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Isolation and expansion of mesenchymal stem cells from umbilical cord of goats under distinct body condition scores

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Mesenchymal stem cells (MSCs) from the umbilical cord (UC) of newborns are usually used in animals and humans as a source of stem cells for different purposes. Nevertheless, very little information is available concerning the impact of maternal nutrition on growth and differentiation of MSCs. The aim of this work was to evaluate the effect of the body condition score of goats on the isolation and expansion capacity of MSCs from UC. Nineteen adult, pluriparous crossbred goats were grouped according to body condition score (BCS) at kidding, assigning a score from 1-5, at quartile intervals: (LG) group with lower BCS, (2.3 ± 0.1, LG, n= 9), and (HG) group with higher BCS (2.9 ± 0.1, HG, n=10). All goats were from the same farm and maintained under similar feeding and management conditions over the course of pregnancy. During delivery, 5- to 7-cm long UC fragments were collected, and rinsed in 0.9% saline solution supplemented with 2% penicillin-streptomycin and 1% amphotericin. Each fragment was sectioned into 1 cm^2 explants of Wharton jelly, which were *in vitro*-cultured in 30-mm cell culture dishes in 1 mL DMEM® medium supplemented with 15% fetal bovine serum (FBS), penicillin/streptomycin (2%) and amphotericin (1%), in incubator at 38.5ºC, high humidity and at 5% CO_2. Exchange of the medium was performed every two days, and the explants were evaluated daily for cell proliferation and confluence. Upon reaching 80% confluence, explants were removed, cells were trypsinized and quantified in a Neubauer Chamber, and a sample was evaluated for viability by staining with 0.4% Trypan blue prior to seeding into the second passage (P2). Data were subjected to Kruskal-Wallis ANOVA test, with BCS group (LG, HG) as main effect. Differences between means were analyzed using the Mann-Whitney test for independent groups. No significant effect of BCS was found for the parameters considered. Appearance of MSCs from primary culture occurred at 7.5 ± 0.6 days. At Day 0 of culture, the mean number of MSCs was 7,111 ± 952 cells. Interval between MSCs appearance and P2 was 19.4 ± 0.9 days. In this period, the daily mean proliferation was 11,747 ± 2,379 cells/day. We conclude that maternal BCS did not show effects in goats on appearance and proliferation of neonatal UC-derived MSCs under *in vitro* culture.

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Effects of a pre-IVM culture with steroids and NPPC for 12 and 24 hours on chromatin configuration of bovine oocytes

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Oocyte quality is crucial for assisted reproductive technologies applied to animals and humans. *In vitro* matured oocytes are less competent to become fertilized and advance to the blastocyst stage in relation to *in vivo* matured oocyte. We have recently proposed a two-step culture system for IVM that was able to hold germinal vesicle breakdown (GVBD) for 9 hours during the first culture step and to improve embryo quality after IVM/IVF. In this study, aiming to test the efficacy of this culture system to hold oocyte nuclear maturation, we assessed chromatin configuration and GVBD rates after 0, 12 and 24 hours of the pre-IVM culture step. Bovine ovaries (predominantly Nellore, *Bos indicus*) were obtained from a local abattoir, COCs were aspirated from follicles of 3–8mm and pools of 20-30 oocytes were divided in three groups to be cultured for 0h (control), 12h and 24h. The pre-IVM was cultured in basic medium (TCM 199 supplemented with BSA, amikacin, pyruvate natriuretic peptide C (NPPC), estradiol, progesterone, androstenedione and r-hFSH (PCT Patent No. 201690005). After culture, oocytes were mechanically denuded, fixed and stained with Hoechst 33342 for chromatin configuration evaluation with fluorescence microscopy. Germinal vesicle (GV) was classified as GV0, GV1, GV2, GV3, GVBD, MI (metaphase I), MII (metaphase II) and DEG (degenerate). Data were arcsine transformed and groups compared by the Tukey's test or Wilcoxon's test when data were non-parametric. Differences were considered significant when $P< 0.05$. Oocytes examined at 0h (control group; n=110 oocytes from 4 replicates) were GV1 2.5%, GV2 40.1%, GV3 54.5%, GVBD 1.0% and DEG 1.9%. After pre-IVM for 12h (n=107 oocytes from 4 replicates), 0.9% of the oocytes were in GV1, 27.1% in GV2, 49.5% in GV3, 20.7% in GVBD and 1.8% were degenerated. After pre-IVM for 24h (n=92 oocytes from 4 replicates), 2.8% of the oocytes were in GV1, 17.4% in GV2, 6.4% in GV3, 32.3% in GVBD and 41.1% were degenerated. Therefore, culture time did not alter the percentage of oocytes at GV1, but decreased the percentage of oocytes at GV2 stage, which was significantly higher at 0h in comparison with 24h. After pre-IVM for 24h there was a decrease in the percentage of GV3 oocytes, while the GVBD rate increased gradually with time. In conclusion, the pre-IVM culture step tested is not capable to entirely prevent GVDB for 12h in bovine oocytes from abattoir. In addition, pre-IVM culture for 24h leads to degeneration of the chromatin and is not suitable for IVM/IVF.

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Anticipation of the first ovulation of the year in mares

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Research on equine breeding has focused on understanding better the mechanisms that determine reproductive seasonality. Successful protocols that stimulate ovarian cyclicity in mares to overcome winter anestrus and/or prolonged transition phase concern to equine industry. Several strategies aiming the anticipation of first ovulation (OV) have been tested, in order to anticipate the breeding season. The aim of this study was to evaluate whether the ultrasound guided transvaginal follicular aspiration technique, associated with the administration of PGF2α during the spring transition season, was able to induce cyclicity in mares. The experiment was carried out in the Animal Reproduction area of the DRAA/IZ/UFRRJ, during the spring transitional season (August and September) of 2015. We selected 18 mares (Mangalarga Marchador breed), aged 5-12 years, weighing between 350-450 kg. Mares were randomly assigned into two groups: Group 1 - Control (GI; n = 9), without treatment; Group 2 - Transvaginal follicular aspiration + PGF2α (Lutalyse®, Zoetis, Campinas, Brazil) (GII; n = 9), largest follicle aspirated (> 25mm) and seven days later, administration of 7.5 mg Dinoprost (PGF2α), IM. Only mares with absence of corpus luteum, as well as presence of a follicle>25 mm were used. The mean follicular diameter at the beginning of treatment was 29.1±0.7mm and 28.9±1.1 mm, for GI and GII, respectively. All mares were monitored every 48 hours until the time of the second OV following treatment. The GII group was more efficient ($P < 0.05$) in promoting the anticipation of the first OV of the year comparing to GI group. For GII, 66.7% (6/9) of the mares ovulated between 14 and 16 days after starting the treatment, while in the GI, no animal ovulated. All mares (9/9) from GII ovulated up to 18 days after treatment. On the other hand, in GI 22.2% (2/9), 66.7% (6/9) 88.9% (8/9), 100% (9/9) ovulated up to 18 days, 24 days, 30 days, and 42 days after treatment, respectively. The time until the first OV was 24.9±7.5 and 16±1.2 days for GI and GII, respectively. An anticipation of 8.9 days in OV was observed in GII when compared to GI. All mares ovulated normally, demonstrating that none of them returned to the transitional period, and that the average days between the first and second OV did not differ between groups ($P > 0.05$). The results of the present study allow to conclude that the follicular aspiration technique during the spring transition period associated with administration of 7.5mg of PGF2α seven days later was able to induce cyclicity in mares.

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ISG15 expression in peripheral blood mononuclear cells as an indicator to identity and monitor embryonic signaling in beef heifers subjected to fixed-time artificial insemination

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In ruminants, maternal recognition of pregnancy is characterized by secretion of a protein called interferon tau (IFNT) produced by trophoblast cells. The endocrine action of IFNT on blood cells increases the expression of interferon-stimulated genes (ISGs), such as Interferon stimulated gene 15 (ISG15). The objective of this study was to evaluate the expression of *ISG15* in peripheral blood mononuclear cells (PBMC) in beef heifers subjected to fixed-time artificial insemination (FTAI). FTAI protocol was performed on 13 beef heifers. On Day -10, estradiol benzoate (2mg/IM) was injected and a progesterone intravaginal device (IVD) (1g) was inserted. On Day -2 the IVD was removed and prostaglandin and estradiol cypionate analog were injected. FTAI occurred 48 hours after IVD withdrawal (n=9; Day 0). The non-bred group was not inseminated (n=4). On Days 16 and 18 following FTAI, blood from the coccygeal vein was collected to isolate blood cells using 4mL tubes containing EDTA, then blood was diluted in 0.9% NaCl. In a 15 mL tube containing Ficoll-Paque PREMIUM® was slowly added the blood solution. Centrifugation was performed at 400XG for 20 minutes at room temperature. Thereafter, the following fractions were identified and separated: plasma, erythrocytes, mononuclear and polymorphonuclear cells. Next to the separation of the fractions, mononuclear cells (PBMC) were collected and stored at -80°C in a cryotube. Subsequently, mRNA extraction, cDNA and qPCR were performed. *RPL19* and *RN18S1* were used as housekeeping genes. After isolation of PBMC fraction, a glass-slide fraction-film was prepared to determine the purity of the fraction. The purity was accessed based on cell morphology. The percentage of PBMC present was determined and samples above 95% were included in the study. On the 29th and 60th days following FTAI, the pregnancy diagnosis was performed by ultrasonography, in order to allocate them in one of the 02 groups: pregnant (n=6), non-pregnant (n=3), and non-bred (n=4) heifers. Data were analyzed by ANOVA and multiple comparisons were performed by Tukey's test. Differences were considered at $P \leq 0.05$. The results revealed that on Day 16 following FTAI, there was no difference in *ISG15* expression in PBMCs from pregnant, non-pregnant and non-bred heifers. No difference was found between non-pregnant and non-bred groups. However, results from the Day 18 showed significant difference between pregnant (upregulation) compared to the other groups. The results from Day 18 compared to Day 16 following FTAI revealed an upregulation in ISG15 expression in pregnant heifers (5 fold change). It suggests that the expression of ISG15 in mononuclear cells can be used as a tool not only to identify, but also monitor embryonic signaling along pregnancy in beef heifers. This research was funded by CNPq and CAPES.

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Dynamic changes in miRNAs levels during early embryonic development *in vivo*: From bovine oocytes to blastocysts

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The early embryonic development is a crucial stage for the determination of a successful pregnancy. One of the most important events of this period is the transition from maternal to embryonic genome activation (EGA), which occurs around 8-16 cells stage in the bovine. During this period of the developmental process, the maternal messenger RNAs (mRNAs) are replaced by embryonic mRNAs that regulate signaling pathways involved in cell differentiation, proliferation and metabolism. Recent studies showed that microRNAs (miRNAs), short non-coding RNAs, can regulate gene expression through post transcriptional mechanisms. Consequently, miRNAs can play a critical role regulating transcripts such as the maternal mRNA during and after the major maternal-to-embryonic transition. The aim of this study was to determine the dynamic of miRNAs levels during early bovine embryonic development *in vivo*. For this, bovine cumulus-oocyte-complexes and embryos produced by artificial insemination (AI) were collected through ovum-pick-up and oviduct flush. Samples of denuded mature oocytes as well as 4 cell embryos, 8-16 cell embryos, morulae and blastocysts were collected for real-time PCR analysis of 380 bovine miRNAs. The day of AI was considered day 0 for embryo collection. Samples were grouped in 3 polls of 10 oocytes; 5 embryos collected on day 2 (D2), containing 4 cells; 5 embryos collected on day 3 (D3), containing 8 to 16 cells; 10 morulae and 10 blastocysts in each poll. Reverse transcription was performed with 100ng of total RNA using the miScript PCR System kit (Qiagen). Real-Time PCR analysis of the miRNAs was performed with a custom miRNA profiler plate. Data were normalized by the geometric mean of 3 endogenous small RNAs (RNU43snoRNA, bta-miR-99b and Hm/MS/Rt T1). The relative levels ΔCt were analyzed by ANOVA followed by Tukey-Kramer HSD test. A total of 175 miRNAs were commonly identified from oocyte to blastocyst stage. The total number of miRNAs identified in each stage was 274 in oocytes, 263 in D2, 272 in D3, 302 in morulae and 292 in blastocyst. The total number of miRNAs identified as unique for each stage were four for oocytes, none for D2, one for D3, seven for morulae and eight for blastocyst. A total of 42 miRNAs were differentially expressed comparing from oocyte throughout blastocyst stage. Additionally, a total of 32 miRNAs were differently expressed comparing oocytes, D2 and D3 embryos. Bioinformatics analysis identified regulated pathways that play an important role modulating cell differentiation and metabolism such as Hippo signaling, TGF-beta, Cell cycle and ErbB signaling. Thus, is important to understand how miRNAs levels are dynamic changing during early embryonic development and their predict impact modulating different pathways during each developmental stage *in vivo*, which can be applied on the assisted reproduction industry in cattle and humans. Financial support: FAPESP 2014/22887-0 and 2015/21674-5.

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Functionality evaluation of two extenders for Puma concolor sperm cryopreservation by interspecific *in vitro* fertilization with domestic cat oocytes

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Despite some advance in the cryopreservation of sperm from endangered felids, little is known about suitable protocols to cryopreserve sperm from *Puma concolor* (PC). In the present study, sperm obtained by electroejaculation from five different males were cryopreserved in either a Tes-Tris- or a lactose-based diluent. The objectives were 1) to compare *in vitro* motility and acrosome status of PC sperm cryopreserved in both extenders and 2) to test functionality of PC sperm cryopreserved in both extenders through their ability to fertilize mature domestic cat oocytes. Straws were thawed by exposing them to air for 10s and then immersing them in a water bath at 37°C for 30s. The contents of the straws were poured into a sterile 1.5-mL microtube pre-warmed to 37°C. The sperm suspension was diluted (1:3 v/v) by the slow (drop by drop) addition of a modified Tyrode's solution. Sperm parameters, percentage of motile spermatozoa and quality of motility was assessed and sperm motility index (SMI) was calculated as follows: [% motile sperm + (quality x20)]/2. Acrosome integrity (AI) was assessed by staining with Coomassie brilliant blue. For IVF, *in vitro* matured domestic cat oocytes (n=256 Tes-Tris, n=274 lactose) were co-incubated with 0.5×10^5 motile spermatozoa mL^{-1} under 5%CO_2 in air at 38.5ºC for 18–20h. Presumptive zygotes were cultured *in vitro* in 50 µl drops of modified Tyrode's medium in 5%CO_2, 5%O_2, 90%N_2 at 38.5°C. Cleavage was assessed 48h post fertilization, and 5% FBS was added at day 5 of *in vitro* culture. Blastocyst stage was evaluated at Day 8. Results, mean (± s.e.m.), showed that SMI and AI (pre&post thawing) was similar for both extenders: pre-thawed (SMI: 66 ± 2.4 vs. 64 ± 2.6; AI: 66 ± 6.1% vs. 60 ± 7.4%), and post-thawed (SMI: 66 ± 7.5 vs. 74 ± 3.7; AI: 40 ± 7.3% vs. 39 ± 6.0%) Tes-Tris vs. lactose respectively. For IVF, results showed a high cleavage rate in both groups (141/256, 55±2.4% vs. 148/274, 54±5.2%), and a high development to morula (110/256, 43±4.3% vs. 114/274, 42±2.9%), and to the blastocyst stage (70/256, 27±3.6% vs. 76/274, 28±2.6%) for all males (Tes-Tris vs. lactose respectively). There were no significant differences between groups at any development stage. In conclusion, we found that both extenders can be used to cryopreserve PC sperm maintaining functional conditions and that fertilizing capacity can be tested using *in vitro*-matured domestic cat oocytes.

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Effect of time after slaughter on large-scale chromatin configuration in cattle

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The oocyte cumulus complex (COC) when removed from its follicular environment undergoes spontaneous and early resumption of meiosis (Zhang *et al.*, Molecular Human Reproduction, 15:399-409, 2009), which is one of the reasons why *in vitro* matured oocytes have lower developmental competence when compared to *in vivo* matured oocytes. However, there are no previous studies describing the dynamics of chromatin configuration after slaughter, this is particularly relevant to studies aiming to evaluate chromatin status in oocytes recovered from ovaries obtained in abattoirs. The aim of this study was to assess the effect of time after slaughter on chromatin configuration of bovine oocytes. COCs were aspirated from abbatoir ovaries at 2 (n = 384) or 4 hours (n = 314) after slaughter. To control effect of time, the averages of time of slaughter waiting, transport and manipulation of oocytes were 40min, 15min and 1h, respectively, for the group of 2hs; and 2h20min, 40min and 1h, respectively, for the group of 4hs. The ovaries were transported in a thermal vessel containing sterile saline solution (0.9% NaCl) heated at 35-37°C. In the laboratory, ovaries were washed in heated saline solution, sterilized with 70% alcohol, and follicles 3-8mm in diameter were aspirated for oocyte recovery. Oocytes were denuded, fixed in methanol 60%, washed in PBS solution, stained with 1µg/ml Hoechst 33342 and chromatin configuration was evaluated in a fluorescence microscope (Nikon Eclipse 80i) to be classified in 4 germinal vesicle (GV) stages with increasing chromatin compaction (GV0, GV1, GV2 and GV3) or as germinal vesicle breakdown (GVBD) and degenerate (DEG) oocytes. The data were arcsine transformed and groups were compared by the Wilcoxon test, considering values of $P < 0.05$ as significant differences. Two hours after slaughter, percentages of GV0, GV1, GV2, GV3, GVBD and DEG oocytes were, respectively, 0%, 7.4%, 46.4%, 35.4%, 0.9% and 9.9%. The correspondent percentages after 4h post mortem were 0.7%, 1%, 43%, 43.7%, 0.1% and 11.5%. The distribution pattern of oocyte classes did not differ between 2 and 4 hours after slaughter. These data suggest that as long as the oocyte remain in its physiological microenvironment, chromatin configuration does not significantly change until 4 hours after slaughter.

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Morphometry after implantation of bovine embryos submitted to thermal stress during IVP

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Induced thermotolerance is a biological phenomenon that consists of cellular resistance after heat treatment and our hypothesis is that this phenomenon can be used for the benefit of embryos in periods of thermal stress. The objective of this study was to induce thermotolerance in bovine embryos and to analyze their development after implantation in the recipients. For this purpose, F1 Holstein-Gir cows were aspirated to obtain oocytes and embryos were produced by *in vitro* fertilization using conventional protocols. The embryos of the TT group were submitted to the temperature increase curve varying from 39 to 40.5°C, for 6h, 144 hpi. 168hpi blastocysts (N 33, 56TT and 77C) were transferred to crossbred Holstein-Gir recipients, between November/2017 and February/2018, in four replicates. The gestations were followed from days 31 to 55, every 6 days, by ultrasonography, measurements were made of the area of the embryonic vesicle (VES); caudal skull length (CCC); biparietal diameter (DBP); and fetal heart rate (HR). CEUA 3956180316. The means of the measurements were analyzed statistically through the Student's T-Test. There was no differences between gestation rates of the gropus (C=22, TT=18, P=0.70, Ficher exact test). At 31 days of gestation (D31) there was no difference between the areas of VES between groups (C=9.71 ± 1.61mm2, TT=9.68 ± 1.14 mm2, P=0.9495). In the D37 the VES area was higher in the TT group (C=13.71 ± 1.67 mm2, TT=15.81 ± 2.41 mm2, P=0.0062). In D43, D49 and D55, there were no differences between VES area between groups (D43, C=20.98 ± 2.37 mm2, TT=21.87 ± 2.47 mm2, P=0.2781, D49, C=28.8 ± 2, 35 mm2, TT=30.04 ± 2.50 mm2, P=0.1377, D55, C=36.43 ± 3.77 mm2, TT=37.87 ± 3.58 mm2, P=0.2580). The CCC did not differ on days D31, D37, D43, D49 and D55 between the groups (D31, C=9.38 ± 1.43 mm, TT=9.50 ± 0.96 mm, P=0.7976, D37, C=15.62 ± 2,02mm, TT=16.05 ± 1.87mm, P=0.5073, D43=23.03 ± 1.72mm, TT=23.06 ± 1.97mm, P=0.9628, D49, C=31.95 ± 1.78mm, TT=31.85 ± 1.65mm, P=0.8649, D55, C=45.76 ± 3.79mm, TT=45.38 ± 2.72mm, P=0.7321). The DBP did not differ on days D43, D49 and D55 between groups (D43, C=8.06 ± 0.70 mm, TT=8.37 ± 0.47 mm, P=0.1198, D49, C=10.59 ± 0.83 mm, TT=10.62 ± 0.55 mm, P=0.9155, D55, C=12.50 ± 0.87 mm, TT=12.43 ± 1.20 mm, P=0.8530). The HR did not differ on days D43, D49 and D55 between the groups (D43, C=183.23 ± 6.94bpm, TT=185.53 ± 6.39bpm, P=0.3547, D49, C=181.05 ± 3.76bpm, TT=182 ± 4.74bpm, P=0.5626, D55, C=173.93 ± 11.06bpm, TT=178.07 ± 6.35bpm, P=0.2188). Although direct effects on pregnancy rates and fetal growth were not observed, the heat treatment performed was safe and did not affect fetal implantation and development. The increase in size of the germinal vesicle in the TT group suggests benefit to the embryos treated in the initial stages of formation of the appendages and fetal membranes, possibly through an adaptive response. Developments in this study are ongoing.

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Recipient cows carrying IVF or SCNT conceptus have different miRNAs profile in the corpus luteum during maternal recognition of pregnancy

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In mammals, corpus luteum (CL) secretes P4 that is necessary to establish and maintain pregnancy. Normal luteal function requires an intricate molecular regulation, which involves transcription and translation of several genes. MicroRNAs are small non-coding RNA molecules involved in post-transcriptional regulation of target genes leading to mRNA degradation or translation repression and may play a role in regulating CL function during maternal recognition of pregnancy. Previous studies have demonstrated an increased rate of embryo loss in SCNT due to embryonic developmental problems leading to impaired pregnancy recognition. This study tested the hypothesis that IVF and SCNT-derived conceptuses elicit a different miRNA profile in the corpus luteum of recipient cows. Slaughterhouse ovaries were collected to make SCNT embryos. IVF embryos were performed by commercial laboratory. On day 7, Nellore cows previously synchronized received one IFV or SCNT embryo. The CL samples from six cows were collected on day 19 of pregnancy, in animals carrying a single embryo produced by IVF (n=3) or SCNT (n=3). P4 concentration in serum were measured on days 9, 14 and 19 following embryo transfer. Total miRNA reverse transcription for mature miRNAs was performed in CL samples from recipient cows carrying IVF or SCNT embryos using miScript HiSpec Buffer. We evaluate the relative levels of 384 bovine miRNAs. Geometric mean of miR-99b, RNU43 snoRNA and Hm/Ms/Rt U1 snRNA was used to normalize the data and differences in relative levels were determined by Student's t-test. Progesterone concentration in serum was similar between IVF (11.40 ± 0.5ng/mL; 12.90 ± 2.1ng/mL; 16.97 ± 2.28ng/mL) and SCNT (10.97 ± 4.32ng/mL; 17.07 ± 6.87ng/mL; 16.40 ± 2.40ng/mL) groups on days 9, 14 and 19, respectively. A total of 288 mature miRNAs were identified in IVF and SCNT CLs, with 10 miRNAs differently detected between the groups. In CL from SCNT pregnancies, 8 miRNAs were downregulated, while 2 miRNAs were upregulated when compared with CL from IVF group. Bioinformatics analysis with DIANA (TarBase v7.0) was used to determine enriched pathways regulated by these miRNAs. The increased miRNAs (miR-130b, miR-149-5p, miR-222, miR-218, miR-454, miR-485, miR-490 and miR-584) in CL from IVF group regulated signaling pathways such as FoxO, TNF, estrogen, TGF-beta, mTOR and steroid biosynthesis. Furthermore, bta-miR-101 and bta-miR-33a, that were upregulated in CL from SCNT modulates MAPK, FoxO and TGF-beta pathways. Some individual miRNAs were previously identified as involved in modulation of angiogenesis, steroidogenesis and CL regression. The results suggest that on day 19, molecular changes may occur in CL of IVF and SCNT embryo recipients cows possibly caused by conceptus of different origins and may contribute to impaired pregnancy recognition during this time.

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Induction of survival mechanisms to thermal stress in bovine embryos

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Thermal stress during embryonic development is associated with increased gestational losses in cattle. An alternative to avoid the negative effects of thermal stress is to induce thermotolerance, a phenomenon that involves the expression of HSP proteins and makes the cells resistant to subsequent stress. The aim of this study was to develop a protocol for the thermal treatment of Girolando (3/4) IVF embryos, which would induce thermotolerance without causing negative effects over development. For this, embryos produced *in vitro* from Girolando cows (1/2) were used (CEUA-EGL 3956180316). After some tests, we standardized a temperature increase curve ranging from 38.5 to 40.5ºC for 6 hours, applied in morulae (144hpi) – TT group, which did not alter the production of blastocysts/ morulae at d7 (C 70.20%, TT 71.67%, p = 0.82, Fisher exact test, n = 371 morulae, 173-198 per group). Immunofluorescence analysis showed that the treatment induced increased nuclear expression of HSP70 protein after 12h (C 43.27 ± 9.22, TT 62.80 ± 18.10, p = 0.01, T Test, n = 19 blastocysts, 9- 10 per group), measured by fluorescence intensity using Photoshop software (pixels 0-255). There was no effect on the blastocyst total cell number (C 70.53 ± 20.06, TT 78.00 ± 14.87, T-test, n = 27 blastocysts, 12 to 15 per group). In a second experiment, the TT embryos were exposed to thermal shock at 40.5 ° C for 2 hours at d7, and survived 15% more than control embryos (C 71.42%, TT 86.66%, p = 0.004, Fisher's exact test, n = 263 blastocysts, 124-139 per group). The effects of treatment after implantation are described in another abstract. The future prospects of the study include embryo transfer of TT embryos in lactating cows, during the summer, to validate the thermotolerance of the embryos, and to follow gestations till birth to prove the safety of this treatment. Therefore it is concluded that the induction of survival mechanisms can be an important strategy to mitigate the effects of thermal stress.
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Calcium serum levels and calcium supplementation at the time of delivery on uterine quality in dairy cows

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The objectives of the study were 1) to correlate plasma levels of calcium in early postpartum with the incidence of subclinical endometritis in the late postpartum period and; 2) to evaluate the effect of calcium formate supplementation on uterine health. The study was carried on in two stages using 73 multiparous and primiparous cows from Jersey and Holstein breeds. In the first experiment the calcium level at 24 hours after calving was correlated with the percentage of neutrophils and the incidence of subclinical endometritis. Blood samples were collected after delivery to determine the serum calcium concentration and cytology for determination of subclinical endometritis was performed 34 to 40 days after delivery by Cytobrush method. The percentage of neutrophils was affected by calcium concentration in the first 24 hours postpartum ($P \leq 0.01$). The second experiment was carried out to evaluate the effect of calcium formate supplementation in animals with different plasma levels of calcium on the incidence of subclinical endometritis. The animals were classified according to calcium concentration (normocalcemic: Ca ≥ 8.5mg/dL or hypocalcemic: Ca<8.5mgdL) and were randomly assigned to receive (Treated group) or not (Control group) calcium formate supplementation at 6 and 30 hours after delivery. Calcium formate treatment reduced neutrophil counts only in normocalcemic cows ($P<0.05$). In addition, calcium formate treatment decreased the frequency of animals with subclinical endometritis between days 34 and 40 postpartum ($P <0.05$). In this study we demonstrated that calcium concentrations in first 24h postpartum affects the percentage of endometrial neutrophils 34 to 40 days later. Moreover, we can conclude that supplementation of two doses of calcium formate at 6 and 30 hours postpartum reduces the incidence of subclinical endometritis in normocalcemic cows. Cows with calcium concentration higher than 8mg/dL in the first 24h postpartum have lower incidence of subclinical endometritis.

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Effect of epigenetic modulators on development of bovine embryos derived from heat-shocked oocytes

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High temperatures during bovine oocyte maturation have been associated with changes in gene expression and on embryonic chromatin organization. This study aimed to analyze the effect of two epigenetic modulators on the development of embryos derived from oocytes submitted to heat shock: 1) Scriptaid, an inhibitor of histone deacetylase and 2) 5-Aza-2'deoxycytidine (AZA), an inhibitor of DNA methylation. Bovine oocytes from slaughterhouse ovaries were matured *in vitro* at conventional temperature (38ºC) for 24h (group with no heat shock: NHS) or at 41.5ºC for 12h followed by 38ºC for a further 12h (heat shock group: HS) under the same conditions of the NHS group. Afterwards, the oocytes from both groups were fertilized *in vitro* for 20h and after the end of fertilization the presumptive zygotes were denuded and randomly exposed to 500 nM Scriptaid or 10 nM AZA for 0h or 24h, comprising six treatments: NHS-0h (n=258), NHS+24hScriptaid (n=242), NHS+24hAZA (n=255), HS-0h (n=263), HS+24hScriptaid (n=261) and HS+24hAZA (n=276). Embryos were cultured in CR2aa medium supplemented with 2.5% FBS at 38.5°C with 5% CO_2, 5% O_2, 90% N_2. Five replicates were performed and data (mean+SEM) was analyzed by logistic regression (Pro Logistic, SAS). Cleavage rates at day three and blastocysts rates at day eight post-fertilization were compared among treatments. Higher cleavage rates ($P<0.05$) were found in all NHS groups (NHS-0h: 67.4±3.8%; NHS+24hAZA: 60.5±5.7%; NHS+24hScriptaid: 76.2±3.3%) when compared to heat shock groups (HS-0h: 53.0±10.6%; HS+24hAZA: 45.3±9.3%; HS+24hScriptaid: 53.2±13.8%). The use of both epigenetic modulators in embryos derived from oocytes with no heat shock (NHS+24hAZA: 22.8±2.6%, NHS+24hScripatid: 24.8±4.9%) reduced ($P <0.05$) blastocyst rate when compared with NHS-0h (38.1±5.2%). However, the same effect was not observed when the comparison was performed among embryos derived from oocytes submitted to heat shock. There was no difference ($P> 0.05$) between HS+24hAZA (16.4±3.1%), HS+24hScriptaid (15.2±3.8%) and HS-0h (17.1±4.5%) treatments. In conclusion, AZA and Scriptaid have a negative effect on the development of embryos derived from oocytes matured *in vitro* under conventional temperature, but that same effect is not observed when embryos are derived from oocytes matured under heat shock conditions. It is suggested that the effect of heat shock during *in vitro* maturation overlaps the effects of AZA or Scriptaid on embryonic development. Further analyzes should be performed to identify possible differences in chromatin organization among treatments. Financial support: CNPq and Fapemig.

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Association of two epigenetic modulators on the initial development of female bovine embryos

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Female embryos are more sensitive to embryo culture *in vitro* and X-chromosome inactivation seems to be one of the epigenetic events related to this fragility. The supplementation of the culture medium with agents that aim to align this process is presented as an alternative to stimulate the embryonic development. The present study aimed to identify the influence of the association of two epigenetic modulators (Tricostatin A and Folic Acid - TSA + AF, at concentrations of 10μM and 5 nM, respectively) in the initial development of female bovine embryos, since the use of these separately showed no influence on embryonic development and blastocyst rate (unpublished data). Oocytes were obtained from slaughtered ovaries, matured, fertilized *in vitro* (d0) with sexed semen (X chromosome) and cultivated (d1) in embryo culture system in pools, allowing individual monitoring of structures during development (*homemade chamber*). Two groups according to the time of development (4 days -Gd4 and 5 days -Gd5) were used for the time of supplementation. The percentages of cleavage and blastocysts were compared using Fisher's Exact Test, and the embryonic development analyzes were submitted to the normality test and evaluated using the Mann-Whitney U Test (5%, Graphpad Instat Demo). The number of cells estimated for analysis was established based on previous results produced in our laboratory, in which embryos smaller than 16 cells were categorized containing 14.3 cells, 30 morulae and d10 blastocysts 101.1. In Gd4, the cleavage rate did not differ (TSA + AF: 74% -n = 47; C: 63% -n = 48) between groups. The blastocyst rate was significantly higher (p = 0.048) in the control group (TSA + AF: 17% -n = 13; C: 31% -n = 20 **), and the modulators did not influence in the number of cells blastocysts recovered at d7 (TSA + AF: 21.14 ± 4.69; C: 41.22 ± 6.70). At time d5, the cleavage rate did not differ (TSA + AF: 59% -n = 82; C: 59% -n = 66). The blastocyst rate was significantly higher (p = 0.0125) in the control group (TSA + AF: 18% -n = 26; C: 33% -n = 37 **), and the supplement showed (p = 0496) a negative influence on the number of blastocyst cells recovered at d7 (TSA + AF: 18.61 ± 5.77; C: 29.99 ± 8.617 **). Thus, we concluded that in the concentrations used, the association between folic acid and Trichostatin A was not beneficial for delayed female embryos, and the time of treatment did not seem to influence the effects caused by supplementation in the medium. Acknowledgments: FAPEMIG, EMBRAPA, CAPES, CNPq.

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Transcriptome of isolated bovine Inner cell mass and trophectoderm from single embryos

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In cattle, sex selection has economic and genetic value in systems in which productivity is favored by the progeny of one sex. Due to this fact in the *in vitro* embryo production (IVP), were developed methods to separate populations of X and Y bearing sperm. Sex can influence embryo development as early as blastocyst stage, moreover sperm sexing process by flow cytometry can lead to DNA damage and changes on the transcription profile of embryos produced with this type of sperm. Furthermore, it could impair blastocyst development in the IVP. In this study we aimed to identify those transcript changes on Trofectoderm (TE) or inner cell mass (ICM) of male and female embryos from blastocysts produced *in vitro* with either conventional or sorted spermatozoa by flow cytometry. We used sorted (X and Y) and conventional semen on IVP from the same bull, known for fertility. Cleavage rate at 48 hours and blastocyst rate and embryos were collected on day 7.5. TE were isolated from embryos by microsurgery, immunosurgery were applied on ICM from the same embryo to remove the remaining TE cells. Immunosurgery-removed TE cells were them used to verify and confirm the embryo sex by PCR. We evaluate the cell isolation process with immunostaining for SOX2 (for ICM) and CDX2 (for TE). ICM viability were analyzed by culturing *in vitro* on TeSR1 modified media. We than used four paired TE and ICM a total of 32 samples, male and female, produced by sorted and conventional semen for RNA-seq. There was no difference on cleavage rate and embryo production. Immunostaining showed that was possible to accurately isolate ICM and TE cells, maintaining cell viability on *in vitro* culture. In this work, we were able to identify embryo sex using the remaining TE cells. For conventional semen, we had 60% male and 40% female, for sorted semen we had an accuracy of 80% for both sex. Furthermore, using R package DESeq2 (adjusted p value>0.05 and log2foldchange>2 or<-2), were identified 2065 differential expressed genes between ICM (1101 genes) and TE (964 genes). Important genes for the maintenance of cellular pluripotency (NANOG) and the development of inner cell mass (SOX2) were identified. Enrichment analysis showed that, for ICM, the most enriched pathways belong to biological process with 134 genes, when compared with TE. For TE, were found that the most enriched pathways belong to cellular components with 230 genes. Using SRY gene and Y chromosome homologous genes (EIF1AY, UTY, ZRSR2y, DDX3Y, EIF2S3Y) we were able to confirm embryo sex. However, it was not possible to accurately identify transcription differences between the embryos produced with or without sorted semen, nor between the male and female ICM and TEs, although it was possible to accurately isolate these factors. These results contribute with robust information about cell differentiation during bovine blastocyst stage.

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Bacterial prevalence in mares with subclinical endometritis

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The objective of this study was to observe the prevalence of bacteria isolated from uterus of mares and to associate this prevalence with cytology results. The study was carried out in an equine breeding farm, located in Seropédica-RJ (Brazil), during the breeding season of 2017/2018. Sixteen cyclic recipient mares (Mangalarga Marchador breed), with absence of apparent abnormalities of reproductive tract verified by ultrasonography (US) during estrus, with recent history of one or more negative pregnancy diagnoses, were used. Mares estrus cycle were monitored every 48 hours, through US in B-mode using Mindray Medical International Limited, model Z5 VET, with 7.5 Mhz rectal linear transducer. Sample collection for microbiological culture and endometrial cytology were performed when a follicle ≥ 35 mm of diameter and uterine ecotexture classified as ≥ 3 was observed. The method for collection, storage, and inoculation in petri dish for microbiological culture were performed as previously described by Oliveira et al. (Veterinária e Zootecnia, 17 (1): 43-46, 2010). Sample collection, preparation and endometrial cytological slide reading were followed as described by Alvarenga, et al. (Medical Journal of Minas Gerais, 5: 132, 1995). Cytologies were classified according to degree of inflammation as absent, moderate and severe, when 0-2, 3-5, >5 neutrophils per field were found, respectively (Leblanc&Causey, Reproduction of Domestic Animals, 44: 10-22, 2009). Out of 16 cytologies performed, 43.75% were negative and 56.25% were positive, 50% with moderate inflammation and 6.25% with severe inflammation. Out of 16 microbiological cultures, 12.5% were negative and 87.5% were positive for one or two bacteria. We found a prevalence of 37.5%, 6.25%, and 6.25% when the cultures were positive for only Escherichia coli, Streptococcus spp, and Proteus spp, respectively. We also found a prevalence of 12.5%, 12.5%, 6.25% and 6.25% when cultures were positive for E. coli/Klesibiella pneumonia, E. coli/Staphylococcus spp, Proteus spp/Klesibiella pneumoniae and E. coli/Streptococcus spp, respectively. Out of nine mares, 11.11% were positive for endometrial cytology and negative for microbiological culture and 85.71% were negative for endometrial cytology and positive for microbiological culture. Out of 16 mares without clinical symptoms for endometritis, 14 (87.5%) presented bacterial presence in the endometrium and 56.25% (9/16) with positive cytology, possibly confirming bacterial endometritis. The most isolated bacteria was E. coli with 68.75% (11/16) of frequency. Considering that, some mares were positive for cytology and negative for culture and vice versa, and a high number of positive for cytology and culture, we concluded that the use of uterine cytology associated with bacterial culture is crucial for a more effective diagnosis of subclinical endometritis in mares during breeding season.

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Effect of heat stress at the beginning of gestation of dairy cows on reproductive indexes of daughters

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The objective was to evaluate the effect of heat stress at the beginning of gestation on reproductive index of offspring in dairy cows. Retrospective data related to reproductive indexes of daughters of Holsteins cows that conceived during summer (December to February) or winter (June to August) were analyzed (n=318 and n=642 respectively). Conception dates for inclusion of the animals in the Summer or Winter groups were estimated by subtracting 280 days from the date of birth of the daughters. In a subgroup of animals, ultrasound evaluations were performed to count antral follicles (Summer - n=36 and Winter - n=39). The study was carried out in three commercial dairy farms located in the south region of Minas Gerais state, with cows born between 2007 and 2015, kept in a free-stall system, voluntary waiting period of 42 days and inseminated after ovulation synchronization protocol. Based on weather data from the nearest weather station available on the website of the National Meteorological Institute (INMET), it was verified that the mean temperature-humidity index (THI) from 2010 to 2017 calculated by the formula: [THI = temperature of the dry bulb + (0.36 * dew point temperature) + 41.2] in the Summer group was 70.1 and in the Winter group was 60.6. All data were analyzed by GLIMMIX procedure of SAS and continuous variables were presented by mean ± standard error. Variation factors for daughters were included in the statistical model and multivariate analysis was used to verify the effect of heat stress on the studied variables. There was no interaction between farms and heat stress for the studied variables. It was verified that daughters of cows conceived in winter presented greater age at first insemination (Winter - 475.3 ± 4.1 days and Summer - 469.3 ± 8.1 days, P=0.01) and at first calving (Winter - 798.8 ± 5.1 days and Summer - 789.1 ± 8.8 days, P=0.02). However, daughters of cows conceived during winter period presented lower interval calving/first insemination (Winter - 66.7 ± 1.3 days and Summer - 75, 5 ± 2.8 days; P=0.01). There was no difference between groups for pregnancy rates at first insemination [Winter - 50.3% (323/642) and Summer - 54.4% (173/318); P=0.15], number of services per conception at first (Winter - 1.9 ± 0.1 services and Summer -1.9 ± 0.1 services, P=0.41), and second calving (winter - 3.2 ± 0.1 services and summer - 3.0 ± 0.2 services, P=0.82), interval between first and second calving (Winter - 507.8 ± 10.4 and Summer - 484.5 ± 13.3 days, P=0.13), calving numbers (Winter - 2.3 ± 0.1 and Summer - 2.3 ± 0.1, P=0.43) and antral follicles counting (Winter - 31.5±2.8 follicles and Summer - 37.0±2.9 follicles; P=0,14). It is concluded that daughters of cows conceived during winter are older at first insemination and at first calving, but are inseminated sooner after first calving.
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Promoter-specific expression of the imprinted IGF2 gene in bovine oocytes and preimplantation embryos

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The splicing of messenger RNA precursors to mature mRNA is a critical component of the gene regulation process that can encode distinct proteins or affect mRNA stability, localization, storage, and translation. Correlations between higher embryonic mortality with embryonic and fetal growth retardation, reduced placental IGF2 expression patterns and fetal abnormalities have already been described in *in vitro*-produced (IVP) conceptus. The IGF2 *locus* is a complex genomic region with multiple alternative splicing transcripts from several leader exons controlled by four distinct promoters (P1, P2, P3, and P4). The present study aimed to evaluate the IGF2 promoter-specific expression pattern in bovine oocytes and in preimplantation embryos as a means of unraveling some aspects of the developmental physiology of bovine embryos produced *in vitro*. For that, immature and matured oocytes, and preimplantation embryos at distinct stages of development (1-, 2-, 4-, 8-, 16-cell stage embryos; morula, compact morula, blastocyst and expanded blastocyst stage embryos) were collected in pools of five structures per stage, after three IVP procedures. Total RNA was extracted from each pool and reverse transcribed into cDNA, which was subjected to qPCR, using a series of IGF2 promoter-specific primers for the four known bovine isoforms. Amplified fragments were sequenced for confirmation. Data on expression of the IGF2 isoforms were analyzed by ANCOVA, using the β-actin endogenous control gene as co-variate, with pairwise comparisons between stages done by LSM, for $P<0.05$. The expression of the IGF2 driven by the P2 and P4 promoters followed patterns similar as previously reported for bovine embryos. An initial expression peak was detected in early development, between matured oocytes and 2-cell stage embryos, mostly from transcript accumulation prior to fertilization, followed by a decrease in transcript abundance until embryo genome activation (EGA), at the 8-cell stage embryo. The P2- and P4-derived IGF2 splicing variants were also detected at compaction and cavitation. IGF2 expression driven by the P1 promoter was negligible at the initial stages, increasing after EGA, especially during compaction, prior to cavitation. The P3 activity was not detected at any stage. Our findings provide some further understanding of bovine *in vitro* embryo development, with the P1 and P2 promoters likely having secondary roles during early stages of development, whereas the P3 promoter could be relevant later on, as shown by others. The P4-driven IGF2 expression seems to be the main pathway for IGF2 synthesis during fetal development in most species, and our results corroborate with such concept. We suggest that promoter P4, if genetic manipulated, could be used to influence *in vitro* embryo production in studies for the modulation of embryo growth and development.

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Morfofunctional and endocrine aspects related to cloprostenol-induced luteolysis in bovine females

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The objective of this study was to evaluate variables related to morphological, functional and endocrinological alterations related to cloprostenol sodium induced luteolysis in bovine females. Twenty-five crossbred (*Bos taurus x Bos indicus*) non-lactating females, heifers (12) and cows (13), weighing between 318 and 457 kg, aged between 26 and 54 months, between days 7 and 16 of the estrous cycle were used. After selection, (0h) the animals' blood was collected using vacuum collection tubes without anticoagulant. On the same day, ultrasound evaluation of the ovaries was performed using B-mode and color Doppler technology (Mindray-M5™). They were recorded from each ovary that had the corpus luteum (CL), a sequence of 252 frames in B mode and 150 frames in Doppler mode. Immediately after the evaluations, 0.5mg of Cloprostenol sodium (Estron®-Agener União, Brazil) was applied IM. Blood samples and the same ultrasonographic evaluations were done 24, 48 and 72 hours after luteolytic application. Mode B images were used to measure the perimeter and area of the corpus luteum (CL). The color Doppler images were used to determine the vascularization score on a scale of 1 to 4. Serum obtained from the blood samples were used for the measurement of progesterone (P4) via Electrochemiluminescence (ECL) using Cobas E411 equipment and commercial Elecsys ™ kits Progesterone III (Roche™). The B mode CL measurements and P4 concentrations were accessed by Anova and compared between the days using Tukey's test. Vascularization scores between the different days were compared by the Kruskal Wallis test. Significant probabilities less than 5% were considered. The intra-assay coefficient of P4 dosages was 1.67%. The mean P4 concentrations were 7.65 ± 2.80^{a}; 4.12 ± 1.49^{b}; 0.53 ± 0.29^{c} and 1.04±0.21c ng/mL for times 0, 24, 48 and 72 hours (P> 0.05). The mean CL circumferences were 5.78 ± 1.23^{a}; 5.24 ± 1.09^{a}; 3.98 ± 0.94^{b} and $2.20+0.59^{c}$ cm and area 2.43 ± 0.80^{a}; 1.97 ± 0.64^{ab}; $1.29+0.44^{b}$ and $0.61+0.24^{c}$ cm^2 for the times 0, 24, 48 and 72 hours (P<0.05). The mean values of CL vascularization score were 3.7^{a}; $1,3^{b}$; $0.8b^{c}$ and 0.5^{c} for 0, 24, 48 and 72 hours, respectively (P<0.05). The efficiency of luteolysis was 100%. The reduction of vascularization and concentration of P4 was observed at 24 hours, that is, at the 1st evaluation after the application of the product. On the other hand, the morphological regression of CL occurred only in the 2nd evaluation, at 48 hours, one day later than the functional and endocrinological regression. It is concluded that the product used is efficient in causing luteolysis in bovine females. Functional regression and reduction of P4 concentrations precede morphological changes in CL.

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Association between serum paraoxonase-1 activity, ovarian dynamics and steroid hormone concentration in cattle

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Paraoxonase-1 (PON1) is an enzyme synthesized by the liver and found in serum and follicular fluid in cows. PON1 has antioxidant activity and it is positively related to fertility. The objective of this study was to evaluate the association between PON1, serum estradiol concentration and follicular diameter, as well as luteal size and serum progesterone concentration in cows submitted to an E2/P4-based FTAI protocol. Fourteen Aberdeen Angus cows (*Bos taurus,* BCS: 2.9 ± 0.2, 48 months, 450 kg, non-lactating and non-pregnant) received an intravaginal progesterone-releasing device (1g, Primer®, Agener União, São Paulo) plus 2 mg i.m. of estradiol benzoate (RIC-BE®, Agener União) on Day 0. On Day 8, device was removal and it was administered 0.150 mg i.m. of *d*-cloprostenol (Prolise®, Agener União) and 1 mg i.m. of estradiol cypionate (ECP®, Zoetis, NJ, USA). Cows were evaluated by ultrasonography each 12 hours to measure follicular size and to detect the ovulation, seven days after ovulation was performed luteal size mensuration. Blood samples were collected after device removal, ovulation and seven days after ovulation to evaluate serum estradiol and progesterone concentration and PON1 activity. Steroid hormones were evaluated by chemiluminescence method. Activity of PON1 was evaluated by spectrophotometry. Cows were classified in two groups according serum PON1 activity: higher activity (HA; >105.0 U/mL) or lower activity (LA; <105.0 U/mL). Results were evaluated by software GraphPad® 6.01 (GraphPad software, Inc., CA, USA). Groups were compared by T test. In addition, PON1 levels were correlated by simple linear regression with the variables. PON1 activity was higher on ovulation moment than 7 days after ovulation. Estradiol concentration was not different between groups on day 8 (LA: 32.8±4.9 pg/mL; HA: 35.8±6.0 pg/mL; R^2=0.06) and on ovulation time (LA: 42.0±6.0 pg/mL; HA: 42.9±5.3 pg/mL; R^2=0.02). Progesterone concentration was higher in LA group (LA: 4.9±1.5 ng/mL; HA: 3.2±0.3 ng/mL, *P*=0.0022; Linear regression: R^2=0.3, *P*=0.05) on day 8, however did not differ on ovulation moment (LA: 0.37±0.05 ng/mL; HA: 0.41±0.06 ng/mL; R^2=0.002) and 7 days after ovulation (LA: 7.7±0.8 ng/mL; HA: 8.2±0.4 ng/Ml; R^2=0.14). Follicular size on day 8 (LA: 8.8±0.5 mm; HÁ: 9.4±0.4 mm; R^2=0.07) and on ovulation moment (LA: 12.19±0.49 mm; HA: 12.9±0.3 mm; R^2=0.001); and luteal size (LA: 5.75±0.99 cm^3; HA: 6.86±1.3 cm^3; R^2=0.0007) did not differ between groups. In conclusion, PON1 activity was associated with serum progesterone concentration at device removal, however more studies are needed to clarify the interaction between these factors.

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Heat stress model decreases cleavage and development rates in bovine embryos produced *in vitro*

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Heat stress (HS) represents one of the most important causes of bovine productive and reproductive losses, especially in dairy farms. Our hypothesis is that heat stress alters *in vitro* embryo development. Therefore, the aim of this study was to evaluate induced heat stress effects on early embryo development. The experiment was divided into five groups: (1) Control; (2) Oocytes matured under HS conditions (IVM HS); (3) Oocytes fertilized under HS conditions (IVF HS); (4) Zygotes cultured on the first day under HS conditions (IVC HS); and (5) Embryos submitted to HS during 3 days of embryo production (IVM, IVF and IVC HS). For HS treatments, the temperature was gradually increased until 40.5ºC, and remained for 6h. Bovine ovaries were obtained from slaughterhouse. Oocytes were aspirated from follicles 3-8mm in diameter and matured (50 oocytes per group) for 22h under 5% CO_2 atmosphere. Matured oocytes were fertilized using spermatozoa selected with Percoll® gradient. Spermatozoa and matured oocytes were co-cultured in Fert medium for 18h under 5% CO_2 atmosphere. IVF day was considered as day 0 of embryo production. After 18h of IVF, zygotes had cumulus cells removed by 2 minutes of vortex. Then, zygotes were cultured in SOF medium under 5% CO_2, 5% O_2 and 90% N_2 at 38.5ºC up to blastocyst stage (day 7). The study was completed in four replicates. Data were statistically analyzed using JMP Software (13.1.0; SAS Institute Inc.). Cleavage and developmental rates of bovine embryos of different treatment groups were analyzed by multiple pairwise comparison (Tukey test). Results are presented as means ± S.E.M., and they were considered significant at $P \leq 0.05$. Cleavage rates were determined 2 days after IVF. The cleavage rate was significantly greater ($P<0.05$) in control group (89.3% ± 0.91) than in HS groups: IVM HS (63.3% ± 8.34), IVF HS (63.3% ± 5.77), IVC (62% ± 1.93) and IVM, IVF and IVC HS (29% ± 2.05). IVM HS, IVF HS and IVC HS groups were not different from each other on cleavage rates. The IVM, IVF and IVC HS cleavage rates were significantly lower. Blastocyst rates were evaluated 7 days after IVF. Blastocyst rate (%) was not different ($P>0.05$) between control (31.6% ± 1.10) and IVM HS (29% ± 1.29) groups. However, IVF HS (21.66% ± 1.37) and IVC HS (18% ± 0.86) presented a reduced blastocyst rates (%) compared to control. The IMV, IVF and IVC HS group had the smallest blastocyst production (12% ±1.31). In conclusion, heat stress during IVP induces cellular detrimental effects confirmed by the reduction of cleavage and blastocyst rates.

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Vaginal cytology as a tool to predict the time of ovulation in goats and sheep

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The detection of ovulation is of great importance for the use of reproductive biotechnologies in small ruminants. The ovulation is efficiently determined by ultrasound (US), equipment that is not always available and of relatively high cost. Therefore, the aim of this study was to identify the efficacy of vaginal cytology as a tool to determine the ovulation time in these species. The study was carried out during the non-breeding season, in Coronel Pacheco, Minas Gerais (21°35'S and 43°15'W). Nine goats and 11 ewes (all pluriparous), ageing on average three years old, under intensive system were used. All females received a short-term estrous induction treatment, with 0.3 g progesterone (CIDR®, Pfizer Aninal Health, São Paulo, Brazil) for six days, and 24 h before its removal, 30 µg d-cloprostenol (Prolise®, Syntex, Buenos Aires, Argentina) and 200 IU eCG (Novormon® 5000, Syntex) i.m. were administered. After CIDR removal, every 12 h until ovulation detection, two procedures were performed: 1) vaginal smear with swab, stained with Fast Panoptic kit (Laborclin Ltda, São Paulo, Brazil), where 100 cells were counted in each moment and 2) transrectal US (7.5 MHz probe; Mindray®, Modelo M5 Vet, Mainland, China). Nonparametric data were analyzed by Mann Whitney, Kruskal Wallis and Dunn test, while parametric data were compared by Student t test and ANOVA. In the comparison between the cytological profile and US, were calculated the negative and positive predictive value, sensitivity and specificity. All analyses were performed by Bioestat 5.0 program and the confidence level was 5%. Ovulation rate was 88% (8/9) in goats and 100% (11/11) in sheep. In order to determine the cell standard to be selected (parabasal, intermediate, superficial and anucleated), analysis of their averages was performed, every 12 h. This analysis aimed to identify which standard differed from the previous one and also from the the other cells at the moment of ovulation. Thus, the chosen cell standard in goats was the superficial ($P < 0.05$) and this was characterized by a low coefficient of variation (CV) of 6%. The specificity found at 60 to 48 h before ovulation was 100%; from 36 to 24 h was 88%; and 12 h was 75%. The sensitivity at the moment of ovulation was 88%. The negative predictive value (NPV) was 97%, higher than the 64% found for positive predictive value (PPV), resulting in an accuracy of 89.6%. In sheep, the standard chosen was anucleated, but it had a high CV (23.7%), which led to non-high accuracy (66.7%). Therefore, PPV and NPV were 26% and 88%, respectively; the specificity was 45% and 64% at 24 h and 12 h before ovulation respectively; and finally, the sensitivity at the moment of ovulation was 55%. It can be concluded that the vaginal cytology may be an efficient tool to determine the moment of ovulation in goats, however it is less accurate in sheep.

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Evaluation of cell death type of canine luteal cells in cyclic and gestational diestrus

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The estral cycle of bitches is distinct of other domestic animals, especially regarding luteal phase, because cyclic and gestacional corpora lutea (C.L) have similarities acting for long periods in both cases and is the only structure responsible for maintenance of gestation. The aim of this research was to investigate the mechanism of cell death (apoptosis / necrosis) in cyclic and gestational luteal cells on final third of diestrus, after cell culture, being the proposal approved by CEUA- UNESP: 168/2013. Twenty healthy mixed breed bitches were used, with age and weight equivalents, which were divided in 02 groups: Cyclic diestrus (n = 10), formed by females that were submitted to OSH, 40 and 60 days after LH pre-ovulatory surge and pregnant (n=10), formed by females inseminated artificially and submitted to OSH, 40 and 60 days after LH preovulatory surge. The ovulations was estimated based on serum progesterone concentration, (4 to 5 ng /ml) and percentage of superficial cells (≥ 90%) in vaginal cytology (Hase et al.,Theriogenology, 62:243-48. 2000). After OSH, the ovaries were collected and the C.Ls were dissected. The cell culture included steps of C.L processing, enzymatic digestion with collagenase type I (1mg/ml, C0130, Sigma Aldrich, USA), serial centrifugation (3x), 220G/10 minutes at 20°C for obtaining cellular and plating. The culture medium used was constituted by DMEN high glucose (41965-039, Sigma, Aldrich USA), 5% fetal bovine serum (Gibco, USA), plus L-glutamine (G7513, Sigma), 10,000 IU of penicillin, 10 mg of streptomycin, 25 µg of amphotericin B and 0,25 mg of amikacin and this was changed 24 hours after culture. After 60 hours of cell culture the trypsinization step (12604021, TrypLE™ Express Enzyme, Thermo fisher, USA) was performed to obtain luteal cells, which were cryopreserved at -80°C. The cells were thawed slowly and resuspended in nucleotide releasing buffer (Biovision, CA) at 37°C, resulting in a final solution containing on average 6000 cells/well. Evaluation of cell death type was performed by measuring the ADP: ATP ratio using the ADP/ATP Ratio Bioluminescent Assay Kit (K255-200, Biovision, CA), which classifies cells in proliferation, growth arrest, necrosis or cell apoptosis phase. For luminescence reading, Biotek Synergy 4 equipment (Bio-tec, USA) with sensitivity adjustment for 135 was used. For statistical analysis was used GLM program of the SAS, applying ANOVA and student t Test, considering significant when $p < 0.05$. The ADP/ATP ratio showed no significant difference between evaluated groups (cyclic X pregnant, P= 0.6670) and timepoints evaluated (40 x 60 days, P=0.2316). In the cyclic diestrus group, at the time of 40 days, the luteal cells were in a growth arrest, presenting apoptosis only at 60 days, whereas in the pregnant group in both 40 and 60 days luteal cells presented apoptosis, suggesting that cell death by apoptosis begins earlier in gestational diestrus.

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Effect of caloric restriction and rapamycin on weight gain, insulin sensitivity and ovarian oxidative stress in mice

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Caloric restriction (CR), a method known to increase longevity in several species, has also been associated with preservation of the ovarian reserve (Xiang et al., Gene, 1: 77-82, 2012). Similarly, the drug rapamycin has been shown to have effects similar to those of CR, including preservation of the ovarian reserve (Li et al., Reproduction Science, 1:60-7, 2015). The objective of this study was to evaluate the effect of CR and rapamycin on weight gain, feed intake, insulin resistance and ovarian oxidative stress in female mice. For this, 36 female C57BL/6 mice maintained with standard diet and water *ad libitum* were used, kept under controlled conditions of light and temperature. The mice were divided in 3 groups of 12 animals each (control group, group treated with intraperitoneal rapamycin every 2 days at a dose of 2mg/kg of body weight and the group submitted to CR of 30% in relation to the control group). Weight data were collected weekly and food intake data daily. At 85 days of treatment, peripheral insulin sensitivity was assessed through the insulin tolerance test (ITT) and insulin resistance through the glucose tolerance test (GTT). At 93 days of treatment the mice were anesthetized and euthanized, and the ovaries were collected. The formation of reactive oxygen species (ROS) of the ovaries was evaluated through a method already described and adapted (Ali, et al., Neurotoxicology, 13: 637-648, 1992). Statistical analyzes were performed using Graphpad Prism 7 software. ITT and GTT were compared by repeated measures ANOVA and ROS were compared by one-way ANOVA. Values of $P<0.05$ were considered significant. Lower weight gain was observed in CR females ($P<0.05$), but there was no difference in weight between the control and rapamycin groups ($P>0.05$). Regarding dietary intake, there was no difference between the rapamycin and control groups, but there was lower intake in the CR group ($P<0.05$). In the ITT the females of the rapamycin group were more resistant to insulin whereas those of the CR showed higher insulin sensitivity ($P=0.003$). In the GTT the females of the rapamycin were more intolerant to the glucose when compared with the ones of the RC and control ($P=0.04$). In the quantification of ROS the females of the rapamycin (174 ± 50) group had higher levels of ovarian ROS when compared to the CR (142 ± 20) and control group ($110 \pm 10P<0.05$). Therefore, the females treated with rapamycin were more resistant to insulin and had more oxidative stress at the ovarian level, whereas the CR females of CR had lower weight gain, greater insulin sensitivity, without any changes at the ovarian level.

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Presence of bovine conceptus modulates interferon-tau stimulated genes expression in peripheral blood polymorphonuclear cells at the beginning of pregnancy

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We aimed to compare the expression of type I interferon stimulated genes (ISGs) in peripheral blood polymorphonuclear cells (PMNs) between pregnant and non-pregnant heifers on days 14 and 18 after FTAI. Twenty-six Nelore heifers were synchronized by pharmacological treatment based on P4 and E2, and the day of FTAI was designated as D0. Pregnancy diagnosis was made by ultrasonography on days 25 and 28 through the detection of the embryonic vesicle and heartbeats. On days 14 and 18, 25mL of blood was collected in heparinized tubes by puncture of the jugular vein for the isolation of PMNs. The isolation was made by Ficoll®Paque Plus gradient (GE Healthcare – São Paulo, Brazil), in an adapted methodology from that described by Jiemtaweeboon et al. (Reproductive Biology and Endocrinology, 9: 79-89; 2011). PMNs samples from 5 pregnant and 6 non-pregnant heifers were subjected to total RNA extraction using the Direct-Zol RNA Miniprep kit (Zymo Research- Irvine, USA) according to the manufacturer's instructions. Five reference genes (GAPDH, PPIA, 18S, RPL30 and ACTB) were quantified by real-time polymerase chain reaction (qPCR), and through the NormFinder software, the two genes with most stable expression (GAPDH and ACTB) were selected. The expression of the target genes (OAS-1, MX2 and ISG15) evaluated by qPCR was normalized in relation to the two reference genes by the comparative Ct method (Pfaffl, Nucleic Acids Research, 29:2001-2007, 2001). The abundance of transcripts was evaluated by analysis of variance (ANOVA) with repeated measures of time, considering the random effect of the heifer and the fixed effects of the group (pregnant or non-pregnant), day and group by day interaction using the PROC MIXED SAS software (Version 9.2; SAS Institute). For the OAS-1 gene, effects of time (P=0.03), group (P=0.03) and interaction between time and group (P=0.0002) were detected. The abundance of this transcript did not differ (P=0.88) between pregnant (1.43±0.76) and non-pregnant (1.09±0.32) females on D14, but it was higher in pregnant heifers on D18 (P=0.0003; 7.11±3.24 vs. 0.30±0.07). There was a 7-fold increase (P=0.02) in the expression of this transcript between D14 and D18 in the pregnant heifer group, while in non-pregnant, there was a 0.57-fold decrease (P=0.05). A group effect (P=0.03) was observed for the MX2 and ISG15 genes, indicating a 3 and 7-fold increase, respectively, in the abundance of these transcripts in pregnant females. It is possible to conclude that a viable bovine conceptus stimulates the greater abundance of transcripts for the evaluated ISGs in circulating PMNs, with a most evident effect on D18 for the OAS-1 gene. Further studies are needed to elucidate the expression profile of ISGs in PMNs aiming the use of these genes as possible markers of gestation and as a form of early pregnancy diagnosis.

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Induction of cyclicity in equine embryos recipients

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The aim of the study was to investigate the effect of an intravaginal progesterone device in the first ovulation of mares on seasonal anestrous at the begining of reproductive season. The experiment was carried out at Fazenda Escola / BIOTEC, from Unicesumar, Maringá, Paraná, in the period from 08/20/2017 to 10/20/2017. Nineteen crossbred mares, evaluated by ultrasound examination (Aloka SSD-500 ™) twice, with a 10-day interval, were used to verify the conditions of the uterus and ovaries, as well as to observe the presence or absence of corpus luteum, characterizing or not the anestrous. The diameter of the follicles was measured through the mean of their horizontal and vertical measurements, which allowed to classify the animals in the two treatments studied: group 1 (T1), composed of 11 animals, with the presence of follicles less than or equal to 20 mm, and group 2 (T2), with 8 animals with follicles greater than or equal to 20 mm in diameter. All animals received bovine intravaginal device (DIB ® - Zoetis, São Paulo - SP, Brazil) containing 1g of progesterone. After seven days, the two groups underwent daily sonographic examinations to verify follicular growth. Implants were removed when the animals had a follicle greater than or equal to 35 mm. Thus, the animals remained implanted for different times according to their follicular growth, and one day after implant withdrawal, all animals had their ovulation induced by an association of 1500 IU of hCG (Vetecor®, Hertape, Juatuba - MG, Brazil) applied intravenously with 750 µg of deslorelin (GnRH - Sincrorrelin ®, Ourofino, Cravinhos - SP, Brazil), administered intramuscularly. The data were tabulated and the statistical analyzes of the variables were performed using the procedure PROC GENMOD of the statistical program SAS (2000), version 8.01, and showed that the percentage of animals that responded to the treatment and had a dominant follicle greater than 35 mm was significantly ($P < 0.05$) in T2 animals, totaling 62.5% (5/8) than in T1, which presented 36.3% of animals with dominant follicle (4/11). The mean time, in days, for the appearance of the dominant follicle was also higher ($P<0.05$) for the T2 group when compared to T1 (8.0 ± 2.23 and 9.50 ± 0.57, respectively). The ovulation rate of the females in this study was calculated in relation to the number of mares that presented a follicle of 35 mm in diameter and was not altered ($P>0.05$) by the use of the P4 implant, 80.0% ovulation (4/5) and in T2, 100,0% (5/5). According to the results obtained in this research, it is concluded that the use of progesterone devices did not contribute to the anticipation of the first ovulation in the studied animals, but they helped induce the cyclicity of the recipients, showing that this technique can make the management of females equines more efficient, productive and sustainable from the economic and environmental point of view.

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Transcriptional evaluation of bovine embryos submitted to long chain Acil-CoA synthetase modulators

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The long-chain acyl-coa synthetase (ACSL) is composed by a family of enzymes that activate fatty acids and participate in virtually all pathways of lipid synthesis. Triacsin C is a metabolic regulator that acts as a competitive selective inhibitor of ACLS 1, 3 and 4 inhibiting triacylglycerol and phospholipid synthesis. GW3965 is an agonist capable of inducing increased expression of ACSL3. We have evaluated the effect of a stimulator (GW3965 / ACS +) and an inhibitor (Triacsin C / ACS-) of ACSL in the development of *in vitro* produced bovine embryos and on the mRNA transcripts abundance for genes associated with lipid metabolism and embryo quality. Oocytes containing homogeneous cytoplasm and more than three layers of cumulus cells were matured and fertilized (Day 0). Presumptive zygotes were cultured in SOFaaci containing 2.5% FCS. On day 4, embryos were randomly distributed on four groups: control, ACS- (0,1 uM), ACS+ (10 uM), and association of both modulators (ACS±). The concentrations used were pre-determined in a pilot study. Expanded blastocysts were collected in pools of 5 embryos for RNA extraction with the PicoPure RNA kit (Life Technologies). Complementary DNA was prepared using High Capacity cDNA Reverse transcription Kit (Applied Biosystems). Samples were preamplified and transferred to an integrated fluidic circuit board. A panel of genes on the 96.96 Dynamic Array ™ Integrated Fluid Circuits (Fluidigm) chip was used for data collection. The thermal cycling qPCR was performed in the Biomark HD System (Fluidigm) with TaqMan GE 96x96 Standard protocol. To analyze the relative expression of the target genes, the Pfaffl method (Pfaffl, 2001) was used, with the logarithmic transformation of the relative frequencies of gene expression. The GLIMMIX procedure of SAS was used for analysis of variance (ANOVA) by the least squares method. Expression of 36 genes was significantly affected by at least one of the treatments. Of these, 18 genes were differentially ($P < 0.05$) expressed only in the ACS± group, a result that still needs to be better explored. In general, no remarkable number of differentially expressed genes was identified comparing control group with ACS+ (4 genes) and ACS- (1 gene) treatments. It was observed an increase ($P < 0.05$) of the mRNA transcript abundance for ACSL3 in ACS+ and its reduction in ACS-, validating the modulatory effect of the drugs used. In addition, the ACSL6 mRNA level was reduced ($P < 0.05$) in the ACS- and ACS± groups, whereas the abundance of mRNA for HSP90AA1 was increase ($P < 0.05$) in the ACS+, compared to control. In conclusion, the use of Triacsin C and GW3965 was effective for modulating ASCL3 transcript levels on embryonic cells; however, further analysis investigating the effect of ACSL3 modulation and its reflects on embryo phenotype are essential.

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Knockdown of the histone lysine demethylase 7A mRNA impairs cell differentiation and development of porcine embryos

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Epigenetic modifications regulate important mechanisms during early embryo development including embryo genome activation (EGA) and cell differentiation. Although several lysine demethylases (KDMs) were shown to be implicated in these processes, the role of many other KDMs remains to be characterized. The KDM7A, which regulates mono- and di-methylation of H3K9 and H3K27, was shown to have a peak of expression during the EGA transition in bovine and porcine embryos (Glanzner et al., 2018. Biol. Repro. doi: 10.1093/biolre/ioy054). However, no previous studies have evaluated if the KDM7A regulates normal embryo development. In this study, two Dicer-substrate interference RNAs (DsiRNAs) targeting KDM7A mRNA or Control (Scrambled sequences) were microinjected (~10 pl of 25 mM diluted) in pig oocytes (~450 oocytes/treatment/method) and effects on embryo development (~90 embryos/treatment), cell differentiation and gene expression (10 embryos x 3 replicates) were evaluated. Statistical differences between treatments were tested by LSMeans student *t test* and considered significant when $P<0.05$. The knockdown efficiency, evaluated by RT-qPCR, in parthenogenetically activated (PA) embryos was 72.1% (D3), 79.2% (D5) and 35% (D7) compared with controls. Embryos produced by *in vitro* fertilization (IVF), somatic cell nuclear transfer (SCNT) and PA were used to test the effect of KDM7A knockdown on development. Microinjections were performed at MII stage, after fertilization (5-6h after the beginning of sperm/oocyte co-incubation), or after activation (5-6h after cell fusion) for PA, IVF and SCNT embryos, respectively. Cleavage rates (D2) were similar, but development to the blastocyst stage (D7) was significantly decreased by 48.4% in PA (29.7±3.3% vs. 57.3±3.6%), 69.2% in IVF (9.3±6.3% vs. 30.3±10.2%), and 48.1% in SCNT (13.6±1.3% vs. 26.3±3.4%) KDM7A knockdown compared to control embryos. KDM7A knockdown reduced total number of cells in blastocysts in PA (32.1±4.4 vs. 52.2±4.1), IVF (19.7±2.3 vs. 38.3±5), and SCNT (19.4±6 vs. 29.7±3.4) compared to control embryos. Moreover, the ratio ICM/total number of cells was reduced 45.5% (8.4±1.9% vs. 15.5±1.3%) in KDM7A knockdown compared to control blastocysts, as determined by SOX-2/DAPI counterstaining. The average number of cells on D3 and D5 of development and the relative mRNA expression of *eIF1A* (an important EGA marker), were not affected by KDM7A knockdown. Higher immunofluorescence signal for H3K27me2 on D3 and D5, and for H3K27me1 on D7 was detected in KDM7A knockdown compared to control embryos. KDM7A knockdown decreased H3K9me1, H3K9me2 and H3K9me3 on D3 embryos, which was correlated with the upregulation of *KDM3C* and *KDM4B* mRNA expression. The relative mRNA expression of NANOG (D5 and D7) and OCT4 (D7) was decreased, and CDX2 (D3) increased in KDM7A knockdown compared to control embryos. Together, these findings revealed that KDM7A regulates cell differentiation and development of porcine embryos.

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MiRNA contents in small extracellular vesicles are modified according to ovarian follicular progesterone levels in bovine

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Small extracellular vesicles (EVs) are nanoparticles that carry bioactive molecules such as microRNAs (miRNAs). Extracellular vesicles participate of intercellular communication and are capable of modulating biological processes, such as follicular cells communication as well as oocytes and the follicular environment. During estrous cycle the ovarian follicle environment is influenced by the stage of corpus luteum (CL) development, leading to low and high intrafollicular progesterone (P4) levels. These changes in hormonal levels significantly impact transcription of different genes in the organism as well as within the follicular environment. Thus, the hypothesis of this study is that miRNAs content in extracellular vesicles present in ovarian follicular fluid (FF) is modulated by intrafollicular progesterone levels. To test this hypothesis, slaughterhouse ovaries were collected in pairs and classified according to the appearance of the CL and P4 concentration in low P4 (early CL; n=4 repetitions) and high P4 (late CL; n=5 repetitions) groups. Follicles between 3-6 mm in diameter of each group were punctured to obtain FF. The progesterone concentration in FF was 63.62 ± 6.79 ng/ml in low P4 group and 158.8 ± 17.47 ng/ml in high P4 group (P=0.002). To isolate EVs the FF was centrifuged and filtered (0.22µM) to eliminate larger particles followed by ultracentrifugation (120.000xg, 70 minutes, twice). After EVs isolation, total RNA was isolated and used for the relative expression analysis of 384 miRNAs precursor/mature or just mature forms by RT-PCR. The data were submitted to Student's T-test considering significance level of 5%. We detected a total of 161 precursor/mature miRNAs up-regulated in low P4 group compared to high P4 group (P<0.05). This result suggests greater post-transcriptional modulation by miRNAs found in EVs of growing follicles during low P4 levels compared to high P4. Mature miRNA analysis demonstrated nine differently expressed miRNAs between groups; three miRNAs were up-regulated in low P4 group while six were up-regulated in high P4 group. The comparative analysis between precursors/matures and just matures miRNAs within each group demonstrated higher levels of precursor/mature forms in EVs from low P4 group: 98.59% as well as in high P4 group: 95.86% (P<0.05). Bioinformatics analysis of the up-regulated miRNAs suggested possible regulation of pathways like RNA transport, PI3K-Akt, oocyte meiosis and oocyte maturation mediated by progesterone. In conclusion, the EVs content is modified according to intrafollicular progesterone levels, which was confirmed by the majority of the miRNAs identified in the precursor form. Therefore, this study suggests that EVs present within FF contains different miRNAs that could modulate follicular development, possibly influenced by different endocrine environment, as well as affecting oocyte maturation and quality. Supported by FAPESP (2014/22887-0; 2015/21829-9; 2017/02037-0).

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ISG15 expression in peripheral blood polymorphonuclear cells as an indicator of embryonic signaling in beef heifers on Day 16 after fixed-time artificial insemination

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In ruminants, maternal recognition of pregnancy is characterized by secretion of a protein called interferon tau (IFNT) produced by trophoblast cells. The endocrine action of IFNT on blood cells elevates the expression of interferon-stimulated genes (ISGs), such as Interferon stimulated gene 15 (ISG15). The objective of this study was to evaluate the expression of *ISG15* in peripheral blood polymorphonuclear cells in beef heifers subjected to fixed-time artificial insemination (FTAI). FTAI protocol was performed on 12 beef heifers. Following FTAI, an ultrasonographic evaluation was performed to allocate the animals in 03 groups: pregnant (P), non-pregnant (NP) and non-bred (NB) On Day -10, estradiol benzoate (2mg / IM) was injected and a progesterone intravaginal device (IVD) (1g) was inserted. On Day -2 the IVD was removed and prostaglandin and estradiol cypionate analog were injected. FTAI occurred 48 hours after IVD withdrawal (Day 0). On the 16th day after FTAI, blood from the coccygeal vein was collected to isolate blood cells using 4mL tubes containing EDTA, where blood was diluted in 0.9% NaCl. In a 15 mL tube containing Ficoll-Paque PREMIUM® was slowly added the blood solution. Centrifugation was performed at 400XG for 15 minutes at room temperature. Thereafter, the following fractions were identified and separated: plasma, erythrocytes, mononuclear and polymorphonuclear cells. Next to the separation of the fractions, polymorphonuclear cells (PMN) was collected and stored at -80°C in a cryotube. Subsequently, mRNA extraction, cDNA and qPCR were performed. After isolation of PMN fraction, a glass-slide fraction-film was prepared to determine the purity of the fraction. The purity was accessed based on cell morphology. The percentage of PMN present was determined and samples above 95% were included in the study. On the 29th and 60th days following FTAI, the pregnancy diagnosis was performed by ultrasonography, in order to allocate them in two groups: pregnant (P, n=4) and non-pregnant (NP, n=4). The results revealed that in the 16th day after FTAI, there was no difference in *ISG15* expression in PMN between P (6.85±0.9) and NP heifers (4.53±0.82), however it shows significant difference between pregnant (6.85 ±0.9) and non-inseminated heifers (1.65 ±SE 0.41) ($p < 0.05$). The expression of ISG15 can be used as a tool to identify embryonic signaling at 16 days of gestation in beef heifers. This research was funded by CNPq and CAPES.

A211 Embryology, Developmental Biology and Physiology of Reproduction

Dysregulation of histone lysine demethylases KDM5B and KDM5C impairs embryo genome activation and stability, and development in pigs

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In a recent study, we observed that the mRNA expression of the histone 3 lysine 4- (H3K4) related histone demethylases *KDM5B* and *KDM5C* was dramatically increased in bovine and porcine embryos during the period when the embryo genome is activated (EGA), i.e. D3 and D4 for porcine and bovine embryos, respectively (Glanzner et al., 2018. Biol. Repro.doi: 10.1093/biolre/ioy054). In somatic cells, KDM5B is recruited to sites of DNA double strand breaks (DSBs) and contributes to regulate DNA damage response. However, the role of KDM5B and KDM5C during embryo development has not been elucidated. In this study, Dicer-substrate interference RNAs (DsiRNAs) were used to investigate KDM5B and KDM5C roles on development of porcine embryos. Two DsiRNAs targeting each KDM5B or KDM5C mRNA, or Control DsiRNA (scrambled sequence) were microinjected (~10 pl of 25 mM diluted) into porcine oocytes (~800 oocytes/treatment/method) after fertilization (IVF), parthenogenetic activation (PA) or somatic cell nuclear transfer (SCNT). Injected embryos were cultured in PZM-3 medium and used to evaluate transcriptional activity (15 embryos/treatment), gene expression (10 embryos/treatment/replicate), DNA damage (15 embryos/treatment) and embryo development (30 embryos/treatment/replicate). Statistical differences between treatments were tested by LSMeans student t test and considered significant when $P<0.05$. Knockdown efficiency in PA embryos, evaluated by RT-qPCR, was 75% and 75% on D3, and 61% and 59% on D5, for KDM5B and KDM5C, respectively. Embryo cleavage (D2) was not affected by treatment, but blastocyst rates (D7) were decreased in PA: 55.1%, 19.0%, 29.8%, and 31.2%; IVF: 30.7%, 4.3%, 8.2% and 3.9%; and SCNT: 23%, 5.2%, 12.1% and 3.4% embryos injected with Control, KDM5B, KDM5C or KDM5B+KDM5C DsiRNAs, respectively. Total cell number in D7 blastocysts was not affected by KDM5B, KDM5C or KDM5B+KDM5C knockdown. The mRNA expression of *Eif1ax* (an important EGA marker) was ~3-fold decreased on D3 embryos injected with KDM5B or KDM5C compared with Control DsiRNAs. The proportion of cells presenting a positive signal for mRNA synthesis, as assessed using the Click-iT® EU RNA Imaging Kit (Life Technologies), was ~4-fold decreased on D4 embryos injected with KDM5B or KDM5C compared with Control DsiRNAs. Levels of H3K4m2 and H3K4m3, as assessed by immunofluorescence, were increased in KDM5B knockdown, but not in KDM5C knockdown embryos on D3 and D5 of development. KDM5C knockdown increased H3K9m1 on D3 and D5 embryos, and H3K9m3 on D3 embryos. The incidence of DSBs, as assessed by the number of immunofluorescent foci for H2AX139ph, was ~2-fold increased in KDM5B and KDM5C knockdown compared with control embryos, which was correlated with a significant decrease in the mRNA levels of genes involved in DNA repair (e.g., *ATM*, *Brca*1). Together, these findings revealed that KDM5B and KDM5C are important for EGA transition, genome stability and development of porcine embryos.

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In vitro viability of bovine IVP blastocysts after exposure to high gaseous pressure

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The use of stressor agents may be an alternative to increase the viability of cryopreserved IVP bovine embryos. Embryo exposure to sublethal stress causes a genomic cellular response, conferring cellular resistance to a subsequent stress, such as cryopreservation. The aim of this study was to evaluate the *in vitro* survival rate after exposure of bovine IVP blastocyst to high gaseous pressure (HGP) (for review see Gonsioroski., 2018: http://hdl.handle.net/10183/179278). Cumulus-oocyte complexes (COC) obtained from slaughterhouse ovaries were morphologically selected based on the cytoplasm and cell vestment, with only grades I and II COCs submitted to IVM for 24 h at 38.5°C and 5% CO_2 in humidified air. Then, IVF was performed using cryopreserved semen of a tested bull. Sperm cells were selected by descontinuous Percoll® gradient (90/45%). At 22 h post-insemination, presumptive zygotes were denuded and cultured *in vitro* during 7 days. On Day 5, cleavage rate was evaluated and embryo feeding was performed. On Day 7, morphologically viable blastocysts were randomly distributed into two experimental groups: Controls, with blastocysts transferred to the IVC medium; and HGP, with embryos exposed to 27.6 MPa HGP for 2 h. Then, all blastocysts were maintained under similar IVC conditions during 48 h. Embryo hatching rate was evaluated on Day 9, and data after eight replicates were analized by the Chi-square test ($P<0.05$). The blastocyst hatching rate was higher in the HGP group (79.6%, 82/103) compared to the Control group (54.6%, 65/119). Bovine IVP blastocysts survived well to the 2-h exposure to HGP at 27.6 Mpa, improving hatching rates on Day 9 of development.

A213 Embryology, Developmental Biology and Physiology of Reproduction

Antral follicles cout during gestation of Holstein cows

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The population of antral follicles or antral follicles counting (AFC) has been identified as one of the main factors that influence the efficiency of reproductive biotechniques. AFC is described as being a variable parameter among different cows but with high repeatability in the same animal. However, there are no studies reporting of the AFC during pregnancy. Thus, the present study aimed to compare the AFC of Holstein cows at the time of AI and at two momentos of gestation (30 and 60 days) to investigate whether the number of antral follicles is altered during this period. For this, 54 Holstein females (*Bos taurus taurus*) were selected, with an average of 5 years, confined in a free stall system, were submitted to the same handling and feeding conditions. The same technician performed AI of the females, after detection of natural or induced estrus with 25 mg of Dinoprost (Lutalyse®, Zoetis, Brazil) intramuscularly (IM). AFC (antral follicles ≥ 2 mm in diameter) was established using a convex intravaginal transducer of 7.5 mHz (Aquila PRO, Pie Medical, Maastricht, The Netherlands) at the time of AI at 30 and 60 days of pregnancy. Systematically, the same females were evaluated at three different times by the same veterinarian. For statistical analysis, the number of antral follicles was analyzed by repeated measurements employing the generalized linear model. In the presence of a significant effect, the means were compared by the Tukey test. A P-value ≤ 0.05 was used to indicate a significant effect and their interactions. We found that AFC increased during pregnancy, from 55.1 ± 3.1^{c} at AI, to 82.6 ± 4.2^{b} at 30 days, and 115.2 ± 5.1^{a} at 60 days of pregnancy (P = 0.0001). It was possible to conclude that AFC is higher during pregnancy than at AI moment. Thus, we can suggest the initial third of gestation as an alternative to produce embryos due to increased AFC, perhaps to increase the efficiency of OPU / PIV in low AFC animals, since the antral follicle population is quantitatively related to production of embryos.

A214 Embryology, Developmental Biology and Physiology of Reproduction

Intravaginal progesterone treatment suppresses follicular growth in a hormonal protocol for estrus synchronization and fixed-time ovulation in mares

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Previous studies have shown that estrus synchronization protocols in mares have presented satisfactory results, nevertheless, a protocol for fixed-time ovulation induction remains a challenge. Our hypothesis suggested treatment with P4 causes suppression of follicular growth and ovulation induction occurs at 42 ± 6 hours after treatment in at least 75% of mares treated with a combination of GnRH and hCG. This study aimed to develop a hormonal protocol to synchronize estrus and induce ovulation in mares. Four experiments were conducted using 20 non-defined cyclic mares, aged 6 to 14 years. In experiment I, animals were distributed in two groups and progesterone device removal on day 7 and not on day 9 was justified due to greater suppression of follicular growth by the action of P4. Experiment II tested whether the efficiency of hormone treatment depends on the estrus cycle stage of the mare and established the best time for ovulation induction. Ovulation day detection was performed and hormonal treatments were initiated on days 5, 10 or 15 (G1, G2 and G3, respectively) after ovulation, representing different phases of the estrous cycle. The difference in follicular growth rates between the animals was calculated during maintenance of the intravaginal device and after removal until the day before ovulation. Differences in follicular growth rates were similar (P> 0.05) between G1, G2 and G3 and mean values were respectively 1.4; 1.02 and 1.3 mm/day. Most ovulations occurred between days 10 and 13 (62%, 36/58) with only two spontaneous ovulations before predicted (3%, 2/58) and ovulation induction was defined on days 10 and 12. In experiments III and IV, hormone treatment started simulating a field condition. On Day 0, mares received 0.25mg IM of PGF2α (Sincrocio, Animal Ouro Fino Saúde) and 1.44g P4 with the intravaginal device (Inovare Biotecnologia e Saúde Animal). On days 6 and 8, mares received 0.25 mg IM PGF2α. At day 7, the P4 device was removed. On day 10, ovulation was induced with 1000UI of hCG (Chorulon, MSD Agroline) combined with 0.75mg of GnRH (Sincrorrelin, Fine Animal Health Gold) in mares displaying a follicle ≥35mm in diameter. The remaining mares had the same hormonal treatment at day 12, regardless follicular size. This protocol proved to be effective in suppressing follicular growth during the reproductive season; however, follicular atresia did not occur. The efficiency of this protocol was 81.1%, considering the 53 ovulation inductions, 43 (81.1%) occurred between D10 and D12 and only 10 (18.9%) did not ovulate in the predicted period. Further studies should be performed on a larger number of animals to confirm our results. In conclusion, 1.44g of the intravaginal P4 device suppressed follicular growth during the reproductive season.

A215 Embryology, Developmental Biology and Physiology of Reproduction

Effect of downregulating ZSCAN4 transcript on early development and gene expression of bovine embryos

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The Zinc finger and SCAN domain containing 4 (*ZSCAN4*) gene encodes a protein that defies cellular senescence and maintains normal karyotype for many cell divisions in mouse embryonic stem cells, markedly enhancing the generation of induced pluripotent stem cells (iPSCs). ZSCAN4 may play an important role in iPSCs generation through epigenetic regulation, but underlying molecular mechanisms are still unclear. In mice, *Zscan4* is enriched in 2-cell stage embryos, and reduction of transcript produces blastocysts that fail to implant or proliferate in outgrowth culture. While ZSCAN4 plays a potentially important role in mammalian embryo development, its expression and role in the preimplantation stages of bovine embryos is unknown. Therefore, the aims of this study were investigating the expression status and role of ZSCAN4 in bovine preimplantation development. *ZSCAN4* transcript expression levels in unfertilized oocytes and early embryos at 1-cell to blastocyst stages were first evaluated. Then, the effects of ZSCAN4 suppression on developmental competencies were assessed after attempted downregulation by short interfering RNA (siRNA). *In vitro* matured oocytes were inseminated for 6 h, after which some embryos were injected with either 10 pl of 50 μM specific ZSCAN4-siRNA or nonsilencing siRNA (Control-siRNA) duplexes, while some were not injected (Uninjected). Embryos siRNA injected or uninjected were cultured in modified TALP medium by day 7 (IVF=day 0). *HDAC1* transcript levels were measured at the 2-, 4-, 8- and 16-cell stages. The level of *ZSCAN4* mRNA was low throughout the oocyte to 4-cell stage embryos, increased at 8-cell, then significantly decreased at Morula stage of embryos. At the 16-cell stage, gene expression in embryos injected with ZSCAN4-siRNA significantly reduced ($P<0.05$) compared to controls (Control-siRNA injected and Uninjected). No difference in developmental rates for the 2- to 8-cell stages was observed. Although 40.5% of ZSCAN4-siRNA injected embryos developed to the 16-cell stage, 32-cell developmental rate was significantly lower ($P<0.01$) than controls at day 4. Moreover, the day 5 Morula and day 7 blastocyst developmental rates of ZSCAN4-siRNA injected embryos (3.4 and 2.7%, respectively) were significantly lower ($P<0.01$) than those of Uninjected (40.3 and 45.0%, respectively) and Control-siRNA injected embryos (36.9 and 46.0%, respectively). The ZSCAN4 downregulation by specific siRNA injection resulted in a significant increase ($P<0.05$) of *HDAC1* expression at the 4-cell stage, but not in 8- and 16-cell stages. Our results indicate the critical importance of ZSCAN4 for early development of bovine embryos, highlighting it as a pivotal factor for regulation of gene expression through epigenetic modification.

A216 Embryology, Developmental Biology and Physiology of Reproduction

Effect of exposure of bovine cumulus-oocyte complexes to high gaseous pressure on *in vitro* embryo development

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Sublethal stress has been reported as gamete and embryo genomic response inducer, providing cell protection to a subsequent stress. Within such observation, researchers started to experiment sublethal stress as a means to improve bovine *in vitro* embryo production. This experiment aimed to test the effect of the exposure of immature bovine oocytes to high gaseous pressure (HGP – 4000 PSI during 120 min) on *in vitro* embryo development to the blastocyst stage after IVP procedures. A total of 510 bovine cumulus-oocyte complexes (COCs), after four replicates, were used in this study. Ovaries were obtained from a slaughterhouse and were transported to the laboratory in saline solution. Selected grades 1 and 2 COCs were randomly distributed in three groups: HGP, with 172 COCs placed in mPBS+ 0.1% polyvinylpyrrolidone, and then exposed to HGP; Control 1, with 157 COCs maintained in mPBS + 0.4% BSA at RT for 120 min; and Control 2, with 181 COCs used immediately for embryo production, according the our established IVP protocol. In all groups, IVM was carried out during 24 h at 38.5ºC using TCM-199 supplemented medium (25 mM sodium bicarbonate, 0.22 mM sodium pyruvate, 50 µM cysteamine, 5 µg/mL LH, 5 µg/mL FSH, 1 µg/mL estradiol, 0.1 µg/mL EGF) + 10% Fetal Calf Serum (FCS), followed by 20 h IVF into FERT-TALP + 0.6% BSA using frozen-thawed bovine sperm cells segregated by Percoll® gradient, at the $1x10^6$/ml inseminating dose. Then, presumptive zygotes were *in vitro*-cultured in SOFaaci + 5% FCS at 38.5ºC, 5% CO_2, 5% O_2 and 90% N_2, in saturated air humidity. Blastocyst rates were observed on D8 after IVC and compared by the Chi-square test ($P<0.05$). Blastocyst rates were significantly lower ($P = 0.0131$) in the Control 1 Group (11.0%, 16/145) than the Control 2 Group (19.8%, 32/162), with the HGP group being similar to both (18.7%, 25/134). In conclusion, results indicate that exposure of immature COCs to HGP prior to IVM did not affect embryo development to the blastocyst stage, but maintaining COCs at RT for the same exposure time decreased embryo development in comparison to COCs subjected to immediate IVP procedures. Studies are ongoing in our laboratory to further understand the effect of exposure of gametes and embryos to HGP.