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Embryo-induced alterations in the endometrial transcriptome of prepubertal bovine heifers

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Keywords: endometrium, prepubertal, calves.

Advancing the age at which puberty is reached in replacement heifers is central to the financial and environmental sustainability of cattle production systems. Puberty onset is regulated by a complex network of biochemical processes and involves interaction between key metabolic, neuroendocrine and reproductive tissues. Most components that regulate the hypothalamic-pituitary-ovarian axis are in place before the occurrence of puberty. However, it is unclear if the prepubertal uterus is capable of responding to the presence of an embryo or conceptus. Thus, the objectives of this study were to determine the response of the endometrium of 5-month-old prepubertal heifers to i) Day (D) 7 blastocysts (Experiment 1), and ii) a D14 conceptus or 100 ng/ml of interferon tau (IFNT) (Experiment 2), and to compare this response to that of a postpubertal endometrium. Angus X Holstein-Friesian heifer calves (prepubertal group; n= 9) were euthanized at 21 weeks of age. Reproductive tracts were recovered to obtain endometrial explants. For Experiment 1, crossbred postpubertal beef heifers (n= 5) were synchronized and slaughtered on D7 of the cycle (D0 = expected ovulation) to obtain endometrial explants. Twenty D7 in vitro produced (IVP) blastocysts were placed on top of an explant from prepubertal (PreP-D7; n= 5) or postpubertal heifers (PostP-D7; n= 5), and co-cultured for 6 h. For Experiment 2, crossbred postpubertal beef heifers were synchronized and either used to generate D14 conceptuses following the transfer of IVP blastocysts on D7 (n= 9; 15 embryos/recipient) or were used to obtain D14 endometrial explants (n= 5). Conceptuses were recovered on D14 by post-mortem uterine flushing and placed individually on top of explants from prepubertal (PreP-D14; n= 4) or postpubertal heifers (PostP-D14; n= 5) and co-cultured for 6 h. In both experiments, endometrial explants were cultured with medium alone as a negative control (PreP-CTRL and PostP-CTRL; n= 4-5 /group). All explants were snap frozen for subsequent RNA-seq. Despite a large number of differentially expressed genes (DEG) between PreP-CTRL and PostP-CTRL on D7 (n= 6063), the response to D7 blastocysts was similar: 27 DEG between PreP-D7 and PreP-CTRL and 5 DEG between PostP-D7 and PostP-CTRL (all 5 also upregulated in the prepubertal endometrium). All D7 embryo-induced DEG were interferon-stimulated genes (ISG). Similarly, while a comparison between PreP-CTRL and PostP-CTRL on D14 revealed 3544 DEG, endometrial response to a D14 conceptus was similar: 42 DEG in PreP-D14 and 61 DEG in PostP-D14 (37 genes in common). All genes upregulated in PreP-D14 and 57 of the 61 DEG in PostP-D14 were ISG. Exposure to exogenous IFNT increased the expression of a similar number of genes (165 in PrepP-IFNT and 168 in PostP-IFNT, relative to the controls, 113 of which were shared). Of the 27 DEG induced in PreP endometrium by a D7 blastocyst, 26 were common with those induced by a D14 conceptus. In conclusion, prepubertal endometrium is capable of responding to D7 blastocysts, a D14 conceptus, and IFNT in a manner similar to that of postpubertal endometrium.

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Anti-cancer potential of pomegranate peel on human ovarian carcinoma cells OVCAR-3

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Keywords: pomegranate, cancer, ovary.

Pomegranate (*Punica granatum*) is a unique and potent source of biologically active substances including flavonoids, anthocyanins, and especially ellagitannins and punicalagins with many beneficial properties. Furthermore, several scientific studies have focused on the bioactivity of pomegranate peel extracts, which possess remarkable antioxidant, antibacterial, anti-inflammatory and anti-cancer activities. In accordance with anti-cancer potential of pomegranate fruits, the aim of our study was to examine the *in vitro* effect of pomegranate peel extract at the different concentrations (0; 25; 50; 100; 200 μg/ml), in short-term application (for 24 h) on a human ovarian carcinoma cell model system (OVCAR-3). Analysis were focused on cell viability, production of reactive oxygen species (ROS), and expression of NAD-dependent deacetylase SIRT1 and histone γ-H2AX as a marker DNA double strand breaks. For this experiment, the ethanol extract from lyophilized pomegranate peel was prepared. Cells treated with ethanol in an amount corresponding to the highest used concentration of extract were used as positive controls (+Control) for the experiments. The metabolic activity was evaluated by AlamarBlue™ cell viability assay; the ROS production was quantified by chemiluminescence and the protein expression was detected by Western Blot analysis. Band intensity was quantified using Image Lab™ software (Bio-Rad, CA, US). Statistical significances were established by using One-way ANOVA along with Dunnett's test. All experiments were done in triplicate. The pomegranate peel extract significantly ($P \leq 0.001$) inhibited the viability of OVCAR-3 cells at all used concentrations in comparison to control. Moreover, ROS generation was significantly ($P \leq 0.01$) increased at all used concentrations of pomegranate peel extract in a dose-dependent manner. Interestingly, evaluation of the level of SIRT1 showed significant ($P \leq 0.05$) decrease in ovarian cancer cells OVCAR-3 in comparison to healthy cells human ovarian granulosa cells (HGL5). On the other hand, SIRT1 expression was significantly increase after pomegranate peel extract treatment at the concentrations 100 and 200 μg/ml in OVCAR-3 cells ($P \leq 0.05$). Additionally, all used concentrations of pomegranate peel extract led to significant ($P \leq 0.01$) γ-H2AX over-expression in ovarian cancer cells OVCAR-3 as a response to DNA damage. In conclusion, our data suggested that oxidative stress due to pomegranate-induced ROS production resulted in a decrease in the number of viable OVCAR-3 cells. The results show dose-dependent effect of pomegranate peel extract on human ovarian carcinoma cells OVCAR-3 and the potential role of pomegranate in the prevention or treatment of cancer by regulation of various signal pathways. Further studies are essential to understanding the therapeutic potential of pomegranate peel extract, however, it might serve to be a potential chemoprotective agent.

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A proteomic approach to decipher embryo-maternal interactions in the oviduct

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Keywords*:* oviductin, mass spectrometry, morula.

In vivo, the bovine embryo develops in contact with the oviductal fluid (OF) up to the 8-cell or morula stage. Oviduct proteins are known to be highly regulated across the estrous cycle. However, up to now, using immunohistochemistry, only few proteins, such as oviductin and osteopontin, have been identified as interacting with the developing embryo. The aim of this study was to use two complementary proteomic approaches: (i) bottom-up using nanoliquid chromatography coupled to tandem MS (nanoLC-MS/MS), and (ii) profiling by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) mass spectrometry (MS), to characterize new OF proteins interacting with the early bovine embryo. Pairs of bovine oviducts were collected at a local slaughterhouse and transported to the lab on ice. Only oviducts at the post-ovulatory stage (small hemorrhagic *corpus luteum* (CL)) and ipsilateral to the CL were used for OF collection by squeezing (n=22 cows). After 2 centrifugations (2000 g, 10 min then 12000 g, 10 min, 4°C), the OFs were pooled, aliquoted in small volumes and stored at −80°C. *In vitro* matured oocytes from slaughterhouse ovaries were fertilized with frozen semen. Zygotes were then cultured in SOF medium without proteins for 5 days. Pools of 25 embryos at the morula stage were incubated in 25 µL of OF (treated) or SOF (control group) for 6 h at 37°C then rinsed 3 times and stored at −80°C before MS analyses. For each proteomic approach, morulas from four replicates were analyzed. Bottom-up analyses were performed on pools of 25 embryos after protein extraction and trypsin digestion (n=4 pools/condition) and nanoLC-MS/MS (Tims-TOF, Bruker). Profiling analyses by MALDI-TOF (UltrafleXtreme, Bruker) in the 2-30 kDa mass range were performed on intact individual embryos (n=40 embryos/group). In parallel, the OF was analyzed both by nanoLC-MS/MS and MALDI-TOF MS. Proteins were considered as embryo-interacting proteins if they were detected in the OF and detected in treated but not in control embryos, or detected with significantly higher abundance in treated *vs*. control embryos (fold-change of mean normalized spectral counts > 2; p-value of t-tests < 0.05). By the bottom-up approach, a total of 561 proteins were identified, among which 21 OF interacting with embryos, including oviductin (OVGP1), galectin-3, transgelin-2, and several annexins (ANXA1, 2, 4). Among interacting proteins, seven had a signal peptide or were reported as secreted via non-classical secretory pathways. By the profiling approach on single embryos, a total of 221 masses were detected, among which five OF interacting with embryos. These masses were annotated as gluthatione S-transferase and several ribosomal proteins. In conclusion, high throughout proteomic methods were successfully used to identify embryo-interacting proteins originating from OF. Further analyses are requested to specify in which embryo compartments (zona pellucida, perivitelline space, blastomeres) these proteins are localized and which roles these interactions could play.

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Sexual dimorphism during early embryo development in the bovine: differential gene expression in relation with oxidative stress and culture conditions

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Keywords: bovine blastocyst, RT-qPCR, sex ratio.

Female and male mammalian embryos differ from the onset of the embryonic genome. This is mainly due to differential gene expression related to sexual chromosomes. Indeed, large parts of X-linked genes are overexpressed in female embryos up to the inactivation of one X chromosome. Therefore, metabolism and adaptation to environmental conditions differ between sexes. Using the IVP bovine embryo as a model, we showed that culture conditions and induced oxidative stress differentially impact male and female embryos at the early blastocyst stage: male embryos survived better an oxidative stress induced by 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) in the presence of serum (FCS medium; sex ratio: 61 vs 46% in control embryos; $p<0.05$), while female embryos were more resistant than males in serum-free conditions (BSA-ITS medium; sex ratio: 44 vs 59% in control embryos; $p<0.05$). The quality of the surviving blastocysts, i.e the apoptotic rates, also differed between sexes (Dallemagne et al., Theriogenology, 2018, 117, 49). In order to decipher the origin of those differences, the relative level of expression of several genes was evaluated by RT-qPCR on single blastocysts obtained in the same culture conditions. Briefly, bovine embryos were cultured in FCS or BSA-ITS medium. Oxidative stress was induced from D5 post-insemination (pi) using 0.1 or 1mM AAPH, respectively. At D7 pi, blastocysts were recovered and individually snap frozen in liquid nitrogen until RNA extraction. RNA extraction and reverse transcription were performed using the RNeasy Plus Micro kit from Qiagen and the iScript cDNA Synthesis kit from Bio-Rad, respectively. PCR was first performed on *DDX3Y* gene to sex the embryos. Then the samples were submitted to qPCR for 2 reference genes and 5 genes of interest (StepOnePlus, Applied Biosystem; 109 embryos; 3 replicates; 3 to 15 embryos per condition). The two reference genes, *YWHAZ* and *H2AFZ*, were selected for their stable expression whatever the condition (female or male, with or without oxidative stress, in FCS or BSA-ITS medium). Four X-linked genes (*AIFM1, XIAP, G6PD, HPRT*) and one autosomal gene (*BAX*) were selected based on the literature for their roles in the control of apoptosis or oxidative and their potential implication in the observed differences between the tested conditions. Statistical analysis was performed with the Standard Least Squares method (fixed effects: stress, sex, stress*sex; random effect: replication). All X-linked genes showed a higher expression in female embryos, whatever the culture medium ($p<0.01$; between 1.4 and 2.5 fold). AAPH treatment significantly decreased the expression of *XIAP* only in FCS containing medium (on average 0.78 fold), while it increased the expression of *BAX* (1.3 fold) and *HPRT* (1.3 fold) only in BSA-ITS medium ($p<0.01$). In this last medium, the impact of stress on *AIFM1* expression tended to depend on the sex of the embryo ($p=0.068$; females: on average 1.2 fold increase vs male 0.9 fold). In conclusion, the study confirms the higher expression of the tested X-linked genes in female embryos. The few differences observed between culture and stress conditions did not allow linking the expression of the studied genes to the sexual dimorphism observed for the developmental and apoptotic rates in the tested conditions.

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Trolox during *in vitro* maturation of bovine oocytes can protect embryos from palmitic acid induced lipotoxicity during development: effects on mRNA transcript abundance

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Keywords*:* fatt acids, trolox, in vitro production of bovine embryos.

Maternal metabolic disorders are associated with elevated concentrations of palmitic acid (PA), which is known to jeopardize bovine oocyte and embryo development and quality. Molecular analyses of PA exposed bovine oocytes and embryos point towards oxidative stress (OS) pathways. Previous research has shown that the detrimental effects of PA-exposure during oocyte IVM cannot be alleviated by antioxidant (AO) supplementation, e.g. Trolox (TR, water soluble VitE), during IVM or IVC. Exposing oocytes with TR during IVM protected subsequent embryo development under PA conditions (De Bie *et al.* 2018, AETE). In the present study, we examined the effects of TR on the quality of the produced blastocysts at the transcriptome level. Bovine COCs were matured, fertilized and cultured in 2 different experiments (min 3 repeats each). In EXP1, COCs (n=1565) were exposed to pathophysiological follicular PA concentrations (150 µM, Sigma-Aldrich, BE), subsequent embryos were cultured under solvent control (ethanol) conditions (PA-SC). TR was added during IVM or IVC (100 µM, Thermo Fisher, BE; PATR-SC, PA-TR). In EXP2, COCs (n=1477) were matured under solvent control conditions, subsequent embryos were exposed to pathophysiological oviductal PA concentrations (230 µM; SC-PA). TR was added during IVM or IVC (100 µM; TR-PA, SC-PATR). In each experiment, a solvent control was included (SC-SC). Pools of min 10 day 8 blastocysts per treatment were examined for relative transcript abundance of genes (normalized to *H2AFZ* and *YWHAZ*) involved in OS (*CAT, GPX, SOD1, SOD2, PRDX1, PRDX3, NRF2*), mitochondrial function (*TFAM, HSPD1*), lipid metabolism (*PPARg*) and apoptosis (*BAX*) and analyzed by one-way ANOVA.A significant increase in *NRF2* and *TFAM* was found in blastocysts from PA exposed COCs (PA-SC) and embryos (SC-PA) compared with controls (SC-SC). Increased *NRF2* in blastocysts from PA exposed COCs (PA-SC) returned to control levels when TR was added during IVM or IVC (PATR-SC, PA-TR). In contrast, when embryos were exposed to elevated PA (SC-PA), adding TR during IVM or IVC (TR-PA, SC-PATR) was not able to alleviate elevated *NRF2* expression to control levels, suggesting activation of OS defense mechanisms. The addition of TR in each EXP significantly reduced *TFAM* gene expression to levels similar to controls (SC-SC), suggesting normalization of mitochondrial biogenesis. In EXP1, a significant increase in *CAT* was found in PA exposed oocytes (PA-SC) compared with their control counterparts. Adding TR during IVM or IVC (PATR-SC, PA-TR) significantly reduced blastocyst *CAT* expression to levels lower than controls. No significant PA-induced changes were found in the expression of other genes. In conclusion, the enhancement of the developmental capacity of PA-exposed bovine oocytes and embryos by TR is most promising when oocytes are protected by TR prior to the PA insult. Moreover, subsequent blastocysts appear to have control levels of expression of genes related to OS and mitochondrial function and increased expression of genes involved in OS relief.

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Effect of Temopl on *in vitro* oocytes maturation in Egyptian Buffalo

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Keywords*:* Tempol, in vitro maturation, Buffalo oocytes.

Oxidative stress is a major biological threat that negatively affect oocytes quality and subsequent maturity competence. Cellular antioxidant system, like superoxide dismutase (SOD), plays a substantial role for maintaining redox balance against the excessive accumulation of reactive oxygen species (ROS). Consequently, the antioxidants are frequently used in the in vitro culture system to promote the oocyte's maturation. Tempol is is a single chemical compound that facilitates hydrogen peroxide metabolism, scavening ROS, and functionally similar to SOD. Therefore, the current study was conducted to study the effect of 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol) as an exogenous antioxidant on in vitro oocyte maturation rate of Egyptian buffalo. Cumulus-oocyte complexes (COCs) were recovered from animals slaughtered at local abattoir and grade A and B were used in this study. The maturation rate was assessed by nuclear status (oocytes reached metaphase II stage) and cumulus expansion of the oocytes as well as on the molecular level. Three different concentrations (0.5, 1 and 2 µM) of Tempol (Sigma-Aldrich, Cat no 176141,) were added to the buffalo oocyte maturation medium (Tissue Culture Medium, TCM-199 HEPES medium supplemented with 2% fetal bovine serum, 5µg/ml of FSH, 1µg/ml estradiol-17β, 0.15 mg/ml glutamine, 22µg/ml Na-pyruvate, 50µg/ml gentamycin). By the end of maturation period (22-24 h), COCs expansion rate and nuclear maturation rates were evaluated. After oocytes denudation, the oocytes from all experimental groups were stored at – 80°C for further genetic analysis. Two candidate genes regulating metabolic activity (CPT2) and antioxidant status (NFE2L2) were profiled using Real-Time PCR find out molecular action of Tempol on COCs during maturation. The GAPDH was used as a reference gene for relative expression quantification. The results revealed that 0.5 µM of Tempol (88.0±4.0 %) enhances buffalo COCs expansion rate comparing to control (75.7±3.0 %) and the other two concentrations of Tempol (1 and 2 µM were 79.9±4.1 and 68.1±4.2 %, respectively). While, the differences were significant ($P \leq 0.05$) between the higher and lower concentration of Tempol. Moreover, the metaphase II (indicator of maturation rate) oocytes were higher in 0.5 µM concentration (84.4 %) in comparison to 1 and 2 µM concentrations (68.9 and 76.7 %, respectively) and control group (75.8 %). For the molecular analyses, the transcriptional abundance of CPT2 showed a significant ($P \leq 0.05$) decline trend with the increase of Tempol concentration in the maturation medium being in addition significantly lower than control group. While, for NEF2L2, the expression level of NEF2L2 gene was comparable between the control group and the groups of oocytes matured with 0.5 and 1 µM of Tempol, However, 2 µM of Tempol concentration showed lower level of expression of NEF2L2 in comparison with all experimental groups. Moreover, the expression pattern of CPT2 and NEF2L2 genes strengthened the results of oocyte maturation rate in vitro. In conclusion, the 0.5 µM concentration of Tempol revealed potential significance for oocyte maturation competence as well as on the molecular level.

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Embryo vitrification device has consequences at birth and adulthood

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Keywords*:* embryo, vitrification, phenotype.

The development of assisted reproductive technologies (ART) over the past decades has provided tremendous advantages in livestock. Cryopreservation of reproductive cells is the second most used ART, which allows for long-term storage gametes/embryos by cooling them to subzero temperatures. This technique has become essential to enable the banking and the transport of embryos from high genetic value animals around the world. In this field a progressive replacement of slow freezing by vitrification methodologies has occurred. However, rather than a standardized method, an explosion of vitrification methods has been appeared over the last decade, using extremely variable vitrification media and more than 25 different vitrification devices. Moreover, it is increasingly common to find evidences that ART conditions can affect embryo development and, ultimately, the adult phenotype via epigenetic mechanisms that vary depending on the nature of the procedure (embryonic developmental plasticity). Therefore, using the rabbit as a model, the aim of this study was evaluate the effect over the growth performance of two vitrification devices, both based on the use of the minimum volume strategy but composed of different material (metal or plastic), which brings different cooling/warming rates. To assess this issue, 72-hours embryos (late morula/early blastocyst) were vitrified using metallic loops (n=102; ML) and Cryotop® (n=100; CP). Embryos were vitrified in a two-step addition procedure; equilibrium (10% EG + 10% DMSO + 10% Dextran) for 2 minutes and vitrification (20% EG + 20% DMSO + 10% Dextran) for 1 minutes. After thawing, embryos were transferred into the oviducts of 16 foster mothers. Birth rate (animals born / transferred embryos) was recorded and the pups were identified and weekly weighted until adulthood. Gompertz growth curve equation [$y= a \cdot EXP(-b \cdot EXP(-k \cdot t)]$] was used to determine the growth rate (k). Statistical analysis was performed through a general lineal model (considering $p<0.05$). The results showed that ML birth rate (44.1±4.67%) was lower compared to CP group (65.0±4.72%; $p<0.05$). However, CP animals were smaller (-9.6±2.69g; $p<0.05$) at birth, even after using the litter size as covariable. Finally, although growth rate was similar between both groups (average k value: 0.16±0.01), data suggest that differences at birth cannot be restored later in life. Therefore, CP animals showed lower body weight (-318.9±103.86g; $p<0.05$) at adulthood. In conclusion, the vitrification device does not seem to be trivial. Our results increase the studies that reports a significant effect of the vitrification device in the achievement of a successful vitrification. In addition, our findings provide evidences of an embryonic plasticity in response to the vitrification device, which modify the birth weight and the late growth performance. Therefore, we show for the first time to our knowledge that phenotype can change in response to a vitrification device.
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Culture under the physiological temperature registered along the reproductive tract of female pigs improves the blastocysts yield *in vitro*

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Keywords*:* temperature, assisted reproductive technology, pig.

Despite temperature being one of the main external environmental factors that affects gene expression, thereby influencing the way an organism develops (Lobo, I. Nature Education vol. 1(1), p39, 2008), its oscillation pattern is barely taken into account in *in vitro* embryo production (IVP). Few studies obtaining direct measurements of temperature within the reproductive tract of the female pig have been reported and the procedures used in those previous studies have been surpassed today by the use of cutting-edge devices. While no reference temperature values have been published to date in the pig uterus, a temperature gradient within oviduct and ovary was described (Hunter et al., Reprod Biomed, vol. 24 (4), p. 377, 2012). We hypothesized that transferring physiological temperature conditions given in nature to IVP could help to reduce the prevalence of polyspermy after insemination in swine and improve the efficiency of that biotechnology in this species. Hence, we aimed to measure temperature within the isthmus, ampulla and uterus of sows (n=15) and use these values in IVP protocols. To this end, oviductal and uterine temperature was monitored adopting a laparo-endoscopic single-site surgery assisted approach along with a flexible and thin miniaturized probe previously used by López-Gatius and Hunter (López-Gatius and Hunter, Reprod Dom Anim, vol. 52(3), p. 366, 2017). The same temperature was retrieved in ampulla and isthmus (37.0 ± 0.5ºC) whereas a significantly higher value was found in uterus (38.7 ± 0.1ºC).This finding suggest that a lower temperature is required during IVF, while a higher temperature is needed during the embryo development. To test this assumption, *in vitro* matured oocytes were inseminated at two different temperature conditions: the routinely used value in pig IVP (38.5ºC) and a lower value (37ºC), recorded in the oviduct. At 18-20 hours post-insemination (hpi), putative zygotes were transferred to embryo culture medium and maintained at 38.5ºC in both groups. A sample of presumptive zygotes (n=218) was fixed and stained to assess the fertilization rates. At 180 hpi, development to blastocyst stage was evaluated. Data were analysed by one-way ANOVA. A P-value <0.05 was considered to denote statistical significance. Monospermy rate was significantly higher at 37ºC compared to 38.5ºC (65.0 ± 6.1% *vs.* 46.0 ± 6.1%), not being enough to improve the IVF yield. However, an increase in blastocyst yield when embryos were fertilized at 37ºC (39.0 ± 3.6%) was observed compared to those fertilized at 38.5ºC (24.0 ± 2.8%). Our study supports the recent data published by Hino and Yanagimachi in mice (Hino and Yanagimachi, Biol Reprod, in press, 2019) claiming that peristaltic movement within the oviduct and the continue ad-ovarian transport of oviductal fluid make a temperature gradient within the oviduct unlikely existent. In contrast, we found a temperature gradient between oviduct and uterus.

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Immunohistochemical identification of CIRBP in bovine ovary and testicle

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Keywords*:* CIRBP, stress-induced protein, cattle.

The cold-inducible RNA-binding protein (CIRBP) is a highly conserved stress-induced protein that helps cells to resist adverse environmental conditions via stabilizing specific mRNAs and facilitating their translation. CIRBP participates in anti-apoptotic and anti-senescent cytoprotective processes. In relation with gametes, CIRBP improves the developmental competence in vitrified-warmed yak oocytes and exerts a protective effect against spermatogenic injury caused by heat stress and cryptorchidism in mice. The purpose of this study was to identify the expression of CIRBP on different cell populations in adult bovine ovary and testicle. Tissues were obtained from healthy slaughtered animals (non-pregnant heifers and sexually matured males). Paraffin blocks containing tissue sections of ovary and testicle were processed for immunohistochemistry. Tissue sections were dewaxed, blocked for intrinsic peroxidase (15 min, 3% H_20_2 in methanol), and subjected to antigen retrieval (10 mM sodium citrate, pH 6.0, 30 min, 95°C). Nonspecific binding was blocked with 10% normal donkey serum (30 min). Tissues were incubated with two dilutions (1/250, 1/500) of two primary antibodies against CIRBP overnight at 4°C: monoclonal rabbit anti-CIRBP [EPR18783] (ab191885, Abcam) or polyclonal goat anti-CIRBP (ab106230, Abcam). Antibodies were detected with a commercial peroxidase kit and diaminobenzidine. Sections were lightly counterstained with hematoxylin. Some sections were processed for immunofluorescence and analyzed through confocal microscopy, using two secondary antibodies at 1/600: Alexa Fluor® 546 donkey anti-rabbit IgG or Alexa Fluor® 633 donkey anti-goat IgG (Invitrogen). Fluorescent sections were counterstained with DAPI. Sections stained on the absence of primary antibody (negative controls) demonstrated a lack of unspecific binding for the detection system. Both immunohistochemistry and immunofluorescent sections were analyzed with ImageJ Software. Two-Way ANOVA analysis was performed with GraphPad Prism Version 8.0.2. *Post hoc* comparisons were performed using the Tukey test. In ovary, CIRBP was present in follicular cells of primordial follicles and in the granulosa and theca cells of the subsequent follicular stages. Oocytes presented less intensity compared to follicular cells. Granulosa cells presented more intensity than theca cells. In testicle, CIRBP was present in Leydig, Sertoli, and spermatogenic cells but not in mature spermatozoa. Dilutions at 1/250 presented better-contrasted images in both primary antibodies. In conclusion, CIRBP was present in bovine male and female gonads as seen in other species. Slight differences can be found using monoclonal or polyclonal antibodies against CIRBP in both tissues. Despite CIRBP being known to play an important role in spermatogenesis, little is known about its role in folliculogenesis and developmental competence of oocytes. Further studies are needed on the function of CIRBP on bovine follicles, oocytes, and developing embryos.

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Metabolomic profiling of oviductal extracellular vesicles across the estrous cycle in cattle

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Keywords*:* Oviduct, extracellular vesicles, metabolomics.

Extracellular vesicles (EVs) in oviductal secretions have been suggested to play major roles in the cross-talk between gametes/embryo and the oviduct. The aims of the present study were to determine the metabolomic profile of bovine oviductal EVs and to examine whether the metabolic content of oviductal EVs varies according to the stage of the estrous cycle and the side relative to ovulation.
Bovine oviducts were collected at a local slaughterhouse, transported on ice, and classified into 4 stages of the estrous cycle according to the ovarian and corpus luteum morphologies (n=34-54 cows/stage): post-ovulatory (Post-ov; Days 1-4 of estrous cycle, coinciding with the time of embryo presence in the oviduct), mid luteal (Mid-lut; Days 5-11), late luteal (Late-lut; Days 11-17) and pre-ovulatory (Pre-ov; Days 18-20, coincinding with the time of estrus). Additionally, follicular fluid was collected from the Pre-ov follicles to exclude animals with cystic follicles (intra-follicular concentrations of progesterone > 160 ng/ml and estradiol < 40 ng/ml). Oviductal fluids (OF) were collected from contra- and ipsi-lateral oviducts by squeezing. Then, OF was separated from cells and cell debris by centrifugation (10 min at 2,000 g then 15 min at 12,000 g). Oviductal EVs were isolated from pools of OF (8-19 cows per pool; 3-4 pools per stage and side; 397 ± 15 µl of OF per pool) by ultracentrifugation (90 min at 100,000 g twice) and resuspended in PBS. Finally, EV samples (with not trace of oviductal fluid) were assayed for protein concentration (12.9 ± 0.5 mg/ml per pool) and stored at – 80°C for metabolic analysis. Samples of EVs were analyzed by proton nuclear magnetic resonance spectroscopy (NMR) as previously described (Lamy et al. Reprod. Fertil. Dev. 2018). The concentration of each metabolite was normalized to 1 mg of protein. Normalized values were compared between stages of the cycle and sides relative to ovulation using two-way analysis of variance (ANOVA) followed by Tukey's tests with $P < 0.05$ considered significant. NMR identified 22 metabolites in oviductal EVs, from which 15 could be quantified. Among them, 5 were amino acids (alanine, glycine, isoleucine, methionine and valine) and 9 energy substrates including lactate, myoinositol, glucose-1-phosphate and maltose as the most abundant metabolites. With the exception of maltose, all metabolites identified in oviductal EVs were previously identified in the OF (Lamy et al. 2018). Except for maltose, the side relative to ovulation had no effect on metabolite concentrations. Interestingly, levels of methionine were significantly higher at Pre-ov compared to Late-lut ($P<0.05$). Furthermore, glucose-1-phosphate and maltose concentrations were greatly affected by the stage of the estrous cycle ($P<0.0001$), showing 10- to 40-fold higher levels at Mid-lut and Late-lut than at Pre-ov and Post-ov. The metabolites identified in the present study could be taken up by gametes/embryos via EVs and play key roles in gamete maturation, fertilization and/or embryo development.
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Parental contribution of splicing factors ZRSR1 and ZRSR2 in early embryo development

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Keywords*:* minor splicing, embryogenesis, zygotic gene activation.

ZRSR1/2 have been implicated in 3' splice site recognition of U12 introns, a minor intron class (<0.4% of all introns) which is conserved across eukaryotic taxa with important roles in developmental processes. *Zrsr1* is a imprinting gene which is paternally expressed in mice, and *Zrsr2* is in the X-chromosome in all mammalian species analyzed. To determine the implications of minor splicing in early embryo development, CRISPR technology was used to produce *Zrsr1/2* mutant mice (*Zrsr1*^{mu} *and* *Zrsr2*^{mu}) that were viable with normal lifespan. We crossed homozygous *Zrsr2*^{mu} female with *Zrsr1*^{mu} male, being the double mutation (Zrsr1/2^{mu}) lethal, giving rise to embryos that stopped developing mainly between the 2- and 4-cells stages, just after zygotic gene activation (ZGA). This indicates that embryos need at least one normal *Zrsr1* allele from the father or one *Zrsr2* allele from the mother to survive. Rescue experiments in which *Zrsr1* mRNA was injected into 1-cell Zrsr1/2^{mu} embryos allowed the development of mutant embryos to blastocyst stage, revealing that minor splicing is essential for ZGA. To investigate the molecular basis of impairing the minor spliceosome machinery during embryo development, 3 pools of 100 *Zrsr1/2*^{mu} 2-cell embryos and 2 pools of 100 wild-type 2-cell embryos were used to purify their RNA and perform RNA-seq analysis. Differential gene expression (DGE) was evaluated with two independent software (DESeq2 and edgeR) (adjusted p-value <0.01) to improve the reliability of our findings. Differential alternative splicing (AS) events was determined using vast-tools software, considering events as modified when the difference in their average inclusion levels was above 10%. DGE analysis showed 3423 upregulated and 1446 downregulated genes in Zrsr1/2^{mu} embryos, which could indicate that the degradation of the maternal mRNAs is impaired. Genes with lower expression in Zrsr1/2^{mu} embryos were enriched in translation, rRNA processing and splicing, and cell cycle GO terms, indicating an essential role of minor splicing during ZGA. Differential AS analysis revealed 2645 upregulated and 1717 downregulated events in *Zrsr1/2*^{mu} embryos, being all categories of alternative splicing affected. Intron retention events were then checked to determine if they were U2- or U12-type intron events, as well as those U2 intron events that occur in genes that contain U12 introns. Seven percent of the intron retention events identified correspond to U12-introns, which represents a significant enrichment when compared with the overall proportion of U12 introns in the mouse genome (7% vs 0.04% expected), showing a crucial role of the U12-type introns during early embryo development. This study identifies paternal ZRSR1 and maternal ZRSR2 as essential factors for efficient U12 intron splicing, highlighting their crucial role on early preimplantation embryo development. Research supported by the Spanish Ministry of Economy and Competitiveness through the project AGL2015-66145-R and BES-2016-077794 grant.

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Ovulation mediated changes in the transcriptomics of the rabbit isthmus

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Keywords*:* isthmus, transcriptomics, rabbit.

The isthmus is the section of the oviduct where sperm is stored forming the oviductal reservoir, before capacitation takes place and spermatozoa are freed to fecundate the oocytes within the ampulla. This study shows transcriptomics changes in the isthmus shedding light on the molecular processes taking place near the ovulation in rabbit species (*Oryctolagus cuniculus*). Six sexually mature New Zealand rabbit does received 0.02 mg Gonadorelin (im; Fertagyl, Merck & Co., Inc., Kenilworth, USA) at insemination time (t=0 h) to induce ovulation. Tissues were collected after euthanasia at 10 h (pre-ovulatory; n=3) and 20 h (post-ovulatory; n=3). RNA was extracted and samples with a RNA integrity number > 7 were analyzed by microarray platform GeneAtlas System (chip Rabbit Gene 1.1 ST Array Strip, Affymetrix). Data analysis was done with the Partek Genomics Suite software, raw intensities were background corrected and RMA normalized. Differentially expressed genes (DEGs) were defined as those with a fold change > |1.5| and FDR < 0.05 obtained through a variance analysis, and false discovery rate adjustment with RankProd. Biological meaning was assessed with Gene Ontology (GO) enrichment, and pathway analysis with KEGG database. There were 86 genes upregulated at the pre-ovulatory stage. Pathway analysis was enriched in terms such as "protein processing in endoplasmic reticulum", "ECM-receptor interaction" and "MAPK signalling pathway". GO analysis revealed enrichment in extracellular proteins, with 18 gen products labelled as “extracellular exosome” and 28 being secreted proteins. Among the extracellular proteins there were some previously reported as oviductal fluid (OF) proteins in rabbit and other species (cow, pig and mouse). There were upregulated chaperones (HSPA5, DNAJB11, HYOU1 and HSPB7), integrins (ITGA5, IGTA2 and IGTB3), neuropeptide Y (NPY), and protease inhibitor SERPINE1. On the other hand, there was an upregulation of 33 genes at the post-ovulatory stage. GO anotation revealed the presence of eight secreted proteins, among the two proteins associated with fertility, PLAT and SPP1. PLAT was previously reported to be necessary for the success of *in vitro* fertilization in mice, while osteopontin (SPP1) has shown a positive effect on spermatozoa, *in vitro* fertilization and embryos in cattle. In the present study, an overexpression of exosomal proteins was reported for the pre-ovulatory stage. Enrichment in proteins previously detected in murine oviductosomes (OVs), including those integrins mediating spermatozoa-OVs fusion, suggest that this molecular mechanism might be conserved in rabbits, and thus participate in modification of the spermatozoa located in the isthmus. Further experiments are required to characterize these oviductosomes their evolution over the doe reproductive cycle, and investigate their role in the modulation of the spermatozoa fertilization ability. Moreover, there are secreted proteins in the rabbit oviductal fluid previously detected by proteomics that show a differential expression pattern before and after ovulation whose role in reproduction remains unknown, requiring additional functional studies.
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Selected physicochemical parameters of serum and fluid from the cavity of corpora lutea and their connection to the mechanisms of its formation in cattle

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Keywords*:* cavitary fluid, serum, corpus luteum with a cavity, progesterone, cattle.

It is well known that cows have two morphologically different forms of the corpus luteum - solid and cavernous. In the studies presented in previous years, among others in the AETE meeting (Jaskowski 2016), we have shown that the presence of the cavity is accompanied by an increased level of progesterone in the blood and better results of conception. Perhaps the presence of the cavity is an additional factor that increases the probability of pregnancy in its early stage. The assessment of the nature and some of the fluid components could help to explain the positive effect of cavities on the CL function. The aim of the study was to determine some physicochemical parameters of the fluid from the CL cavities and serum in connection with the presence of the cavernous form of the corpus luteum. The study material from 30 Polish Holstein Friesian cows aged from 43 to 148 months, obtained in one of the cattle slaughterhouses in Poland, was used. After ultrasound examination performed before slaughter and finding the cavity inside CL, blood was collected (tail vein, 7.5 ml tubes, EDTA), and after slaughter liquid from cavities of corpora lutea (eppendorf tubes). The samples were transported to the laboratory under chilled conditions, and the analyzes were held at -80 ° C. The specific gravity, total protein content (Danlab refractometer), glucose concentration (Reflovet Plus, Roche) and progesterone (RIA) were determined in the fluid and serum samples. Data were analysed by logistic regression using the STATISTICA 9.0 software PL. The correlation between the size of cavities and the specific gravity, protein and glucose concentration was clearly established. While the increase in cavity diameter corresponded to the increase in glucose concentration (0.5116, p=0.015), specific gravity and protein concentration were negatively correlated with the size of the cavity (-0,5192, p=0.013 and -04813, p=0.023, respectively). There were no significant correlations between the fluid from the cavities and the amount of luteal tissue, as well as serum and fluid from the cavities. There was no correlation between the concentration of progesterone in the cavity and serum fluid, however the mean P4 values obtained from cavitary fluid were significantly higher than from serum (4612±3847.6 to 28.6±15.2 ng/ml, p<0.001). It is known that progesterone has an autocrine luteotropic effect on the luteal cells positively affecting its own concentration measured in blood. The P4 values in the cavities which is many times higher than in serum may explain the higher concentrations of P4 in the blood of cows with cavitary corpora lutea in relation to those with solid counterparts. Physical parameters of the fluid from the cavities suggest its exudative character. Higher specific gravity and protein concentration in smaller sized cavities compared to the large ones may indicate the angiogenesis related background of cavity formation.

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Mono (2-ethylhexyl) phthalate induces transcriptomic and proteomic alterations in bovine oocytes

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Keywords*:* phthalates, oocyte developmental competence, transcriptome.

Phthalates are plasticizers, used in a variety of industrial plastics. Di(2-ethylhexyl) phthalate (DEHP), the most commonly used plasticizer, and its main metabolite, mono(2-ethylhexyl) phthalate (MEHP), are known reproductive toxicants. A residual concentration of MEHP (~20 nM) has been found in follicular fluid aspirated from IVF-treated women and DEHP-treated cows. Previously we have reported that exposure of oocyte during maturation to MEHP impairs nuclear maturation, reduces clevage and blastocyst formation rates. However, the effect of 20 nM MEHP on the transcriptomic profile of oocytes and their derived blastocyts is not entirely clear. Bovine oocytes were in-vitro matured with or without 20 nM MEHP for 22 h. At the end of maturation, they were collected for transcriptomic (by microarray; n = 20 per sample; 4 replicates) and proteomic (n = 200 per group) analyses to examine a possible direct effect of MEHP on the oocyte transcriptomic and proteomic profiles. The remaining oocytes were in-vitro fertilized and embryonic development was recorded 42–44 h and 7 days postfertilization. Blastocysts were also collected for microarray analysis (n = 10 per sample; 4 replicates). Transcriptomic data were analyzed using Partek Genomics Suite software. Control probes were removed; signals were log2 transformed followed by interslide quantile normalization. Genes were considered differentially expressed if the *P*-value by one-way ANOVA was lower than 0.05 and absolute fold change was 1.5 between the control and MEHP-treated group. Proteomic raw data were imported into Expressionist® followed by Mascot software. Data were searched against the bovine sequences from UniProtKB. Proteins were considered differentially expressed at a fold change of ±1.5 with at least 2 unique peptides. Oocyte transcriptome analysis revealed MEHP-induced alterations in the expression of 456 genes. The differentially expressed genes were associated with actin cytoskeleton (n = 47 genes; e.g., *ACTG1*), metabolic pathway (n = 43) including oxidative phosphorylation (n = 12; e.g., *ND5*), oocyte maturation (n = 9; e.g., *PIK3CA*), and embryonic development (n = 14; e.g., *SOX10, NOTCH*); 191 proteins were affected by MEHP in mature oocytes, associated with methylation and acetylation (n = 51), metabolic pathway (n = 33) including mitochondrial oxidative phosphorylation (n = 7; e.g., ATP5E), and cytoskeleton structure (n = 32; e.g., ACTN1, EGFR). In control vs. MEHP-derived blastocysts, 290 genes were differentially expressed, associated with transcription process, cytoskeleton regulation and metabolic pathway; 9 of these genes were impaired in both oocytes exposed to MEHP (i.e., direct effect) and blastocysts developed from those oocytes (i.e., carryover effect). The study explores, for the first time, the risk associated with exposing oocytes to relevant MEHP concentrations (i.e., those found in the follicular fluid) to the maternal transcripts. Although it was the oocytes that were exposed to MEHP, alterations carried over to the blastocyst stage, following embryonic genome activation, implying that these embryos are of low quality.

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Atrazine-induced DNA fragmentation in bovine spermatozoa is associated with alterations in the transcriptome profile of in-vitro-derived embryos

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Keywords*:* ATZ, spermatozoa, microarray.

Atrazine (ATZ) is an extensively used herbicide, considered a ubiquitous environmental contaminant. ATZ and its major metabolite, diaminochlorotriazine (DACT), cause several cellular and functional alterations in spermatozoa, involving viability, acrosome integrity, and mitochondrial membrane potential. Our aim was to examine the effect of ATZ/DACT exposure on DNA integrity of bovine spermatozoa, fertilization competence, embryonic development and transcriptome profile of in-vitro-produced embryos derived from fertilization with pre-exposed sperm. Three experiments were performed with fresh semen. Statistical analysis was performed using JMP-13 software. Comparison of treatments was performed by one-way ANOVA followed by *t*-test. In the first experiment, spermatozoa (25 x 10^6 cells/mL) were capacitated (4 h) in presence of 0.01% DMSO (solvent; control), 0.1 µM ATZ or 1 µM DACT and were examined for DNA fragmentation (acridine orange dye). Exposure to ATZ and DACT increased the proportion of cells with fragmented DNA compared to control (26 and 25.8 *vs* 84.5% respectively; $P < 0.0001$). Spermatozoa were separated using annexin V micro-bead kit. DNA-fragmentation index was higher among annexin-positive (AV+) vs. negative (AV-) spermatozoa (99.5 ± 0.05 *vs* 20.8 ± 4.29 % respectively; $P < 0.0001$). In the second experiment, fertilization competence of AV+ and AV- spermatozoa was examined with in-vitro-matured (22 h) oocytes (1,051 oocytes, 4 replicates). In the control group, AV+ spermatozoa gave lower cleavage rate (31.3 ± 2.92 %) relative to AV- spermatozoa (78.6 ± 2.18 %; $P < 0.0001$). No blastocysts were recorded in the AV+ group. ATZ/DACT-treated spermatozoa defined as AV- had a significantly lower cleavage rates relative to the control (59.8 ± 4.04 and 65.3 ± 3.33 *vs* 78.6 ± 2.18 %, respectively; $P < 0.005$). The proportion of the developed blastocysts did not differ between groups. In the third experiment, in-vitro fertilization was performed with non-separated spermatozoa. Blastocysts (n=4 per sample; 4 replicates) were collected on day 7 post fertilization and subjected to microarray analysis to identify differentially expressed genes (DAVID). Transcriptome analysis revealed that 139 and 230 genes were differentially expressed (up- or down-regulated) in blastocysts derived from spermatozoa treated with ATZ and DACT, respectively. In particular, alterations were found in genes involved in pregnancy (*IFNT2*, *IFNT3*, *IGFBF5*) and in-utero embryonic development (*YBX3*, *ANKRD11*, *PDGFRA*, *VIM*), pluripotency (*MYF5*), apoptosis (*THEM4*, *BCAD29*, *EIF2AK2*), and methylation and acetylation (*H2B*, *RAB27B*, *H4*, *HIST1H1C*, *LOC616868*). In conclusion, DNA damage induced by ATZ and DACT might explain, in part, the reduced fertilization competence of treated spermatozoa, reflected by lower cleavage rates. Given that blastocyst-formation rate did not differ between groups, other mechanisms cannot be ruled out. Here we report on alterations in the genomic profile of embryos developed from ATZ/DACT-treated spermatozoa, suggesting alterations in some cellular processes, including genetic and epigenetic modifications.

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Endoplasmic reticulum stress: is it induced in heat-shocked bovine oocytes?

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Keywords*:* heat stress, salubrinal, in vitro maturation.

The endoplasmic reticulum (ER) is a multifunctional organelle that plays a role in protein synthesis and modification, calcium homeostasis, and lipid synthesis. It also acts as a sensor of environmental stress. Accumulation of unfolded protein in the ER lumen leads to ER stress and activation of the Unfolded Protein Response. The bovine oocyte is very susceptible to environmental stress such as elevated temperature. However, the role of ER stress on heat-shocked oocytes has not been investigated. Therefore, the objective of this study was to determine the role of ER stress on bovine COCs exposed to heat shock (HS) during IVM. The first experiment was conducted to determine whether HS induces ER stress on bovine oocytes. COCs obtained from slaughterhouse ovaries were distributed on the following groups: Control (IVM at 38.5ºC for 22 h) and Heat Shock (IVM at 41ºC for 16 h followed by 6 h at 38.5ºC). After IVM, oocytes were denuded and stored at -80ºC for Western Blotting analysis. This experiment was replicated 5 times using 60 oocytes/treatment/replicate. Exposure of bovine oocytes to HS increased ($P<0.05$) the abundance of spliced X-box-binding protein-1 (sXBP1: ER stress marker) compared to control group, indicating the occurrence of ER stress. The second series of experiments (experiment 2, 3 and 4) were conducted to determine the role of ER stress on fertilization, kinetics and developmental competence of heat-shocked oocytes. COCs were matured in IVM medium containing 0 or 400 nM Salubrinal (ER stress inhibitor) under control and HS temperatures. After IVM, COCs were submitted to IVF and IVC. The fertilization rate was determined at 18 h after insemination (hai) and preimplantation developmental kinetics was determined by evaluating the cell number of each embryo at 26, 29, 32, 35, 38, 41 and 48 hai. These experiments were replicated 5 times using 30 COCs/treatment/replicate. There was not effect of temperature on fertilization rate. However, ER stress inhibition at 38.5ºC increased ($P<0.05$) fertilization rate compared to HS in the absence of salubrinal. Moreover, exposure of oocytes to HS in IVM medium caused a delay on embryonic developmental kinetics reducing ($P<0.05$) the percentage of 2-cell embryos at 29, 35 and 38 hai, as well as the percentage of 4-8 cell embryos at 41 hai and >8-cell embryos at 48 hai. Consequently, HS impaired cleavage rate ($P<0.05$) at 32-48 hai. On days 3 and 8 after insemination, cleavage and blastocyst rates were also reduced ($P<0.05$) in heat-shocked oocytes. Addition of Salubrinal to heat-shocked oocytes had a negative effect on embryonic development reducing ($P<0.05$) the percentage of 3-cell embryos at 38 hai and cleavage rate from 32 to 41 hai compared to 38.5ºC. However, cleavage and blastocyst rates at days 3 and 8 were not different between those groups. In conclusion, the present study demonstrated for the first time that HS induces ER stress on bovine oocytes during IVM. Moreover, HS retarded kinetics of embryonic development, reducing cleavage and blastocyst rates. ER stress inhibition on heat-shocked oocytes with Salubrinal was not as benefic as expected, but further studies are necessary to determine the importance of ER stress on bovine oocytes exposed to HS during IVM.

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Bta-miR-10b secreted by bovine embryos negatively impacts preimplantation embryo development

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Keywords*:* Bovine embryos, Secreted miR-10b, HOXA1.

In a previous study, we found miR-10b to be more abundant in the conditioned culture medium of degenerate embryos compared to that of blastocysts. In this study, miR-10b mimics, double-stranded and chemically synthesized RNAs ordered from the company, were supplemented to the culture medium (SOF+BSA+ITS) at 21-hours post insemination with a final concentration of 1 μM. The expression of miR-10b in embryos was evaluated by RT-qPCR and was found to be approximately 70 times higher expressed in embryos treated with mimics compared to the control embryos, indicating that miR-10b mimics can be taken up by embryos. Additionally, this uptake results in an increase in embryonic cell apoptosis (2.15 times) using TUNEL staining and aberrant expression of DNA methyltransferases (*DNMTs*) using RT-qPCR. Using several computational methods Homeobox A1 (*HOXA1*) was identified as one of the potential miR-10b target genes and dual-luciferase assay, which measures firefly and Renilla luciferase, confirmed *HOXA1* as a direct target of miR-10b in bovine embryos. Microinjection of si-*HOXA1* into embryos also resulted in an increase in embryonic cell apoptosis (4.44 times) and downregulation of *DNMTs*. Overall, this work demonstrates that miR-10b negatively influences embryonic development and might do this by targeting *HOXA1* and/or influencing DNA methylation.

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Effects of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEndMSCs) on porcine embryo development *in vitro*

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Keywords: porcine, IVF, embryo culture.

In vitro fertilization in pigs is an assisted reproductive biotechnology that is still developing to optimize its efficiency. Porcine IVF is a very labour-intensive technique that yields inconsistent results between IVF sessions due to low initial oocyte quality, polyspermy and suboptimal composition of embryo culture media among others. Many different approaches are being tested to increase the quality and quantity of embryos produced by IVF in this species. Specific commercial embryo culture media are not available in pigs, being the North Carolina State University 23 (NCSU-23) the most commonly used medium. Combinations of NCSU-23 and different macromolecules, growth factors, hormones or oviductal fluid have been shown to improve blastocysts yield *in vitro*. In this regard, supplementation of extracellular vesicles derived from human endometrial mesenchymal stem cells (evEnd-MSCs) to the zygote culture medium has demonstrated to increase embryo yield and quality in mice (Marinaro F. et al., Biology of Reproduction, 2019, 100(5), 1180–1192). Therefore, in the present work we aimed to improve blastocyst formation in swine using evEnd-MSCs in the embryo culture medium. To test this, EndMSCs were isolated from menstrual blood from four healthy women and characterized according to multipotentiality and surface marker expression. Extracellular vesicles from 4 donors were pooled, purified and characterized by nanoparticle tracking and CD9/CD63 expression by flow cytometry. Porcine oocytes were retrieved at a slaughterhouse, matured *in vitro* and fertilized with 1 x 10^5 spermatozoa/ml for 4 hours in a humidified atmosphere at 38.5°C in a 5%CO_2/95% air incubator. After IVF, the presumptive zygotes were randomly allocated to one of the following groups and cultured for 7 further days: A) Bovine specific medium: BO-IVC medium (IVF Bioscience, Barcelona, Spain); B) BO-IVC + evEnd-MSCs: BO-IVC added with 40 µg/ml of evEnd-MSCs; C) NCSU-23 and D) NCSU-23 + evEnd-MSCs: NCSU-23 added with 40 µg/ml of evEnd-MSCs. The evEnd-MSCs batch used was the same for all the experiments. Four IVF trials were performed and a minimum of 82 oocytes per group were evaluated. Blastocyst rates relative to initial oocyte number were compared among groups by a Chi-Square test, $P < 0.05$ was considered significant; results are presented as embryos obtained/initial oocyte number. Blastocyst rates were: (8/87) for BO-IVC, (5/82) for BO-IVC + evEnd-MSCs, (13/90) for NCSU-23 and (20/87) for NCSU-23 + evEnd-MSCs; no statistically significant differences were observed among groups ($P > 0.05$). In our setting, the use of evEnd-MSCs seemed to yield better results when combined with media specifically developed for porcine, not being able to help to overcome suboptimal culture conditions. More experiments need to be performed to fully corroborate our observations.

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Protective effects of Mitoquinone during *in vitro* maturation of bovine oocytes under lipotoxic conditions

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Keywords*:* Mitochondria, antioxidants, embryo development.

Oxidative stress and mitochondrial dysfunction in oocytes play a central role in the pathogenesis of several conditions associated with infertility. Upregulated lipolysis during negative energy balance can directly increase oxidative stress and alter mitochondrial functions in oocytes. Furthermore, *in vitro* maturation (IVM) following ovum pick up has been shown to increase gene expression of markers of cellular stress in oocytes. This leads to reduced developmental competence and reduced production efficiency. Mitochondrial targeted treatments containing co-enzyme Q10 are used to increase the anti-oxidative capacity within the mitochondrial matrix and enhance mitochondrial activity, however their efficiency in assisted reproduction to enhance oocyte developmental competence has not been investigated. In the present study, we tested the effect of different concentrations of Mitoquinone (MitoQ; 0, 0.1, 0.5, 1.0 μM) during bovine oocyte IVM, then we tested the effect of MitoQ (0.1 μM) in the presence or absence of palmitic acid (PA)-induced lipotoxicity (150μM) as a model (Marei *et al.* 2019, Sci. Rep. 9:3673). The effect of the carrier molecule of MitoQ, triphenyl-phosphonium (TPP) was also tested. A total of 2823 bovine oocytes from slaughterhouse ovaries were used. All data were derived from at least three replicates and were compared by linear logistic regression (categorical data) or ANOVA (numerical data) with Bonferroni post-hoc corrections. MitoQ supplementation at 1 μM significantly ($P<0.05$) reduced cleavage (50.8±6.81 vs. 78.7±5.17), and blastocyst rates (6.7±0.98 vs. 27.4±6.07) compared with solvent control (ethanol 0.01%). TPP (1 μM) also induced similar toxic effect ($P<0.05$). This was associated with, and probably caused by, a reduced mitochondrial inner membrane potential (J-aggregates: monomer intensity ratio of JC-1 staining) ($P<0.05$). Lower concentrations of MitoQ and TPP had no effects on developmental competence. PA increased the levels of oxidative stress in oocytes (43±2.39 vs. 28.4±2.36, CellRox Deep Red pixel intensity) and reduced cleavage (56.6% vs. 69%) and blastocyst (13.9% vs. 24%) rates compared with the controls ($P<0.05$). These negative effects were ameliorated in the presence of 0.1 μM MitoQ (CellRox, 30.5±2.30; cleavage, 69.4%; and blastocysts, 24.2%, $P<0.05$). In contrast, 0.1 μM TPP alone did not enhance cleavage (55.8%) and blastocysts rates (20.2%) compared to the PA group ($P>0.1$). In conclusion, low concentrations of MitoQ can protect against induced oxidative stress during oocyte IVM, and enhance developmental competence under lipotoxic conditions. These effects are specific to the CoQ10 content of MitoQ since the carrier molecule TPP had no protective effects. In contrast, higher doses of MitoQ and TPP are toxic for oocytes.

A175E Embryology, developmental biology and physiology of reproduction

Prolonged transportation of ovaries negatively affects oocyte quality and *in vitro* embryo production in sheep

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Keywords*:* sheep, oocyte quality, ovary transport.

The first step during *in vitro* embryo production (IVP) is the collection of developmentally competent oocytes. For wild species, such as Iberian red deer, the recovery of oocytes represents a problem because the slaughtering usually takes place far away from the laboratories and transport times are usually much longer. This may negatively influence developmental competence of immature oocytes and, therefore, their quality. The aim of this work was to examine the effects of different ovary storage times and media composition, using sheep as a model, with a view to achieving better results for wild species in which long transport times are inevitable. Adult sheep ovaries were recovered and randomly assigned to the Control (saline solution) or TCM (medium TCM199) groups and maintained for 13 h at 30 ºC. At 3, 7 and 13 h since ovary collection, sixty cumulus–oocyte complexes (COCs) were denuded and late stages of apoptosis were detected by TUNEL staining. Remaining 889 COCs (467 for Control and 422 for TCM) were matured, fertilized and cultured *in vitro* in order to examine oocyte maturation and sperm penetration rates by staining with Hoechst 3342 fluorescent dye, cleavage and blastocyst rates. Generalized linear model was used to study the influence of medium composition and storage time on oocyte quality and embryo production. When the analysis revealed a significant effect ($P<0.05$), a post hoc test with Bonferroni correction was carried out. Immature oocytes retrieved from ovaries stored during 13 h showed higher apoptosis ($P<0.05$), regardless of the medium composition. After fertilization, the proportion of inseminated oocytes with two pronuclei (2PN) was significantly higher ($P<0.05$) in the Control group compared to TCM (29.43% ± 4.54 vs 13.33% ± 4.54) and after 3 h of storage ($P<0.05$) compared to 7 and 13 h (47.58% ± 5.57 vs 14.46% ± 5.57 and 2.08% ± 5.57, respectively). Although maturation and embryo production did not show differences ($P>0.05$) in terms of medium composition and between 7 and 13 h of ovary storage, the percentage of Metaphase II (MII)-oocytes, cleavage and blastocyst rates were significantly higher ($P<0.05$) when ovaries were stored during a short period of time (3 h) compared to long periods of 7 and 13 h (maturation: 70.83% ± 7.96 vs 32.64% ± 7.96 and 6.25% ± 7.96; cleavage: 73.17% ± 8.43 vs 18.20% ± 8.43 and 1.02% ± 8.43; blastocyst rate: 32.78% ± 2.21 vs 5.82% ± 2.21 and 1.02% ± 2.21, respectively). In summary, although ovary storage medium composition had an influence on oocyte quality, the most prominent effect was found with transport times. Prolonged transportation of ovaries increased oocyte apoptosis and decreased maturation, sperm penetration, cleavage and blastocyst rates. Therefore, for wild species such as Iberian red deer, the optimization of these conditions is necessary to maintain oocyte quality and ensure a successful outcome during IVP.

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A176E Embryology, developmental biology and physiology of reproduction

Evaluation of the cumulus cells viability and its oxidative state by flow cytometry from ovaries subjected to prolonged storage in sheep

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Keywords*:* cumulus cells, sheep, long storage.

Cumulus cells (CCs) have an important role during oocyte maturation and competence acquisition. Assessment of viability and oxidative status of CCs may be potential non-invasive predictors of oocyte quality in *in vitro* maturation (IVM) routines. In addition, the time intervals required by transport of ovaries from the slaughterhouse to the laboratory may adversely affect the oocyte quality. In this context, the current study aimed to evaluated the impact of ovaries storage duration on viability and oxidative status of CCs during *IVM* in sheep oocytes. Adult sheep ovaries were collected in saline solution with antibiotics at 30ºC and storage for 3, 7 or 13 h. Cumulus-oocytes complexes (COCs, n= 2436, four replicates) were collected after these times and placed in TCM199 supplemented with 10 ng/mL FSH/LH, 100 µM cysteamine and 10% fetal calf serum for 24 h. After IVM, COCs were denuded and CCs were collected. Denuded oocytes were stained with Hoechst3342 to assess *in vitro* maturation rate and CCs were evaluated with flow cytometry using specific fluorophores for viability, intracellular generation of reactive oxygen species (ROS), glutation content (GSH) and mitochondria activity. A general linear model was used to study the influence of storage time on CCs quality and IVM rates. When the analysis revealed a significant effect ($P<0.05$), values were compared by Bonferroni test. The percentage of live CCs was greater ($P<0.05$) when ovaries were stored for 3 h (63.0 ± 8.0) compared to 7 h (19.4 ± 8.0) and 13 h (22.4 ± 8.0) of storage while, at 13 h (56.1 ± 6.5) the dead CCs percentages was significantly higher than for 3h (20.9 ± 6.5) and 7 h (35.6 ± 6.5). After 7h of ovary storage, a significantly higher ($P<0.05$) percentage of apoptosis was observed compared to 3h of storage (45.0 ± 6.6 and 16.2 ± 6.6, respectively). The storage of ovaries for long time period (13 h, 779.3 ± 78.8) produced less ROS levels in CCs than short time periods, 3 h (1 067.8 ± 78.8) and 7 h (1 052.0 ± 78.8). However, no difference in GSH content (670.2 ± 54.3, 687.7 ± 54.3, 543.5 ± 54.3) and mitochondria activity (10.7 ± 2.8, 6. 5± 2.8, 4.7 ± 2.8) was shown for CCs from ovaries stored for 3, 7 and 13 h, respectively. In addition, *in vitro* maturation rate was found significantly higher after 3 h (69.2 ± 6.5) of storage followed by 7 h (34.0 ± 6) and 13 h (4.68 ± 6.5), respectively) ($P<0.001$). In conclusion, ovary storage time negatively influenced CCs viability that may be responsible for alters in oocyte quality and the *in vitro* maturation parameters in sheep.

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A177E Embryology, developmental biology and physiology of reproduction

Effect of vitrification of prepubertal goat oocytes matured with melatonin on embryo development after Parthenogenic Activation

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Keywords*:* Melatonin, Vitrification, Goat oocytes.

It is known that vitrification negatively affects oocyte quality. Melatonin added to the *in vitro* maturation (IVM) medium helps to improve embryo development of prepubertal goat oocytes (Soto-Heras, Reprod Fertil Dev;30(2):253-261.2018). The aim of this study was to assess the effects of vitrification of IVM-oocytes matured with melatonin on; a) intra-oocyte Reactive Oxygen Species (ROS) levels, b) oocyte apoptosis and c) blastocyst development after Parthenogenic activation (PA). Prepubertal goat oocytes were subjected to IVM in our conventional conditions. Three experimental groups were designed: 1) Control group (CG): oocytes after IVM. 2) Vitrified Group (VG): oocytes vitrified after conventional IVM and 3) Melatonin Vitrified group (MVG): oocytes matured with 10^{-7} M melatonin and vitrified after IVM. After 22 h of IVM, oocytes from VG and MVG groups were vitrified in an open-system using CVM™ Cryologic devices (IVF Bioscience; UK) and vitrification and warming protocol and solutions as described by Kuwayama (Kuwayama, Reprod Biomed Online;11:300-8. 2005). At the end of IVM, intra-oocyte ROS level was measured by staining denuded oocytes during 30 min with 10 μM 2',7' dichlorodihydrofluorescein diacetate (Molecular Probes Inc., OR, USA) (36-37 oocytes per group in 3 replicates). Oocyte apoptosis was analysed using eBioscience™ Annexin V Apoptosis Detection kit (Invitrogen. USA). 98-118 oocytes per group in 5 replicates were classified as viable (unstained), early apoptotic (stained with annexin), dead non-apoptotic (stained with propidium iodide) and necrotic (stained with both: annexin and propidium iodide). PA of mature oocytes was performed by 4 min incubation with 5 μM Ionomicyn followed by 4 h incubation with 2 mM 6-(Dimethylamino) purine (Sigma-Aldrich®Chemical,St. Louis.USA) (83-151 oocytes per group in 4 replicates). Presumptive zygotes were *in vitro* cultured in BO-IVC medium (IVF Bioscience; UK) for 8 days. Data were analyzed by two-way ANOVA followed by Tukey's multiple-comparison test (SAS® software version 9.4). ROS levels were lower in CG than VG oocytes (21327 ± 3309. vs 36959 ± 4336; $P<0.05$). No differences were found between VG and MVG groups. The percentage of viable oocytes after IVM was significantly higher ($P<0.05$) in the CG (67.0%±3.5%) than in both VG (50.8%±2.9) and MVG (39.0%±5.6) vitrified groups. After PA, CG showed higher cleavage rate than VG (80.1 ± 5.3 vs 53.4 ± 9.9, $P<0.05$) and a tendency to higher cleavage rate than MVG (55.6 ± 1.2 %, $P=0.055$), but no differences were found between vitrified groups. However, we did not find any blastocyst development in any of the vitrified groups regardless of the presence of melatonin, whereas in the CG we obtained 19.72%±3.38 blastocysts per oocytes. In conclusion, the vitrification process increased ROS levels and apoptosis in prepubertal goat oocytes and hinder the blastocyst development. Melatonin supplementation during IVM did not prevent these negative effects.

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A178E Embryology, developmental biology and physiology of reproduction

In silico prospection reveals evolutionary divergence in the cattle DNA methylation pathway

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Keywords*:* bioinformatics, bovine, epigenetics, thermoregulation.

DNA methylation is an epigenetic mechanism that controls gene activity by bookmarking CpG-enriched DNA sequences which recruits methyl-binding trans-acting factors mostly for gene silencing. Multiple reports have demonstrated compelling data that environmental factors – such as heat stress - affect the DNA methylation patterns of germ cells and early embryos. Nevertheless, little is known about the intrinsic epigenetic divergences between taurine - *Bos taurus taurus (B. taurus*) and zebu - *Bos taurus indicus (B. indicus*) cattle, in spite of the greater adaptability of *B. indicus* to harsh environments. Therefore, a preliminary study using *in silico* tools was conducted to prospect evolutionary differences in the components of the DNA methylation pathway (DNA methyl-transferases, transcription factors, and co-factors) by sequence homology approaches between *B. taurus* and *B. indicus*. Initially, DNMTs were scrutinized for fishing possible divergences in DNA methylation activity. The genome anchorage carried out using the "genome data viewer" was similar between subspecies, where DNMT1 is localized on chromosome 7, DNMT2 and DNMT3B on chromosome 13, DNMT3A on chromosome 11, and DNMT3L on chromosome 1. Both *B. taurus* and *B. indicus* hold an identical number of DNMT isoforms (DNMT2: 2, DNMT3A: 2 and DNMT3B: 6), except DNMT1 (*B. taurus*: 10 vs. *B. indicus*: 1) and DNMT3L (B. taurus: 2 vs. *B. indicus*: 1). DNMTs display similar size and sequence between subspecies (DNMT2: 391 Aa, DNMT3A: 909 Aa, DNMT3L: 417 Aa). However, one DNMT3L isoform in *B. indicus* displays four additional amino acids (Aa) in the N-terminus. Six DNMT3B isoforms were found with variable size (*B. taurus*: 773 - 844 Aa vs. *B. indicus*: 733 - 842), with a divergent 10-Aa stretch in the N-terminus and another of 100 Aa in the C-terminus. The larger enzyme is DNMT1 (*B. taurus*: 1,611 Aa vs. *B. indicus*: 1,644). Remarkably, the *B. indicus* DNMT1 N-terminus displays an alanine-rich sequence, which may confer greater structural or thermal stability. No difference was found at the protein domain level of DNMTs between *B. taurus* and *B. indicus* by the CD-search tool. Additional DNA methylation pathway components involved in DNA methylation maintenance were thus addressed. The UHRF1, ZFP57, DPPA3/STELLA, and ZNF445 genes did not show any sequence difference between *B. taurus* and *B. indicus* for the aforementioned protein traits. There is evidence that the expression of DNMT1 is enriched in *B. taurus* spleen and testis within nine tissues investigated (expression atlas - www.ebi.ac.uk). Moreover, DNMT1 mRNA levels have been shown to fluctuate in *B. indicus in vitro* produced embryos and in oocytes and embryos of unknown genotype under heat shock. Even though Dnmt1 protein is found throughout oocyte maturation, it is only present in the cytoplasm of early embryos up to the 8-to-16 cell stage when genome activation is established, probably contributing to imprinting maintenance. In conclusion, the components of the DNA methylation pathway are highly conserved between *B. taurus* and *B. indicus*, although DNMT1 displays evolutionary-driven variation that deserves further experimental investigation.

A179E Embryology, developmental biology and physiology of reproduction

The potential role of isoquercitrin by interfering with cellular reactive oxygen species and growth factors in human ovarian cancer cells

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Keywords*:* isoquercitrin, OVCAR-3, ROS production.

Nowadays, many studies have reported that beneficial properties of flavonoid compounds from medicinal plants can be attributed to isoquercitrin. Isoquercitrin is powerful phytocompound that have been shown to exhibit disease prevention and health promotion properties. The aim of our study was to investigate the impact of isoquercitrin (purity 96.5 %, prepared by selective enzymatic derhamnosylation of rutin) treatment at the concentrations 5, 10, 25, 50, and 100 μg/mL on a human ovarian carcinoma cell culture (OVCAR-3) *in vitro.* Cell viability, cell death, apoptosis, the release of human epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), insulin-like growth factor I (IGF-I), and the production of reactive oxygen species (ROS) by cells after short-term application of 24 h were analyzed. The metabolic activity was determined by AlamarBlue^{TM} assay, the apoptotic assay using flow cytometry, presence of growth factors was detected by ELISA method, the ROS production was quantified by chemiluminescence. One-way ANOVA along with Dunnett's test was used to establish statistical significance at $P<0.05$. All experiments were done in triplicate. Isoquercitrin caused any significant changes neither in metabolic activity of OVCAR-3 cells, nor in the proportion of live, dead and apoptotic cells, nor in the release of EGF, TGF-β1, and IGF-I ($P>0.05$). However, tendency of a slight increase of TGF-β1 level after isoquercitrin application at the highest concentration 100 μg/mL was detected. Interestingly, our results showed, that lower concentrations (5, 10 and 25 μg/mL) significantly ($P<0.01$) inhibited the production ROS. On the other hand, ROS production observed in OVCAR-3 cells after isoquercitrin treatment was significantly ($P<0.001$) increased at the high concentrations (50 and 100 μg/mL). In conclusion, the results of our *in vitro* study show antioxidant and pro-oxidant activities of isoquercitrin in dose-dependent manner. ROS production by OVCAR-3 cells was increased at hight concentrations of isoquercitrin and decreased at lower concentrations. Isoquercitrin at any used concentrations did not interfere with growth factors in human ovarian carcinoma cells, however, further studies are required to better understandig of biological actions of isoquercitrin.

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A180E Embryology, developmental biology and physiology of reproduction

Inhibition of miR-152 during *in vitro* maturation enhances the developmental potential of porcine oocyte

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Keywords*:* oocyte, transfection, miRNA.

Oocyte and embryo development are regulated by complex molecular mechanisms. Several molecules are involved in these regulation mechanisms including microRNAs (miRNAs). MiR-152 is well known as a tumor suppressor in human cancer by inhibiting cell proliferation and suppressing the PI3K/Akt and MAPK signaling. We have shown previously that miR-152 is upregulated in fully-grown compared to growing oocytes. Several genes required for progression through different stages of meiosis are putatively targeted by this miRNA. However, its specific role in oocyte and embryo development is still unknown. In this study, we evaluated the developmental potential of porcine oocytes after manipulation of miR-152 abundance during in vitro maturation using mimic, inhibitor or random sequence miRNA as negative control. Cumulus-oocytes complexes isolated from 3 – 6 mm follicles of premature gilts have been cultivated in maturation medium TCM 199 (Sigma-Aldrich, Munich, Germany) without hormonal stimulation but with dibutyryladenosine cyclic monophosphate (dbcAMP) and miR-152 mimic (40nM), inhibitor (600nM) or negative control (40nM) in the presence of Lipofectamine 3000 (Thermo Fisher Scientific, Massachusetts, USA) for 4 hours. Then transfection was continued in TCM 199 with hormonal stimulation for 44 hours and metaphase II (MII) rate was calculated. Some of the matured oocytes were parthenogenetically activated and cultivated in porcine zygote medium 3 (PZM 3) for 6 days until blastocyst stage. The abundance of miR-152 was analyzed in MII oocytes using TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) on droplet digital PCR (ddPCR) system (Bio-Rad Inc.). Our preliminary results showed that mimic and inhibitor treatments change the abundance of miR-152 in MII oocytes by 2.2 and -2.5 folds, respectively, compared to the negative control group. There were no differences in MII rate among negative control, mimic and inhibitor group (92±1%, 92±2%, and 91±5%, respectively) or in cleavage rate of parthenotes of the same treatment groups (71±12%, 77±3%, and 82±7%, respectively). On the other hand, the blastocyst rate of parthenotes was significantly higher in the inhibitor group (48±3%) compared to negative control (28±5%) or mimic group (22±9%). In conclusion, inhibition of miR-152 during oocyte maturation could enhance the developmental rate of porcine parthenotes. More studies are required to understand the exact function of miR-152 during oocyte and embryo development.

A181E Embryology, developmental biology and physiology of reproduction

Establishment of a single embryo sequential culture system for cell free DNA (cfDNA) based genetic screening in cattle

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Keywords*:* Culture media, sexing PCR, blastocyst.

Recent studies have demonstrated the natural release of genomic DNA by embryo during culture. This cell free DNA (cfDNA) could be employed in whole genome amplification (WGA) strategies to obtain representative quantities of embryonic DNA without the need for invasive techniques, minimising damage to the blastocyst intended for embryo transfer, and provides an easily scalable platform for cattle breeders interested in screening large numbers of *in vitro* produced (IVP) embryos for sex or genetic value, a growing trend in the current market. As a result, the development of efficient single embryo culture systems to obtain cfDNA from spent culture media has immense potential for the genetic screening of IVP blastocysts. The present study aims to optimise a culture system, free from foreign DNA contaminants, for the *in vitro* culture of cattle zygotes, to demonstrate presence of amplifiable quantities of cfDNA in this system, and to obtain sexing diagnoses by PCR. Oocytes from abattoir material across two replicates (n=146) were matured for 22 h and fertilised with frozen/thawed bull sperm. Presumptive zygotes derived from the same dam were co-cultured for 3 days in 100 µl of Synthetic Oviduct Fluid (SOF) medium supplemented with 6 mg/ml BSA. The zygotes were then cultured in individual SOF droplets of 25 µl for a further 4 days when blastulation rates were recorded. Following this, individual media samples of different volumes (2 or 5 µl) from each drop and the corresponding blastocysts (n=5) were collected separately on day 7 post-IVF and subjected to WGA by DOPlify® kit (Perkin-Elmer, Waltham, MA), as per manufacturer's instructions. Quantification of the WGA products was obtained by Qubit dsDNA HS fluorometry assay (Thermo Fisher Scientific, Waltham, MA) as per manufacturer's instructions using a 1:200 dilution of the original WGA sample. Following amplification, sexing was performed by PCR using chromosome Y (SRY primers) and bovine autosomal primers (BSPF primers). In this pilot study, culture in single droplets produced a blastocyst rate of 36% per cleaved embryo (n=21 blastocysts and n=58 cleaved embryos). The average cfDNA yield per 25 µl following WGA was 0.92 ± 0.15 µg, while whole blastocysts produced a similar amount of 1.08 ± 0.33 µg (t-test, P= 0.66). There was no statistically significant difference in the total yield of DNA amplified when either 2 or 5µl of spent culture medium were used (paired t-test, P=0.31). The proportion of WGA samples producing a sexing PCR result was 50% and the concordance for sex determination between the blastocyst and its medium was 25% (n=12). While presence of cattle specific DNA was confirmed by PCR, the results presented suggest DNA cross-contamination may affect WGA and PCR efficiency. To address this, future tests will replace BSA with recombinant human serum albumin (HSA) and will include media changes during culture. To improve PCR specificity we will employ shorter amplicons since cfDNA is known to be of very low molecular weight. Following optimisation, we will use these cfDNA samples for single nucleotide polymorphism typing to develop a non-invasive aneuploidy screening strategy for cattle IVP.

A182E Embryology, developmental biology and physiology of reproduction

Timing of pronucleus formation and first DNA replication in porcine IVP zygotes using frozen-thawed spermatozoa

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Keywords*:* pig, embryo, DNA replication.

Improvements in the efficiency and repeatability of porcine IVP is necessary for the application and optimization of new technologies as gene edition of embryos by CRISPR/Cas9. Our lab has optimized IVF procedures like sperm selection by swim-up, the addition of natural fluids (Cánovas, eLife. 6:e23670, 2017) and variations of atmosphere conditions in IVF (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). For future experimental designs and optimize the IVF system could be useful to know the kinetic of penetration, pronuclear formation and DNA replication. To determinate starting of DNA replication 441 porcine oocytes were *in vitro* matured in NCSU-37 medium (Cánovas, eLife. 6:e23670, 2017) and were inseminated in TALP with frozen-thawed boar spermatozoa selected with NaturARTs-Pig sperm swim-up medium (EmbryoCloud, Murcia, Spain) and cultured at 38ºC, 5% CO_2 and 7% O_2 (García-Martínez, Mol Hum Reprod. 1;24(5):260-270, 2018). Groups of 25 oocytes were fixed every hour from 4 hours to 12 hours and 24 hours after IVF and were staining with Click-iT™ EdU Alexa Fluor™ Imaging Kit (Invitrogen, Spain) to determinate penetration rate (PEN), spermatozoa/oocyte (S/O), male pronucleus formation (MPF) and rate of zygotes with DNA replication. PEN already started at 4h after IVF (15.79%) and was increasing to 55.00-66.67% at 5-8h until reaching 80.43% at 9h and without significant differences with the following hours. Pronuclei formation started at 6h after IVF (MPF = 12.00%), increasing to 52.78% at 7h and near 100% since 8h (93.33%). DNA replication was not detected until 10h after IVF (20.00%) with a slight increase at 11h (37.78%) and 12h (43.48%) to reach 100% at 24h. The kinetic of penetration and MPF depend on various factors like male, sperm preservation (fresh or frozen-thawed), sperm origin (epididymal or ejaculated), sperm selection and capacitation method. Our swim-up method has similar time of penetration rate and pronucleus formation respect other fresh sperm preparation and capacitation method described in the literature but Percoll gradient advance both parameters (Matás, Reproduction. 125(1), 133-41, 2003). Regarding the beginning of DNA replication, our study had a delay of 2h respect the commence of DNA replication in porcine embryos produced by IVF previously reported (Jeong, Dev Dyn 236(6), 1509-16, 2007). Know the time when first DNA replication takes place in the zygote is important to plan strategies of technology applications like genetic engineering, for which gene edition previous DNA replication is an important objective to reduce mosaicism in the future organism. Supported by MINECO-FEDER (AGL 2015-66341-R), Fundación Séneca 20040/GERM/16 and FPU fellowship (FPU16/04480) from the Spanish Ministry of Education, Culture and Sport.

A183E Embryology, developmental biology and physiology of reproduction

In vitro production of the first reindeer (Rangifer tarandus tarandus) blastocysts

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Keywords*:* ruminant, ex situ conservation.

Reindeer herding is economically, societally, culturally and ecologically important livelihood in northern Eurasia. In the future, climate and socio-economical changes in the arctic region may challenge the vitality of the reindeer populations. Optimized reproductive technologies could be used both for the conservation of the reindeer genetic resources and to facilitate successful breeding of reindeer in the future. The aim of this study was to test the suitability of optimized bovine *in vitro* embryo production protocol for reindeer embryo production *in vitro.* Reindeer ovaries were collected during the period of cyclic ovarian activity which extends from September to February in reindeer females. At the slaughterhouse, reindeer ovaries were sliced after collection in EMCARE^{TM} Biofree Flushing solution (ICPbio Reproduction, Spring valley, WI, USA). Recovered and washed oocytes were placed in tubes containing Hepes-buffered TCM199 supplemented with glutaMAX-I (100 × stock solution, Gibco™; Life Technologies Limited, Paisley, UK), 0.25 mM Na-pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 ng/ml FSH (Puregon, Organon, Oss, Netherlands), 1 µg/ml β-estradiol (E-2257) and 10% heat inactivated FBS (Gibco™, New Zealand) for maturation for 24 h at 38.5°C in air while transported to laboratory. Matured oocytes were fertilized for 20 h with Sperm-TL washed (2×4 ml) frozen-thawed semen (Lindeberg H, Nikitkina E, Nagy Sz, Krutikova A, Kumpula J, Holand Ø 2019, Abstract book of the 10th Circumpolar Agriculture Conference, 13th – 15th March 2019, Rovaniemi, Finland. p. 87) in IVF-TL supplemented with heparin (10 µg/ml) and PHE having 1×10^6 spermatozoa/ml as a final concentration. Denuded zygotes were cultured in G1/G2 media (Vitrolife, Göteborg, Sweden) supplemented with FAFBSA (4 mg/ml) and L-carnitine (1.5 mM) at 38.5°C in maximal humidity in 5% O_2, 5% CO_2 and 90% N_2. Cleavage rates were recorded at 42 hpi. Blastocysts were recorded on days 7 and 8 (IVF=day 0). Unless otherwise stated all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The average cleavage rate was 28.4% for the 162 best quality oocytes selected for maturation during the two slaughterhouse visits, one in December and the other in January. Altogether four and two blastocysts (3.7%) were produced by day 7 and 8, respectively. In conclusion, bovine *in vitro* embryo production protocol may also be used to produce reindeer embryos *in vitro*. However, the challenge is the oocyte quality as most of the slaughtered females are either prepubertal or old.

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A184E Embryology, developmental biology and physiology of reproduction

Gene expression profiles of bovine genital ridges during sex determination and early differentiation of the gonads

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Keywords*:* sex determination, Rna-seq, cattle.

Sex determination in mammals depends on a complex interplay of signals that promote the bipotential fetal gonad to develop as either a testis or an ovary. Most knowledge of this process has arisen from experiments in the mouse model. (Sekido and Lovell-Badge 2008; Kim et al. 2007). In mice, the differentiation of supporting cell progenitors into male-specific Sertoli cells or female-specific granulosa cells is controlled by SOX9 presence or absence. However, there is scarce information concerning the process of sex determination in livestock species, especially in cattle. In order to clarify the process of sex determination in cattle, we used an RNA sequencing (RNA-seq) strategy to analyze the transcriptome landscape of male and female bovine fetal gonads collected *in vivo* at important developmental stages before, during, and after *SRY* activation. The estrous cycles of cross-breed heifers were synchronized followed by AI, and heifers were slaughtered at 35 (n=12; bipotential gonad formation), 39 (n=12; *SRY* peak of expression) and 43 (n=9; early gonad differentiation) days later. At each time-point, genital ridges were dissected from mesonephros and RNA was extracted using a Direct-zol^{TM} RNA MiniPrep Kit (Zymo Research, CA, USA) following the manufacturer's protocol. After PCR sexing of the fetuses, RNA-seq libraries were prepared from 3 male and female samples and were sequenced using a HiSeq2500 v4 chemistry system at the Centre of Genomic Regulation (Barcelona, Spain). Differential gene expression analyses were performed independently using DESeq2 v.1.20 (adjusted p-value < 0.05). Firstly, we identified the differentially expressed genes (DEGs) between male and female gonads (sex analysis at D35, D39 and D43). Secondly, we identified DEGs during the period of transition between sex determination and differentiation within each sex (time-couse analysis between D35 and D39, and between D39 and 43). We also used a hierarchical clustering approach to cluster genes with similar expression profiles during the period from D35, D39 and D43. We performed western blot and immunofluorescence analysis of SOX9 and SOX10 to check if expression was also evident at the protein level. Gene analysis identified 143, 96 and 658 DEGs between males and females at D35, D39 and D43, respectively. Regarding the time-course analysis, 767 DEGs were identified in the comparison between D35 vs D39 male gonads, and 545 DEGs in the female gonads. In the comparison between D39 vs D43, 3157 DEGs were identified in males, and 2008 DEGS were identified in females. We found expression of several Y chromosome genes before SRY (that are absent in mice and human), *SOX9* and *SOX10* expression in both somatic and germinal cell lineages in the XY genital ridge during sex determination, the nuclear and higher expression of SOX10 instead of SOX9 in Sertoli cells during male determination and early differentiation, a lack of nuclear internalization of SOX9 in Sertoli cells during early sex differentiation and no early expression of the WNT/β-catenin pathway repressing *SOX9* in gonads. In conclusion, our data indicate that sex determination and early gonad differentiation in cattle exhibit some unique characteristics.

A185E Embryology, developmental biology and physiology of reproduction

Metabolic changes in uterine fluid collected from cyclic heifers at different stages of embryo receptivity

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Keywords*:* Metabolism, elongation, uterine fluid.

Preimplantation embryo development relies on the metabolites, hormones and growth factors present in oviductal and uterine fluids. *In vitro* culture conditions partially recapitulate the natural milieu where embryos develop, but a culture system capable of supporting bovine embryo development beyond the blastocyst has not been yet established, suggesting that medium composition requires further optimization. The objective of this study was to characterize the changes in biochemical compounds in bovine uterine fluid (UF) at different stages of embryo receptivity. To achieve this, 22 crossbred heifers were synchronized and UF was collected post-mortem by uterine flushing using 20 ml of DPBS at specific days after ovulation: 1) Day 0, when UF does not support embryo development, 2) Day 7, when UF supports blastocyst development, 3) Day 10, just prior to when UF triggers conceptus elongation, and 4) Day 14, when UF supports the exponential growth phase during conceptus elongation. UF (n=6, 5, 6 and 5 samples for Days 0, 7, 10 and 14, respectively) were centrifuged at 1500 g for 15 min at 4ºC to remove cell debris and supernatants were kept at -80 ºC until analyses. Metabolomic analyses were performed by Metabolon Inc., which provides an unbiased metabolite analyses based on Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). Welch´s two-sample t-test was used to identify biochemicals that differed significantly between groups ($p<0.05$). The analysis identified 359 compounds of known identity. The most dramatic changes in the abundance of different compounds occurred between D7 and D14, suggesting that elongation requires significant modifications in UF composition. Biochemicals related to glucose metabolism showed significant changes over time. For instance, glucose, fructose, mannitol/sorbitol and pyruvate increased over time (1.5-, 2.3-, 190- and 5-fold increases between D7 and D14). Significant changes were also noted for glutamate metabolism, with a steady increase of beta-citrylglutamate (24-fold increase between D7 and D14). Compounds involved in Krebs cycle also exhibited significant variation between days, with increases in citrate, aconitate and 2-methylcitrate/homocitrate (8-, 12- and 11-fold increases between D7 and D14). These results highlight that embryo metabolic requirements vary greatly between blastocyst hatching and conceptus elongation and provide relevant insights to develop an *in vitro* system to achieve the cell proliferation, differentiation and migration events involved in this process.
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A186E Embryology, developmental biology and physiology of reproduction

Post-hatching *in vitro* bovine embryo development inside agarose tunnels or without physical constriction

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Keywords*:* Elongation, conceptus, hypoblast.

The greatest gestational losses in cattle occur during the second week of pregnancy, when critical developmental events take place: hypoblast migrates to cover the entire inner surface of the embryo, and epiblast forms a flat embryonic disk. Previous studies have established an *in vitro* post-hatching development system based on agarose gel tunnels and glucose-enriched medium. This system achieves some expansion of the trophoblast and hypoblast proliferation. However, embryonic disc formation is not achieved and it remains unclear whether the hypoblast covers entirely the inner surface of the embryo. An open question about this system is whether embryo culture inside tunnels is actually required for development or it just shapes the embryo to a tubular shape by mechanical constriction. The objective of this study has been to compare post-hatching development inside agarose tunnels or free-floating in an agarose-coated dish. *In vitro*-produced E11 blastocysts were measured and cultured in Synthetic Oviduct Fluid (SOF) supplemented with 27.7 mM glucose and 10 % FCS inside ~1mm agarose tunnels or over an agarose surface until E15. At the end of the culture period, embryo area was calculated using Fiji software and the development of specific lineages was assessed by immunostaining for SOX2 and NANOG (epiblast), SOX17 (hypoblast), and CDX2 (trophectoderm). No differences were found on embryo survival until E15 and the main factor determining survival was the initial embryo size at E11. In particular, when <0.5 mm E11 were cultured, only 1/16 (6 %) embryos cultured free-floating or 1/18 (6 %) cultured in tunnel survived, while when E11 embryo diameter was ≥ 0.5 mm, 12/17 (71 %) and 16/21 (76 %) survived when cultured free-floating or inside tunnel, respectively. Surviving embryos showed a cylindrical shape when they developed inside the tunnels and spherical when they develop without physical constriction, but area and volume were significantly smaller in embryos cultured inside a tunnel (2.19±0.21 vs. 4.76±1.14 mm^2 and 1.71±0.40 vs. 9.32±2.92 mm^3, for embryos cultured in tunnel or free-floating, respectively, t-test $p \leq 0.05$). A layer of hypoblast cells (SOX17+) was detected inside the trophectoderm, but, irrespective of the culture system, that layer did not cover the entire inner surface of the embryo. Similarly, although a compact cell structure was detected in some developmentally advanced embryos, no SOX2 or NANOG-positive epiblast cells were detected in any of the culture systems. In summary, post-hatching blastocyst culture inside agarose tunnels shapes embryo morphology by physical constriction, but it may restrict embryo growth and does not seem to provide any significant advantage in terms of development of hypoblast and epiblast lineages. The partial hypoblast migration and the absence of embryonic disc highlight that post-hatching culture conditions still requires significant optimization.
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A187E Embryology, developmental biology and physiology of reproduction

Changes in the transcriptome of ovine MII oocytes caused by lipopolysaccharide

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Keywords*:* ovine oocyte, lipopolysaccharide, differential gene expression.

Increasing low fiber high fermentable carbohydrate diets increase the ruminal lipopolysaccharide (LPS) derived from gram negative bacteria cell walls. LPS was detected in plasma and follicular fluid of ruminants with endometritis and mastitis and disturbed the reproduction performance. While several studies have examined the effect of LPS on oocyte maturation and developmental competence, limited knowledge is available on potential effect of LPS on transcript aboundance of ovine oocytes. Thus, transcriptome profiles of MII oocytes matured in presence or absence of LPS were compared using 3'tag digital gene expression method. Cumulus oocyte complexes collected from salaughterhouse-derived ovaries were matured either in media supplemented with 0.1 μg/mL, 1 μg/mL, 10 μg/mL of LPS (Sigma Aldrich Inc) or in media without LPS (control). After in vitro maturation, the cleavage and blastocyst formation following parthenogenetic activation were determined for each group. Subsequently, three biological replicates of 36 oocytes cultured in 1 μg/mL LPS and controls were subjected to 3'tag digital gene expression profiling. Differential expression analysis was performed using the R Statistical Programming Language and limma package (using voom method, an animal as a blocking factor and treatment in the model). Functional enrichment analysis of the differentially expressed genes was further performed using Enricher database. Our results showed that maturation rate (determined based on first polar body extrusion), was not significantly different between the groups. The lowest LPS dose that significantly affected developmental competence to blastocyst stage was 1 μg/mL of LPS (unpublished data). After culturing ovine oocytes in vitro for 22 hours in the presence of 0 μg/mL (control) and 1 μg/mL, a total of 7887 gene transcripts were detected and only eight genes were differentially expressed. Of these, seven genes were down-regulated (two-fold or greater) in LPS-treated group (adjusted $p< 0.05$). Down regulated genes were the following: (Tripartite motif containing 25 (TRIM25), Tripartite motif containing 26 (TRIM26), Zona Pellucida glycoprotein 3(ZP3), Family with sequence similarity 50-member A (FAM50), Glyoxalate and hydroxy pyruvate reductase (GRHPR), cornichon family AMPA receptor auxiliary protein 4 (CNIH4) and NADH ubiquinase oxireductase subunit A8 (NDUFA). Functional analysis showed that these genes were significantly enriched in immune response, oxidation-reduction process as well as oocyte development. It is worth to note that *TRIM25* and*TRIM26* were reported as important genes in innate immune response. In addition, *ZP3* is well known as a positive regulator of inflammation, interferon gamma and interleukin 4 production, oocyte and blastocyst development. Moreover, NDUFA8 is involved in electron transport process which could be related to decreased mitochondrial membrane potential in matured oocytes in the presence of LPS. Accordingly, LPS is associated with impaired developmental competence.In conclusion,our results expand our knowledge of the genes transcribed of non-LPS and LPS treated in MII oocytes, which can shed light on molecular mechanisms of LPS-induced infertility in ruminants.

A188E Embryology, developmental biology and physiology of reproduction

Comparing the ability of epididymal or ejaculated sperm to elicit endometrial transcriptomic changes in cattle

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Keywords*:* seminal plasma, sperm, endometrium.

In mice and pigs there is robust evidence of seminal plasma (SP) modulating the maternal environment, which positively impacts embryo survival and development. However, similar evidence in cattle is sparse. Both mice and boars deposit the ejaculate inside the uterus, while bovine semen deposition takes place in the vagina, and it is questionable whether any SP reaches the uterus. However, at ejaculation, sperm encounter SP, leading to proteins binding tightly to their plasma membrane, which probably allows them to act as a vehicle for SP proteins. Based on the beneficial effect of SP observed in other species, we hypothesised that ejaculated sperm, through SP proteins, elicit a different response in the endometrium than epididymal sperm (which have never been exposed to SP). To test this, a model of endometrial explants, which has been previously used to study embryo-maternal interaction, was used. Six crossbreed heifers were oestrous synchronised and slaughtered 12 h after the onset of oestrus. Three explants from the uterine horn ipsilateral to the preovulatory follicle were obtained from each animal. Epididymal sperm were collected and pooled from the cauda epididymis of three beef bulls slaughtered in a commercial abattoir. In addition, ejaculates were collected by artificial vagina from three Holstein bulls. After pooling, they were washed through a density gradient to isolate ejaculated sperm. Endometrial explants were incubated for 6 h with: 1) medium alone (control); 2) epididymal sperm (10^6 sperm/ml) or 3) ejaculated sperm (10^6 ejaculated sperm/ml). After incubation, they were snap frozen for subsequent RNA sequencing. Strikingly, explants exposed to ejaculated sperm had no differentially expressed genes (DEG) in comparison with control explants. In contrast, explants incubated with epididymal sperm exhibited 48 DEG (32 down and 16 up) in comparison with control explants. For the annotated genes (n=35), the most represented Gene Ontology (GO) terms were “binding” (n=12; 9 down and 3 up) and “catalytic activity” (n=13; 10 down and 3 up) for the molecular function, whereas “cellular process” (n=13; 10 down and 3 up) was the highest represented term in the biological process category. When explants exposed to ejaculated sperm were compared with those exposed to epididymal sperm, 80 DEG were identified (72 up and 8 down). For the 64 annotated genes, “binding” (n=21; 19 up and 2 down), “biological process” (n=29; 26 up and 3 down) were again the most represented GO terms, together with “catalytic activity” (n=17; 16 up and 1 down), and “biological regulation” (n=13; 13 up and 0 down) in the biological process category. In conclusion, these data do not support a role for bovine SP in the regulation of endometrial function. In contrast, the results suggest that SP may mask sperm surface proteins, inhibiting their interaction with the endometrium. Because the media used in this experiment does not support sperm capacitation, it remains to be determined whether this process, which is triggered in the female reproductive tract, can reshape the sperm surface in a way that it enables sperm-endometrium interaction.

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A189E Embryology, developmental biology and physiology of reproduction

Improvement of pig embryonic development after the addition of haptoglobin (Hp) to the culture medium

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Keywords*:* embryo, haptoglobin, porcine.

Haptoglobin (Hp) is an acute phase protein recently detected in female reproductive structures (ovary, endometrium and decidua) and fluids (vaginal and amniotic). We previously described the presence of mRNA and detection by immunohistochemistry of HP in the porcine oviduct along of the oestrous cycle, especially in postovulatory and luteal phases. The upregulation of HP gene in these phases could indicate a function in embryo development. The aim of this study was to evaluate the effect of Hp protein on pig embryonic development. Oocytes were matured in NCSU-37 medium at 38.5ºC, 5% CO_2 and 95% humidity. Later on, *in vitro* matured oocytes were mechanically stripped, transferred to TALP medium and co-incubated at 38.5°C, 5% CO_2 with $1x10^4$ spz/ml porcine sperm selected by a discontinuous Percoll® (Pharmacia, Uppsala, Sweden) gradient (45/90%). At 18h post-insemination putative zygotes were transferred to NCSU23a medium supplemented and incubated for 22-24h. Subsequently, the percentage of cleavage was evaluated and only those zygotes that presented 2 to 4 cells were transferred to NCSU23b medium (in which the sodium pyruvate and lactate of the NCSU23a medium were replaced by D-glucose 5.55mM) for another 120h under the same conditions previously mentioned, to complete a development of 7 days post insemination. In the case of the Hp group, TALP and NCSU23a culture media were supplemented with purified pig Hp protein (HGLB12-N-25. Alfa Diagnostic Internacional, San Antonio, EEUU) at a final concentration of 10 µg/ml. Blastocysts obtained were photographed and their diameter was evaluated by ImageJ® program. Finally, blastocysts were stained with Hoechst 33342 to evaluate the total number of cells per blastocyst. The data were analyzed by Chi-square test ($p<0.05$). Our results showed that cell division was similar in both experimental groups (control: 35.60% vs. Hp: 32.97%). However, the blastocyst development was higher in the Hp group in comparison with the control (control: 37.83 % vs. Hp: 64.50 %). In the case of the embryo quality, both the diameter (control: 357.86 ± 8.98 µm vs. Hp: 373.13 ± 5.40 µm) and the number of cells per blastocyst (control: 52.46 ± 2.73 vs. Hp: 56.11 ± 2.50) were identical in both groups (control: N=14 and Hp: N=20 blastocysts evaluated respectively). In conclusion, adding Hp protein into the culture medium increases the number of embryos that reach the blastocyst stage.
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A190E Embryology, developmental biology and physiology of reproduction

Immunofluorescence analysis of NR3C1 receptor following cortisol exposure during bovine *in vitro* oocyte maturation

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Keywords*:* oocyte, cortisol, NR3C1.

Glucocorticoid hormones (GCs) play a key role in a various set of important cellular and physiological functions such as stress signaling, lipid and carbohydrate metabolism, apoptosis and mitochondrial activity. However, the mechanisms by which stress and glucocorticoids damage or protect the oocyte are largely unknown. Current knowledge has reported differences in the effect of cortisol exposure depending on the species. As completely opposite examples, pig oocyte in vitro maturation (IVM) was inhibited by cortisol (Yang, Biol Reprod 60:929–936, 1999), whereas equine was not impaired (Scarlet, Dom Anim Endocrin 59:11-22, 2017) and a previous study in bovine reported beneficial effect of cortisol in the IVM medium on blastocyst rate (daCosta, Theriogenology 85(2):323-329, 2016). Here we studied the nuclear maturation rates and the levels of the glucocorticoid receptor (NR3C1) after exposure of bovine oocytes to cortisol during IVM. Briefly, cumulus-oocyte complexes (COCs) were cultured for 24h with cortisol (Control (n=374) (C): 0µg/mL, Control vehicle (n=371) (CV): ethanol, CORT1(n=372): 0.1µg/mL, CORT2 (n=370): 0.25µg/mL) at 38.5ºC in an atmosphere of 5% CO2 in humidified air. After 24 h of IVM, oocytes were denuded and fixed in 4% paraformaldehyde (30 min at 38.5ºC) and permeated (0.3% Triton-X100, 30 min, room temperature). For nuclear maturation assessment, oocytes were mounted in DAPI medium (Vector labs, Burlingame, USA) for chromosome staining and coverslipped. Metaphase II achievement status was checked. For immunofluorescence determination of NR3C1 glucocorticoid receptor presence, permeated oocytes were incubated overnight at 4ºC with anti-rabbit primary antibody (1:500) (GR/NR3C1 NBP2-42221, Novus Bio, Centennial, USA), washed 5x in PBS and then incubated with goat anti-rabbit IgG H&L (Alexa Fluor® 488) secondary antibody (1:1000) for 1h at room temperature for subsequent washing and immunofluorescence semiquantitative assessment. Results showed that nuclear maturation of cortisol treated groups was improved (C: 61.5±1.5; CV: 59.8±3.7; CORT1: 75.3±2.0; CORT2: 76.8±0.5; $p < 0.01$) compared to control ones. NR3C1 expression was 40.1% and 40.9% times more expressed in CORT1 and CORT2, respectively, compared to control groups; while whole intensity of the oocyte was 5.7% and 5.4% increased. Cortisol seems to play a role in the oocyte developmental competence and may be acting directly on oocyte maturation. We hypothesize that this may be due to the preparation of the oocyte for the following stressing phenomenons, acting as a sublethal stress for the acquisition of stress tolerance (Pribenszky, Biol Reprod 563;83:690-7, 2010). Further studies are needed in order to elucidate the specific mechanism by which the glucocorticoid receptor affect the development of oocyte competence on different species.

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A191E Embryology, developmental biology and physiology of reproduction

Production of cloned and fertilized embryos of a cloned bull of buffalo (Bubalus bubalis)

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Keywords*:* buffalo, re-cloning, IVF, and embryos.

Buffalo cloning has gained popularity in India as a valuable tool to make genetically identical copies of superior bulls to mitigate demand of quality semen for breeding schemes. However, it is unknown how the somatic cells of cloned buffalo will behave in re-cloning experiments and fertility of cloned bulls. Also, no data is available about the in vitro fertilization success rate using cloned bull semen. As a step towards answering these apprehensions, we performed re-cloning of the cloned bull that was produced in 2015 by us, and the semen of this cloned bull was used to produce in vitro fertilized embryos. Briefly, the somatic cells of cloned bull and its donor were used for nuclear transfer experiments. Three independent experiment data were used and data were analyzed by Student's t-test using SPSS software (SPSS.com). Following cloning, the blastocyst rate (39.6±1.1 vs 41.2 ±1.2), total cell number (322.0 ±18.2 vs 333.1±28.8) and apoptotic index (3.9±0.5 vs 3.1±0.3) of blastocysts were similar between the donor and cloned bull, respectively. Similarly, there was comparable blastocyst rate (17.5±1.4 vs 16.5±4.8), total cell number (234.8±30.9 vs 202±22.5), and apoptotic index (2.4±0.3 vs 2.5±0.4) of blastocysts produced from IVF procedure using donor and cloned bull semen, respectively. In addition, at the time abstract submission, we established one pregnancy from the transfer of cloned blastocyst of cloned bull and 8 pregnancies following artificial insemination using cloned bull semen. In conclusion, the somatic cells of a cloned bull can be used for re-cloning experiments; whereas, sperms can be used to produce in-vitro fertilized embryos and to impregnate females using artificial insemination.

A192E Embryology, developmental biology and physiology of reproduction

In vivo characterization of pH, CO2 and O2 analytes in the bovine uterus: preliminary study

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Keywords*:* uterus, pH, oxygen.

A precise knowledge of the physiological level of certain environmental parameters such as pH, CO_2 and O_2 are of high relevance for in vitro production of embryos (IVP). The uterus is the site for the transport of sperm, early embryonic development and gestation. However, its physiological environment is poorly defined yet, and in cattle only pH values have been reported (Hugentobler et al., Theriogenology 61, 1419, 2004). By new cutting-edge devices and a non-invasive approach, we aimed to define a new method to record *in vivo* data of pH, CO_2 and O_2 in cows during the ovulation day (Ovul) and luteal (5 days after ovulation, Lut) phases. For this, 3 multiparous Holstein females of 6-8 years under the same conditions of feeding and handling were used. The synchronization of ovulation in all the animals was induced through administration of GnRH at day 0 (0.2 mg i.m., Dalmarelin®, Fatro Ibérica, Barcelona, Spain), together with the application of a progesterone-releasing intravaginal device (1.38 g, CIDR®, Zoetis, Madrid, Spain). On day 7 the intravaginal device was removed and PGF2α (25 mg i.m., Dinolytic®, Zoetis, Madrid, Spain) was administered twice (days 7 and 8, 24 hours interval), plus a final injection of GnRH i.m. (day 9) to induce ovulation. Monitorization of the estrous cycle was verified on a daily basis to detect ovulation time and the existence of corpora lutea through a portable ultrasound scanner (ImaGo®, ECM, Angoulême, France) equipped with a linear transducer from 5 to 7.5 MHz. To facilitate the measurement procedure, all the animals were immobilized in a cattle cage and calmed with 0.20 ml/100 kg of xylazine i.m. 2% (Nerfasin®, Fatro Ibérica, Barcelona, Spain), followed by lidocaine 2% (Anesvet®, Laboratorios Ovejero S.A., León, Spain) epidural (5 ml/animal). Miniaturized (0.3 mm diameter) luminescent probes of pH, CO_2 and O_2 (PreSens®, Regensburg, Germany) were inserted in the caudal-middle part of the ipsilateral uterus horn to ovulation through an insemination steel catheter of 70 cm and 6 mm outer diameter. At Ovul y Lut stages instant values of these parameters were taken simultaneously every 5 seconds for a total of 15 minutes in one cycle per animal. Data were then processed for basic statistics (mean±SD). No inference tests were used due to the low number of animals. The continuous records of pH, CO_2 and O_2 showed variable oscillatory patterns over time. Average pH was 7.13±0.11 (6.99-7.23 range) and 6.98±0.04 (6.95-7.0 range) at Ovul and Lut phases, respectively; CO_2 was 4.21±0.74 % (3.19-4.81 % range) and 5.75±1.55 % (3.67-6.27 % range); and O_2 was 4.35±0.56 % (2.89-5.21 %) and 10.98±0.78 % (8.64-12.30 %). These preliminary results showed that the methodology used can provide an effective characterization of the uterine environmentt of cattle with minimal iatrogenesis. Increasing the number of measurements is necessary to better define the oscillatory patterns of each parameter and ascertain potential differences between the stages of the estrous cycle. Finally, this information might be helpful to optimize IVP protocols in cattle. Acknowledgements.
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A193E Embryology, developmental biology and physiology of reproduction

Embryo quality in relation to endometrial health in cows with problematic reproductive anamnesis intended for MOET

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Keywords*:* embryo quality, subclinical endometritis, somatic cell count.

The quality of embryos is strongly related to the donor cow's overall and reproductive health, age and management. Poor health of the endometrium is a detrimental factor for embryo development, quality and harvest. Periparturient reproductive problems may predispose dairy cows to subclinical endometritis (SE). The diagnosis of SE is proven if more than 5% of polymorphonuclear leucocytes (PMNL) are present in the cytological sample of endometrium >8 weeks post parturition (pp) and >14% of PMNL 4 weeks pp. An inflamed uterus is a poor environment for the developing embryo. The aim of this study was to investigate embryo harvest in Latvian native breed donor-cows with problematic reproductive anamnesis in relation to endometrial health and somatic cell count (SCC) in the milk. Ten Latvian native breed donor-cows which had a problematic reproductive anamnesis (repeated artificial insemination, difficult parturition, stillbirth or elevated somatic cell count in the milk (SCC) and had no any signs of illness were included in this study. Milk recording data from Agricultural Data Centre of Latvia were used to establish productivity and milk quality. Cytological samples were obtained using a uterobrush (Mekalasi, SAXO, Finland) and blood samples were taken to establish count of white blood cells (WBC) after the embryo flushing procedure (7^{th} day after AI). Diff-Quick stain (Sysmex, Japan) was used to visualize cells (PMNL, epithelial cells, lymphocytes, eosinophils (Eo), monocytes). Cytological samples at 400x magnification using immersion oil were investigated. One hundred somatic cells were counted and the percentage of cells was determined in each sample. Results were analysed in relation to obtained transferable and damaged embryos in healthy cows and cows with SE. Average ± standard deviation (SD) was calculated, two-independent samples t-test (Mann-Whitney U test), two-tailed bivariate correlation was performed using *SPSS 17*. Cows were 6.3±2.71 (average±SD) years old (min. 3, max. 12 years), in 3.5±1.90 lactation (min.1 and max. 6 lactation). Productivity was 18.7±6.16 kg/day, milk fat 4.7±0.84%, milk protein 3.6±0.63%, SCC 821.4± 1505.15 thousand/ml (min. 50.0 and max. 5010.0 thousand/ml). Total embryo harvest from all cows was 8.0±6.67 embryos per cow (min. 0, max.18 embryos per cow); 4.7±5.3 embryos per cow were transferable (min. 0, max. 13 embryos) and 2.1±4.10 embryos per cow (min. 0, max. 13 embryos) were degenerated. Subclinical endometritis was diagnosed in 70% of cows on the embryo flushing day. Healthy cows, in comparison to SE cows, had no significant differences regarding WBC in blood (8.0±5.05 vs 7.1±1.40 $x10^3/mm^3$), age (4.8±2.26 vs 6.9±2.80 years), productivity (19.9±6.8 vs 18.2±6.40 kg/day), milk fat (4.9±1.35 vs 4.6±0.65%) and milk protein (3.6±0.25 vs 3.6±0.75%) ($P>0.05$). Cows with SE, in comparison to healthy cows, had statistically significant lower total embryo count (6.1±6.57 vs 12.3±5.50 embryos), transferable embryo count (2.6±4.76 vs 9.7±2.52 embryos) and higher degenerated embryo count (3.6±4.50 vs 1.3±2.30 embryos) ($P<0.05$). SCC in milk strongly correlated with PMNL in the endometrium ($r=0.99$; $P<0.05$) despite an optimal count of WBC in all cows' blood (7.4±2.80 $x10^3/mm^3$). SCC in cows without SE was 253.3±135.24 thousand/ml, but in cows with SE it was 1064.9±1778.09 thousand/ml ($P>0.05$). The count of degenerated embryos correlated with Eo in endometrium ($r=0.97$; $P<0.05$). In conclusion, a cytological investigation of the endometrium should be performed before cows with problematic reproductive anamnesis are considered for use as embryo donors. Studies must be continued to establish at what level increased SCC in milk affects the quality of the embryos in donor cows, because it may be an early marker for successful MOET.

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A194E Embryology, developmental biology and physiology of reproduction

SNP based Preimplantation Genetic Testing for Aneuploidy (PGT-A) to improve pregnancy outcomes in cattle IVP: a blind retrospective study

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Keywords*:* bovine, pregnancy rates, blastocyst.

Currently, the ability to produce Genomic Estimated Breeding Values (GEBVs) from Single Nucleotide Polymorphism (SNP) data acquired from live animals plays a key role in guiding the selection process operated by the cattle breeding industry. Increasingly, this technology is being applied to *in vitro* produced (IVP) embryos as a way to increase genetic gain rates and avoid the birth of unwanted animals. However, a significant proportion of the recipients of a SNP typed embryo will not become pregnant, resulting in a waste of time and resources for breeders. Aneuploidy is the most common cause of early developmental arrest and implantation failure in IVP embryos. Thus, supplementing GEBVs with the use of preimplantation genetic testing for aneuploidy (PGT-A) may ensure the selection of embryos with desirable traits which stand a high chance of returning a pregnancy. Here we employed a new PGT-A algorithm (Handyside *et al*. 2010, J Med Genet, 47:651-8) to obtain ploidy diagnoses from the same SNP data used to establish GEBVs. Heterozygous loci in one parent that are homozygous in the other were used as markers to trace chromosome inheritance across generations. The analysis of haploblock patterns in the chosen embryo was made possible by comparing embryonic and parental SNP information with data acquired from a full sibling (either another embryo or a live-borne). To test the hypothesis that the selection of euploid embryos by our PGT-A algorithm would benefit pregnancy rates, we performed a blind retrospective study analysing the SNP and pregnancy data provided by two commercial cattle breeders: Boviteq (Saint-Hyacinthe, Canada) and Activf-ET (Carlisle, UK). The analysis of 66 embryos revealed that 18.2% of them were aneuploid. When individually transferred, 75.0% of the aneuploid embryos did not result in pregnancy, compared to a rate of just 46.3% for euploid embryos (chi square, P=0.05). One of the pregnancies from aneuploid embryos (n=3) resulted in a miscarriage, effectively increasing aneuploid embryo failure rate to 83.3%. If only euploid embryos had been transferred in this cohort, the average pregnancy rate would have increased from 48.5% to 53.7%. When an embryo transfer resulted in a pregnancy, our PGT-A algorithm identified an euploid embryo in 90.6% of cases; conversely, when there was no pregnancy, aneuploidy was identified in 26.5% of cases. In conclusion, when embryos are euploid, our PGT-A algorithm cannot reliably predict pregnancy outcomes as ploidy is just one aspect of a complex system. Nevertheless, our preliminary study seems to suggest that euploid embryos are more developmentally competent and their elective transfer might offer better value for money to breeders. However, our statistical analysis did not provide a robust answer in this pilot study, and the test of a larger sample is likely necessary to achieve clear statistical significance. In our future work, we will analyse a much larger sample database for a more in-depth analysis. We will then investigate whether certain chromosomal abnormalities are more often associated with reductions in pregnancy rates, and which, if any, can be tolerated by the embryo.

A195E Embryology, developmental biology and physiology of reproduction

Long-term antiapoptotic action of progesterone on bovine oocytes during the second phase of IVM is not mediated through the Bax/Bcl2 pathway

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Keywords*:* progesterone, oocyte apoptosis, Bax/Bcl2 pathway.

Endogenous progesterone (P4) secreted by cumulus cells exerts an antiapoptotic effect on bovine oocytes during IVM through cumulus-expressed proteins (O'Shea et al., Biol Reprod, 89:146, 2013). However, the action of exogenous P4 on the oocytes is not so obvious and may be dependent on the stage of the meiotic maturation. Therefore, the present work was aimed to study: (1) a pattern and duration of effects of P4 and luteotropic hormone prolactin (PRL) on bovine oocyte apoptosis during the second phase of IVM (from M-I to M-II) and (2) a role of the Bax/Bcl2 pathway in these effects. In one-step IVM, bovine cumulus-oocyte complexes (COCs) were matured for 24 h in TCM 199 containing 10% fetal calf serum (FCS), 10 μg/ml FSH, and 10 μg/ml LH (1st IVM medium). In two-step IVM, COCs were cultured in the 1st IVM medium for 12 h and then transferred to TCM 199 containing 10% FCS (2nd IVM medium) and cultured for next 12 h. The 2nd IVM medium was either free of additives (Control) or supplemented with 50 ng/ml P4 or 50 ng/ml bovine PRL (Research Center for Endocrinology, Moscow, Russia). After one-step and two-step IVM, a half of COCs was cultured for additional 24 h in TCM 199 containing 10% FCS to test long-term hormonal effects during aging. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). At the end of culture, oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI. The expression of apoptosis-related genes (*Bax* and *Bcl2*) in oocytes was analyzed by qPCR following RNA isolation by Trizol method and reverse transcription to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA). Levels of the gene expression were normalized by the reference gene *GAPDH*. The data for apoptosis (n=5-6, 87-113 oocytes per treatment) and gene expression (n=3, 30 oocytes per each replicate for each treatment) were analyzed by ANOVA. The rate of M-II oocytes was similar in all the compared groups (83.3-94.3%). Following 24 and 48 h of culture, the apoptosis frequency and the expression of *Bax* and *Bcl2* genes in oocytes did not differ between one-step IVM and the control group of two-step IVM. During 24 h of oocyte aging, the apoptosis frequency increased 2-3 times ($P<0.001$) in all the groups, whereas the relative levels of the transcripts changed only slightly. The addition of P4 (but not PRL) to the 2nd IVM medium resulted in the reduction ($P<0.05$) in the rate of apoptotic oocytes from 11.7±1.2 to 5.9±1.7% after 24 h of maturation. Furthermore, this rate in the P4-treated group was lower than in one-step IVM (17.6±1.6 vs. 24.3±0.4%, $P<0.05$) after 24 h of aging. Meanwhile, P4 did not affect the expression of *Bax* and *Bcl2* genes in matured or aged oocytes. Thus, during the second phase of IVM, exogenous P4 can exert the long-term antiapoptotic effect on bovine oocytes that is not related to modulation of the Bax/Bcl2 pathway. The role of the AVEN-associated pathway is currently under consideration.

A196E Embryology, developmental biology and physiology of reproduction

Bovine oocyte quality when cultured in one-step and different two-step IVM systems

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Keywords*:* two-step IVM system, MII chromosomes, Bax/Bcl2 pathway of oocyte apoptosis.

In vitro matured oocytes are widely used for commercial and research purposes. The quality of oocytes acquired during in vitro maturation is the main limitative factor affecting their capacity for further development. The aim of the present research was to study effects of different conditions of IVM on the state of M-II chromosomes and apoptosis of bovine oocytes. Cumulus-enclosed oocytes (CEOs) were matured in either one-step or two-step IVM systems. In the case of the one-step protocol, CEOs were cultured for 24 h in TCM 199 supplemented with 10% fetal calf serum (FCS), 10 μg/ml porcine FSH, and 10 μg/ml ovine LH (standard medium) at 38.5°C and 5% CO2. In the case of the two-step procedure, CEOs were first cultured for 16 h in the standard medium and then transferred to one of three experimental media and cultured for additional 8 h. The following media for the two-step IVM system were tested: (1) TCM 199 containing 10% FCS (Group 1), (2) TCM 199 containing 3 mg/ml BSA (Group 2), or (3) Fert-TALP medium supplemented with 6 mg/ml BSA (Group 3). Fert-TALP with traditional for IVF concentration of BSA was selected because it can potentially be used throughout maturation and fertilization. At the end of culture, the state of the oocyte nuclear material was evaluated by the Tarkowski's method (N=251). Oocyte apoptosis was detected using the TUNEL kit (Roche, Indianapolis, USA); nuclei were stained with DAPI (N=212). The expression of involved in apoptosis genes *Bax* and *Bcl-2* in oocytes was analyzed by real-time RT-PCR (N=332). The data (4 replicates, 69-114 oocytes per treatment) were analysed by ANOVA. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rate of M-II oocytes was similar in all groups and reached 84.3-87.7%. No effects of the systems on the frequency of M-II chromosome abnormalities (decondensation, adherence, clumping) were observed, with the frequency after culture was: 29.5±3.0 (one-step IVM), 36.5±3.1 (Group 1), 31.7±2.7 (Group 2) and 33.6±1.9% (Group 3). In the one-step system, the rate of matured oocytes with apoptotic signs was 15.1±2.0%. Transfer of CEOs after 16 h of incubation in the standard medium to TCM 199 containing BSA (Group 2) caused a decrease in the rate of oocyte apoptosis to 6.9±1.3% ($p<0.05$). Moreover, the rate of apoptotic oocytes in Group 2 was lower than in Group 1 (19.4 ± 1.1%, $p<0.01$) and in Group 3 (14.5 ± 2.7%, $p<0.05$). The expression level of pro-apoptotic gene *Bax* after oocyte maturation did not differ between groups or systems. Meanwhile, oocyte culture in Group 2 (but not in Group 1 and 3) led to an increase in the transcript abundance for anti-apoptotic gene *Bcl-2* and a decrease in the ratio of *Bax* and *Bcl-2* transcript levels as compared to the one-step system ($p<0.05$). Our data indicate that bovine oocyte culture in the two-step IVM system including oocyte transfer to TCM 199 containing BSA can increase the apoptosis resistance of the oocytes by enhancing expression of anti-apoptotic gene *Bcl-2* and may be used as an alternative for the standard one-step IVM.

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A197E Embryology, developmental biology and physiology of reproduction

Effect of different culture conditions on bovine embryos derived from metabolically compromised oocytes

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Keywords*:* FFA-exposed oocytes, in vitro culture conditions, supplementation.

Metabolic disorders e.g. obesity lead to elevated saturated (stearic; SA, palmitic; PA) and mono-unsaturated (oleic; OA) free fatty acids (FFAs) in serum and follicular fluid. Exposure of maturing oocytes to these FFAs, particularly to PA, hampers embryo development. Supplementation of embryo culture media with Insulin-Transferrin-Selenium (ITS) or serum is used to enhance embryo production; however the effect of such enrichment on development of metabolically compromised oocytes has not been investigated. Here, bovine oocytes (n=3737) were exposed to either 1) pathophysiological high PA, SA and OA concentrations (150, 75, 200 µM, respectively; **HFA**); or 2) high PA, basal SA and basal OA (150, 28 and 21 µM; **HPA**); compared to 3) basal PA, SA and OA (23, 28, 21 µM; **BASAL**) as a physiological control. Zygotes were cultured in SOF medium containing 1) BSA (2%) only or supplemented with 2) ITS (10 µg/mL insulin; 5,5 µg/mL transferrin and 6,7 ng/mL selenium) or with 3) serum (5%). Cleavage (48h) and blastocyst rates (day 7 (D7) and D8 post insemination) were recorded. D8 blastocysts were analyzed for apoptotic cell indexes (ACI) (caspase-3 immunostaining), embryo metabolism (glucose consumption and lactate production), or mRNA expression of genes involved in ER unfolded protein responses (UPR^{er}) (*Atf4, Atf6*), oxidative stress (*SOD2, GPx, CAT*) mitochondrial UPR (*HSP10, HSP60*) and mitochondrial biogenesis (*TFAM*). Categorical and numerical data were analysed using binary logistic regression and ANOVA, respectively, and were Bonferroni corrected. Cleavage rate was significantly (P<0.05) reduced in HPA embryos compared with BASAL when cultured in BSA. However, ITS or Serum in culture alleviated this negative effect. Compared with BASAL, HPA exposed oocytes showed significant lower D7 and D8 blastocyst rates after culture in BSA and Serum, but not in ITS containing SOF medium. Within the PA-treated group, ITS significantly increased D7 and D8 blastocyst rates compared with BSA. HFA did not have significant effects on development under all IVC conditions. For embryo quality, ACI was not different among BASAL, HFA and HPA groups in BSA culture. Surprisingly, supplementation of ITS during IVC significantly increased ACI of HPA and HFA embryos compared to BASAL (P<0.05). Serum supplementation also increased ACI of HPA embryos compared with HFA and BASAL (P<0.05). Regardless of IVM treatment, embryos cultured in Serum showed increased lactate/2glucose ratio compared with BSA and ITS, confirming the reported preference for Warburg metabolism. In contrast, HPA-derived embryos cultured in ITS or Serum had significantly lower lactate/2glucose ratio compared to BASAL and HFA. At the blastocyst transcriptomic level, HPA increased *HSP60* expression compared to BASAL when cultured in BSA, indicating activation of mitochondrial stress responses. ITS and Serum alleviated this increase in *HSP60.* In conclusion, enrichment of embryo culture media with ITS or serum can improve developmental competence of oocytes after maturation in lipotoxic conditions. However, the surviving blastocysts exhibit higher apoptosis and altered metabolism indicating inferior quality.

A198E Embryology, developmental biology and physiology of reproduction

Relationship between nitric oxide in follicular fluid and ovarian response among oocyte donors

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Keywords*:* nitric oxide, follicular fluid, oocyte.

The identification of a family of enzymes catalyzing the synthesis of nitric oxide (NO) in the oviduct, oocytes and cumulus cells of several species [Rosselli M, Mol Hum Reprod. 2(8):607-12, 1996; Lapointe J, Endocrinology. 147(12):5600-10, 2006; Tao Y, Mol Cell Endocrinol. 222(1-2):93-103, 2004], suggested that NO is a key component of the oocyte microenvironment [Romero-Aguirregomezcorta J, PLoS One. 9(12):e115044, 2014]. Interestingly, it has been reported that NO modulates the granulosa cell function, follicular maturation and ovulation (Yalçınkaya E, J Turk Ger Gynecol Assoc. 14(3):136-41, 2013). The present work aimed to determine if the NO levels in human follicular fluid (FF) correlate with the number of total and MII oocytes retrieved from donors. Seventy-two women participating in the oocyte donation program at IVI-RMA Global Murcia (Spain) took part in this study. FF was obtained at oocyte retrieval in 93 donation cycles to measure the levels of stable-end products of NO oxidation, nitrite (NO_2) and nitrate (NO_3). For each donor, demographic, lifestyle and donation cycle-related data were also recorded. NO_2 and NO_3 were determined by HPLC-UV/VIS. Multivariate mixed Poisson and logistic regression models with random slopes to account for repeated observations within woman were used to compare total and MII oocyte yields for women across tertiles of NO_2, NO_3, total NO and NO_3/NO_2 ratio while adjusting for age, body mass index, hours of sleep, coffee intake, smoking and physical activity. NO_2 levels ranged from 0.7 to 96.1 µM, NO_3 levels ranged from 4.9 to 39.7 µM, total NO levels ranged from 5.6 to 109.5 µM and NO_3/NO_2 ratio ranged from 0.1 to 31.5. NO_2 and NO_3 concentrations were unrelated to each other (r=-0.01). FF NO_2, NO_3, total NO or NO_3/NO_2 ratio were unrelated to total or mature oocyte yield. The multivariable-adjusted MII yield (95% CI) for women in the lowest and highest tertiles of NO_2 was 12.4 (10.2, 15.1) and 13.2 (10.9, 16.0) (p=0.53); 14.1 (11.7, 17.1) and 12.2 (9.9, 15.0) for NO_3 (p=0.21); 13.7 (11.4, 16.5) and 12.2 (10.1, 14.6) for total NO (p=0.26); and 14.1 (11.7, 16.9) and 12.2 (10.1, 14.8) for the NO_3/NO_2 ratio (p=0.15). When MII oocytes were considered as the proportion of total oocytes, however, the proportion of MII oocytes increased with increasing FF NO_2 levels but decreased with increasing NO_3 levels. The adjusted proportion (95%CI) of MII oocytes for women in the lowest and highest FF levels of NO_2 were 68% (58-77%) and 79% (70-85%) (p, linear trend=0.02); whereas the proportion of MII oocytes for women in extreme tertiles of FF NO_3 levels were 79% (70-85%) and 68% (57-77%) (p, linear trend=0.03). In conclusion, NO and its metabolites did not predict the number of mature oocytes retrieved from donors, but NO_2 and NO_3 correlated with the MII proportion. The fertilization rate, embryo quality and pregnancy rates should be analyzed in patients who received these oocytes to determine any correlations with NO levels in FF.
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A199E Embryology, developmental biology and physiology of reproduction

Effect of month of birth on the development of Belgian Blue calves

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Keywords*:* month of birth, Belgian Blue calves, organs.

Intra-uterine growth is important in beef cattle since it determines weight and morphometrics of neonatal calves and hence contributes to ease of calving. Earlier, we have shown in dairy cattle that environmental circumstances like for example season of birth and parity of the mother significantly influence birth weight of calves. The present study aimed to evaluate the effect of month of birth on the intra-uterine development of Belgian Blue calves based on the measurement of 10 body parts shortly after birth: body weight (BW), withers height (WH), oblique length (OL), heart girth (HG), width of the back (WB), shoulders width (SW), circumference of the head (CH), diameter of the head (DH), length of the metatarsus (LM), and length of the underarm at the front leg (LA). Furthermore, we also investigated the effect of month of birth within the sex of calves. The data include 73 records of calves born in the Clinic of Reproduction and Obstetrics at the Faculty of Veterinary Medicine, Ghent University (Belgium), collected between 2016 and 2017. The results show that the average BW was 52.46 kg, WH 70.08 cm, OL 64.78 cm, HG 80.08 cm, WB 25.95 cm, SW 24.45 cm, CH 50.49 cm, DH 13.39 cm, LM 30.57 cm, and LA 25.28 cm. There was a significant association between the month of birth and the length of the metatarsus as well as the length of the underarm at the front leg. In comparison to calves born in winter, calves that were born in autumn had both a longer metatarsus (31.13 versus 29.82 cm; $P<0.05$) and a longer underarm at the front leg (26.13 versus 24.14 cm; $P<0.05$). Furthermore, the data showed an effect of the gender of the calves, as the male calves had a significantly ($P= 0.026$) bigger HG (81.69 cm) than their female counterparts (78.67 cm). Based on these results, it appears that the season of birth and the gender of the calves both have a significant effect on some of the neonatal morphometrics in Belgian Blue calves, which is important to know in terms of calving ease.

A200E Embryology, developmental biology and physiology of reproduction

Dynamic transcriptome changes during embryonic diapause and reactivation in the embryo and endometrial epithelium of the European roe deer

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Keywords*:* diapause, embryo, endometrium.

The European roe deer pre-implantation embryo development is characterized by a four-month period of embryonic diapause, after which the embryo rapidly elongates and implants. Pre-elongation developmental pace is 10 times slower than in cattle. In roe deer, endometrial secretions at implantation are 1.5-fold higher than during diapause, and morphological changes of the embryo coincide with changes of the uterine fluid composition. To identify the reactivation initiating mechanism, we investigated the embryonic and endometrial transcriptome changes during diapause and following reactivation. Samples were collected at regular huntings between September and January 2015-2017. A total of 360 animals was sampled and 537 pre-implantation embryos were collected (77% recovery rate). A group of six day 14 *ex vivo* flushed embryos from captive roe deer represent the early blastocysts. Embryonic DNA was extracted for DNA content determination and cell number estimation. Endometrial luminal epithelial was collected by laser-capture micro-dissection. Total embryonic RNA from 87 embryos and total endometrial RNA from 56 different females was subjected to RNA-sequencing. Raw sequence reads were analyzed using a customized Galaxy pipeline. A pseudotime analysis (CellTree) was performed to gain insight into the transcriptome dynamics of diapausing embryos, in which the number of embryonic cells was used as proxy for developmental progression. Differentially expressed transcripts (DET) were identified in a time-course dependent manner with the ImpulseDE2 algorithm with an FDR <1%. To elucidate dynamic gene expression changes, a self-organizing tree algorithm (SOTA) was used. Gene set enrichment analysis and gene ontology were used to identify enriched hallmarks between diapause and elongation. As determined by a rise in DNA content, embryonic cells divide every two weeks during diapause. With developmental progression, an overall increase in the number of embryonically expressed transcripts was observed. The pseudotime analysis of both embryos and luminal epithelium showed grouping of the early blastocysts on one end of the trajectory, the elongated embryos on the other end with diapausing embryos dispersed heterogeneously in between. Embryonic time-course analysis revealed 13,193 DET out of 29,575 transcripts. The DET grouped into 7 SOTA clusters. Gene set enrichment analysis and gene ontology revealed an enrichment of MYC targets, MTORC1 signaling, PI3K, AKT and MTOR signaling, unfolded protein response, peroxisome and the glycolytic pathway in elongated embryos. In the luminal epithelial cells, changes were less dynamic; 2,754 DET grouped into 2 clusters and lacked any enriched biological pathway. Taken together, roe deer embryos divide at a slow pace and are transcriptionally active during diapause. Enriched pathways indicate cell proliferation following reactivation. Targeted transcript analyses will emphasize on the identification of diapause-related regulatory pathways and aim at identifying conserved mechanisms of cell cycle control.

A201E Embryology, developmental biology and physiology of reproduction

Effect of the zona pellucida removal on the developmental competence of domestic cat embryos generated by *in vitro* fertilization

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Keywords*:* felids, in vitro embryo production, zona pellucida.

The domestic cat is a valuable model for the development of assisted reproductive techniques that might be used in the conservation of endangered felids. However, the efficiency of the *in vitro* embryo production in the domestic cat remains low compared to other species. In the bovine and equine, the zona pellucida removal enhance the developmental competence of the embryos generated *in vitro*, allowing the birth of live offspring (Gambini *et al*., 2012; Rodriguez *et al*., 2008). The objective of this research was to evaluate the effects of zona pellucida removal in domestic cat embryos generated by in vitro fertilization (IVF). To achieve this purpose, two experimental groups were made, 1) domestic cat embryos generated by IVF and *in vitro* cultured (Zona-included), 2) domestic cat embryos generated by IVF and cultured without zona pellucida (Zona-Free). To evaluate the effect of the zona removal, the developmental capacity and morphological quality of the embryos generated in the Zona-free group were compared against the Zona-included group. For this, the ovaries of domestic cats were collected by ovariohysterectomy and the cumulus-oocyte complexes (COCs) were recovered by slicing. The COCs were *in vitro* matured in supplemented TCM-199 Earle's salts medium for 26-28 hours, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC. The IVF was realized using epididymal refrigerated sperm. 1.5 – 2.5 x 10^6 spermatozoa /mL were incubated with 20-30 COCs in supplemented TALP medium for 18 hours, in a 5% CO_2 atmosphere, at 38.5ºC. After cumulus cell removal, the zona pellucida of the presumed zygotes was removed by 2-4 minutes incubation in 2 mg/mL of pronase (Sigma-Aldrich, P8811, USA). The presumed zygotes were cultured using the well of the well system (Vajta *et al*., 2000) in supplemented SOF medium, in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5ºC, for 8 days. The cleavage, morulae and blastocysts rates were estimated at day 5 and 8. The Zona-included and Zona-free groups were performed at different times, with nine and six replicates respectively. The diameter and total cell number of the blastocysts were evaluated. The Wilcoxon non-parametric test was used to analyze the developmental competence and the t-test was used to analyze the diameter and total cell number. Regarding to the results, no statistical differences were observed between the Zona-included and Zona-free groups in the cleavage rate: 155/239 (64.9%) and 116/177 (65.5%), morulae rate: 115/155 (74.2%) and 68/116 (58.6%), and blastocysts rate: 51/155 (32.9%) and 36/116 (31.0), respectively ($P > 0.05$). No differences were observed in the total cell number (mean ± SD) of the blastocysts generated in the Zona-included (279.9 ± 148.1) and Zona-free group (313.1 ± 164.9) ($P > 0.05$). Finally, the diameter (mean ± SD) of the blastocysts from the Zona-free group (253.4 ± 83.3 μm) was significantly higher than the diameter of the blastocysts from the Zona-included group (210.5 ± 78.5 μm). In conclusion, the zona pellucida removal did not affect negatively the morphological quality and developmental competence of domestic cat embryos in our cultured conditions. However, more studies are needed to evaluate the *in vivo* competence of these embryos.

A202E Embryology, developmental biology and physiology of reproduction

Effects of serum and serum substitutes on *in vitro* maturation (IVM) and embryo development of porcine oocytes

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Keywords*:* Embryo development, blastocyst, pig.

Porcine *in vitro* embryo production (IVP) protocols have traditionally relied on the use of follicular fluid and serum, which results in undefined media with undetermined levels of growth (and other) factors. Media composition can alter the efficiency of embryo development, and more importantly gives rise to a potential biohazard. Moreover, the use of serum in IVP has been linked to alterations in embryo transcriptional activity (Oliveira *et al.* 2006, Reprod Domest Anim. 41:129-36). Hence, our aim was to establish whether a serum substitute (formed by a combination of cytokines currently under NDA) could be used efficiently in pig IVP. Here, we compared the use of this serum substitute during *in vitro* maturation (IVM) vs. sow follicular fluid (sFF) as stand alone treatments or in combination, and followed the development of the resulting zygotes. Oocytes collected from abattoir-derived ovaries were matured for 44 h in supplemented or non-supplemented Porcine Oocyte Medium (POM). There were four treatment groups: 1) Non-supplemented (control), 2), 10% sFF, 3) serum substitute, or 4) combination of 10% sFF + serum substitute (n=100 oocytes/group). Fertilisation of matured oocytes was carried out using extended boar semen (JSR, Driffield, UK). *In vitro* culture (IVC) of zygotes across treatment groups remained consistent, and occurred in defined Porcine Zygote Medium 5 (PZM5) supplemented with our serum substitute and with partial media changes at 48 h and 96 h post-IVF. Cleavage and blastocyst rates were assessed at 48 h and 144 h post-IVF, respectively. Differences between the groups were analysed using a comparative General Linear Model followed by Tukey's *post-hoc* test. Cleavage rates, as compared to the control group (36.7%), were significantly higher in both serum substitute (57.4%, p=0.02) and combination groups (70.4%, $p=6x10^{-6}$). There was no significant effect of using 10% sFF compared to controls (50.4%, p=0.25). Interestingly, there was no significant difference in the proportion of blastocysts per cleaved embryo between the control (30.3%), 10% sFF (26.8%), serum substitute (26.8%) and combination groups (30.9%)(F_3=1.85, p=0.14). The use of serum in combination with cytokines might have resulted in a higher proportion of growth factors, providing a possible explanation to the combinatory effect here described for cleavage rates. Moreover, the fact that cleavage but not blastocyst rates differed between these groups advocates that the effects of the supplementation in IVM did not extend to IVC. Certain IVM treatment groups produced a higher proportion of developmentally competent oocytes, as highlighted by the difference in cleavage rates. At the same time, the use of a standardised IVC protocol following IVM might have had a role in ensuring consistent development to the blastocyst stage following successful fertilisation. To further characterise this observation, future experiments will assess the effects of supplementation with sFF and serum substitutes on IVC, while IVM will be kept consistent. While our serum substitute appeared suitable for IVM and IVC, the combinatory effect observed suggests that the development of a more complex and efficient serum substitute should be possible.

A203E Embryology, developmental biology and physiology of reproduction

Using a time-lapse system to study the morphokinetics of blastocysts derived from heat-shocked oocytes

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Keywords*:* heat shock, embryo morphokinetics, time-lapse system.

Embryonic development involves multiple dynamic events that are remarkably sensitive to environmental changes. We used a non-invasive time-lapse system (TLS) to continuously monitor the kinetics of embryonic development. The objectives were to: (1) compare embryonic development in a conventional incubator and in an incubator equipped with a TLS; (2) characterize the precise cleavage time and duration (i.e., morphokinetics) of individual in-vitro-derived embryos; (3) examine whether exposing oocytes during maturation to heat shock affects the morphokinetics of the developing embryo. Ovaries were collected from a local abattoir. Cumulus-oocyte complexes (COCs) were aspirated, matured (22 h) and fertilized (18 h) in a conventional incubator (humidified air, 5% CO_2 at 38.5°C). Statistical analysis was performed using JMP-13 software. In the first experiment, putative zygotes were cultured in a conventional incubator (n = 192, control) or an incubator equipped with a TLS (n = 126, TLS) for 8 days. The proportion of oocytes that cleaved to the 4-cell stage (74.3±7 vs 89.2±2.3%, respectively) and that of blastocysts (20.6±6.9 vs 25.9±5.8%, respectively) did not significantly differ between control and TLS groups. In the second experiment, the morphokinetics of embryos (n = 427) cultured in the TLS-equipped incubator were individually recorded. Findings revealed that the median of cleavage into 2-cells stage was 27.5 h post fertilization (pf): from 2- to 4-cell stage was 37.5 h pf, from 4- to 8-cell stage was 50.5 h pf and to the blastocyst stage was 127.5 h pf. In the third experiment, COCs (n = 421) were matured for 22 h in an incubator under normothermic conditions (5% CO_2, 38.5°C, control) or exposed to heat shock (6% CO_2, 41.5°C, HS) in a conventional incubator. Following fertilization (18 h), embryos were cultured for 7 days under normothermic conditions in the TLS-equipped incubator and embryo morphokinetics was recorded. In the control group, embryo cleavage was characterized by two waves of divisions; the first between 22 and 28 h pf and the second between 28 and 36 h pf. In the HS group, cleavage was characterized by one wave of divisions that occurred between 28 to 36 h pf. The median of the cleavage into 2-cell stage was 31.5h pf and 27.5h pf for HS- and control groups, indicating a delay in cleavage.Blastocysts from both control and HS groups were collected for real-time PCR assay to evaluate the expression of selected transcripts (*OCT4*, *NANOG*, *SOX2*, *DNMT1*, *PTGS2*, *GDF9*, *STAT3*). In summary, use of a TLS adds to our understanding of the mechanisms by which heat stress can impair oocyte developmental competence. We documented the precise morphokinetics of bovine embryo development from oocytes matured under normothermic or heat-shock conditions during maturation. These were associated with reduced developmental competence in the HS group. PCR findings will enable a comparison of gene expression in blastocysts developed from control and heat-shocked oocytes.