofluorescence using an anti-EGFR antibody (Invitrogen, USA). Fluorescence was quantified with ImageJ software. Data were statistically analyzed by two-way ANOVA followed by Tukey's correction. The results of blastocyst development of BMP15 (380 IVF-oocytes) and control (353 IVF-oocytes) groups showed no significant differences (8.8% ± 3.0 and 7.4% ± 2.8, respectively). The TZP density at 6h IVM of BMP15 COCs was higher ($p<0.05$) than control COCs (20.7 ± 1.6 and 11.9 ±1.6 arbitrary units, respectively). There was an increase ($p<0.05$) in the BMP15 group at 6h compared with 0h (14.9 ± 1.2) that was not observed in control group. There were no significant differences between groups at 12h or 24h. The expression of EGFR was higher ($p<0.05$) in MII-oocytes treated with BMP15 than control ones (22.5 ± 0.7 and 18.3 ± 0.8 arbitrary units, respectively). In conclusion, adding BMP15 to IVM medium of oocytes from prepubertal goats promoted EGFR expression and increased TZP density at 6h after follicular recovery, suggesting an enhanced cumulus-oocyte communication. However, it did not affect in vitro development after IVF.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Effect of a biphasic in vitro maturation system with c-type natriuretic peptide and oestrogens on the quality of prepubertal lamb oocytes

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Keywords: IVM, pre-maturation culture phase, lamb

One of the problems presented by oocytes from prepubertal animals in vitro matured is a lack of synchronization in nuclear and cytoplasmic maturation (Kochhar et al., Reprod Dom Anim 37:19-25, 2002). Biphasic maturation with meiotic inhibitors has shown to increase developmental competence of prepubertal goat oocytes (Soto et al., Plos One 23;14(8):e0221663, 2020). The aim of this study was to assess the effect of C-type natriuretic peptide (CNP) and 17ß-estradiol (E2) on lamb oocytes 'quality. Ovaries from lambs (1-5 months old) were recovered at a local slaughterhouse. Cumulus-oocyte complexes (COCs) were collected by ovary slicing and selected using HEPES-buffered (25 mM) TCM-199 medium with the meiotic inhibitor 3-Isobutyl-1-methylxanthine (500µM) and heparin. COCs were cultured in TCM-199 with 200 nM CNP and 10 nM E2 for 6h and then in conventional IVM medium (TCM-199 with FBS, FSH, LH, oestradiol and EGF) for 24 hours (biphasic group) at 38.5ºC with 5% CO2. Oocytes in the control group were cultured in conventional IVM medium for 24 hrs under the same conditions. After 24 hrs (control group) and 30 hrs (biphasic group) of culture, a sample of COCs were denuded and stained for assessing reactive oxygen species (ROS) and glutathione (GSH) levels and with Brilliant Cresyl Blue (BCB) stain for evaluating the growing of oocytes. BCB determines the intracellular activity of G6PDH enzyme which is active in growing oocytes and degrades the dye (BCB-). After BCB staining, all the oocytes classified in BCB+ (fully grown) and BCB- (growing oocytes) were parthenogenically activated (PA) with ionomycin and DMAP and cultured in BO-IVC medium (Bioscience, UK) for 8 days at 38.5ºC with 5% CO2 and 5% O2 .The rates of BCB stained oocytes, oocyte cleavage at 28 hrs and blastocysts at day 8 after PA were statistically analyzed by Chi Square test with Yates correction test. The data from ROS and GSH levels were analyzed using unpaired T-test. The rate of oocytes that had reached their growth phase (BCB+) were higher ($p<0.01$) in biphasic group (91.4%; n=105) than control group (63.5%; n=104), while both ROS (30 oocytes per treatment) and GSH (30 oocytes per treatment) levels were lower ($p<0.01$) in biphasic group than control group. The IVM group showed 1.8 and 1.18 more intensity than biphasic group in ROS and GSH stain, respectively. After PA of 153 oocytes from biphasic group and 163 oocytes from control group, oocyte cleavage rate was higher ($p<0.01$) in biphasic group (52%) compared to control group (35%) although there was no significant difference between both groups ($p=0.1081$) in blastocyst production at day 8 of in vitro embryo culture (12% and 6% of the total oocytes activated from biphasic and control group, respectively). In conclusion, the results from this study show that biphasic maturation increases the quality of oocyte and the rate of embryo produced after parthenogenetic activation in prepubertal lamb, but it does not affect blastocyst production. Further experiments are required to explain these results.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

GATA3 is dispensable for ovine blastocyst formation and first lineage differentiation, but plays a role in epiblast development

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Keywords: ovine, blastocyst, GATA3

The first lineage differentiation in mammalian embryos leads to the formation of the inner cell mass (ICM) and the trophectoderm (TE). In mice, GATA3 drives TE fate and regulates other TE genes such as *Cdx2*, although its ablation does not abrogate blastocyst formation. In bovine, a recent report has described that GATA3 deletion downregulates the core pluripotency factor NANOG. The objective of this study has been to elucidate the role of *GATA3* in ovine embryos by evaluating the developmental potential of *GATA3* KO embryos generated by CRISPR. *In vitro* matured oocytes were microinjected with Cas9 mRNA and a sgRNA against *GATA3* (C+G) or with Cas9 alone as microinjection control (C). Microinjected oocytes were fertilized and cultured *in vitro* up to Day (D) 8 (in SOF medium) or D12 (in N2B27 medium from D6/7), when pictures were taken and embryos were fixed and immunostained to detect GATA3, CDX2 (TE marker), SOX2 and NANOG (ICM/epiblast markers). Embryo genotyping in C+G group was performed by deep sequencing. Blastocyst rate was similar in the group containing *GATA3* KO embryos (C+G) and in the control group (C) (26.3±1.1% vs. 27.2±3.3%, mean ± s.e.m, 5 replicates, t-test; $p>0.05$). In C+G group, 23/55 (41.8%) blastocysts genotyped were *GATA3* KO (containing only frame-shift alleles). GATA3 protein was not detected in KO embryos, which showed normal morphology and expressed CDX2. *GATA3* ablation did not affect CDX2, SOX2 or total cell numbers (CDX2+: 80.9±22.6 vs. 94.5±18.6; SOX2+: 15±3.5 vs. 17.4±2.4; total: 139.2±24.5 vs. 156.5±22.9; mean±s.e.m for KO vs. WT). However, the number of NANOG+ cells was significantly reduced in KO embryos (9.7±6.1 vs. 16.2±3.1; mean±s.e.m for KO vs. WT, Mann-Whitney test $p<0.05$). Embryo survival from D6/7 to D12 in a post-hatching culture system was similar between C+G and C groups (93.03±2.98% vs. 83.93±6.83%, mean ± s.e.m, 4 replicates). No differences were found in embryo area between KO and WT (0.34±0.05 vs. 0.32±0.03 mm^2, mean ± s.e.m for KO vs. WT), but the number of embryos showing surviving epiblast cells (12/30 [40%] vs. 16/20 [80%]; KO vs. WT) and SOX2+ cell number (10.97±3.64 vs. 18.25±4.03; KO vs. WT) were significantly reduced in KO embryos (Chi-square and Mann-Whitney tests; $p<0.05$). No differences were found in the number of embryos developing an embryonic disc (4/12 [33.33%] vs. 5/16 [31.25%]; KO vs. WT). In conclusion, *GATA3* is dispensable for ovine blastocyst formation and TE vs. ICM specification, but its ablation impairs proper NANOG expression and further epiblast development *in vitro*.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

MEK signalling pathway is required for hypoblast specification in ovine blastocysts in vitro

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Keywords: ovine, embryo, MEK pathway

Proper development of the first cell lineages is critical for embryo survival. The second lineage specification differentiates the inner cell mass cells into epiblast, which will form the proper foetus, and hypoblast, which together with the trophectoderm will form the extraembryonic membranes and the foetal part of the placenta. In the mouse, hypoblast differentiation is induced through the MEK signalling pathway, and MEK inhibition with 1 µM PD0325901 (PD) impairs hypoblast specification. However, whether hypoblast differentiation in ungulates completely depends on MEK pathway remains unknown. Controversial results have been reported in bovine and porcine embryos treated with 0.4 to 10 µM PD and analysed with diverse hypoblast markers. However, the effect of MEK inhibition in ovine embryos remains to be studied. The aim of this work was to analyse whether hypoblast differentiation depends on MEK signalling in ovine, by culturing embryos in different doses of PD. First, to determine the specificity of lineages development markers, the reliability of epiblast (SOX2 and NANOG; n = 14) and hypoblast (SOX17, FOXA2 and GATA6; n = 11) markers was tested by immunofluorescence in day (D) 8 *in vitro*-produced blastocysts. SOX2 signal was consistent with an epiblast marker, as all SOX2+ cells allocated to the ICM region. Only 41.9±6.2% of the SOX2+ cells were co-labelled by NANOG, indicating that NANOG expression might appear later along epiblast development. SOX17 and FOXA2 labelling was restricted to hypoblast cells, being highly concordant (>94% of the cells labelled by one of them were co-labelled by the other). In contrast, GATA6 was expressed by both hypoblast and TE cells. Next, D5 *in vitro* embryos were randomly cultured in N2B27 medium supplemented with 1) 0.4 µl/ml DMSO (Control, n = 124); 2) 0.5 µM PD (0.5PD, n = 125); 3) 2.5 µM PD (2.5PD, n = 126) or 4) 10 µM PD (10PD, n = 126) until D8, when blastocyst rates were recorded and lineages development was analysed by immunofluorescence for SOX2 (epiblast), SOX17 and FOXA2 (hypoblast). No significant differences were detected in blastocyst rates at D8 between Control (35.9±2.9%); 0.5PD (30.3±9.1%); 2.5PD (25.9±3.2%) and 10PD (29±4.5%) (mean±s.e.m, ANOVA $p>0.05$). MEK inhibition did not affect either SOX2+ epiblast (27.9±3.6 vs. 31.6±3.9 vs. 23.1±3.3 vs. 16.6±2.2 for control, 0.5PD, 2.5PD and 10PD, respectively) or total cell number (239.5±38.9 vs. 208.5±27.2 vs. 138.3±16.5 vs. 131.6±15 for control, 0.5PD, 2.5PD and 10PD, respectively) (mean±s.e.m; ANOVA $p>0.05$). However, the number of SOX17+ (83.2±19.9 vs. 57.5±15.7 vs. 1±0.7 vs. 0±0 for control, 0.5PD, 2.5PD and 10PD, respectively) and FOXA2+ hypoblast cells (139.5±28.6 vs. 78.4±23.7 vs. 0±0 vs. 0±0 for control, 0.5PD, 2.5PD and 10PD, respectively) was significantly reduced in 2.5PD and 10PD (mean±s.e.m; ANOVA $p<0.05$). In conclusion, MEK signalling pathway is required for hypoblast specification in ovine. Revealing the key signalling pathways involved in early lineages development could help to design strategies to increase preimplantation embryo survival.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Characterisation of oviduct epithelium spheroids for the study of embryo-maternal communication in cattle

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Keywords: oviduct epithelium spheroids, spheroid characterization, Bos Taurus

Oviduct epithelial cells (OEC) cultured *in vitro* have been used for a long time to gain insights into early embryo-maternal communications. Most previous *in vitro* models included OEC monolayers grown on plastic dishes or on inserts in air-liquid interphase (ALI) systems (Schoen and Chen, 2018, DOI: 10.21451/1984-3143-AR2018-0012). However, OEC adherent to plastic rapidly dedifferentiate while the ALI system is technically challenging and takes three weeks before OEC differentiation. Hence, easy-to-use and physiological *in vitro* models are still needed. The aims of this study were (i) to characterise bovine oviduct epithelial spheroids (bOES) cultured in suspension under different culture conditions, and (ii) to test the impact of co-cultured embryos on bOES morphology. Isthmic mucosal fragments were isolated from peri-ovulatory oviducts obtained at a local slaughterhouse and were cultured for 3 days in TCM-199 HEPES + 10% FBS (M199) at 38.8°C under 5% CO2 in air. At Day 3, groups of 25 bOES of 100-200 µm in diameter and with ciliary beating were allocated to one of the following condition: 1) 500 µl of M199 (M199/500); 2) 25 µL droplet of M199 under mineral oil (M199/25); 3) 25 µL droplet of SOF + 5% FBS (SOF/25); or 4) 25 µL droplet of SOF + 5% FBS + 25 presumptive IVF zygotes (SOF/25/E). All groups were cultured for 10 days at 38.8°C under 5% CO2 (4 replicates). BOES were evaluated for morphology, movement, cell viability (ethidium homodimer/Hoechst 33342 staining) and immunodetection of cytokeratin and vimentin. Moreover, bOES on Days 3, 6 and 13 in M199 were analysed by qRT-PCR for gene expression of *ESR1, ESR2, PGR, OVGP1, ANXA1, VMAC, HSPA1A,* and *HSC70* as target genes, and *GAPDH, PPIA and YWAHZ* as reference genes. Normalized relative gene expression was calculated using the delta-delta-Ct method. Data were compared between groups by one-way ANOVA or Kruskal-Wallis test followed by Dunn's post-hoc-tests if appropriate. Normal bOES are defined as vesicle-shaped, 100-200 µm diameter and lined by a semi columnar epithelium layer including ciliated cells, with the apical pole and ciliary beating outside. The proportion of viable cells in bOES was not affected by culture conditions or time and remained high (>80%) up to Day 13. Numbers of morphologically normal bOES (with a cavity) and their movement amplitude decreased over time in all culture conditions ($P < 0.0001$), although 100% of morphologically normal bOES were still moving at Day 13. Proportions of normal BOES were higher in M199/500 (43%) and SOF/25 (27%) than in M199/25 (13%) ($P<0.05$). Co-culture with embryos in SOF/25 increased the proportion of normal bOES at Day 13 (47% vs. 27%, $P < 0.05$). OES cells displayed a positive signal for cytokeratin and a negative signal for vimentin. Relative gene expression of *ESR1, ANXA1, HSPA1A* and *HSC70* in bOES remained stable during culture while that of *ESR2, PGR* and *OVGP1* decreased from Day 3 to Day 13 ($P<0.05$) and *VMAC* was below the detection threshold. To conclude, bOES constitute an innovative easy-to-use and physiological model to study embryo-maternal interactions. Developing embryos in the vicinity of bOES supported spheroid morphology by mechanisms that remain to be investigated.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Oviduct epithelium spheroids support embryo development under oxidative stress conditions in cattle

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Keywords: oviduct epithelium spheroids, embryo development, oxidative stress

Monolayers of bovine oviduct epithelial cells (OEC) have been shown to improve development rate and quality of bovine blastocysts co-cultured under stress conditions (20% O_2), although the cells were rapidly dedifferentiating after reaching confluence (*Schmaltz-Panneau et al.,* 2015, https://doi.org/10.1111/rda.12556). Here, we tested bovine oviduct epithelium spheroids (bOES) with more stable OEC differentiation status (Pranomphon et al., Characterisation of oviduct epithelial spheroids for the study of embryo-maternal communications in cattle; submitted AETE abstract number X) as a new co-culture approach to support embryo development under usual (5% O_2) and oxidative stress (20% O_2) culture conditions. We hypothesized that bOES co-culture up to embryo genome activation (5 days post-IVF) would be sufficient to overcome oxidative stress conditions. Ovaries were collected from a local slaughterhouse. Oocytes were aspirated and selected for maturation for 22-23 h at 38.8°C under 5% CO_2 in air. IVF was performed using frozen-thawed Percoll-washed semen from two Holstein bulls of proven fertility at a final concentration of $2x10^6$ spermatozoa/mL for 18 h at 38.8°C under 5% CO_2 in air. After IVF (day 0), groups of 25 presumptive zygotes were allocated to one of the 6 following conditions: in 25 µL droplets of SOF medium + 5% FBS at 38.8°C without bOES for 8 days under 5% CO_2 and 5% O_2 (control-5%) or 20% O_2 (control-20%); under 5% O_2 with 25 bOES up to day 5 (5dBOES-5%) or day 8 (8dBOES-5%); and under 20% O_2 up to day 5 (5dBOES-20%) or day 8 (8dBOES-20%). Cleavage rates were evaluated on Day 2 and blastocyst formation rates on Days 6, 7, and 8. Blastocysts on Days 7 and 8 were fixed and stained with Hoechst 33342 for evaluation of cell number. Data were analysed by one-way or two-way ANOVA followed by Tukey's post-tests if appropriate (significant differences with a p-value < 0.05) using RStudio (R software version 4.2.2). The cleavage rates did not change between treatments, ranging from 71% to 78% (4 replicates). Under 5% O_2, the presence of bOES for 5 or 8 days did not affect the blastocyst rates at Day 7 (19 to 23%) and Day 8 (26 to 31%). However, under 20% O_2, the presence of bOES significantly increased the rate of development at Day 7 (16 vs. 26%) and Day 8 (18 vs. 29%) compared to control without bOES. The presence of bOES during 5 or 8 days had similar positive effects on embryo development. Furthermore, the blastocyst cell numbers were significantly increased compared to controls in 8dBOES-5% (102.6 ± 8.4 vs. 134.7 ± 10.2; $P<0.05$) and in 5dBOES-20% and 8dBOES-20% (82.1 ± 4.5 vs. 112.7 ± 7.8. and 138.1 ± 10.5, respectively; $P<0.0001$). In conclusion, bOES were able to overcome the negative effect of high oxygen level during bovine embryo development *in vitro*. Their presence during the first 5 days of culture, i.e., during major embryo genome activation, was sufficient to produce this positive effect.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Mitochondrial dysfunction during oocyte maturation and its impact on embryo epigenetic programming: mechanistic insights

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Keywords: oocyte, mitochondria, ATP

During final oocyte maturation, mitochondrial activity increases and epigenetic changes are highly dynamic, making this a sensitive window to metabolic and environmental stressors. This may impact fertility and offspring health. Somatic cell studies show that mitochondrial ATP is important for the biosynthesis of substrates and activation of regulatory (co)enzymes required for epigenetic programming. The extent by which early epigenetic programming is dependent on oocyte mitochondrial ATP is not known. Therefore, we aimed to reduce oocyte mitochondrial ATP production using a specific ATP synthase inhibitor (oligomycin A, OM) during bovine *in vitro* maturation (IVM) and assess the impact on global DNA methylation and histone modifications in the exposed oocytes and resultant embryos. For this, a bovine in vitro production model was used, where cumulus-oocyte complexes were exposed to control media (CONT; TCM-199 with 0.4mM L-glutamine, 0.2mM sodium pyruvate, 50µg/ml gentamicin, 0.1µM cysteamine and 20ng/ml epidermal growth factor) or CONT + 5nM OM during IVM (24h). *In vitro* fertilization (IVF) was performed in Fert-TALP + 0.72U/ml heparin (20h) and presumptive zygotes were cultured in synthetic oviductal fluid + 2% bovine serum albumin (7d) (14 replicates, 2110 COCs/treatment). Oocytes, zygotes, 4-cell embryos and morulae were collected at 24h IVM, 20h post insemination (p.i.), 48h p.i. and 4.7d p.i., respectively. Oocytes (34/treatment, 3 replicates), zygotes (26/treatment, 3 replicates) and morulae (30/treatment, 4 replicates) were fixed for 5mC and H3K9ac/H3K9me2 immunostaining and confocal microscopy to assess global DNA methylation and histone acetylation/methylation, respectively. Mitochondrial ATP production rate was measured in oocytes and 4-cell embryos using a Seahorse XF HS Mini Analyzer (Agilent, ATP rate assay kit) (20/pool, 3 replicates). Data were analyzed with t-test or Mann-Whitney U test depending on homogeneity of variance. ATP production rate was significantly reduced (by 33.8%, P=0.018) in OM-exposed oocytes (at 24h IVM), but returned to normal levels in the produced 4-cell embryos (P=0.547), compared to the CONT group. OM-exposure significantly decreased 5mC staining intensity in oocytes (9.8% reduction, P=0.019) but increased 5mC in zygotes (22.3% increase, P<0.001) and morulae (10.3% increase, P=0.041). H3K9ac staining was not detectable in oocytes. OM significantly increased H3K9ac in zygotes (10.3% increase, P=0.023) but not in morulae (P=0.414). Finally, H3K9me2 staining intensity was increased in OM-exposed oocytes (10.9% increase, P=0.024), but was not changed in zygotes (P=0.819) and morulae (P=0.509). We conclude that oocyte and embryo epigenetic patterns are dependent on oocyte mitochondrial ATP production. Maturation under metabolic stress conditions that are known to impact oocyte mitochondrial functions may thus result in persistent oocyte and embryo epigenetic alterations even if the stress is alleviated and mitochondrial functions are restored at subsequent stages.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Sex steroid determination during periestrous and peridiestrous period in saliva of sows derived from assisted reproductive techniques

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Keywords: sex steroids, saliva, pig

Emerging evidence indicates a long-lasting effect of *in vitro* fertilization on molecular physiology and metabolic dysfunction (Feuer and Rinaudo, J Dev Orig Health Dis 8, 2017). In pigs, clear evidence of phenotypical differences between artificial insemination and in vitro-derived animals have been reported (París-Oller et al., Res Vet Sci 142, 2021; Paris-Oller et al., J Dev Orig Health Dis 13, 2022). These differences might be extended to reproductive phenotype. Therefore, the reproductive stage and the concentrations of 17ß-oestradiol (E2) and progesterone (P4) were monitored in saliva samples of 4-year-old sows obtained from a previous study (Paris-Oller et al., J Anim Sci Biotech 12, 2021). Animals were born after artificial insemination (AI group; n=8) and surgical transfer of in vitro-produced embryos (IVP group; n=12), housed in an open pen and fed under the same conditions. Sows' interest in the male was recorded by a boar station (Compident) that registered the number and duration of visits to the station. Simultaneously, sows were subjected to the back pressure test. Estrus (d1 estrous cycle) was defined as the day of longer residence time at the station together with immobilization response to the back pressure test. Saliva samples were collected using Salivette® tubes containing the polystyrene sponge previously chewed by the sows for 10 seconds. Tubes were centrifuged (1.000 g, 5 min) and samples stored (-80ºC) until hormone analysis by quimioluminescence (Immulite). Concentration of E2 and P4 was determined in samples collected from days 1-3 (periestrous period; PEP) and 13-15 (peridiestrous period, PDP). Data (mean±SD) for number of visits to boar station (n), duration of the visits (sec), E2 (pg/ml) and P4 (ng/ml) were normalized and then analyzed by one-way repeated-measurements ANOVA followed by uncorrected Fisher's to compare variables between groups. $P < 0.05$ was considered significant. During PEP, sows from IVP group visited the boar station more times (10.60±1.91 *vs.* 3.30±1.05) and for longer time (239.77±43.12 secs *vs.* 77.69±26.01 secs) than AI sows. This observation is attributed to the higher hierarchy of IVP animals in this herd. No differences were found in E2 concentration (108.03±2.50 pg/ml and 106.81±4.95 pg/ml, respectively for IVP and AI animals). However, P4 concentration was higher in IVP (0.23±0.03 ng/ml) than AI sows (0.12±0.01 ng/ml). During PDP, only IVP sows visited the boar station (10.80±8.60 times for 265.0± 213.13 secs) and no differences between groups were found in E2 concentration (121.16±5.91 pg/ml and 119.11±3.40 pg/ml) neither P4 (0.21±0.06 ng/ml and 0.32±0.05 ng/ml; respectively for AI and IVP sows). Surprisingly, E2 levels in saliva were higher during PDP than PEP in IVP-derived animals but not in AI-derived animals (P=0.06). As for P4, no differences were found between concentration during PEP and PDP in any group of animals. Further analysis of saliva samples collected from whole estrous cycle will help to interpretation of data.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

MicroRNA-181d secreted by extracellular vesicles from bovine uterine fluid improve quality of in vitro produced embryos

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Keywords: extracellular vesicles, microRNAs, bovine blastocyst

Extracellular vesicles (EVs) contain proteins, lipids, metabolites, and microRNAs which rule cell-to-cell communication. We have demonstrated that bta-mir-181d was upregulated in uterine fluid EVs, suggesting that this miRNA may be involved in the arrival of the embryo to the uterus. Bta-mir-181d exerts its regulatory effects through modulating several pathways associated with blastocyst formation and embryo lineage segregation as: PI3K/AKT, TGFβ, Wnt, and Notch (Leal et al., J Animal Sci Biotechnol, v. 13, p. 116, 2022). We evaluated the effects of miR-181d mimic and its inhibitor on the development and quality of bovine in vitro produced embryos. Presumptive zygotes (n=1512) were cultured in SOF+0.3% BSA (Control) or supplemented with: 1 µM miR-181d mimic (miR181d), or 1 µM miR-181d control mimic (CM), or 1 µM of its inhibitor (Inh181d), or 1 µM of control inhibitor (CInh), in different time frames based on the physiological location of the embryo in the reproductive tract: i) days 1-4 (miR181d-OV or Inh181d-OV: representing miRNA effect in the oviduct), ii) days 4-7 (miR181d-UT or Inh181d-UT: representing miRNA effect in the uterus), or iii) days 1-7 of culture (miR181d or Inh181d or CM or Control). The miR-181d mimic, inhibitor, and their corresponding controls were purchased from Qiagen's miRCURY LNA miRNA line. Embryo developmental rates were recorded on days 4 (≥16-cell) and 7 (blastocysts: BD7) of culture and representative number from both stages were collected to assess their quality (mitochondrial activity by MitoTracker Deep Red; lipid droplet content by Bodipy). One-way ANOVA and Tukey test was used for all analyses. At day 4 of culture the proportion of embryos with ≥16-cell was lower ($p<0.05$) in Inh181d, CInh and Inh181d-OV compared to the other groups. At day 7, blastocyst yield was higher ($p<0.05$) in Control: 25.4±0.9%, CM: 25.0± 0.7%, miR181d: 24.5±0.9%, miR181d-OV: 24.1±1.0% and miR181d-UT: 24.9±0.8%, compared to CInh: 14.7±0.5%, Inh181d: 14.7±0.5%, Inh181d-OV: 14.0±0.4% and Inh181d-UT: 14.5±0.8%. No differences were found in mitochondrial activity and lipid droplets in embryos at ≥16-cell. Mitochondrial activity in BD7 from miR181d and miR181d-UT was increased ($p<0.05$) compared to all other groups. BD7 from miR181d-OV, Control and CM had higher mitochondrial activity than them from CInh and inhibitor groups, while BD7 from CInh had higher mitochondrial activity compared to both inhibitor groups. Lipid droplets content was decreased ($p<0.05$) in BD7 from miR181d and miR181d-UT compared to other groups. While BD7 from miR181d-OV, Control and CM had lower ($p<0.05$) lipid droplets compared to them from CInh and inhibitor groups. In conclusion, supplementation of miR-181d between days 4 to 7 or the entire period of IVC modulates blastocysts mitochondrial activity and lipid content, suggesting that miR-181d may play a role in embryo-uterine interaction.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

MicroRNA-133b from oviductal extracellular vesicles of pregnant cows improve in vitro embryo quality

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Keywords: extracellular vesicles, microRNA, blastocyst quality

Extracellular vesicles (EVs) are present in reproductive fluids and play an important role in cell-to-cell communication through their cargoes, especially microRNAs. We have detected mir-133b in oviductal fluid EVs from pregnant cows (Mazzarella et al., Front Vet Sci., v. 8, p. 639752, 2021), which is related to embryo development through the TGFβ pathway. We aimed to evaluate the effects of miR-133b mimic and its inhibitor, on the development and quality of bovine in vitro produced embryos. Presumptive zygotes (n=1844) were cultured in SOF+0.3% BSA (Control) or supplemented with: 1 µM miR-133b mimic (miR133b: designed for mimicking mature endogenous miR-133b), or 1 µM Mimic's negative control (CM), or 1 µM of its inhibitor (Inh133b), or 1 µM of control inhibitor (CInh), in different time frames based on the physiological location of the embryo in the reproductive tract: i) days 1-4 (miR133b-OV or Inh133b-OV: representing miRNA effect in the oviduct), ii) days 4-7 (miR133b-UT or Inh133b-UT: representing miRNA effect in the uterus), or iii) days 1-7 of culture (miR133b or Inh133b or CM or Control). The miR-133b mimic (339173), inhibitor (339131), and their corresponding controls (CM: 339136 and CInh: 339131) were purchased from Qiagen's miRCURY LNA miRNA line. Embryo developmental rates were recorded on days 4 (≥16-cell) and 7 (blastocysts: BD7) of culture and representative number from both stages were collected to assess their quality (mitochondrial activity by MitoTracker Deep Red; lipid droplet content by Bodipy). One-way ANOVA was used for all analyses. At day 4 of culture the proportion of embryos with ≥16-cell was lower ($p<0.05$) in Inh133b, CInh and Inh133b-OV compared to the other groups. At day 7 blastocyst yields were significantly higher ($p<0.05$) in Control: 29.2±0.6%, CM: 27.8± 0.6%, miR133b: 26.8±0.6%, miR133b-OV: 26.9±0.7% and miR133b-UT: 27.7±0.5%, when compared to CInh: 15.7±0.4%, Inh133b: 14.5±0.8%, Inh133b-OV: 15.2±0.5%, Inh133b-UT: 15.1±0.7%. MiR133b and miR133b-OV groups showed decreased mitochondrial activity ($p<0.05$) in ≥16-cell embryos and BD7, compared to all groups, while CInh group showed increased mitochondrial activity ($p<0.05$) only in BD7 compared to all other groups. Control, CM and miR133b-UT groups showed lower mitochondrial activity ($p<0.05$) compared to CInh, Inh133b and Inh133b-OV groups both in ≥16-cell and BD7. At BD7, Inh133b-UT increased their mitochondrial activity compared to Control, CM, and mimic groups. Lipid droplets in BD7 from MiR133b and miR133b-OV were significantly reduced ($p<0.05$) compared to the other groups, while them from miR133b-UT, Control and CM had reduced lipid droplets compared to CInh and inhibitor groups. In conclusion, medium supplementation with miR-133b during days 1 to 4 or the entire period of IVC modulates blastocysts mitochondrial activity and lipid content, highlighting the potential role of miR-133b in embryo-oviduct interaction.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Nobiletin supplementation to the culture medium increases in vitro porcine embryo development

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Keywords: nobiletin, porcine embryo, quality

Due to its potential as an antioxidant, anti-apoptotic, and free-radical scavenger, Nobiletin (Nob) has been used to promote *in vitro* oocyte maturation and embryo development in bovine (Cajas et al., Int J Mol Sci, v.21, p.5340, 2020; Cajas et al., Biol Reprod, v. 105, p. 1427, 2021). However, the effects of Nob on porcine *in vitro* embryo development are still unknown. This study aimed to evaluate the influence of Nob on the developmental kinetics and quality of *in vitro*-produced porcine embryos. Immature cumulus-oocytes complexes (COCs) were collected from prepubertal gilts and cultured in 500 µL of in vitro maturation (IVM) medium for 44 h. First 22 h, the medium was supplemented with 10 IU/mL eCG and 10 IU/mL hCG, then COCs were transferred to fresh IVM medium without hormone supplementation for an additional 22h. Mature COCs were denuded and co-incubated with thawed sperm (3000 spermatozoa per oocyte) in 100 µL drops of fertilization medium for 5 h. Presumtive zygotes were incubated in 500 µL of in vitro culture (IVC) medium supplemented with 2.5 or 5 µM Nob (MedChemExpress) (N2.5; n=220 and N5; n=234, respectively), or with 0.03% dimethyl sulfoxide (DMSO; vehicle for nobiletin dilution; n=213) or without any supplementation (Control; n=226) for 2 days. Then, embryos were cultured for 5 d in fresh IVC without Nob supplementation. Embryo developmental rates were evaluated at 48, 144, and 168 h of IVC, and their quality was assessed by i) mitochondrial activity (staining with MitoTracker Deep Red) and ii) ROS and glutathione (GSH) content using CellROX Deep Red Reagent and CellTracker fluorescent, respectively. One-way ANOVA and Tukey test was used for all analyses. No differences among groups were found in the cleavage rates at 48 h. However, at 144 and 168 h of IVC, N2.5 increased ($p<0.05$) blastocyst rates (45.0±1.4% and 48.0±1.1%, respectively), in comparison with N5 (32.4±0.6% and 35.2±1.1%, respectively), DMSO and Control groups (33.4±0.7% and 37.9±1.7%; and 34.8±1.0% and 38.2±1.15% respectively). In addition, no differences among groups were found in the mitochondrial activity, ROS and GSH levels of embryos at 48 h of IVC; however, at 144 h of IVC mitochondrial activity was increased ($p<0.05$) in blastocysts from the N2.5 group compared to the N5, DMSO, and Control groups. In addition, N2.5 group induced a significant reduction ($p<0.05$) of ROS and GSH content in blastocysts at 144 h of IVC when compared to all other groups. This result can be attributed to the higher mitochondrial activity observed in the embryos from the N2.5 group, leading to increased electron exchange in the inner mitochondrial membrane. This observation suggests that GSH was consumed to mitigate the detrimental effects of the elevated ROS levels. In conclusion, IVC medium supplementation with 2.5 µM Nob to the embryo culture medium increased porcine blastocysts development rates and enhanced embryo quality, thus improving the efficiency of the system.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

3D-organization and spatial localization of chromatin and epigenetic marks in relation to nucleolar activity in porcine oocytes

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Keywords: Epigenetics, porcine oocytes, chromatin conformation

This study employed porcine oocyte developmental biology as translational model investigating epigenetics in oocyte maturation. Previously, studies in various species confirmed that the chromatin of oocytes is subjected to large-scale modifications correlated to transcriptional silencing during final maturation. These modifications seem essential both for completion of the oocyte's meiosis and subsequent embryonic developmental success. In the present study, we focused on a putative interconnection between nucleolar transcriptional activity and spatial chromatin organization towards completion of oocyte growth. Porcine cumulus-oocyte-complexes (COCs) were aspirated from >3mm follicles of abattoir material. 300 COCs were then divided into 2 groups, pre-categorized by supravital brilliant-cresyl-blue (BCB) staining into fully mature (BCB+) and still maturating (BCB-). Following denuding and fixation, oocytes, in the same ratio of BCB- to BCB+, were processed for 3D-immunofluorescence (n=140) using antibodies against upstream binding factor (UBF; nucleolar activity), H3K9me3 and centromeric protein A and B (CENP) as epigenetic heterochromatin markers, as well as for 3D-DNA-FISH (n=140) with fluorescent oligonucleotides specific for porcine meta- and acrocentric heterochromatin sequences. Finally, the remaining oocytes (n=20) were prepared for TEM according to standard protocol. Analysis by high-resolution microscopy included confocal and TEM. Qualitative assessment of cellular ultrastructure by TEM (as previously described by P. Hyttel, Oocyte Maturation and Fertilization: A Long History for a Short Event, Chapter 1, 2011, pp 1-37) revealed distinct differences in manifestation of the perivitelline space, organelle abundance, shape and localization, as well as chromatin organization and localization between BCB+ and BCB- oocytes, supporting BCB-staining as viable method for rough categorization regarding maturation status. Immunostaining and labeling of chromatin allowed to detect all chromatin conformations (as previously described by Pan et al., Biology of Reproduction, Volume 99, Issue 6, 2018, pp 1149–1158), from non-surrounded nucleolus (NSN) to surrounded nucleolus (SN) and their intermediate conformations (pNSN, pSN) in both BCB groups. However, the BCB+ group contained a higher percentage of oocytes expressing chromatin conformations categorized as mature, whereas the opposite was true for the BCB- group. UBF-activity was only present in NSN and pNSN categorized oocytes and detected significantly more often in the BCB**-** group. The distribution of centromeric (CENP) and pericentromeric chromatin (H3K9me3) as well as repeated sequences DNA-FISH signal displayed distinct changes in their 3D-organization between NSN an SN conformation, characterized by significant signal-condensation around the nucleolus towards final maturation. Altogether these results demonstrated that there is an evident interconnection between nucleolar transcriptional silencing and the display of specific spatial 3D-chromatin organization patterns of both oligonucleotide sequences and epigenetic marks for constitutive heterochromatin, characterizing mature oocytes with higher competency for embryonic development.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Comparison between liquid and lyophilized media for bovine embryo production

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Keywords: lyophilization, IVC medium, cattle

Each year, more than one million bovine embryos are produced in vitro worldwide. Traditional in vitro culture media consist in either homemade freshly prepared medium or liquid commercial medium. Liquid media formulations involve limited lifespan and high transportation costs. As an alternative, there are ready-to-use lyophilized media in the market offering the advantages of easier transportation and longer lifespan. It has been reported that lyophilization of bovine IVC medium yields the same blastocysts rates as freshly prepared medium (Rubessa et al., RFD 2017). However, no information is available about the quality of the resulting embryos. The aim of the study was to compare the performance of a ready-to-use commercial lyophilized embryo culture medium to the traditional homemade medium, in terms of blastocyst development and quality. Blastocyst quality was evaluated by the total cell number (TCN), the inner cell mass (ICM) ratio and the survival after vitrification. Bovine oocytes were matured in TCM199 supplemented with 20ng/mL of Epidermal Growth Factor (EGF). Presumed zygotes (n=292, 4 replicates) were randomly allocated to either freshly prepared SOFaa supplemented with 0.3% BSA (Liquid group) or to COW-IVC-LYO (EmbryoCloud, Murcia, Spain; LYO group) a semi-defined lyophilized medium supplemented with BSA, and cultured for 8 days in groups of 25 embryos in 50µL drops. Blastocyst evaluation occurred 7- and 8-days post insemination (dpi). Blastocyst rates were calculated as the proportion of blastocysts out of zygotes placed in culture. Hatching rates were measured as the proportion of hatching/hatched blastocysts out of total 8 dpi blastocysts. At 8 dpi, all hatching and hatched blastocysts were fixed in 4% paraformaldehyde for differential staining, consisting in Hoechst and anti-CDX2 antibody labeling TCN and trophectoderm, respectively. The rest of the Q1 and 2 blastocysts were vitrified. Developmental and vitrification data were analyzed using a binary logistic regression model, while data concerning cell numbers were analyzed using an independent samples t-test. Differences at $p<0.05$ were considered significant. The results showed similar blastocyst rates at 7 dpi (Liquid 17.2%, LYO 19.7%) and 8 dpi (Liquid 29%, LYO 21.8%). And no differences were found either in hatching rates (Liquid 19%, LYO 18.2%). Concerning quality, there were no differences in TCN (Liquid 195.88 ± 16.18, LYO 170.33 ± 24.65) or ICM ratio (Liquid 0.29 ± 0.04, LYO 0.30 ± 0.03). Finally, regarding survival after vitrification, no differences were found in re-expansion 24h after warming (Liquid 90.9%, LYO 83.3%), and hatching rates 24h (Liquid 27.3%, LYO 27.8%) and 48h (Liquid 31.8%, LYO 38.9%) after warming. In conclusion, the tested lyophilized embryo culture medium supports embryo development reaching similar blastocyst rates to the freshly prepared traditional medium. Moreover, the blastocyst produced using lyophilized medium were of similar quality to the blastocysts produced in the traditional medium, in terms of TCN, ICM ratio and survival after vitrification.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Transforming growth factor beta (TGFβ) pathway is required for the proliferation of bovine extra-embryonic membranes following blastocyst hatching

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Keywords: Conceptus elongation, embryonic disc, hypoblast, embryo culture.

Following blastocyst hatching, ungulate embryos undergo a prolonged preimplantation period termed conceptus elongation that is characterized by the proliferation of extraembryonic membranes (hypoblast and trophectoderm) and the formation of an embryonic disc by epiblast cells. This is the most susceptible period for embryonic loss, but the study of the processes occurring during elongation has been limited by the lack of *in vitro* systems. Luckily, a recent culture system based on N2B27 medium allows post-hatching embryo development including complete hypoblast migration and the formation of an embryonic disc (ED)-like structure. Transforming growth factor beta (TGFβ) signalling pathway plays a relevant role in epiblast and hypoblast development in mice, but its role in ungulates is not well characterized. The objective of this study has been to test the effect of the inhibition of the TGFβ pathway on post-hatching bovine embryo development up to D12 *in vitro.* To that aim, Day 7 (D7) bovine blastocysts produced *in vitro* were transferred to N2B27 medium supplemented with TGFβ inhibitor (SB431542, TGFβi) at 200 μM (TGFβi-200, n=30), 20 μM (TGFβi-20, n=79) or 10 μM (TGFβi-10, n=54). As a control group, 68 D7 blastocyst were transferred to N2B27 medium without inhibitor (C). Surviving embryos at D12 were fixed to determine hypoblast migration and epiblast survival rates by immunostaining for *SOX17* and *SOX2*, respectively. Supplementation of TGFβi at 200 μM completely abrogated embryo development to D12 *In vitro.* Survival rates in TGFβi-20 were significantly lower compared to the control group, whereas no significant reduction was observed in TGFβi-10 group (81.2±5.7% vs. 74.2±7.6% vs. 58.7±5.6%, mean±s.e.m. for C vs. TGFβi-10 vs. TGFβi-20, ANOVA $p<0.05$). SB supplementation at 10 or 20 μM significantly reduced the proliferation of extra-extraembryonic membranes as evidenced by a reduced embryo diameter (0.76±0.05 vs. 0.46±0.05 vs. 0.49±0.03 mm, mean±s.e.m. for C vs. TGFβi-10 vs. TGFβi-20, ANOVA $p<0.05$). Hypoblast migration rates were reduced in embryos exposed to TGFβi (32/50 vs. 14/39 vs. 18/44, for C vs. TGFβi-10 vs. TGFβi-20, Chi-square $p<0.05$) but TGFβi had no effect on epiblast survival (30/50 vs. 21/39 vs. 31/44, for C vs. TGFβi-10 vs. TGFβi-20, respectively, Chi-square $p>0.05$). SOX2+ cell number was similar between groups (51.9±10.1 vs. 45.1±8.2 vs. 36.7±7.5 mean±s.e.m. for C vs. TGFβi-10 vs. TGFβi-20, ANOVA $p<0.05$), and ED-like formation rates were significantly reduced in TGFβi-10 compared with C (22/50 vs. 8/35 vs. 13/44, for C vs. TGFβi-10 vs. TGFβi-20, Chi-square $p<0.05$). In conclusion, TGFβ inhibition impairs the proliferation of extra-embryonic membranes at early post-hatching bovine embryo development.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

The replacement of N2 and B27 supplements by ITS-X reduces epiblast proliferation during bovine post-hatching development

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Keywords: Conceptus elongation, culture medium, supplement, epiblast.

During early post-hatching embryo development the inner cell mass cells complete cellular differentiation into two lineages: epiblast and hypoblast. Epiblast cells proliferate forming an embryonic disc, whereas hypoblast cells expand and migrate to cover the inner surface of the trophectoderm. These developmental landmarks can be achieved *in vitro* upon culture in N2B27 media, composed by a 1:1 mix of Neurobasal and DMEM/F12 media and N2 and B27 supplements. N2 and B27 supplements are particularly complex, adding 17 compounds to the Neurobasal:DMEM/F12 basal medium (NDF12) and it is unknown which of these components are essential for embryo development. To elucidate if those compounds are required by the embryo, we have evaluated the developmental effects of substituting N2 and B27 supplements by ITS-X, keeping NDF12 as basal medium. ITS-X supplement is composed by 4 of the compounds present in N2+B27 mix (insulin, transferrin, selenium and ethanolamine). To that aim, Day 7 *in vitro* produced blastocysts were produced following conventional pre-hatching culture protocols (SOF medium) and subsequently cultured in NDF12 medium supplemented with N2+B27 (N2B27 group) or with ITS-X at the recommended (ITSX-1X group) or doubled (ITSX-2X group) concentration up to Day 12. No statistically significant differences (ANOVA $p>0.05$) were observed in survival rates from D7 to D12 in 4 independent replicates (82.5±8 vs. 60±7.6 vs. 59.5±9.5%, mean±s.e.m. for N2B27 vs. ITSX-1X vs. ITSX-2X, 4 replicates). At D12 embryo diameter was measured and embryos were fixed to analyze hypoblast migration and epiblast survival by immunohistochemistry for epiblast (SOX2), hypoblast (SOX17) and trophectoderm (CDX2) markers. Embryo size, complete hypoblast migration and epiblast survival rates did not displayed significant differences between groups (size: 732±57 vs. 521±41 vs. 629±41 mm, mean±s.e.m.; hypoblast migration: 24/48 vs. 12/43 vs. 21/44; epiblast survival: 29/48 vs. 20/43 vs. 30/40 for N2B27 vs. ITSX-1X vs. ITSX-2X; ANOVA or Chi-square $p>0.05$), but the number of epiblast cells was significantly reduced in ITSX groups (47.8±9.3 vs. 21±5.5 vs. 23.4±7.8, mean±s.e.m. for N2B27 vs. ITSX-1X vs. ITSX-2X, ANOVA $p<0.05$). In conclusion, ITS-X can partially substitute N2 and B27 supplements during bovine post-hatching development, but epiblast cells proliferate to a greater extent in N2B27 medium.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

ZP2 is essential for embryo development in golden hamster (Mesocricetus auratus)

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Keywords: CRISPR-Cas9, zona pellucida, fertility

The zona pellucida (ZP) is an extracellular glycoproteic matrix made-up by three to four different glycoproteins (ZP1, ZP2, ZP3 and ZP4) in eutherian mammals that surrounds the oocyte and early embryo, and it is crucial in several mammalian reproductive processes. Genetic ablation of ZP2 in mice, a species whose ZP lacks ZP4, produced oocytes without a ZP, showing an impairment in fertility (Rankin T.L. et al., Develop., 128, 1119–1126, 2001). However, some ZP2 mutations found in infertile women resulted in a thinner ZP surrounding oocytes that could be fertilized by ICSI but not by IVF (Weimin Jia, J Assist Reprod Genet, 39, 1205–1215, 2022). Besides, in KO-ZP2 rat females only 16.5% of ovulated eggs possessed a ZP (Yan Wang et al., Reprod., 160, 353–365, 2020). Due to these contradictory results, we would like to know the relevance of the ZP2 protein in the hamster as, unlike mice, its ZP does not lack ZP4 and can be a useful four-protein-ZP with a present ZP model. Thus, the current research aimed to assess the role of ZP2 protein on hamster ZP formation and fertility. For that, we produced a novel ZP2-KO hamster model using CRISPR-Cas9 technology as in our previously reported protocols (Zhiqiang Fan et al., PLoS ONE, 9 (10): e109755, 1-9, 2014). A ten-nucleotides deletion mutation localized in the fifth exon of genomic hamster ZP2 has been achieved, which produces a change in the reading frame and triggers a premature STOP codon, leading to the translation of a 140-nucleotides long truncated ZP2. Our results showed that female KO hamster were infertile since no offspring was obtained after the observation of 18 mattings of 6 females. On the other hand, ovarian sections were evaluated by histological analysis, and we observed that folliculogenesis was not affected in the KO animals. Moreover, ovulated oocytes were collected by superovulation from oviducts. Oocytes from ZP2-KO females had thinner ZPs (7.33 ± 0.53 µm of thickness) compared to the WT (12.37 ± 0.91 µm). After isolating all ZPs from oocytes, 2 pools of 50 ZPs per genotype (ZP-KO and WT) were analyzed by proteomic identification (LC/ESI-MS/MS) and no ZP2 peptides were found on the KO samples. For the embryo study, we recovered embryos at 0.5-, 1.5-, and 2.5-days post coitum. Zygotes obtained from ZP2-KO females were fertilized, as two pronuclei and two polar bodies could be seen, but 1.5 day-embryos failed to divide (no cleavage was observed), and all 2.5 day-embryos were degenerated with their ZP broken. Moreover, cracked, isolated ZPs were found in the oviducts of every KO female. In conclusion, our results prove that ZP2 is not mandatory for the ZP formation, folliculogenesis and fertilization in the golden hamster. However, ZP2 would be necessary for fertility in this species due to an impairment of the early-embryonic development that compromises cell-division and leads to embryonic loss.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Embryo-induced alterations in the protein profile of bovine oviductal extracellular vesicles

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Keywords: oviductal extracellular vesicles, maternal-embryonic communication, protein signature

The oviduct provides the optimum environment for early embryonic development. Maternal-embryonic communication, which is essential for embryo quality, is mediated partly via extracellular vesicles (EVs). This study aimed to investigate the protein cargo of EVs obtained from the oviductal fluid (OF) of pregnant and cyclic heifers and their implications for maternal-embryonic communication in vivo. Oestrous cycles of crossbred beef heifers were synchronized, following which they were artificially inseminated (pregnant; n=13) or not (cyclic; n= 8) and slaughtered 3.5 days after insemination. The oviduct ipsilateral to the corpus luteum was flushed and the OF was examined to confirm the presence of a 6-8 cell embryo in pregnant animals. OF-EVs were isolated using size exclusion chromatography, concentrated by ultrafiltration, while EVs presence were characterized by flow cytometry using antibodies for specific EV markers (CD63, CD81, and CD44). Proteomic analysis was carried out using nanoLC-MS/MS with spectral counting to identify and quantify the proteins present in the EVs. Five animals from each group were used and statistical analysis was performed using ANOVA for flow cytometry data or T-test for proteomic data, both with a significance level of 5%. Bioinformatic analysis was performed with the DAVID and STRING tools. Flow cytometry analysis confirmed EV presence and no significant differences in EV markers between groups. A total of 1,101 proteins were identified: 5 unique to OF-EVs from cyclic heifers, 611 unique to pregnant heifers, and 485 in common. Among the common proteins, 93 were upregulated and 42 were downregulated in OF-EVs from the pregnant group. Functional enrichment analysis demonstrated that proteins exclusive to pregnant OF-EVs are involved in the Ras and Hippo pathways. Of note, Ras signaling is critical for mouse embryo development at the time of embryonic genome activation, which in cattle occurs in the oviduct during 8- to 16-cells transition. Furthermore, LLGL1, PATJ, and PARD6GB, members of the Hippo pathway exclusively found in pregnant OF-EVs, can regulate cell polarity and establishment of pluripotency. Additional pathways related to unique and upregulated proteins in pregnant OF-EVs include tight junction, cell adhesion molecules, and focal adhesion, which are essential for proper oviductal cell functioning and embryo development. Gene ontology analysis also revealed that upregulated proteins in pregnant OF-EVs, in comparison to EVs from cyclic animals, are associated with the immune response. In conclusion, although our model does not exclude a potential effect of sperm on the OF-EVs in the inseminated group, the study characterized a specific protein signature in OF-EVs from pregnant animals, which is likely due to the interactions established between the mother and the embryo.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Profiling the protein cargo of uterine extracellular vesicles isolated from pregnant and cyclic heifers

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Keywords: uterine extracellular vesicles, proteomics, embryo-maternal communication

Embryo-maternal communication is essential for successful pregnancy establishment in cattle. Over the last decade, interest has grown regarding the extracellular vesicles (EVs) role in these interactions. EVs can mediate cell-to-cell communication through the delivery of their cargo to target cells. This study aimed to compare the protein cargo of EVs isolated from the uterine fluid (UF) of pregnant and cyclic heifers to identify potential mediators of maternal-embryonic communication in cattle. Oestrous cycles of crossbred beef heifers were synchronized and artificially inseminated (pregnant; n=17) or not (cyclic; n= 11) and slaughtered 7 days after insemination. The uterine horn ipsilateral to the corpus luteum was flushed, and the UF was examined to confirm the presence of an blastocyst stage embryo in pregnant animals. UF-EVs were isolated by size exclusion chromatography and concentrated by ultrafiltration. Flow cytometry using antibodies for specific EV markers (CD63, CD81, and CD44) were utilized to identify and differentiate EV populations. NanoLC-MS/MS with spectral counting was utilized for the identification and quantification of proteins found in the UF-EVs. Statistical analysis was conducted on five animals per group, using ANOVA for flow cytometry data and T-test for proteomic data, both with a significance level of 5%. Bioinformatic analysis was performed with the DAVID database. Flow cytometry analysis confirmed the presence of EVs and no differences in the EV markers between groups. A total of 1,376 proteins were identified, 297 of which were exclusively detected in UF-EVs from pregnant heifers, and 101 unique to cyclic animals. Among the 978 common proteins, 22 were upregulated and 34 were downregulated in pregnant UF-EVs. The proteins found exclusively in pregnant heifers are associated with 22 pathways which include signal transduction (8), cellular processes (5) including cellular structure, transport, catabolism, and cell motility; endocrine system (3), metabolism (3) and Immune system (3). Among the pathways related to signal transduction, Wnt and RAS signaling are particularly important for their role on early embryo development involved in cell-fate specification, proliferation, survival, growth, and migration. The immune system pathways highlighted Th1 and Th2 cell differentiation. Importantly, Th1-Th2 shift is an important mechanism that prevents the bovine embryo from undergoing maternal rejection during pregnancy establishment. Of note, JAK3 which is exclusively found in UF-EVs of pregnant heifers, also mediates the suppressive effects of interferon tau on neutrophil function. In conclusion, the presence of an embryo modulates the uterine environment, more specifically the protein profile within UF-EVs. Thus, understanding the expression pattern and related function of these proteins in UF-EVs may help unveil the signals exchanged between the embryo and the mother during early embryo development.

Animal Reproduction

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Impact of Spirulina platensis as a feed supplement of ewes reared in endemic fluorosis areas from late pregnancy to early lactation

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Keywords: endemic fluorosis, pregnant and lactating ewes, Spirulina platensis

In sheep, chronic exposure to the fluoride excess is harmful to teeth, bones, kidneys, liver and heart (Rahim et al, Toxicology, 465, 153025, 2022). Additionally, it was documented that pregnant and lactating ewes are more susceptible to the chronic fluoride toxicity, as a result of their precarious calcium metabolism (Pradhan et al, Int J Pharm Sci Res, 4, 29-40, 2022). *Spirulina platensis* given its richness in bivalent minerals that could chelate fluoride excess, and in antioxidants, mainly c-phycocyanin, which could correct the oxidative stress generated by this halogen. Therefore, this study aimed to evaluate the impact of S*pirulina platensis*, firstly on plasma fluoride levels and oxidative stress in ewes (during late pregnancy and early lactation) reared in endemic fluorosis areas, and secondly on the average daily weight gain of their offspring. The experiment was conducted in El Fokra commune belonging to Khouribga province Morocco, where fluorosis is endemic, and compared to a group of ewes from Settat region, which is free fluorosis. Forty-eight ewes were divided into four equal groups (G1, G2, G3 and G4), G1 and G2 served as controls belonging respectively to free fluorosis (Settat) and endemic fluorosis (El Fokra) areas, while G3 and G4 received respectively 250 and 500 mg/kg bodyweight (BW)/day of *Spirulina platensis,* during late pregnancy and early lactation. Plasma levels of fluoride, glutathione (GSH), ascorbic acid and lipid peroxidation (MDA) as well as erythrocyte enzyme activity of catalase (CAT) and superoxide dismutase (SOD) were evaluated. In addition, the average daily weight gain of their offspring was determined. Statistical analyzes were performed using the JMP SAS 11.0.0 (SAS Institute Inc. Cory, NC, USA) program. In both late pregnancy and early lactation, the results revealed a significant increase in plasma fluoride, GSH and MDA levels as well as a significant decrease in ascorbic acid and enzymatic activities of CAT and SOD in ewes of G2 compared to those of G1. While in both late pregnancy and early lactation, the 250 and 500 mg/kg BW/day of *Spirulina platensis* (G3 and G4, respectively), significantly reduced plasma fluoride levels in ewes, and significantly improved the antioxidant system with a remarkable effect of the second dose. Furthermore, it showed that the average daily weight gain is significantly lower in the offspring of G2 than that of G1. While, offspring of G4 presented an average daily weight gain significantly higher than those of G2 and significantly lower than those of G1. In conclusion, the incorporation of *Spirulina platensis* as a feed supplement is advocated to minimize blood oxidative stress caused by fluoride toxicity in pregnant and lactating ewes and ameliorate the average daily weight gain of their offspring.

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EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Microbiota of the equine placenta

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Keywords: cervical star, pregnant horn, umbilical cord

The presence of a placental microbiome is considered to be controversial, with some authors isolating bacteria whereas others consider their presence to be due to contamination. The aim of this study was to determine which bacteria are present in the equine placenta. Methods: placental samples were obtained from the cervical star (CS) pregnant horn (PH), and the region near the umbilical cord (UC) of 24 foaling mares, aged 6-21 years old, at a Lipizzaner Stud in Hungary during spontaneous parturition. The DNA was extracted followed by 16S rRNA sequencing using the Ion Torrent method to identify the microbiome. Results: All of the parturitions were uneventful with 24 healthy foals born. Expulsion of the placenta occurred within 3 hours in all the mares, mean (± SD) 67 ± 36 minutes. An abundance of bacteria was found. The most abundant phyla were Proteobacteria (42-46.26%), Actinobacteria (26.91-29.96%), Firmicutes (14.19-17.31%) and Bacteroidetes (8.91-9.87%). The top genera found were *Acinetobacter, Brachybacterium, Brevibacterium, Chryseobacterium, Comamonas, Corynebacterium, Devosia, Flavobacterium, Kurthia, Luteimonas, Paracoccus, Planomicrobium, Pseudomonas, Saccharopolyspora, Sphingomonas,* and *Stenotrophomonas*. The diversity of bacterial microbiota was similar in all placental regions at the phylum level but differed at the genus level and alos between placental regions. Thus, Chlamydiae and Fibrobacteres were present only in PH; Ignavibacteriae was present only in UC. Fusobacteria were found in CS and PH, whereas Lentisphaerae and Nitrospirae were found in PH and UC. The results are similar to those obtained by sampling during human, murine and canine caesarian deliveries, although the bacteria were present in greater abundance, indicating possible contamination, However, the localisation of some bacteria to certain areas of the placenta tends to suggest that the healthy equine placenta may indeed have its own microbiome, which may differ according to placental region.

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Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Gonadotropin supplementation improved in vitro developmental capacity of Egyptian goat oocytes by modulating mitochondrial distribution and utilization

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Keywords: Goats, oocytes, hCG, IVM, IVF, cytoplasmic maturation.

Supplementation of gonadotropins to IVM medium has enhanced the developmental potential of oocytes (Dinopoulou et al., 2016; Bahrami and Cottee, 2022). Additionally, cytoplasmic maturation such as mitochondrial distribution impact oocyte competence (Ghanem et al., 2021a; Ghanem et al., 2021b; Reader et al., 2017; Torner et al., 2008). However, the mechanism by which gonadotropins improve oocyte quality is not completely defined. Therefore, the goal of this study was to investigate the effect of non-ruminant gonadotropin (hCG) supplementation on IVM, IVF, and mitochondrial activity of goat oocytes. In total, 2356 morphologically good quality COCs (Wieczorek et al. 2020) were recovered from 476 freshly obtained goat ovaries. Selected COCs were incubated in IVM medium consisting of TCM-199 supplemented with 10% (v/v) FBS, 10 ng/ml epidermal growth factor (EGF), 1 µg/ml estradiol (E2), 0.25 mg/ml Na+ pyruvate and 20 IU pregnant mare serum gonadotropin (PMSG) (Gonaser, 500 IU) according to previous publications (Soto-Heras et al., 2019; Jose et al., 2021; Maksura et al., 2021) for 24 hours at 38.5 °C, 5% CO2 and 95% humidity. The IVM medium was supplemented with hCG (IBSA, choriomon® 5000IU, Egypt) at two different concentrations according to the experimental design which included three experimental groups. The first group (G1) was allocated as a control group. The second (G2) and third (G3) groups were supplemented with 10 and 20 IU/ml hCG, respectively. The maturation rate was calculated after staining COCs with Hoechst 33342® (Sigma-Aldrich, St. Louis, MI, USA) in addition to the cumulus expansion rate which scored from 1-5 based on degree of cells dispersion (Maksura et al., 2021) and the first polar body (1st Pb) extrusion rate. According to Saini et al. (2022), was evaluated fertilization rate. Moreover, mitochondrial activity was assessed using Mito-Tracker green® dye (Invirogen-M7514, USA) fluorescent staining, according to Ghanem et al. (2021). The data obtained in the current experiment were analyzed statistically by SAS Enterprise Guide 4. Moreover, a chi-square test and one-way ANOVA were performed. The results indicated a higher rate ($P \leq 0.05$) of nuclear maturation in G3 (82.5%) than in G2 (65.9%) and the control group (64.8%). Furthermore, the fertilization rate (48 hrs post IVF) was significantly improved in G3 (18.7%) compared to G2 (10.6%) and the control group (9.5%). Notably, the percentage of diffuse pattern of mitochondrial distribution was increased in G3 (73.3%) compared to G2 (13.3%) and the control group (6.7%). This change in oocyte mitochondrial distribution was coupled with enhanced fluorescent mitochondrial intensity in G3 (95%) and G2 (89.2%) compared to the control group (79.9%). Our data indicated that hCG is a good substitute for LH during IVEP (Zombie et al. 2018). In conclusion, supplementing the IVM medium with a high concentration of hCG (20 IU/ml) increased the rate of metaphase II stage, mitochondrial activity and fertilization rate of goat COCs.

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THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Validation of sgRNAs to target GATA3 gene in Bovine cell lines using CRISPR/Cas9 gene editing

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Keywords: CRISPR, Knockout, Bovine fetal fibroblasts, cell lineage specification and embryo development

CRISPR/Cas9 based genome editing has recently become a popular and efficient technique to carry out targeted genetic modifications of any host genome. Knockout or knock-in of a gene of interest by targeted gene disruption is an effective approach of deciphering its function. Here we present an optimization of CRISPR/Cas9 in bovine fetal fibroblasts (BFF) cell line for application to bovine embryos with improved efficiency. These KO cell lines serve as an important tool to investigate the protein function by analysing the consequences of a specific gene loss. We have successfully generated *GATA3* knockout (KO) BFF cells using the CRISPR/Cas9 mediated genome editing. The method involves designing the CRISPR gRNAs targeting different regions of *GATA3* gene, CRISPR cloning into px459 plasmid, delivery of this CRISPR clone into bovine fibroblast cells, screening of knockouts and MiSeq analysis to verify successful disruption of *GATA3* gene. We have designed 11 guides spanning in different regions of bovine *GATA3* gene including the functional domains in Exon 4 and 5 regions and the transcription initiation site in Exon 2. Designed guides were optimized and screened by their cutting efficiency using the in vitro cleavage assay. The guides with best cutting efficiencies were then tested in bovine fetal fibroblast (BFFs) cell line. Bovine fetal fibroblasts cells were transfected with px459 plasmid bearing the sgRNA and Cas9, screened using puromycin based selection following the subsequent MiSeq analysis to verify the successful knockouts. We have identified two effective guides targeting the ZnF functional domains of *GATA3* gene (sgRNA#5 and sgRNA#8 cutting in Exon 4 and Exon 5 respectively) and one in Exon 2 (sgRNA#1) targeting the transcription initiating site of *GATA3* gene. The results showed a maximum indel frequency of 36.8% and 18% for sgRNA5 and sgRNA8 respectively for the bovine GATA3 gene. Overall, MiSeq data for all screened gRNAs showed that insertion of a base pair upstream of the PAM site resulted in introduction of premature stop codon TAA in the downstream region. *GATA3* is expressed in the outer cells of the morula stage during embryonic development and considered as a key driver of TE initiation and regulation of early lineage development in human and bovine embryos. We are currently assessing the efficiency of the *GATA3* knockout by microinjecting these selected sgRNAs and Cas9 protein RNP mix into putative bovine zygotes 12h post fertilization. This study is thus a step forward to apply recent advents of CRISPR/Cas9 system for gene editing to carry out the functional studies.

Animal Reproduction

THEMATIC SECTION: 39TH ANNUAL MEETING OF THE ASSOCIATION OF EMBRYO TECHNOLOGY IN EUROPE (AETE)

EMBRYOLOGY, DEVELOPMENTAL BIOLOGY, AND PHYSIOLOGY OF REPRODUCTION

Investigating the roles of interferon tau stimulated genes in sheep embryo implantation

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Keywords: embryo implantation, interferon tau, sheep

Interferon tau (IFNT) serves as the molecular signal that initiates maternal recognition of pregnancy and modification of the maternal immune system to favour a successful pregnancy in ruminants. It binds to the Type-1 interferon alpha receptors (IFNAR) present in the luminal cells of the endometrium to upregulate the expression of interferon stimulated genes (ISGs) to support embryo implantation. This hypothesis is being tested as a prelude to confirming the roles of immune cells (especially uterine natural killer cells) in the endometrium at this stage of pregnancy. Ewes kept in the experimental unit of the Royal Veterinary College were synchronised to a common oestrus, mating done by allocating 10 ewes per ram which were of the same breed and pregnancy was confirmed by the presence dye on the back and non-return to oestrus. Slices of caruncular and inter-caruncular area of the endometrial tissues were cut and snap-frozen from eight pregnant and six non-pregnant ewes on day 17 after natural mating. Pregnancy was confirmed by the presence of an elongating blastocyst when the uterus was opened alongside presence of corpus luteum on the ipsilateral ovary. Through RNA sequencing, this study has compared the expression of IFNT and ISGs and the related signalling pathways during embryo implantation in sheep. RNA sequencing was performed using 600 ng of the total RNA for analysis by Illumina with paired-end 150 bp sequencing (PE150) to reach over 30 million reads per sample, followed by a gene ontology enrichment analysis (GOEA). The cutoff criteria were P (BH) < 0.05 and absolute fold change ≥ 2.0. The result showed a significant upregulation of the ISGs (MX1, MX2, ISG15, ISG20, RSAD2, LGALS15, B2M, IFI6, IFI35, IFIH1, CXCL9, CXCL10, WNT7A, CTSL, IFIT2, IFIT3, IFIT5, IFI44, BST2, PLAC8, PLAC9, ADAR, DDIT4, DDX58, IFI44L, NAMPT, NT5C3A, PML, RTP4, TRIM25), in pregnant ewes' caruncular endometrium ($p<0.0001$) compared to those of the non-pregnant counterparts. STAT1, STAT2, and IRF9 which are critical transcription factors for the induction of the ISGs were also upregulated in the pregnant ewes compared to the non-pregnant ones. Six biological processes (defence response to virus, negative regulation of viral genome replication, immune response, innate immune response, complement activation classical pathway and inflammatory response) were observed to be related to interferon activation, and the DEGs associated with each of them were identified. These include ISGs involved in defence response to virus, e.g., MX2, STAT2, ISG15, RSAD2, IFI6, CXCL9, or in other biological processes like negative regulation of viral genome replication (IFIH1, RSAD2, MX1, ISG15), immune response (CXCL9, CXCL10, B2M), and innate immune response (MX2, MX1, IFI6, IFI35, IFIH1). It has also shown significant upregulation of the previously acclaimed ISGs involved in embryo implantation including LGALS15, ISG15 and CXCL10. These data suggest the critical role of IFNT and the upregulation of ISGs in regulating endometrial receptivity, embryo implantation and uterine immunity in sheep.