Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Collection of embryos and fluid from the bovine oviduct

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In contrast to in vitro culture systems which are most commonly performed on a static medium culture basis, embryo development in the oviduct is directed by a dynamic supply system. In order to get more insight into stage specific needs of a developing embryo the aim of the present study was to determine, whether embryos and the corresponding tubal fluid can be simultaneously obtained from the bovine oviduct. Fifteen Austrian Fleckvieh heifers were synchronized by two intra muscular injections of PGF2a (Estrumate, Cloprostenol, MSD Animal Health, Austria) 11 days apart and GnRH (Veterelin, Buserelin, Calier, Barcelona, Spain) 48 hrs after each PGF2a injection. Fixed time artificial insemination has been performed using fresh semen. At Day 3 after expected ovulation, oviduct flushing and embryo collection has been done using an endoscopy set for transvaginal access (STORZ, Tuttlingen, Germany). Following epidural anesthesia and genital disinfection a trocar set was placed in the tip of the vagina and introduced via dorsomedial perforation through the vagina wall into the peritoneal cavity. Prior to flushing the ovulation side has been determined and the morphology of the growing corpus luteum has been assessed. Flushing was performed in two steps: first the ampulla was repeatedly flushed using 0.5 ml PBS medium. Finally, 0.2 to 0.4 ml medium/fluid has been recovered, adjusted to 0.5 ml and transferred into an Eppendorf tube and centrifuged for estimation of total protein using colorimetric Protein Quantitation Assay (Pierce BCA Protein Assay Kit, ThermoFisher, Austria). In a second step 50 ml medium (PBS, 1% FCS) was flushed through the oviduct, collected in the tip of the uterine horn via an embryo flushing catheter which has been connected to an Emcon embryo filter. Additionally, 300 ml medium were used to extra flush the uterine horn via the flushing catheter. The medium collected in the filter was checked for the presence of an embryo using a stereo microscope. Overall, 12 heifers have been synchronized 3 times and 3 heifers 2 times (in total: 42 treatments). In 35 cases ovulation has been confirmed and oviduct fluid has been collected successfully. Additionally, 21 embryos (recovery rate 60%) have been recovered at the 4 to 8-cell stage. Protein quantitation analyses of the collected tubal fluid ranged between 0.28 and 1.4 mg/ml total protein. Taken these preliminary results together it is concluded, that is possible to repeatedly collect tubal stage embryos and the corresponding fluid as a prerequisite to analyze the stage-specific environment of the embryo. It was also assumed that the obtained total protein amount was sufficient for further examining tubal components. It is expected that consecutive studies including the collection of tubal fluid and corresponding embryos throughout the migratory phase in the oviduct will provide much more detailed information about the needs of an embryo especially during in vitro culture.

Keywords: embryo, oviduct, cattle

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Embryology, developmental biology, and physiology of reproduction

Effect of serum in maturation medium on the birth weight of Holstein calves

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It has been known for years that IVP calves generally have a higher birth weight as compared to AI calves. Serum in the IVP media is often blamed for this. Indeed removing serum from the IVC medium reduced the amount of heavy calves (Wagtendonk et al. 2000), but the level is still higher than that in AI calves.

The aim of this study was to investigate whether the removal of serum from the maturation medium could further decrease the birth weight and the percentage of heavy calves.

Oocytes were collected from female HF donors between 9 and 24 months of age. The oocytes were incubated for 24 hours at 38.5°C in IVM medium (M199 supplemented with 0.1 mM Cysteamine, 0.01 U/ml FSH (SigmaAldrich) and LH 0.02Ug/ml (SigmaAldrich)) either with 10% Fetal Calf Serum or without (no further additions to compensate for the serum removal). After 24 hours, oocytes were fertilised with different bulls and subsequently cultured for another 7 days. Day 7 embryos were transferred fresh or frozen at different commercial farms throughout The Netherlands. Birth weight of the calves were recorded by the farmers and are a mix of estimated weights and real measured weights.

In total 1198 and 689 different OPU sessions were performed for control and serum free medium respectively. On average 10 oocytes were collected per session.The percentage of transferable day 7 embryos was significantly (Chi-square test, $P<0.05$) smaller in the IVM medium without serum as compared to the control with serum (21% transferable embryos for the control compared to 19% for the serum free IVM medium). Subsequently, 860 control embryos and 620 embryos from the serum free group were transferred. Pregnancy rates (on average 45%) of the embryos from both groups were comparable. The mean birth weight of the 97 animals from the serum free IVM medium was not different from that of the 148 animals in the control group (42.5 and 43.0 kg respectively). Also the percentage of heavy calves (i.e. calves of 50 kg or heavier) was not significantly (Chi-square test) different between the two groups (9% and 7% for the serum free IVM and control respectively). As expected male calves were about 3 kg heavier, which was the same in both groups.

These results indicate that the removal of serum from the IVM medium does not have a significant effect on lowering the birth weight of IVP calves. Therefore further research is needed to reduce the birthweight of the IVP calves. Potentially further modifications in the media are required.

Keywords: IVP, birthweight, serum

Animal Reproduction

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Embryology, developmental biology, and physiology of reproduction

Comparative proteomic analysis of bovine embryos developed in vivo or in vitro up to the blastocyst stage

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Despite many improvements in *in vitro* systems and embryo culture media, *in vitro* derived embryos still display morphologic and metabolic differences making them less viable and cryoresistant compared to their *in vivo* counterparts. To bring knowledge to this issue, we used a quantitative proteomic approach to compare early bovine embryos developed *in vivo* or *in vitro*.

Eleven Holstein females were synchronized for estrus, treated for ovarian superovulation and inseminated twice with frozen-thawed semen. Between days 1.7 and 7.5 after the first artificial insemination, *in vivo* embryos were recovered after slaughter by flushing of the oviducts and uterus. In addition, embryos were produced *in vitro* using slaughterhouse bovine ovaries, the same male semen and a culture medium with no serum or protein supplementation. All embryos were washed three times and stored at −80°C before analysis. Proteins from pools of grade-1 embryos at the 4-6 cells, 8-12 cells, morula, compact morula and blastocyst stages (4 embryos/pool; 3-4 pools/stage, total of 38 pools) were analyzed by nanoliquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Proteins were identified using the UniProt *Bos taurus* database and quantified by label-free spectral counting. Proteins quantified with minimum 2 normalized weighted spectra (NWS) in at least one condition were analyzed using principal component analysis (PCA) and ANOVA. The hierarchical clustering of differentially abundant proteins (DAPs; ANOVA p-value < 0.05) were done using Spearman correlations and the gplots package of RStudio. Functional analysis of DAPs was carried out using the Metascape on-line tool.

A total of 3,028 proteins were identified in embryos, of which 227 were specific to *in vivo* embryos and 49 to *in vitro* embryos. The PCA of the 2,186 proteins quantified with more than 2 NWS showed a clear separation of embryo pools according to their stage of development and origin (*in vivo* vs. *in vitro*). Oviductin, also known as oviduct-specific glycoprotein 1 (OVGP1), and clusterin were among the most overabundant proteins in *in vivo* compared to *in vitro* embryos at all stages. Three clusters of 999 DAPs (ANOVA's p-value <0.05) according to the origin were evidenced: 463 DAPs with higher abundance *in vivo* than *in vitro* across development (cluster 1); 314 DAPs with less abundance *in vivo* than *in vitro* before the morula stage (cluster 2); and 222 DAPs with less abundance *in vivo* than *in vitro* after the morula stage (cluster 3). Proteins in cluster 1 were mainly involved in carbohydrate metabolic pathways, cellular detoxification and cadherin binding. Proteins in cluster 2 were mainly involved in protein synthesis. Proteins in cluster 3 were mainly involved in mitochondria-dependent activity and cytoskeleton organization.

These data provide a first exhaustive proteomic comparison between *in vivo* and *in vitro* embryos in cattle and bring new insights into the molecular contribution of the maternal environment (ovarian follicle, oviduct and uterus) to the preimplantation embryo. Moreover, the DAPs identified constitute valuable markers of embryo quality for the assessment of new *in vitro* systems, closer to *in vivo* conditions.

Keywords: Embryo, proteomics, cattle.

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Embryology, developmental biology, and physiology of reproduction

Sub-optimal paternal diet accelerates pre-implantation embryo development in mice

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The embryo is critically sensitive to altered environmental conditions *in utero*, which can subsequently direct fetal growth and influence offspring health in later life. Emerging evidence suggests the father's pre-conception diet/lifestyle also has a role in regulating fetal development and ultimately alters disease risk in the adult offspring. Defining the early embryonic dynamics that underlie this impact on offspring health is key to improving our understanding of the developmental origins of health and disease. Therefore, the aim of this study was to examine how impaired paternal nutritional status influences early embryo development in a mouse model.

Male C57/BL6J mice were fed either a low-protein (LPD: 9% casein, 24% sugar, 10% fat; n=6), Western (WD: 19% casein, 34% sugar, 21% fat; n=7) or control (CD: 18% casein, 21% sugar, 10% fat; n=6) diet for a minimum of 8 weeks, to ensure all periods of spermatogenesis were exposed to dietary factors. Males were mated to virgin 8-12 week old females (n=6-7 per group), with pregnancy confirmed by the presence of a copulation plug and termed embryonic day (E)0.5. Pregnant dams were euthanized on E1.5 and the oviducts flushed to retrieve 2-cell embryos. Embryos were cultured individually in EmbryoMax® KSOM media (37°C; 5% CO_2) in an EmbryoScope time-lapse incubator, with images acquired every 10 minutes. Embryo cleavage rate and blastomere/blastocyst area were determined using EmbryoViewer software. Fully expanded blastocysts were fixed in 4% paraformaldehyde and stained for quantification of inner cell mass (Oct4) and trophectoderm (Cdx2) cell numbers. Statistical analysis was performed using IBM SPSS Statistics v25. Data were assessed using a linear mixed model or a one-way ANOVA with Tukey's post-test where appropriate.

The sub-optimal paternal diets did not alter number of embryos per dam (CD: 8.2±1.0, LPD: 8.2±0.2, WD: 8.1±0.3). Embryos generated by WD fed-males demonstrated an increased average blastomere size at the 2-cell stage (2282±31μm^2 vs 2108±47μm^2, $p<0.05$) compared to CD. Embryos from both LPD and WD fed males developed through all pre-compaction cell cleavage stages faster than those from CD males (time to 4-cell stage; LPD: 6.0±0.6h, WD: 5.3±0.4h vs CD: 9.8±0.8h; $p<0.01$, $p<0.001$ respectively, and to 8-cell stage; LPD: 19.1±1.0h, WD: 19.0±0.5h vs CD: 22.9±0.8h; $p<0.01$). Post-compaction, no differences were seen between diet groups. Blastocysts from LPD fed males demonstrated a faster time to expansion compared to CD (54.1±1.0h vs 59.5±1.3h, $p<0.01$ (h= hours from start of culture)), yet were found to have a smaller area than both CD and WD blastocysts (6745±127μm^2 vs 7647±278μm^2 and 7886±245μm^2, $p<0.05$, $P<0.01$ respectively). We observed no differences in inner cell mass or trophectoderm cell number.

We found patterns of early embryonic development were accelerated in embryos derived from LPD and WD fed males. Whilst this accelerated rate of developed declined post-compaction, differences in blastocyst formation were still observed. Altered rates of early embryonic development have been linked with impaired implantation, and even altered fetal development later in gestation. Our data suggests that a poor paternal pre-conception diet plays a key role in regulating the rate of early embryonic development, which could have implications for the future health of the offspring.

Keywords: embryo, diet, paternal

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Embryology, developmental biology, and physiology of reproduction

Lipids characteristics in bovine preimplantation embryos originated from in vitro fertilization or parthenogenetic activation

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Lipids are essential elements of the cells since they build biological membranes and are involved in many processes, including energy metabolism. They are stored in the cytoplasm in lipid droplets (LD). Lipid content alters during the preimplantation development of embryos, mainly due to the changes in energy metabolism requirements. Except for classic in vitro fertilization (IVF), embryos have the ability to activate their development without the involvement of male gametes (parthenogenesis, PA). The question arises of whether it affects embryonic lipid content.

The aim of this study was lipid characteristics in embryos: 1. at crucial stages of development, 2. originating from IVF and PA systems.

Bovine oocyte-cumulus complexes were matured in vitro and either in vitro fertilization (bIVF group) or parthenogenetic activation (5uM ionomycin/2mM 6DAMP; bPA group) was performed. Embryos were cultured in SOF medium and collected in the following developmental stages: zygote, 2-cell, 4-cell, 8-16-cell, morula, early and expanded blastocyst (approx. 20 per group). They were stained with BODIPY 493/503 and DAPI for LD and chromatin visualization, respectively, then captured and z-stacked with Zeiss LSM 880 confocal microscope. The following parameters were analyzed in ImageJ Fiji software: total lipid content, LD number, LD size, and % area of LD. Statistics included appropriate tests in the R statistical package.

Our results show that in bIVF group, total lipid content reaches the highest level at the zygote stage, and it drops to the lowest values at the 8-16-stage ($P<0.01$), following a significant increase at the expanded blastocyst stage ($P<0.05$). A similar decrease at the 8-16-cell stage is observed for the LD number (113+/-41 vs 57+/-21 μm^2), LD size (5.15+/-2.59 vs 2.16+/-0.77), and % area occupied by LD (3.3+/-1.14 vs 1.06+/-0.83) ($P<0.01$). When bIVF and bPA at 2-cell, 4-cell, and expanded blastocyst stages are compared, a significantly lower value of total lipid content ($P<0.05$) is observed at the 2-cell stage in bIVF embryos. Yet, there areno differences between expanded blastocysts.

LD parameters decrease at the 8-16-cell stage is observed at the same time-point as the embryo genome activation in cattle. It suggests that early embryos may strongly utilize lipids as a source of energy without the possibility of replenishment due to the lack of transcription. Moreover, others suggest a lower contribution of lipids in the late preimplantation embryo development since embryos switch their energy metabolism into glucose. However, it is inconsistent with our data due to the observed rise of lipid parameters at the blastocyst stage. Our results also indicate that lipid content is altered depending on the embryo origin (IVF or PA) only in the early stages of development (2-cell embryos). It suggests that during further development, embryos are able to compensate for the lipid deficiencies, however further studies in every step of development are necessary.

Keywords: embryo, cattle, lipids

Funding: Faculty of Veterinary Medicine and Animal Sciences, funding for Young researchers 506.534.05.00, Poznan University of Life Sciences.

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Embryology, developmental biology, and physiology of reproduction

Preimplantation genetic testing for aneuploidy (PGT-A) reveals a high incidence of chromosomal errors in in vivo and in vitro pig embryos

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Chromosome errors in embryos can lead to implantation failure, spontaneous abortions, and birth defects; as such, characterising their incidence is of interest not just in humans but also in domestic animals where embryo production is employed for breeding. Recent studies have found an aneuploidy incidence between 14-24% in cattle embryos; however, information on other domestic species is lacking. Here, we present for the first time a characterisation of chromosome errors in both *in vivo* derived (IVD) and *in vitro* produced (IVP) porcine embryos, using single nucleotide polymorphism based PGT-A. Five sows were inseminated and culled at day 4, 5 or 6 of the oestrous cycle (D0 = onset of oestrous) to collect IVD embryos at different development stages by flushing of the distal portion of the uterine horn. Additionally, IVP blastocysts were produced from 10 sows during three fertilisation rounds. For all embryos, the zona pellucida was removed using 0.5% pronase and the samples were stored at -80 °C. Whole genome amplification was performed by using the REPLI-g Advanced DNA Single Cell Kit (Qiagen, Oslo, Norway), and genotyping was completed on a custom Illumina GeneSeek 25K SNP chip (Lincoln, NE, United States). PGT-A diagnosis were obtained by combining Log R ratio (LRR) and B-allele frequency (BAF) graphs to detect copy number variations, and Karyomapping to trace the parental origin of chromosomal errors (maternal or paternal), and to detect triploidy and uniparental disomy. Proportions between groups were analysed by Fisher's exact test and the threshold for statistical significance was set as $p \leq 0.05$. In IVD embryos, the overall incidence of chromosomal errors was 32% (32/101). Although not significantly different ($p > 0.05$), fewer errors were detected at the blastocyst stage as compared to earlier stages: 40% in 4 cell (10/25), 35% in 6-12 cells and morulae (7/20 and 12/34) and 14% in blastocysts (3/22). Conversely, IVP blastocysts showed an 80% incidence for chromosomal errors (n = 51/64). Even with the low sample size achieved, this provides the indication that IVP blastocysts suffer from a higher incidence of chromosomal errors as compared to IVD blastocysts ($p < 0.001$). Triploidy was the most common chromosomal error in IVD embryos (16%, 16/101), followed by whole chromosome errors (10%, 10/101). Surprisingly, two parthenogenetic embryos and one androgenetic embryo were also identified in the IVD embryos. In IVP blastocysts, parthenogenesis affected one in three embryos (21/64). The parthenogenetic embryos arose from just three sows, across two different IVP rounds, suggesting a possible individual effect. The incidence of triploidy in IVP blastocysts was 25% (16/64), and errors arising from either polyspermy (12/16) or meiotic non-disjunction in the oocyte (4/16) were both detected. Errors with a maternal origin were prevalent in IVP embryos (41/57, $p < 0.01$), whereas IVD embryos presented a similar incidence of errors from either parent (17/36 maternal, $p > 0.05$). In conclusion, PGT-A discovered a high incidence of aneuploidy and triploidy in IVD and IVP embryos, suggesting that the future application of this technology might improve embryo transfer success in the pig.

Keywords: Ploidy, porcine, cytogenetics

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Embryology, developmental biology, and physiology of reproduction

Effect of astaxanthin on post-thaw viability of bovine vitrified oocytes: preliminary results

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Although various techniques for cryopreservation of bovine oocytes are known, their developmental potency after thawing is still unsatisfactory. Astaxanthin (AX) is a powerfull antioxidant, which improved post-thaw viability of pig oocytes but its effects on bovine vitrified oocytes are to be examined. Our goal was to examine whether AX, added to the post-thaw medium, can improve the viability of bovine vitrified oocytes. Oocytes, recovered from cow´s ovarian follicles at slaughtering, were *in vitro* matured (IVM) and then vitrified in minimum volume on the nickel electron microscopy grids by ultra-rapid cooling technique. Following several months the oocytes were warmed and incubated 3 hours for post-thaw recovery in the maturation medium (TCM199, 10% FCS, 0.25mmol.l^{-1} sodium pyruvate, 50µg/ml gentamicin, 1/1 I.U FSH/LH (Pluset)) either without AX (Sigma Aldrich, Missouri, USA; 0µM; vitrified group; n=186) or with 2.5µM (the dose was chosen according to the previous reports) of AX (vitrified+AX group; n=179). Fresh IVM oocytes (n=157) served as a control. Afterwards, oocytes of all groups were fertilized *in vitro* using frozen bull semen and cultured in B2 medium (prepared according to Menezo) with 10% FCS, 10mg/ml BSA, 31.25mM sodium bicarbonate and 50µg/ml gentamicin on a monolayer of BRL-1 (Rat epithelial cells; ECACC, UK) cells at 38.5°C and 5% CO_2 until the blastocyst stage (6-8 days). Experiments were performed in four replicates. Total blastocyst rate (D6-D8) was significantly less in vitrified (12.90%) and vitrified+AX (13.41%) groups compared to control group (25.48%), whilst cleavage rate was different only in vitrified group (53.26%), but not in vitrified+AX (55.87%) compared to control (64.33%). However, AX significantly ($p<0.05$) increased (Chi-square test) the proportion of embryos that reached the blastocyst stage earlier (Day 6; 20.83%), compared to the vitrified group without addition of AX (8.33%), thus approaching this value to those in the control group (25.00%). Vitrification led to slight decrease ($p>0.05$; Student´s t-test) in the blastocyst cell number from 103.03±4.42 (control group) to 92.24±6.20 (vitrified), whilst AX reversed this suppressive effect (102.87±6.00). Apoptotic occurrence (TUNEL-index) did not differ significantly among control (10.33±1.84%), vitrified (13.02±3.24%) and vitrified+AX (13.93±3.35%) groups (Student´s t-test). AX indicated a trend to improve quality of actin cytoskeleton by increasing the proportion of embryos with excellent actin quality (grade 1) in vitrified+AX oocytes (82.61%) in comparison to the fresh (64.87%) or vitrified (61.90%) groups, although differences were not significant (Mann-Whitney U-test). In conclusion, astaxanthin, added to vitrified/warmed oocytes during post-thaw recovery period, accelerated development to the blastocyst stage, what was reflected in increased rate of faster developing blastocysts with no effect on the total blastocyst yield.

Keywords: astaxanthin, oocytes, vitrification

Grant support: The Slovak Research and Development Agency, Slovak Republic (grant no. APVV-19-0111).

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Embryology, developmental biology, and physiology of reproduction

HH5 double-carrier bovine embryos show impaired development through elongation

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Genomics analyses in dairy cows have uncovered deleterious haplotypes (alleles) never found in homozygosity at birth and thereby causing pre-term mortality. Between these deleterious haplotypes, Holstein Haplotype 5 (HH5) consists of a large 138 kb deletion causing the ablation of the gene *TFB1M* (Transcription Factor B1, Mitochondrial). Unfortunately, the developmental period when double carrier embryos (DC, i.e., *TFB1M*-null embryos, homozygous for HH5) arrest their development is unknown. Given that the impact of pregnancy loss varies greatly from early losses (before maternal recognition of pregnancy) to later miscarriages, such information is crucial to evaluate the economic losses associated to the inadvertent cross of single carrier (SC, heterozygous) individuals. To solve that question, we have analysed the development of Day 14 conceptuses collected from 3 superovulated SC cows inseminated with a SC bull. Conceptus were recovered by uterine flushing, fixed in 4% paraformaldehyde and subjected to immunohistochemistry to analyse trophectoderm (CDX2), hypoblast (GATA6) and epiblast (SOX2) development. Following fluorescence microscopy analysis, samples were genotyped by PCR. Mendelian inheritance of the allele was observed in the 25 conceptuses retrieved (5:16:7 for WT:SC:DC). Hypoblast migration was observed in all conceptuses, but a significant impairment in the development of the extra-embryonic membranes (hypoblast and trophectoderm) was evident in DC embryos. The development of such membranes is the main responsible of the increase in conceptus length and the change in shape during elongation. DC conceptuses remained spherical, in contrast to the ovoid or elongated WT (non-carrier) or SC conceptuses, and were significantly smaller (26.2±8.5 vs. 30.5±4.9 vs. 0.7±0.1 mm for WT, SC and DC, respectively, mean±s.e.m., ANOVA $p<0.05$). Embryonic disc was formed in all DC embryos, but its diameter was also significantly reduced compared to WT or SC embryos (319±40 vs. 396±27 vs. 223±13 µm for WT, SC and DC, respectively, mean±s.e.m. ANOVA $p<0.05$). In conclusion, the bovine HH5 DC embryos analyzed arrested their development prior to early conceptus elongation and maternal recognition of pregnancy. Supported by StG-757886 from ERC and PID2020-117501RB-I00 from MINECO.

Keywords: Embryo development, conceptus elongation, haplotype, dairy cows, mitochondria.

Animal Reproduction

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Embryology, developmental biology, and physiology of reproduction

Bovine embryos lacking progesterone receptor (PGR) develop normally through early elongation

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Endogenous maternal progesterone levels and exogenous progesterone supplementation have been positively linked with conceptus length and embryo survival in cattle. However, the mechanism by which progesterone enhances conceptus development is unknown. Progesterone may act directly on the embryo or indirectly, by promoting changes in uterine fluid composition favoring conceptus growth. To discriminate between both possibilities, we have analyzed the development of bovine embryos lacking progesterone receptor (PGR) generated by CRISPR technology. To that aim, we have microinjected *in vitro* matured oocytes with mRNA encoding for Cas9 alone (control group, C, solely composed by WT embryos) or combined with sgRNA against *PGR* (C+G group, partially composed by KO embryos). Following fertilization and culture up to Day 7 (D7) blastocyst, subsequent development was analyzed *in vitro* and *in vivo*, assessing lineage development by immunostaining for CDX2 (trophectoderm), SOX17 (hypoblast) and SOX2 (epiblast), and conducting genotyping by miSeq. *In vitro* development from D7 to D12 was similar between groups (85.4±5.7 vs. 81.3±6.3, mean±s.e.m. for C and C+G, respectively, t-test p>0.05). In C+G group, 22/45 D12 embryos analyzed were KO (i.e., contained only KO alleles). Embryo diameter at D12 was not affected by embryo genotype (772±74 vs. 648±64 vs. 731±44 µm, mean±s.e.m. for WT, edited non-KO and KO, respectively, ANOVA p>0.05), and the proportion of embryos attaining complete hypoblast migration was similar in WT (23/32, 72%), edited non-KO (12/20, 60%) and KO (19/22, 86%) embryos (Chi-square p>0.05). No differences were noted either on embryonic disc (ED) formation rate (20/32 63% vs. 6/20 30% vs. 10/22 45% for WT, edited non-KO and KO, respectively Chi-square p>0.05). To assess *in vivo* development, 40 blastocysts from C+G group were transferred to two synchronized recipient ewes. Pregnancy was supported by exogenous progesterone (CIDR-Ovis) and conceptuses were recovered 9 days after embryo transfer at a developmental stage equivalent to day 14 (E14). All intact conceptuses recovered from recipient ewes were edited by CRISPR (23/23) and 10/23 were KO. Conceptus growth was not affected by PGR ablation (1.3±0.3 vs. 3.7±1.3 cm, mean±s.e.m. for edited non-KO and KO, respectively, Two-Way ANOVA p>0.05) and all conceptuses showed hypoblast migration. Embryonic disc was present in 7/10 (70%) KO and 12/13 (92%) edited non-KO conceptuses and embryonic disc size was not affected by the ablation (333±72 vs. 234±43 µm, mean±s.e.m. for KO and edited non-KO, respectively, Two-Way ANOVA p>0.05). In conclusion, the ablation of PGR does not impair embryo development up to E14, suggesting that progesterone-mediated conceptus growth enhancement is indirectly mediated by triggering changes in the uterus. Supported by StG-757886 from ERC and PID2020-117501RB-I00 from MINECO.

Keywords: CRISPR, progesterone, embryo development, conceptus elongation

Animal Reproduction

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Embryology, developmental biology, and physiology of reproduction

Microplastics have a negative effect on sperm and oocytes in vitro

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An annual increase in global plastic production, in addition to poor waste management, represents a massive contamination of the environment. These plastics pollutants can break down to small particles, and when fragmented to a size smaller than 5 mm, are called microplastics (MPs). A decline in fertility was already pointed out by WHO as a major health issue and, considering the lack of studies relating MPs and reproduction, for better understanding of its effects, more studies are necessary. Therefore, the present study aimed to investigate if MPs can affect gametes in vitro. Bovine frozen semen (N = 4 bulls) were thawed and submitted to swim-up separation. 5x106 sperms/mL were then incubated with a range of sizes of polystyrene (PS) beads (SURF-CAL™ particle size standards): 0.05, 0.1, 0.3, and 1.1 µm (1.2 million beads/mL), and CellRox (2 µL/mL) using FERT-TALP for 2 hours at 38.5ºC, 5% CO2, 95% O2. Every 30 minutes an aliquot was collected, checked for motility (N=9 replicates), spread in a SuperFrost slide and fixed with 4% paraformaldehyde (PFA). Fixed samples were stained with Hoechst33342 and FITC-PNA, to check acrosome integrity (FITC-PNA, N=4), bead attachment (N=3 replicates) and oxidative stress (CellRox; N=2 replicates) by fluorescence microscopy. Oocytes were isolated from bovine ovaries by follicle aspiration and only those with a homogeneous cytoplasm and at least three layers of cumulus cells were selected. Oocytes were randomly assigned to 3 groups: (1) 0.3 µm PS beads (1.2 million beads/mL; N=46); (2) 1.1 µm PS beads (1.2 million beads/mL; N=64), and (3) control (N=52) no beads, and matured for 24h at 38.5ºC, 5% CO2, 95% O2; three replicates were performed. COCs were then washed, denuded by pipetting, fixed in 4% PFA, and stained with Hoechst33342 (5 µg/mL) for nuclear stage checking. Oocytes were classified as mature (MT), degenerating (DG), or broken zona pellucida (BZP). All data was analyzed for normality using the Shapiro-Wilk test, a two-way ANOVA was used and the differences checked using Tukey HSD. Both 0.3 and 1.1 µm beads attached to the sperm surface in 6.9±4.8 and 2.1±18% of sperm counted, respectively. Even though a small attachment of beads was identified, they did not affect sperm motility (p>0.05). However, sperm incubated with 1.1 µm beads had reduced acrosome integrity at 2h compared to the control (32.3±6.5 vs 59.1±20.5, respectively, p=0.001). The results for oxidative stress indicate a small increase of ROS production on sperm incubated with MPs, but no significant differences were detected and more replicates need to be done. Oocytes matured in the presence of MPs had a reduced maturation rate (67.5±12.5%, 41.9 ± 18.1 and 37.6 ±20.0%, for control, 0.3 and 1.1 µm, respectively). It was also shown that MPs promoted an increase in oocytes with BZP (2.6±4.4%, 17.8 ± 3.4 and 23.4 ±11.4%, for control, 0.3 and 1.1 µm, respectively; p=0.03). Proteomics of oocytes is currently in progress to better understand the molecular mechanisms by which MPs are damaging oocytes. We have shown, for the first time, that PS MPs exert a negative effect on both male and female gametes in vitro, demonstrating that MPs should be treated as concerning environment toxicants.

Keywords: microplastics, infertility, reproduction, polystyrene

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

First evidence of nanoplastic uptake by the maturing oocyte

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Plastic pollution has become a growing environmental problem as the result of its widespread use and improper treatment of plastic waste. Over time, plastics that leaked into nature will degrade into smaller particles including micro- and nano plastics (MNPs). MNPs can enter the human body through ingestion, inhalation, and dermal contact (Parta et al., Sci Total Environ, 702:134455, 2020). Previous studies have shown that MNPs can induce an immune response and can cause a neurologic, oxidative, and toxicity response in somatic cells, including those of the reproductive tract in both mammals and aquatic organisms (Xie et al., Ecotoxicol Environ Saf, 190:110133, 2020; Liu et al., J Hazard Mater, 424:127629, 2022; Nie et al., Nanotoxicology, 15;885, 2021). However, the impact of MNPs on oocyte and embryonic development remains largely unknown. Furthermore, it is unknown whether oocytes take up MNPs from the environment.

In this study, the uptake of MNPs by developing bovine cumulus-oocyte-complexes (COCs) and their effects on development have been studied. Given the large similarities in reproduction and early development during early embryonic development between human and cow, the bovine model is an excellent model to study human oocyte and early embryo development (Sirard, In: Animal Models and Human Reproduction, 127-144, 2017; Eds: Constantinescu & Schatten; Wiley & Sons Inc.; ISBN:9781118881606). COCs, collected from slaughterhouse ovaries, were exposed to our standard maturation condition without, control condition (NaHCO3-buffered M199 supplemented, with 100 IU/ml Penicillin-streptomycin, 0.05 IU/mL FSH, 0.1 µM cysteamine, and 10 ng/mL EGF) or with green-fluorescent polystyrene (PS) nanoplastics (Polysciences, Inc., Hirschberg an der Bergstrasse, Germany) of 50nm or 200nm (10 µg/mL) during the 23 h *in vitro* maturation (39˚C, 5% CO2 in air). After maturation, COCs were stained with Hoechst (DNA) and phalloidin (actin), to score the nuclear stage of matured oocytes (one-way ANOVA), and uptake of MNPs, respectively.

Confocal microscopy showed that MNPs of 200nm were only taken up by some cumulus cells, while MNPs of 50nm were taken up by cumulus cells and oocytes. In total, 351 oocytes in 3 replicates were analysed, there was no difference between the nuclear maturation status after exposure to MNPs of 200nm (59.9%) and 50nm (46.5%) during maturation, in comparison to control oocytes (60.8%). In conclusion, bovine oocytes are able to take up 50nm PS particles while cumulus cells are able to take up both 50 and 200 nm PS particles. However, the current experiment did not show an effect of the uptake of plastics on the nuclear stage of the oocyte after maturation. Future studies need to unravel whether the uptake of MNPs may impact oocyte competence.

Keywords: micro- and nanoplastics, oocyte, bovine embryo model

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Effects of palmitic acid-induced lipotoxicity on epigenetic programming in zygotes and morulas during bovine in vitro embryo production

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Maternal metabolic disorders are associated with subfertility. Upregulated lipolysis causes a rise in non-esterified fatty acids in the blood, which is reflected in the follicular and oviductal micro-environment. This has a lipotoxic effect on oocyte and embryo development, mainly due to elevated palmitic acid (PA) concentrations. Surviving embryos may exhibit persistent defects in later life due to epigenetic alterations as oocyte maturation and early embryo development involve dynamic changes in epigenetic reprogramming and may therefore be vulnerable to changes in their micro-environment. We hypothesized that short-term exposure to PA during oocyte maturation and early embryo development can alter epigenetic patterns in the resulting embryos. To test this hypothesis, a validated bovine IVP model was used, where oocytes were exposed to standard (CONT) or solvent (SCONT) media, or a pathophysiological concentration of PA (150µM, BSA 7.5 mg/ml) during IVM (24h). Oocytes were then *in vitro* fertilized (for 20h) and presumptive zygotes were cultured in the corresponding CONT, SCONT or PA (230µM, BSA 20 mg/ml) media, respectively (3 groups in total). 12 replicates were performed (858-1630 COC's/treatment). Cleavage rates were recorded at 48h post insemination (p.i.) (12 replicates) and blastocyst rates at 8d p.i. (4 replicates). Zygotes (60/treatment, 6 replicates) were collected at 20h p.i. and morulas (70/treatment, 8 replicates (not used to record blastocyst rates)) were collected at 4.7d p.i. and were fixed for 5mC and H3K9ac/H3K9me2 immunostaining and confocal microscopy to assess global DNA methylation and histone acetylation/methylation, respectively. Developmental competence data were analysed using logistic regression, and numerical data with one-way ANOVA (5mC/H3K9ac) or Kruskal-Wallis test (H3K9me2) with post-hoc Bonferroni correction depending on normality of distribution. For developmental competence, 5mC, and H3K9ac, no effects of the solvent could be detected compared to CONT. H3K9me2 mean gray intensity was significantly lower in SCONT compared to CONT in zygotes (22.2% reduction, P=0.011) and morulas (13.5% reduction, P=0.001). Exposure to PA during IVM and IVC resulted in significant reduction of cleavage (63.9 ± 4.7% vs. 79.5 ± 2.6%, P<0.001) and blastocyst rates (25.2 ± 4.9% vs. 39.2 ± 2.6%, P=0.005) compared to SCONT. 5mC mean grey intensity was not altered in PA-exposed zygotes (P=0.432) but was increased in morulas (27.4% increase, P<0.001). H3K9ac was significantly increased in zygotes exposed to PA (32.5% increase, P<0.001), but not in morulas (P=0.913). H3K9me2 was significantly increased in PA-exposed zygotes (46.3% increase, P<0.001) and morulas (15.5% increase, P=0.039). We conclude that a lipotoxic micro-environment during bovine IVM results in increased histone acetylation and methylation already at the zygote stage. Continued exposure during IVC was associated with increased histone and DNA methylation in the morulas. These upregulated epigenetic marks may cause altered gene expression and imprinting, resulting in aberrant embryonic cell differentiation. We are currently investigating potential mechanisms through which these changes occur.

Keywords: bovine in vitro embryo, epigenetics, lipotoxicity

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Glyphosate affects mRNA expression pattern of bovine oocytes and belonging cumulus cells

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Glyphosate (Roundup®) is a non-selective systemic herbicide widely used. There is some evidence that Roundup® (R-Gly) can act as an endocrine disruptor. Cleavage and developmental rates were significantly decreased for embryos stemming from cumulus-oocyte complexes generated with 300 µg/mL R-Gly during IVM (36.2% ± 16.6; 4.9% ± 4.5) compared to those of two other groups (0 and 30 µg/mL R-Gly; 73.9% ± 11.1, 80.3% ± 7.1; 31.7% ± 11.2; 29.5% ± 11.5). A significant P4 and E2 increase was detected in the maturation medium from the 300 µg/mL group. These data indicate that a supraphysiological R-Gly concentration during IVM affects steroid synthesis of cumulus cells and subsequent embryo development (Blad-Stahl et al. 2020; Reprod Dom Anim, 55, 6-7). The aim of this study was to evaluate the effect of different Roundup® concentrations (0, 30, 300 µg/mL R-Gly) supplemented during in vitro maturation (IVM) on mRNA expression patterns in oocytes and belonging cumulus cells before and after in vitro maturation which means for oocytes at the GV and MII stage, respectively. Gene transcripts were analyzed via a RT-qPCR assay as described previously (Blaschka et al. 2019; Theriogenology 131, 182-192). MessengerRNA (mRNA) from single oocytes and total RNA from the belonging cumulus cells was directly used for the reverse transcription (RT). The relative expression ratio of a target gene is calculated based on efficiency and the crossing point deviation (Ct-values) of an unknown sample versus a reference gene. At least 6 IVM runs and 3 replicates from different IVM for the RNA analyses were undertaken. Selected transcripts are involved in steroid metabolizing processes [cytochrome P450 monooxygenase (CYP19), hydroxysteroid dehydrogenase 3 beta (HSD3β), luteinizing hormone/choriogonadotropin receptor (LHCGR), follicle-stimulating hormone receptor (FSHR), estrogen receptor alpha (ERα), progesterone receptor (PGR), progesterone receptor membrane component 1 (PGRMC1), progesterone receptor membrane component 2 (PGRMC2) in cumulus cells and PGR, PGRMC1, PGRMC2, and steroidogenic acute regulatory protein (STAR) in oocytes]; and in oocyte growth and development [zygote arrest protein 1 (ZAR1), growth differentiation factor 9 (GDF 9), bone morphogenetic protein 15 (BMP15), glucose-6-phosphate dehydrogenase (G6PD9]. In cumulus cells, all transcripts, except PGRMC2, showed a significant increase in the relative abundances after IVM. For PGRMC2, a decrease was detectable. Supplementation of the high R-Gly concentration affected the mRNA expression of HSD3β, LHCGR, FSHR, ERα, PGR and PGRMC1. During IVM of oocytes, a significant decrease of GDF9, ZAR, and STAR transcripts was determined, whereas it was an increase for PGR mRNA. A significant increase was also measurable in oocytes being matured in medium supplemented with the high R-Gly concentration for G6PD, whereas the relative mRNA abundance was similar compared to immature oocytes for GDF9, ZAR, and STAR transcripts.

These data indicate that during IVM a supraphysiological R-Gly concentration affects the mRNA expression of gene transcripts related to steroid metabolism in cumulus cells and that in oocytes especially related to oocyte growth.

Keywords: glyhosate, IVM, bovine

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Effects of a polylactic acid, 3-D printed scaffold, on bovine embryo development in vitro

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The development of 3D printers and the advances in materials science and tissue engineering in the last years have allowed the implementation of this technology in several research areas. So, it has been used as a tool to create a 3D-oviduct-on-a-chip with poly(dimethylsiloxane) that mimics de physiological environment, which has proved to be useful in reducing the differences between in vitro and in vivo produced embryos (Ferraz et al, Nat commun, 9(1), 1-14, 2018). As an alternative, we suggested polylactic acid (PLA) as a great candidate to generate engineered 3D scaffolds due to its high biocompatibility and mechanical properties (Chi et al, BMC chem, 14(1), 1-12, 2020). Our goal was to evaluate for the first time, the feasibility of PLA scaffolds printed by the fused filament fabrication method to support IVF, by means of resulting blastocyst rates and total cell number. IVF was performed under 3 different conditions (N=4 replicates): i) Conventional IVF (Parrish et al., Biol Reprod, 38(5), 1171-1180, 1988) (control group, N=360) ii) IVF in a medium conditioned by the scaffold for 24h (rinse group, N=215) and iii) IVF inside the scaffold used for the rinse group (scaffold group, N=190). Before IVF, the scaffolds were sterilized in 70% ethanol for 1h, washed 4 times for 5 min in PBS and air-dried at room temperature. For IVF, in vitro matured oocytes were washed and cultured in Fert-TALP medium (Parrish, Theriogenology, 81(1), 67-73,2014) for 22h with frozen-thawed bull sperm ($1x10^6$spz/ml) selected by Bovipure gradient (Nidacon, Sweden). Putative zygotes were cultured in microdrops of SOF medium (Holm et al, Theriogenology, 52(4), 683-700, 1999) supplemented with 0.3% BSA (w/v) covered with paraffin oil (Nidoil, Nidacon). Cleavage (48h) and blastocyst rates (day 8) were evaluated. At day 8, blastocysts were fixed in glutaraldehyde and stained with Hoechst 33342 to assess their cell number by fluorescence microscopy. The parameters were analyzed by Kruskal-Wallis one-way ANOVA test when the distribution was not normal and by one-way ANOVA when it was. Differences were considered significant when $p<0.05$. Regarding cleavage rate, it was significantly higher in the control group (79.4±2.1%^{a}) than in the rinse and scaffold groups (30.7±3.2%^{b} and 52.6±3.6%^{c}, respectively). Furthermore, the control group showed a higher blastocyst rate (25.6±2.3%^{a}), than the scaffold and rinse groups (14.1±2.5%^{b} and 2.3±1.0%^{c}, respectively). As for the total cell number, no significant differences were found (78.2±3.6 for control, 89.6±22.3 for rinse, and 79.4±7.0 for scaffold). It's known that the incubation of PLA in PBS causes a rapid drop in pH on the first day, probably due to the release of lactic acid, a known PLA degradation byproduct (Diomede et al, Stem Cell Res Ther, 9(1), 1-21, 2018). This fact could explain the differences between groups, since the rinse group contained the medium that had been in contact with PLA for 24 hours. Further experiments controlling the post-culture pH could corroborate such hypothesis. In conclusion, the current data demonstrates that PLA does not seem te be applicable to use for 3D scaffolds for IVF. Supported by Fundación Séneca reference 21651/PDC/21.

Keywords: Polylactic acid (PLA), IVF, embryo development, bovine

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Can a maternal obesogenic diet influence offspring oocyte lipid droplets and mitochondria?

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Consumption of an obesogenic (OB) diet is linked with infertility. Hyperlipidemia alters the ovarian follicle microenvironment and induces lipotoxicity in the oocytes, mainly characterized by mitochondrial (MT) dysfunction and lipid accumulation. Increased reactive oxygen species (ROS) production by the defective MT leads to oxidative stress and reduced oocyte quality. Since MT are exclusively maternally inherited, transmission of aberrant MT from the oocyte to the embryo may alter MT functions in the offspring germline. In addition, the obesogenic maternal uterine and lactation environment can also impact the developing offspring oogonia, which may lead to defective oocyte MT in newborns. Therefore, we hypothesized that oocyte lipid content and MT are not only affected by an OB diet but also by the mother's obesogenic background.

To test this hypothesis, female Swiss mice were fed a control (C, 10% fat, 7% sugar) or OB diet (60%fat, 20% sugar) for 7 weeks (w), then mated with the same males. Female offspring from each litter were equally weaned on a C or OB diet in a 2x2 factorial design, resulting in 4 treatment groups: C»C, C»OB, OB»C and OB»OB. Per treatment group, at least 5 oocytes per offspring (at least 7 females) from 7-8 C mothers or 6-8 OB mothers were collected at 10w of age after hormonal stimulation (10IU PMSG and 10IU hCG i.p.). Oocytes were stained and imaged (LeicaSP8 Confocal microscope) to quantify lipid droplet (LD) content (BODIPY, $x10^3$ μm^3), MT inner membrane potential (MMP) (JC1) and ROS (CellRox Deep Red) ($x10^3$ pixel intensity). Data were analyzed in SPSS using two-way ANOVA, and shown as mean±SEM. In addition, active MT distribution patterns were categorized as peri-cortical, diffuse or aggregated, analyzed using generalized linear models and presented as mean%±SEM.

No interactions between maternal and offspring diets were observed. However, LD content was affected by offspring diet (*P*=0.000), irrespective of the maternal diet, as it was significantly increased both in C»OB compared to C»C (7.2±0.3 vs 5.7±0.3) and in OB»OB vs OB»C (6.8±0.3 vs 5.5±0.3). Similarly, MMP was significantly increased only by offspring diet (*P*=0.025), both in C»OB vs C»C (55.7±2.9 vs 46.9±2.3) and in OB»OB vs OB»C (56.3±2.7 vs 52.9±3.0). Comparably, ROS accumulation was only affected by offspring diet (*P*=0.007), and was also higher in C»OB vs C»C (31.9±2.6 vs 24.2±2.0) and in OB»OB vs OB»C (30.6±3 vs 25.3±2.4). MT distribution was not affected by offspring diet. In contrast, maternal diet significantly increased the proportion of MT aggregation (*P*=0,016), irrespective of the offspring diet, as it was increased in OB»C vs C»C (6.3%±4.7 vs 0%±0.0) and in OB»OB vs C»OB (7.8%±4.1 vs 0%±0.0). This category exhibited high ROS accumulation and very low MT MMP.

In conclusion, while we could confirm the increase in LD content, MMP and ROS in oocytes upon direct exposure to an OB diet, it appears that oocyte MT in offspring born to obese mothers have more MT aggregation with an increased ROS accumulation and a low MMP. This study stresses the importance of a heathy dietary intake for both mother and offspring to guarantee oocyte quality.

Keywords: intergenerational effects, oocyte quality, obesogenic diet

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Role of secreted proteins and heparan sulfate on sperm binding to oviduct epithelial cells in cattle

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After insemination in mammals, the elite of spermatozoa reaches the first part of the oviduct (isthmus) where they can bind to oviduct epithelial cells (OEC) to form a sperm reservoir (Lefebvre et al., Biol Reprod, 1995). In cattle, sperm receptors on OEC surface include glycoproteins which are also secreted in the oviduct fluid (OF) and interact with sperm as soluble proteins (Lamy et al., Reproduction, 2018). Sulfated glycosaminoglycans (sGAG) are other macromolecules secreted in the OF and known to interact with sperm surface (Plante et al., Cell Tissue Res, 2015). Moreover, heparin, a sGAG which is not present in the bovine OF, was shown to release bovine sperm from OEC (Talevi and Gualteri, Biol Reprod, 2001). How the proteins and sGAG present in the OF before ovulation modulate sperm adhesion to the oviduct reservoir is currently not known. The objective of this study was to assess the roles of soluble proteins and heparan sulfate, one of the most abundant sGAG in the bovine OF, on the binding of sperm to OEC.

Bovine isthmic OEC were collected from pre-ovulatory oviducts at a local slaughterhouse and cultured for 4 days (TCM199, 10% FCS, 38.8°C) to form spheroids with the apical side of OEC oriented outside. Groups of 20 spheroids ~100 µm in diameter were incubated with frozen-thawed Percoll-washed sperm from 3 bulls at 1.10^6/mL for 1 h at 38.8°C in a non-capacitating medium as control (TALP-HEPES + pyruvate/lactate/PVA; pH=7.4) ; OF at 1, 2 and 4 mg/mL of proteins; fractions of OF at 1 mg/mL after ultrafiltration (cut-off at 3 kDa); OF after heating (complement inactivation, 56°C, 30 min); OF after boiling (protein denaturation, 95°C, 5 min) or protein digestion (proteinase K); OF after heparan sulfate lysis (heparinase I/II/III treatment). After incubation, sperm-spheroid complexes were washed, fixed and stained with Hoechst for determination of bound sperm density using confocal microscopy. Sperm viability and motility were analyzed during incubation by flow cytometry and CASA, respectively. ANOVA followed by Tuckey post-tests were used to analyze the data.

Co-incubation of sperm with OF at 1, 2 and 4 mg/mL of proteins had no effect on sperm motility and viability but decreased significantly the sperm density on spheroids compared to controls with a dose effect ($p<0.001$). The OF fraction > 3kDa (containing most proteins and sGAG) but not < 3kDa reproduced the effect of OF on sperm density ($p<0.001$). Complement inactivation and protein denaturation did not reproduce the effect of OF on sperm binding. OF after protein or heparan sulfate digestion tended to reduce sperm binding compared to control ($p=0.08$ but to a lesser extent than native OF ($p<0.01$).

In conclusion, macromolecules in the pre-ovulatory OF decreased the ability of a sperm subpopulation to bind to OEC. Heparan sulfate together with other sGAG/proteoglycans and glycoproteins seem to be responsible of this effect.

Keywords: spermatozoa, oviduct epithelial cells, oviduct fluid

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

MALDI-TOF lipidomic imaging of the oviduct after short and long-term exposure to an obesogenic diet in outbred mice.

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Metabolic disorders associated with the consumption of an obesogenic (high fat/high sugar (HFHS)) diet are strongly linked with reduced fertility in women. Direct detrimental effects of such metabolic alterations on oocyte quality have been documented, however the impact on the oviductal microenvironment where fertilization and early embryo development take place, is less characterised. Furthermore, when such changes appear after the start of a HFHS diet remains unclear. The aim of this study was to test whether the introduction of a HFHS diet in mice can lead to changes in lipid composition in the oviductal epithelial cells (OECs) and when these changes start to appear. Seven week old female outbred Swiss mice were fed with either control (CTRL; 10% fat) or HFHS (60% fat in diet, 20% fructose in drinking water) diet. Mice (n=3 per treatment per time point) were sacrificed and oviducts were collected at 3 days (3d), 1 week (1w), 4w, 8w, 12w and 16w after the start of dietary treatment. MALDI mass spectrometry imaging was performed to image lipids on sections of the oviduct (ampulla) using a Rapiflex MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) with norharmane as matrix. Spectra at mass range of m/z 400-2000 were obtained in positive and negative reflectron modes (10µm resolution). Data sets were analyzed with SCiLS Lab 3D, version 2016b. Spatial segmentation of ion spectra showed differences in spatial distribution of lipid species between the oviductal epithelium and stroma. For further analysis we focused on the OEC layer, which was identified by co-registering the MS images with the optical scans of the H&E staining of the same section. Spectra from the OEC cluster were subjected to Receiver Operating Characteristic (ROC) analysis to calculate discriminative m/z values and determine differentially regulated lipids (DRLs) in the HFHS versus CTRL mice at each time point. Assignments were done from MS/MS spectra. ROC analysis revealed a different lipid profile in HFHS oviducts compared to the CTRL, which was shown by the detection of 303 and 247 discriminative masses (DMs) between HFHS and CTRL mice in negative and positive mode respectively, when including all time points. The total number of detected DMs in both reflectron modes increased over different time points, in negative mode this is from 10 DMs at 3d, 40 at 1w, 44 at 4 and 8w, to 55 at 12w and finally 110 DMs between CTRL and HFHS mice at 16w. The DRLs (across all time points) were focused in specific mass ranges, namely around 700-900 m/z in both modes which indicates differential abundance in phospholipids (phosphatidyl(P)-choline, P-serine, P-ethanolamine, P-inositol) and sphingomyelin. A few DRLs were detected around 1500 m/z suggesting a differential abundance of the mitochondrial lipid cardiolipin. In conclusion, exposure to an obesogenic diet results in changes in lipid profile in the oviductal epithelium even after a short exposure time of only 3 days. These changes progressively increase after longer exposure. Further analysis is ongoing to functionally annotate the detected DRLs and study their potential pathophysiological impact on the oviductal microenvironment and ultimately on the growing embryo.

Keywords: obesogenic diet, oviduct, MALDI imaging

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Monounsaturated oleic acid addition during early embryonic development increases bovine blastocyst rates.

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Metabolic stress, characterized by elevated levels of free fatty acid (FFA), have been linked to reduced female fertility. Saturated FFA (stearic acid (SA)) appears to have a dose-dependent negative effect on oocyte developmental competence while monounsaturated oleic acid (OA) is shown to be harmless. Oocytes are protected against FFA by cumulus cells via stearoyl-CoA desaturase 1 (SCD1) activity, which converts saturated SA into mono-unsaturated OA. To study FFA effects on early embryonic development, embryos were cultured in the presence of SA and OA and SCD activity was analyzed.

Cumulus-oocyte-complexes (COCs), collected from 2-8 mm sized follicles of bovine slaughterhouse ovaries, were *in vitro* matured (n=400/run) and fertilized (Aardema et al., Biol Reprod; 85: 62-69, 2011). During the first five days of embryo culture (day 1-5; i.e. oviductal period), embryos were cultured in SOF without (control) or with FFA (FFA conc.= 25 and 50 µM OA; 25 and 50 µM SA; 25 or 50 µM OA + 25 or 50 µM SA). Fatty acids were complexed to fatty acid free BSA (10 mM) (FFA:BSA ratio of 5:1). FFA was conjugated to albumin, likewise the transport of FFA *in vivo*, to solubilize FFA in an aqueous solution. At day 8 the number of blastocysts was counted. With RT-qPCR the mRNA expression of *SCD1* was measured in all the conditions. The general linear model was used for statistical analysis with SPSS 27.0. The day 8 embryos, COCs (positive control) and oocytes (negative control) were fixated in 4% PFA and incubated with a primary antibody, SCD1, diluted 1:100 in PBST overnight at 4°C for immunostaining. Thereafter, embryos were washed 3 times in PBST for 15 min and incubated with the second antibody, goat anti-rabbit Alexa™ fluor 647, diluted 1:100 in PBST for 1h in the dark at RT. Confocal microscopy was performed using an inverted Nikon A1R confocal microscope to determine the presence of SCD1 protein.

Exposure to 25 and 50 µM SA from day 1-5 resulted in a significantly lower blastocyst rate of respectively 18.9 ± 1.6% and 2.6 ± 4.6%, compared to the control condition 25.9 ± 3.1% (n=3; $p<0.05$). Interestingly, exposure to 25 and 50 µM OA resulted in a significantly higher blastocyst rate, 36.4 ± 6.3% and 34.6 ± 7.3% respectively (n=3; $p<0.05$). Exposure to 25 µM OA + 25 µM SA resulted in a blastocyst rate of 26.0 ± 3.7% comparable to the control condition and was not significantly different from exposure to 50 µM OA + 50 µM SA (25.6 ± 5.5%) (n=3; $p>0.05$). Interestingly, exposure to 50 µM OA + 25 µM SA resulted in a blastocyst rate of 33.7 ± 9.3% comparable to the 50 µM OA condition (n=3; $p<0.05$). SCD1 was faintly expressed at RNA and protein levels in day 8 embryos. Nevertheless, SCD1 was stronger detected in embryos compared to oocytes.

Previously, our group demonstrated no detectable SCD1 protein levels in oocytes, but solely in cumulus cells. We here showed that day 8 embryos express SCD1, which may protect embryos against saturated FFA. The current culture data show that OA counteracted the negative effect of SA on embryos. Future studies should investigate the role of OA and SCD1 in embryos.

Keywords: embryo; free fatty acid; stearoyl-CoA desaturase.

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

The oviduct expresses the protein stearoyl-CoA desaturase 1 that converts saturated into mono-unsaturated fatty acids

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Reduced reproductive performance is an important problem in dairy cattle and has been related with the negative energy balance (NEB) during the first weeks postpartum. A major characteristic of the NEB are elevated concentrations of non-esterified free fatty acids (FFA), after mobilization from body fat reserves, in the blood and follicular fluid. Previous studies demonstrated that saturated FFA, like stearic acid (SA), have a negative effect on the developmental competence of the oocyte. In contrast, unsaturated FFA like oleic acid (OA) can reverse the negative effect of saturated FFA on oocytes (Aardema et al., Biol Reprod, 85, 62-69, 2011). Cumulus cells that surround the oocyte are proven to protect the oocyte for SA stress via the enzyme stearoyl-CoA desaturase 1 (SCD1), which converts saturated into unsaturated fatty acids (Aardema et al., Biol Reprod, 96, 982-992, 2017). However it is not clear whether the embryo is protected after release of the cumulus cells. In this study the focus lays on the oviduct, where the bovine embryo resides during the first 5 days after fertilization. The possibility that SCD1 is present in oviductal cells, and may act as a protector, was tested. Both fresh and cultured bovine oviductal epithelial cells (BOECs) originated from slaughterhouse material, were lysed before Western blot examination for the presence of SCD1. The antibody against SCD1, was kindly provided by Dr. Corl (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA). Fresh oviductal tissue was isolated of bovine genital tracts, treated with liquid nitrogen and the piece of fresh oviductal tissue was smashed with a hammer. Then it was lysed with RIPA buffer (Thermo Fisher Scientific, Waltham, MA, US). Udder material, which was the positive control, was treated the same way. To collect BOECs, epithelial cells were squeezed out of the oviduct and lysed with RIPA buffer. Alternatively, the BOECs were cultured on a porous membrane for two weeks in order to obtain a confluent BOEC monolayer which was confirmed by TEER and Tracer-Flux measurements (Leemans et al., Biol Reprod, 106, 710-729, 2022). After one week BOEC differentiation was initiated by an air-liquid culture period of one week. The BOEC monolayer was lysed with RIPA buffer. The positive control showed the SCD1 specific 37 kDa band, which was also present in the isthmus region of the oviduct. However the band was not detectable in the ampulla region, squeezed epithelial cells and BOECs. However, SCD1 expression was visualized in the BOECs by the Nikon A1R confocal microscope, using the primary antibody of SCD1 by Dr. Corl, and its binding was visualised using goat anti-rabbit Alexa Fluor 647 (Thermo Fisher). Beyond the predicted 37 kDa band, an additional band was observed around 50 kDa in lysates of the isthmus and ampulla region, and the positive control. This 50 kDa band has been described for an commercial anti-SCD1 antibody (ab19862, Abcam, Cambridge, UK). The extra band could indicate an isoform of SCD1 or PTMs. The presence of SCD1 indicates that the oviduct is capable to convert saturated into unsaturated fatty acids, which may protect the bovine embryo during metabolic stress during early development.

Keywords: oviduct, fatty acids, stearoyl-CoA desaturase

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Effects of endocrine disruptors ketoconazole and diethylstilbestrol on BOEC air-liquid interface monolayer culture

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Reproductive disorders, a worldwide public health concern, have been associated with exposure to endocrine disrupting chemicals (EDCs). Two well-known EDCs to influence female reproductive health are diethylstilbestrol (DES; a synthetic estrogen agonist) and ketoconazole (KTZ; a CYP450 steroidogenesis enzyme inhibitor). Here, the effects of DES and KTZ on a bovine oviduct epithelial cell (BOEC) culture, an *in vitro* animal model of the first embryo-maternal contact site (i.e. the oviductal epithelium), are explored. To establish a BOEC monolayer an air-liquid interface (ALI) culture approach was adopted, which supports cell differentiation (Chen et al., Sc. Reports, 7, 2017). Reported KTZ and DES effects include ALI-BOEC monolayer permeability, confluency, and actin organisation.

BOECs were mechanically isolated from the lumen of oviducts, obtained from slaughterhouse cows post-mortem. BOECs ($5x10^5$ cells) were seeded and cultured for 8 days in Transwell® cell culture inserts (Corning, USA, NY, CLS3413, 6.5mm). To introduce an ALI apical media was removed, while basolateral media was maintained. At day 14 of ALI, BOEC monolayers were exposed for 4 days to DES (10^{-9} M, 10^{-7} M, 10^{-5} M) or KTZ (10^{-8} M, 10^{-7} M, 10^{-6} M), or 0.01% v/v DMSO (vehicle). Effects of DES and KTZ on the permeability of the BOEC monolayer were assessed by transepithelial electrical resistance (TEER) measurement and paracellular tracer flux assay. TEER measurements confirmed confluency in all BOEC monolayers, with no significant difference between DES and KTZ treated vs DMSO treated monolayer (one-way ANOVA). The cell-impermeable tracer fluorescein disodium salt (12 µg/mL, 0.4kDa) was used for the tracer flux assay. Apical media was supplemented with the tracer and, after a 2h incubation, the basolateral media was collected to measure fluorescence. There was low percentage (<1.2%) of total tracer transferred from the apical to the basolateral side, and not significantly different between DES- or KTZ-exposed vs vehicle treated monolayers (one-way ANOVA). Consistent with TEER and tracer flux data, confocal microscopy of stained (phalloidin, acetylated α tubulin, Hoechst 33342) BOEC monolayers further supported confluency after DES and KTZ exposure. Clear basolateral cell-cell and cell-membrane adhesion was observed for all monolayers. In contrast to the membrane-adhering side of DES- or KTZ-treated BOECs, lateral cell-cell connections were scarce towards the apical side of the cells. This effect was similar in all doses of DES or KTZ and evidenced by gaps between phalloidin staining of individual cells, and it was not observed in DMSO treated BOECs.

In conclusion, DES and KTZ induced abnormalities in BOEC cytoskeletal organisation. We hypothesize that cell-cell junctional contacts in BOECs are disturbed by these EDCs. This distortion has an effect on cell polarity, and may also change epithelial cell binding and secretion properties. The possibility that this may indirectly cause aberrant early embryo-development, which could differ from direct exposure of these EDCs to the embryo cultures, is under current investigation.

Keywords: oviduct epithelium, endocrine disruptor, cytoskeleton abnormalities

Acknowledgements

The project is funded by the EU Horizon 2020 Research and Innovation Programme Grant No 825100 (FREIA)

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Uptake evaluation of bta-miR-181d present in extracellular vesicles from bovine oviductal and uterine fluids by in vitro produced embryos

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through post-transcriptional mechanisms. We have found that bta-miR-181d was more abundant in extracellular vesicles (EVs) isolated from uterine fluid during mid-luteal phase (Days 5-10 of the estrous cycle) than in EVs from the oviductal fluid recovered during the early luteal phase (Days 1-4 of the estrous cycle) in cattle. In addition, bioinformatic analysis indicated that this miRNA is related to Hippo and WNT biological pathways, both critical for lineage segregation and blastocyst formation. Therefore, we aimed to determine whether bta-miR-181d is uptaken in bovine in vitro produced embryos by passive transfection (gymnosis). Presumptive zygotes (PZ) produced by in vitro maturation and fertilization were cultured in SOF (Control) or supplemented with 1 µM miR-181d mimics (miRCURY LNA miRNA Mimics; Qiagen, Maryland, USA); or 1 µM control mimics fluorescently labeled (miRCURY LNA miRNA Mimic 5'FAM, Nº 339173, Qiagen). Embryos were collected at ≥16-cell (≥16C) and D7 blastocyst (BD7) stages and snap-frozen in LN_2 (3 pools n=10/ group) to examine the expression pattern of miR-181d by qPCR using miRCURY LNA miRNA PCR Assay. To confirm the uptake of control mimics fluorescently labeled, BD7 (n=10/group) were fixed, stained with Hoechst 33342, and observed under a widefield fluorescence microscope. Data were transformed by arcsine square root and tested for normality prior to One Way ANOVA. Embryo development was not affected by the presence of miR-181d or control mimics in the media (cleavage rate/PZ: 88±6.0%, 86±1.6%, 87±1.5% and blastocyst yield/PZ on Day 7: 25±0.8%, 25±2.2%, 24±0.1% for miR-181d mimics, control mimics and control respectively, P>0.05). Fluorescent staining showed that the control mimics can be taken up in blastocysts by gymnosis; however, expression of miR-181d mimics did not differ between groups, suggesting that embryos failed to incorporate this miRNA via this mechanism. Consequently, a second experiment was conducted to test Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, USA) for the delivery of miR-181d mimics. Hence, presumptive zygotes were cultured in SOF (Control) or supplemented with 50 nM miR-181d mimics and 1.5 µL Lipofectamine; or 50 nM control mimics and 1.5 µL Lipofectamine. Embryos (≥16C and BD7) were snap-frozen in LN_2 for future miR-181d expression analyses. Supplementation of Lipofectamine to the culture media did not have any deleterious effect on embryo development (cleavage rate/PZ: 82±4.0%, 81±2.1%, 82±4.1% and blastocyst yield/PZ on Day 7: 25±8.5%, 18±6.4%, 25±3.1% for miR-181d mimics, control mimics and control respectively, P>0.05). In conclusion, despite the fact that the control mimics is uptaken, the miR-181d mimics was not able to internalize in bovine embryos by gymnosis. Lipofectamine does not impair embryo development, hence, it could potentially be used as a carrier for miR-181d. Ongoing work will confirm whether miR-181d is internalized in embryos via this system.

Keywords: mimic miRNAs, early embryo development, bovine

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Abnormalities in centrosome behavior are frequent in the first mitotic division of non-rodent mammalian zygotes

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Post-zygotic or “mosaic” aneuploidy, i.e. the presence of a subset of cells with an aberrant number of chromosomes, is a frequent feature of human preimplantation embryos. A high incidence of aneuploidy within an embryo is recognized as the major cause of developmental arrest and miscarriage. Post-zygotic aneuploidy often arises during the first cell divisions in the embryo and it is not only exclusive of human embryos, but has a similar occurrence in nonhuman primate, bovine, and equine embryos. Despite the wide incidence and often severe developmental consequences of postzygotic aneuploidy, it is still unclear why the early cleavages are so prone to errors.

In somatic cells the centrosomes, formed by two centrioles surrounded by the pericentriolar material, are the two major microtubule-organizing centres (MTOCs) and play an essential role in spindle assembly and chromosomes segregation. Mammalian oocytes lack of centrosomes and, although two centrioles are re-introduced by the spermatozoon at fertilization, we recently showed that centrosomes make only a minor contribution to zygotic spindle assembly. Although not essential for spindle assembly, the role of centrosomes in ensuring fidelity during the zygotic division is still unclear. Here, we evaluated the incidence and consequences of centrosomes abnormalities in the zygotic division of bovine embryos, a species which, similarly to human embryos, inherit centrioles paternally at fertilization. To this end we imaged the first mitotic division in real-time live bovine zygotes (n=55) injected with mRNA encoding for H2B-mCherry and MAP4-eGFP to allow visualization of chromatin an microtubules respectively.

Abnormalities in centrosome behaviour were observed in 40% of the zygotes imaged. The most observed abnormality was failure (15%) or delay (10%; range: 10-30min after nuclear envelope break down) of one of the centrosome to engage to the metaphase spindle. Premature fragmentation of one of both of the centrosomes (10%; range 6-21 min before anaphase) and abnormal positioning of one of the centrosomes (5%) within the mitotic spindle were also observed. Centrosome failure to engage to the mitotic spindle resulted in 60% of the cases in the inability of one or more chromosomes to be captured by spindle microtubules. In contrast, chromosomes lagging after anaphase onset (50%) was observed with the same frequency also in zygotes displaying normal centrosomes behaviour. Non injected zygotes (n=350) fixed at different stages of the first mitotic division showed similar type and frequency of centrosome abnormalities as the ones observed in mRNA injected zygotes. Taken together our observations suggest that centrosomes partially contribute to chromosomes segregation fidelity during early embryonic development, however other players are probably responsible for the high incidence of lagging chromosomes. Further studies are needed to elucidate the reason why in mammalian zygotes centrosomes are less active as MTOCs than in somatic cells.

Keywords: zygote, centrosomes, aneuploidy

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Towards serum-free culture conditions for bovine endometrial explants: some preliminary results

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Bovine endometrial explant culture has emerged as a model for the study of *in vitro* uterine function. Culture medium for endometrial explants is routinely supplemented with fetal bovine serum (FBS) as it contains hormones, vitamins, transport proteins, and growth factors that optimize *in vitro* endometrial function. However, there is an increasing interest in using serum-free conditions as a more defined medium and due to sanitary and animal-welfare concerns (FBS is an animal-derived product). Yet, the optimal serum-free culture medium composition and incubation time window for endometrial explants remain to be elucidated. This study aimed to evaluate the effect of serum and serum-free culture medium and incubation time on endometrial explant viability markers. Five uteri from healthy Belgian Blue cows at the diestrus stage (stage I corpus luteum) with no evident signs of gross inflammation were collected from the slaughterhouse and transported to the laboratory within 1 h. Intact endometrium tissue samples (8 mm in diameter; 1-2 mm in thickness) were obtained using a sterile 8-mm punch biopsy. The explants were cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 50 µg/mL gentamicin and supplemented with, (1) control (no supplementation), (2) 10% FBS, (3) 10% serum replacement (SR) (Knockout™ SR, Gibco), and (4) 1% bovine serum albumin (BSA). Spent culture medium samples were collected at 6, 12, 18, 24, 30, 36, 42, and 48 h of incubation and assayed for interleukins (IL-1β and IL-6) using bovine-specific ELISA kits. Indirect assessment of tissue viability was measured through lactate dehydrogenase (LDH) activity (colorimetric test) in the spent culture media. The effect of culture medium composition, incubation time, and their interaction on IL-1β, IL-6, and LDH concentrations were fitted in linear regression models in RStudio. No differences ($P > 0.05$) within the first 24 h of culture were found among experimental groups for IL-1β. Reduced IL-1β concentrations ($P < 0.05$) were found in FBS compared with SR at 30 and 42 h of incubation. Control and FBS culture medium had lower IL-6 concentrations at 12, 18 and, 24 h compared with the other groups ($P < 0.05$). The LDH activity was higher ($P < 0.05$) for FBS than SR (6 and 18 h) and BSA (12, 18, 24, and 30 h). The present study shows that a serum-free medium is a valid alternative for short-term bovine endometrial explant culture. However, high LDH activity may suggest that endometrial explants viability significantly declines after 24 h of incubation irrespective of culture conditions.

Keywords: Endometrium, explant culture, serum-free médium

Animal Reproduction

Abstracts - 37th Annual Meeting of the Association of Embryo Technology in Europe (AETE)

Embryology, developmental biology, and physiology of reproduction

Overview on the metabolism of buffalo oocyte during in vitro maturation

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The interest in IVEP in buffalo (Bubalus bubalis) has increased worldwide in the last decade, due to the recognized role of this livestock in different environments. It is known that an appropriate oocyte maturation is critical for the acquisition of oocyte competence. The aim of this study was to analyze the variation of metabolite content during IVM of buffalo oocytes, in order to acquire information on oocyte metabolism during maturation. Abattoir-derived buffalo oocytes were divided into two groups (N=50/group, over 5 replicates): immature oocytes (IO) and oocytes in vitro matured (MO) according to standard procedures (Gasparrini et al.,Theriogenology,54,1537-1542, 2000). Immediately after collection and 22h post IVM oocytes were denuded, pooled (N=10) and stored at -80°. To extract the polar fraction, samples were re-suspended in methanol, sonicated for 30 sec for cell lysis, centrifuged (4000 rpm, 10 min) in the presence of chloroform and analyzed by Proton Nuclear Magnetic Resonance (1H-NMR). Pathway analysis on polar metabolites was performed using the Metabo Analyst tool (Xia et al., Nucleic acids research,37,652-660,2009). The Orthogonal Projections to Latent Structures Discriminant Analysis plot evidenced that the two groups clustered separately, suggesting the presence of significant differences in metabolites in relation to maturation stage. The Variable Importance in Projection plot showed the proton signals corresponding to metabolites that showed the greatest variation. Interestingly, among the huge number of detected metabolites, some amino acids such as ornithine, arginine, trimethylamine, asparagine, leucine, proline and alanine were found in lower concentration in MO compared to IO, with the exception of tryptophan that showed an opposite trend. The reduction of the amino acid content may be due to the use of these substrates during maturation for both energetic and biosynthetic processes. After IVM also glucose was reduced while the ATP content was higher, suggesting an increased energy production during maturation. Finally, in MO lower levels of glutathione were found compared to IO, suggesting that oocytes use this thiol to counteract oxidative stress. The pathways enrichment analysis revealed that the metabolites showing variations during IVM are mainly involved in glutathione metabolism, as well as glycine and serine metabolism. These preliminary results can be considered as a starting point to better understand the metabolism of buffalo oocytes during the maturation process. The significantly changes in the metabolite content and the higher ATP levels found in matured oocytes are probably related to the need of producing energy for the subsequent phases of development. However, further studies are needed to deeply investigate buffalo oocyte metabolism, providing information on metabolic needs, useful to improve the IVM system in this species.

Keywords: Buffalo, Oocyte, Metabolomic