A260 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Morphofunctional characteristics of the corpus luteum and early gestation diagnosis in sheep

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The objective was to evaluate the morphological features and blood flow (BF) of the corpus luteum (CL) throughout the estrous cycle and the efficiency of an early pregnancy diagnosis (EPD) of non-pregnant ewe, performed on day 17 with color Doppler ultrasound. In experiment 1, the morphological evaluation of the CL was performed in 34 Dorper x Santa Inês crossbred, which were randomized in groups (G1 - teased and G2 - mated). The estrus cycle was synchronized with intravaginal sponges (60 mg of medroxyprogesterone acetate, Progespon™ - Zoetis, São Paulo, Brazil) insertion on day o (D0). On D7, 400UI of eCG (Novormon™ - Zoetis) and 0.133mg of sodium cloprostenol (Ciosin™ - MSD Animal Health, São Paulo, Brazil) was applied and, the intravaginal sponges were withdrawn two days later on D9. After expected day of ovulation, the overall area and BF area of the CL were daily inspected throughout the 17 days of the estrous cycle by transrectal ultrasonography (-B and –Doppler mode). Blood samples were taken for progesterone (P4) analysis. The data were analyzed using PROC MIXED procedure (SAS 9.3), using T test for group effect and LSD test for day effect and group x day interaction. Probabilities less than 5% were considered significant. For experiment 2, early pregnancy diagnosis (EPD) was performed on day 17 of the estrous cycle in 62 sheep, and pregnant was designed for ewe with BF area detected in at least one CL. Non-pregnant ewe was designed for animals with no apparent BF area. On day 30 of the estrous cycle (D30), the pregnancy diagnosis (DG30) was confirmed by B-mode ultrasonography. Considering the DG30 a Gold pattern, indexes for sensitivity (SEN), specificity (SPE), positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated for the EPD performed on day 17. On experiment 1, The CL area increased to D5 ($P < 0.0001$), with no significant growth on subsequent days, this same pattern was similar between groups. However, a drop on CL area, starting on D13 ($P < 0.0001$) was observed only in G1. The same pattern was observed for the BF area in both groups. A drop on plasma concentrations of P4 starting on D12 ($P < 0.0001$) was also observed only in G1. On experiment 2, the EPD had shown a SEN of 100.0%, SPE of 80.5%, VPN 100.0%, PPV of 73.3% and ACC of 87.1%. There was a low false positive diagnostic (12.9%, 8/62), caused by long cycles and early embryonic losses, and no false negative diagnostic was observed. The Doppler technology is a potential tool to access luteal function and for the early diagnosis of pregnancy in sheep.

Support: Fapemig, Capes and CNPq.

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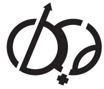

A261 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of thermal stress on the maturation of bovine oocytes, *in vitro* embryo production and expression of genes related to stress response

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The sirtuins are members of the histone deacetylase (HDAC) family and are involved in the regulation of transcription activation and apoptosis. In this regard, the aim of this study was to investigate the influence of different temperatures during *in vitro* maturation (IVM) on the developmental competence of bovine oocyte, as well as mRNA expression of sirtuins. Cumulus-oocyte complexes (COCs) from Simental cows were stained with Brilliant Cresilblue (BCB) and categorized as BCB+ or BCB-. Soon after, they were matured *in vitro* at different temperatures (37°C, 38.5°C and 40°C). After IVM, the oocytes were denuded and evaluated for the extrusion of the first polar body, and the cumulus cells were stored for mRNA expression analyses. Next, *in vitro* embryo production (IVEP) was carried out with the oocytes. Real-time PCR of oocytes and cumulus cells was performed to determine mRNA expression of sirtuins. For parametric data, ANOVA was applied and when a significant difference was found, the Tukey test with 5% probability was performed. For non-parametric data, the Kruskal Wallis statistical test was performed with 5% probability (software R). The different maturation temperatures did not significantly influence the maturation rate and the oocyte cleavage rate. However, COCs matured at 38.5°C (control) had a higher rate of blastocysts (37%), in contrast to those at matured at 37°C and 40°C (33.2% and 21.5%, respectively). At all different temperatures, the rates of blastocysts were higher for BCB+ oocytes than for BCB- oocytes. In the BCB+ oocytes the mRNA expression of SIRT1, SIRT2, SIRT3 and SIRT5 were higher after maturation than in immature oocytes, but no difference was observed in BCB- oocytes. In addition, the maturation temperature proved to have an effect on the expression of SIRT1, SIRT2, SIRT3 and SIRT5 mRNAs in BCB+ oocytes, with the control group been superior to all other treatments. In cumulus cells, only the mRNA expression of the SIRT2 gene had no effect on the different temperatures in the BCB+ oocytes, whereas the expression of the mRNA of the SIRT1 gene presented higher expression at the temperature of 38.5°C. The mRNA expression of the SIRT 3 and 5 was affected by the temperature of 40°C. Cumulus cells from BCB- oocytes had an effect only on the expression of SIRT3 mRNA, which was higher at 40°C. BCB+ oocytes are more resistant to different temperatures than BCB- oocytes. In addition, the IVM temperature influences the mRNA expression of SIRT 1, 2, 3 and 5, which are important for cell protection. The most significant effects were observed in the hottest temperature (40 °C). Therefore, findings from this study indicate that low quality oocytes, according to the BCB selection, are more susceptible to detrimental effects of unideal IVM temperature, which causes irreversible effects on the oocytes and decreases production of blastocysts.

A262 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Epigenetic mapping of *in vitro* cultured fibroblasts: How artificial environment influences the bovine epigenome

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Artificial culture systems require well-controlled conditions to mimic natural environment for cell culture. Components such as fetal bovine serum (FBS) and oxygen tension are essential to establish the optimal condition. Albeit broadly adopted in human and animal cultures, artificial environment has been related to incidence of epigenetic disorders. Components present in FBS were identified as barrier to the epigenetic reprogramming. In malignant cells, O2 concentration seems to be critical for epigenetic regulation, due to the action of demethylases enzymes. These enzymes are dioxygenases involved in the epigenetic control and hypoxia-inducible factor regulation. Nevertheless, little is known about the impact of those environmental factors on the bovine epigenome. In this project, we aimed to use bovine fibroblasts as a model to investigate how artificial environmental factors as serum starvation, cellular confluence, and oxygen tension impact the bovine epigenome. In order to answer it, this project was divided in two experiments. In the first one, our goal was to investigate the influence of growth arrest upon repressive chromatin marks. To this end, fibroblasts were cultured: under regular condition (10% FBS), serum starvation (0.5% FBS) and until reach confluence. After treatment, we analyzed the changes on the histone marks H3K9me2, H3K9me3, and H3K27me3 by immunostaining and ChIP-Seq. Both treatment showed a global reduction in the epigenetic marks levels compared to the control group. Regarding ChIP-Seq, our preliminary bioinformatics analysis revealed substantial changes in the chromatin patterns caused by both treatments. Annotated Peak analysis showed 446, 305, and 505 exclusive genes for H3K9me2; 319, 448, and 275 for H3K9me3; and 1028, 565, and 2154 for H3K27me3 in control, starvation, and confluence group, respectively. Peak intensity analysis showed different patterns on *Transcription Start Sites* associated with the groups and treatments. Additionally, enrichment analysis of biological process showed 134 relevant pathways, where 20 genes were enriched with the three marks, when starvation and confluence were compared to control. In the second one, our goal was to investigate the influence of O2 tension on the epigenetic "erasers" expression. For this, bovine fibroblasts were cultured in low (5%) and high (20%) O2 tension, and collected at different passages for gene expression analysis of *HIF1A, VEGF, KDM3A, KDM4A, KDM4C,* and *TET1*. Among them, only VEGF was differently expressed at the third passage. Analysis of samples in different passages are underway to verify the long-term influence of oxygen tension. Whereas preliminary, these results demonstrate the major influence of artificial environmental factors on the bovine epigenome, and suggests that bovine fibroblasts could be a suitable model to study epigenetic modifications caused by *in vitro* conditions.
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A263 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Can Doppler velocimetric values of dog's testicular artery change according to the measured region?

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Testicular tissue maintenance is performed by the testicular artery, and its hemodynamics can be evaluated using Doppler ultrasonography. The present study aimed to characterize the peak systolic velocity (PSV), diastolic velocity (EDV), the pulsatility index (PI) and resistivity index (RI) of five regions of the testicular artery, three previously described (distal supratesticular, marginal and intratesticular) and two not yet presented in the literature (proximal and middle supratesticular). Twenty-two medium size dogs were used, with an average weight of 11.6 ± 5.5 kg, the PSV, EDV, PI, and RI of the testicular artery were measured in the proximal, middle and distal supratesticular, marginal and intratesticular regions. For regions evaluation the pampiniform plexus was located and the probe was moved cranially to the proximal portion of the inguinal ring (proximal supratesticular region). The portion between the proximal and caudal supratesticular region was characterized by middle supratesticular. The variables were compared between the regions using the Kruskal-Wallis test. Mann-Whitney test was used to compare the regions between the left and right testicles, using SigmaPlot 11.0 software and a significance level of <5%. There was no difference in hemodynamics measured by Doppler between the left and right testicles. The medians for PSV (cm/s), EDV (cm/s), PI and RI in the five regions were respectively: proximal supratesticular region (23.1, 3.7, 2.1 and 0.8); middle supratesticular (17.2, 4.5, 1.5, 0.7); distal supratesticular (12.2, 5.7, 0.8, 0.5); marginal (11.3, 6.5, 0.5, 0.4); and intratesticular (5.7, 3.5, 0.5, 0.4). There was a difference between the PSV of the middle and distal supratesticular regions. There was a difference of PSV, EDV, PI, and IR between the distal supratesticular, marginal and intratesticular regions. Measurements of the PSV, EDV, PI, and RI of the testicular artery of dogs in the five proposed regions showed different results, related to the hemodynamic and morphological differences of the artery during its course in the spermatic cord and the testicles. To the authors' knowledge, this is the first description of Doppler velocimetry of the testicular artery of dogs in the proximal and middle supratesticular regions. Due there is a difference in Doppler values according to the measured region, there is a need to identify the region in Doppler velocimetric evaluations of the testicular artery of dogs.

A264 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Sodium Dodecyl Sulfate on ram sperm cryopreservation

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The addition of Sodium Lauryl Sulfate (SLS), a commercial product, although at an unknown concentration, improves ram sperm quality after thawing. As an alternative, some studies tested the detergent Sodium Dodecyl Sulfate (SDS) as an additive. However, no ideal concentration is defined. This study aimed to determine the best SDS concentration as an additive to Tris-Egg Yolk-Glycerol (TEYG) extender for ram semen. Ejaculates of four adult rams with progressive motility (PM) ≥ 70% were pooled (n = 5), including the same spermatozoa concentration from all rams. The semen was diluted in TEYG (2 x 10^9cells/mL) and split in five groups: control without detergent (CON-); control with of SLS (CON+, 0.5% Equex®); and groups with 0.017% (G1), 0.05% (G2); and 0.15% (G3) of SDS. Diluted semen was packed in 0.25 ml straws, which were cooled at 0.5°C/min to 5°C and kept at this temperature for 90min. Straws were kept at 4 cm of distance from liquid nitrogen for 10 min. Thereafter, straws were submerged in liquid nitrogen and stored until evaluation. Paired samples were thawed and incubated at 37°C. The PM was determined by a Computer Assisted Semen Analysis (CASA, Sperm Vision®), considering four post thawing incubation periods: 10min (T0); 60min (T1); 180min (T2); and 300min (T3). Sperm membrane and acrosome integrity were determined at T0 by flow cytometry (Attune®). The responses were analyzed by ANOVA, with comparisons of means by the Tukey test. At T0, PM was greater (P <0.05) in groups including SDS (60.5%) than in CON- and CON+ (47.3%), which had similar PM (P>0.05). At T1, PM was generally similar between groups, although G3 presented greater PM mean than CON- (P <0.05). At T2, G1 presented similar PM to G2 (39.3%), but greater (P <0.05) than the other groups (30.6%). At T3, PM for G2 (33.8%) was greater (P <0.05) than for G1, CON- and CON+ (24.0%). Spermatozoa from CON+ presented greater acrosome integrity (73% - P <0.05) than CON- (54%), but no other differences were observed among groups. Generally, sperm frozen with SDS presented greater *in vitro* viability compared to the controls. As there was lower PM reduction between T0-T3, we conclude that SDS should be used at 0.05%. The results should be tested *in vivo* to confirm the feasibility of using 0.05% SDS as an additive in ram sperm freezing.

A265 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

PTGS2 expression in cumulus cells is a potential biomarker of oocyte quality independently of patient's clinical variables

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Gene expression in Cumulus cells (CCs) have previously been suggested as a predictive tool for oocyte quality in several studies. Still, there is little consensus about which biomarkers would actually be clinically efficient and applicable (Fragouli, Human Reproduction Update, 20:1-11, 2014). We analyzed CCs gene expression data considering the patient's clinical characteristics and the oocyte's potential, aiming to identify possible biomarkers of oocyte competence independently of patient's clinical characteristics. Pooled CCs samples were obtained from 29 patients submitted to ICSI procedure. The oocytes corresponding to the samples were accompanied until day 5 after ICSI, and samples were divided in Good Quality group (GQ) (n=11) and Poor Quality group (PQ) (n=18) accordingly to blastocyst formation. Each sample was submitted to reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) on StepOnePlus™ (Applied Biosystems, USA). Oligonucleotides were selected to be complementary to the human sequence of Anexin 1 (ANXA1), Prostaglandin-endoperoxide synthase 2 (PTGS2), Glutathione Peroxidase 4 (GPX4) and Glutathione-S-Transferase 1 (GST1). The relative expression was calculated by the 2-ΔΔCT method (Livak and Schmittgen, Methods, 25:402–408, 2001). Patient's clinical data (infertility diagnosis, BMI, age and stimulation protocols) and experimental data were combined using mice package (Buuren and Groothuis-Oudshoorn, Journal of Statistical Software, 45, 2011) in a multiple regression model built using the percent of blastocysts as dependent variable and clinical data as independent variables. Test models composed of clinical variables and each assay data were compared against the baseline model. All procedures and computations were performed in R statistical environment. Differences within the experimental groups were determined by Mann-Whitney test (GraphPad® Software 5.0). When submitted through Mann-Whitney test, only ANXA1 expression shown to be significantly different between GQ and PQ groups ($P<0.05$), being elevated in GQ group. Gene expression results were then submitted to linear regression analysis, which indicated that ANXA1 expression levels were not a potential predictor of oocyte quality when we considered clinical information. Contrarily, PTGS2 expression levels were overexpressed in GQ group ($P<0.05$), and this significance was independent of the clinical variables of each patient. As far as we are aware, this is the first study suggesting biomarkers found in CCs that predicts blastocyst formation potential regardless of patients age, diagnosis, BMI and stimulation protocol.

A266 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Transcriptional profiling of embryo cryosurvival

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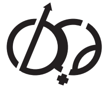
Embryo cryosurvival is a complex process involving dynamic structural and molecular changes. The aim of this work was to evaluate the transcriptional profile of bovine embryos with high and low cryosurvival. Immature oocytes (n=3926) were *in vitro* matured, fertilized and culture under standard conditions. Cleavage and embryo production were recorded on day 3 and 7 after fertilization, respectively. Grade I expanded blastocysts (n=894) were vitrified by the cryotop method. Vitrified blastocysts were warmed and embryo re-expansion and hatching rates were recorded at 6 and 12 h after warming, respectively. A rigorous morphological evaluation was done by experienced technician and embryos were classified as high cryosurvival (HC: blastocysts with the blastocoel completely re-expanded followed by hatching 12 h after warming and without or with mild signs of degeneration), and low cryosurvival (LC: blastocysts with poorly re-expanded blastocoel not followed by hatching 12 h after warming and with severe signs of degeneration) after cryopreservation. HC and LC extracted RNA (PicoPure®) samples (123 blastocysts/group; pool of 41 blastocysts per replicate; n=3) with RNA integrity ≥7.7 were submitted to library preparation and RNAseq (Illumina®). Sequencing data were cleaned, mapped against the *Bos taurus* genome, reads counted, and differential expression analysis was performed (edgeR). For gene enrichment analysis, Ingenuity Pathway Analysis (IPA) was used. An independent set of blastocysts (25 blastocysts/group; pool of 5 blastocysts per replicate; n=5) was evaluated on microfluidic 96.96 dynamic array (Biomark®) qPCR analysis. Cleavage and blastocyst production rates were 73.2% (2873/3926) and 29.0% (1137/3926), respectively. The re-expansion (89.2 *vs*. 73.4%) and hatching (23.7 *vs*. 8.4%) rates were higher (P <0.05) at 12h compared with 6h after warming, respectively. A mean of 17.8 million of reads per sample were uniquely mapped. Blastocyst samples were clearly separated with pronounced group individualization at two- and three-dimensional principal component analysis and heat clustering mapping sustaining the phenotype characterization (high *vs*. low cryosurvival). A total of 9422 genes were identified, 114 were differently expressed genes (p-adjusted <0.05), with 27 and 84 genes up-regulated in HC and LC, respectively. Among the top five biological functions identified (p-adjusted <0.1), organismal survival, cell death and survival, cellular growth and proliferation were predicted to be activated (z-score ≥+2) whereas cellular movement and cell-to-cell signaling were predicted to be inhibited (z-score ≤-2) at HC. At qPCR, 81.5% (65/96) of the genes presented the same transcription pattern of RNAseq. Therefore, the present work provided a comprehensive analysis of the transcriptional profile of bovine embryo cryosurvival and elucidated the involvement of noble biological processes in the embryo development resumption after cryopreservation.



A267 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Sex effect in gene expression of *in vitro*-produced bovine embryos vitrified by cryotop

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Male and female embryos are known to be different in terms of developmental kinectics, metabolism, gene expression and epigenetic patterns, as well as several stress conditions resistance. Consequently, it can be assumed that the response to cryopreservation may also be different between male and female embryos. However, there are no reports in literature evaluating the effect of gender on the response of bovine embryos to vitrification. In this study, the expression of eight genes related to apoptosis and cell damage (FOSL1, HSPB1, CASP3 and CASP8), thermal stress (HSPA5 and HSPA1A) and glucose metabolism (G6PD and PGK1) in IVP bovine embryos were analysed, by qPCR, aiming to determine the difference between gender on the response to cryopreservation. Male and female cryopreserved bovine embryos oocytes obtained from slaughterhouse ovaries were used, then were submitted to 24 hour IVM, inseminated with previously tested bull semen and presumptive zygotes were transferred to *in vitro* culture (IVC) medium, where they remained for 7 days. Cleavage on D2 and blastocysts rates on D6 and D7 were evaluated. On D7, expanded blastocyst embryos were removed from IVC and distributed into two treatments: control (C) and vitrified (V) by *Cryotop* (Cryo-Ingá: Ingamed®, Maringá, Brazil) method. After the warming process, embryos from C and V groups returned to IVC conditions for additional 24 hours. Then, hatched blastocysts were stored individually in DM-PBS solution at -80 ° C for sex determination. Each embryo was submitted to a DNA and RNA extraction process simultaneously using the AllPrep DNA/RNA Mini Kit (Qiagen®, Hilden, Germany). The extracted DNA was used for embryo sex determination, which was performed by PCR and confirmed in 1.5% agarose gel. Embryos were pooled in number of 20 according to the sex into 3 pools of male embryos C and V and 3 pools of female embryos C and V. These pools were used for gene expression quantification by qPCR using Sybr Green FAST Master Mix. ACTB and GAPDH were used as endogenous controls genes. Data were analyzed by t-test, considering $P \leq 0.05$. Among analyzed genes, female and male embryos differed between them in V treatment for HSPA1A ($P = 0.0043$), CASP3 ($P = 0.0037$) and G6PD ($P = 0.0071$) genes and in C group for G6PD ($P = 0.0526$) gene. Results indicate that gender did not affect cryopreservation response, because there was no difference between treatments. Therefore, it was evident that male and female bovine embryos are different, despite being submitted to cryopreservation process or not, and those differences are sex-related, because female embryos showed higher abundance regarding to gene expression compared to the male counterparts.

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A268 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and “omics”

Luteal B-Mode ultrasonography used for early pregnancy diagnosis in goats

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Luteal analysis by ultrasonography (US) was previously demonstrated in cows and sheep as an early and accurate pregnancy diagnosis. This study aimed to establish the best day for early pregnancy diagnosis in dairy goats, using luteal morphological assessment by B-Mode US. Saanen does (n=131) about 2.0±0.5 years old were used. At the first study, after a hormonal protocol for induction of synchronous estrus and artificial insemination, 60 animals were accompanied from Day 15 to Day 23 of the estrous cycle (Day 1 or D1 = ovulation day), by a subjective and scored B-Mode US assessment using a portable equipment (Sonoscape S6, Shenzhen, China) with a 7.5 MHz linear rectal transducer adapted to be used in small ruminants. Luteal morphology score 1 (near anechoic) meant no pregnancy and, score 2 (heterogeneous and hypoechoic) and 3 (homogeneous hyperechoic) meant positive pregnancy. At the second study, 71 does passed through the same protocol and had the ultrasound exam performed at Day 23 (the best day detected by the first study) for the luteal morphological assessment. In both studies, B-Mode uterine US at D30 confirmed pregnancy diagnosis (Gold standard). The performance of the subjective luteal morphology analysis and its agreement with the gold standard outcome in both studies was classified calculating Sensitivity (SEN), Specificity (SPEC), Positive Predictive Value (PPV), Negative Predictive Value (NPV), and Kappa index (κ). In study 1, pregnancy diagnosis by subjective luteal assessment by B-Mode US was not feasible at D15 and D16 (SEN 100%; SPEC 0%; PPV 49%; NPV was incalculable, and κ = 0) as all Corpora Lutea were considered viable (score above 2) and consequently all animals were diagnosed as pregnant. From D17 to D23, the overall performance of the technique progressively increased. D17: SEN 100%; SPEC 4%; PPV 50%; and κ = 0.04; D18: SEN 100%; SPEC 8%; PPV 51%; and κ = 0.08; D19: SEN 100%; SPEC 27%; PPV 56%; and κ = 0.27; D20: SEN 100%; SPEC 50%; PPV 66%; and κ = 0.50; D21: SEN 100%; SPEC 73%; PPV 78%; and κ = 0.73; D22: SEN 100%; SPEC 81%; PPV 83%; and κ = 0.80; D23: SEN 100%; SPEC 92%; PPV 93%; and κ = 0.92; also NPV 100% from D17 to D23. Besides, two animals diagnosed as non-pregnant on Day 30 CL scored as 2 until Day 23. In study 2, the assessment presented a similar pattern of sensibility and specificity observed in study 1 (SEN 100%; SPEC 95%; PPV 94%; NPV 100%, and κ = 0.94). The results showed that subjective luteal morphological assessment by B-Mode US is a reliable tool for early pregnancy diagnosis in goats and can be efficiently be used as early as 23 days post-breading.

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A269 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Impact of the implementation of cryopreservation technologies on the destination of bovine embryos through time in a commercial company

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In Vitro Brasil Ltda. (IVB), a Brazilian company that leads the worldwide market for *in vitro* production of bovine embryos, introduced embryo cryopreservation techniques (vitrification and slow freezing, marketed as Direct Transfer - DT) in the middle of 2014, however, both of them were applied on a large scale only in 2015. The aim of this study was to evaluate the impact of the introduction of new technologies of cryopreservation on the commercial destination of bovine embryos, as well on pregnancy rates obtained from cryopreserved embryos. Data were collected from the company's own laboratories in Brazil. The proportion of embryos designated for both cryopreservation techniques represented 22.94% (21,483/93,660), 45.18% (68,475/151,577), 50.97% (60,399/118,503) and 58.10% (83,654/143,986) of the total number of embryos produced in 2014, 2015, 2016 and 2017, respectively. In addition, because of improvements made to the cryopreservation techniques over the time, there was an inversion in the destination of embryos and more than 50% of the total produced embryos were cryopreserved. Due to this inversion, we decided to verify the possible impact of the cryopreservation techniques on pregnancy rates for embryos transferred fresh (ET), frozen (DT) or vitrified (VIT). Data normality was confirmed with the Lilliefors test ($p = 0.87$), and data analyzes performed by two-way unbalanced ANOVA with interactions. There were no significant differences between cryopreservation techniques on pregnancy rates fresh embryo transfer ($n = 38.163$) 46.39%, slow freezing ($n = 2.826$) 35.60% and vitrification ($n = 20.143$) 44.95% ($p = 0.083$, $SD = 0.069$). In addition, there was no significant difference ($p = 0.37$) between the three techniques when compared in embryos from each of the breeds used for data collection Gyr [45.63% (1,734/3,801), 33.10% (48/145), 36.75% (61/166)], Girolando [41.76% (2,586/6,192), 39.39% (156/396), 43.60% (825/1,892)], Holstein [42.16% (1,081/2,564), 40% (2/5), 36.65% (188/513)], Nelore [49.32% (6,168/12,506), 33.95% (721/2,124), 41.21% (1,669/4,050)] and Senepol [46.84% (6.136/13.100), 50.99% (77/151), 46.67% (6.311/13.522)], ET, DT and VIT, respectively. In conclusion, our results demonstrate that the large-scale use of well-consolidated cryopreservation techniques does not affect pregnancy rates obtained over time compared to fresh embryos transfer. This result could be explained by medium and technical improvements made by the company in the cryopreservation technology, both of them are company's privacy.

A270 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and “omics”

Influence of type-B natriuretic peptide (NPPB) on the lipid content of *in vitro* matured bovine oocytes

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Although *in vitro* produced embryos (IVP) are widely used in research and for commercial purposes in Brazil and the world, these embryos are still inferior in quality compared to those produced *in vivo*. One of the factors that contributes to this is the greater accumulation of lipids, rendering such embryos more sensitive to cryopreservation. Techniques that can cause modifications in the oocyte so that they become blastocysts with greater capacity of cryopreservation are required by professionals who apply this biotechnology in the field. Previous studies have shown that cGMP signaling pathway may be involved in the lipid metabolism of cumulus-oocyte complexes (COCs). The synthesis of cGMP by activation of membrane (mGC) or soluble (sGC) guanylate cyclases. mGC, also called natriuretic peptide receptors (NPR1 and 2), are activated by natriuretic peptides (NPs), types A, B and C (NPPA, NPPB and NPPC). The objective of this study was to verify the influence of different concentrations of NPPB to stimulate the synthesis of cGMP by NPR1 (mGC), on the lipid content of *in vitro* matured bovine COCs. Pools of 25 COCs were submitted to *in vitro* maturation (IVM) in TCM199 with 0.2 mM sodium pyruvate, 10 µg/ml gentamicin, 0.5 µg/ml FSH, 10% fetal bovine serum (FBS) and NPPB (10^{-8}, 10^{-7} and 10^{-6} M). Controls were cultured without NPPB. After 24 h IVM, the cumulus cells were removed and the denuded oocytes (DO) were fixed and permeabilized in 4% paraformaldehyde (PFD) + 0.5% Triton-X100 for 20 minutes and stained with 10µg/ml Hoechst 33342 for 15 min and 1µg/ml Nile Red for 30 min. Then, stained DO were transferred to 13µl Pro Long between a glass slide and a coverslip and analyzed by epifluorescence microscopy to evaluate the nuclear maturation (emission 445-450nm and excitation 475-490nm) and lipid content (emission 590nm and excitation 516-560nm). The images had their fluorescence intensities (FI) measured by ImageJ software. Data for the 4 replicates/group were tested for normality of results and homogeneity of variance and then submitted to statistical analysis by ANOVA followed by Tukey test (GraphPad Prism software) with a significance level of 5%. Nuclear maturation (MII) was not influenced by any of NPPB concentrations, ranging 82 to 87% MII for all groups ($P>0.05$). The groups treated with NPPB presented lower lipid content in relation to the control (0.64 FI/µm^2, $P<0.05$), but did not differ among them ($P>0.05$). The treated groups presented 0.29, 0.28; and 0.34 FI/µm^2 for 10^{-8}, 10^{-7}, and 10^{-6} M NPPB, respectively. In conclusion, stimulation of cGMP synthesis by NPPB during *in vitro* maturation decreased lipid contents in bovine oocytes, showing the relationship of this pathway with lipid metabolism and the lowest concentration (10^{-8} M) was sufficient to cause such an effect without interfering with nuclear maturation.

A271 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

How does uterine hemodynamics respond to a short-time synchronization protocol in ewes?

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Moment and side effects on estimates of uterine hemodynamics in ewes were investigated during a short-time ovulation synchronization protocol. Twenty-eight Santa Inês ewes, were fed on *Panicum maximum cv. Aruana* grass and supplemented with concentrate 280g/ewe/day (soybean meal and ground corn, crude protein of 14.3%) were synchronized with a protocol vaginal device impregnated with 60 mg of medroxyprogesterone (MAP Progespon®, Intervet/Schering-Plough) and intramuscular (IM) injection of 37,5 µg cloprostenol (Prolise®, Tecnopec, Brazil) on a random day of the estrous cycle, considered as day zero (D0). On day 6 (D6) of the protocol, in the morning, vaginal devices were removed and after 24 h (D7), the ewes received 25 µg of lecirelin (GnRH analogue) (Gestran Plus®-Tecnopec). Doppler velocimetry of both uterine arteries was performed by transrectal ultrasonography on D0, D2, D4 and morning on D6 (every 48 h) and from D6 to D8 every 12 h (7:00 am and 7:00 pm). The same operator did all the ultrasound evaluations. We analyze the peak velocity of systole (PVS) (cm/s), end velocity of diastole (ED) (cm/s), time-averaged maximum and median velocity (TAMAX, TAMEAN cm/s), pulsatility index (PI), resistance index (RI), systolic/diastolic ratio (S/D), blood flow volume (BFV) (ml/min) and arterial diameter (AD) (mm). Statistical analyses were performed using the MIXED procedure of SAS software and significant difference was considered when $P<0.05$. Except for TAMAX and PVS, there were differences between the time of P4 insertion and the estimated time for ovulation ($P<0.05$). There were increases in ED (11.29 x 13.25 cm/s) and TAMEAN (7.93 x 9.87 cm/s) ($P<0.05$). The PI and RI were different when comparing the moment of insertion and withdrawal of progesterone device (PI: 2.53 - 1.54 and RI: 0.76 - 0.68) ($P<0.05$). The AD and BFV initially decreased and then increased in the estimates (minimum of 2.26 mm and 25.60 ml/min and maximum of 3.04 mm and 53.38 ml/min, respectively) ($P<0.05$). The TAMAX tended to increase during the protocol ($P = 0.11$). The AD was higher on the left side (2.82 mm) while PS, ED, TAMAX, TAMEAN were higher on the right side. The situation that was analyzed in the study exhibits important contrasts regarding hormonal prevalence in the uterus, specifically the evidence of hemodynamics patterns associated with responds and results post-insemination. In the same way, if the reference values are established, discards can be performed based on hemodynamic abnormalities.

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A272 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Heterotopic ovarian tissue allotransplantation in caprine

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Transplantation of ovarian tissue is a viable way to preserve and/or restore the fertility of women subjected to cancer treatment. Therefore, studies that evaluate the feasibility of grafting sites and their effects on function of ovarian tissue fragments are of great interest. Thus, the aim of this study was to evaluate the effects of intramuscular (IM) and subcutaneous (SC) sites on follicular morphology, class distribution, follicular density, and stromal cell density. Goat ovaries (n=10 pairs) were fragmented and randomly assigned to fresh control (processed immediately) or allograft groups, which were grafted for 7 (SC7 and IM7) or 15 days (SC15 and IM15). As hosts for allografting, goats (n=10) with age between 24 and 36 months were used. During the transplantation period, color Doppler sonographic parameters (number of pixels and perimeter of the perfusion areas adjacent to the graft) were recorded. After 7 or 15 days, the grafts were recovered and histologically processed. All statistical analyses were conducted using Sigma Plot software version 11. Data are presented as mean±SEM and percentage. Statistical significance was defined as $P<0.05$, but P-values >0.05 and ≤ 0.1 indicate differences tending to significance. Doppler sonographic parameters (number of pixels and perimeter of perfusion areas) were positively correlated with the proportion of normal follicles and negatively associated with the percentage of primordial follicles ($P\leq 0.1$). A total of 8,164 preantral follicles were assessed in 16,392 histological sections evaluated. Data showed a decrease ($P<0.05$) of follicular density (number of preantral follicles/cm2) in all grafted groups (IM7, 2.9±0.2; SC7, 21.2±1.2; IM15, 0.05±0.01; SC15, 0.03±0.01) compared to the control (96.1±11.5). At Day 7, the SC site showed a higher follicular density in comparison with IM site. However, a reduction ($P<0.05$) of follicular density after 15 days was observed, regardless of transplantation site. The proportion of developing follicles increased ($P<0.05$) and the mean stromal cell density (number of cell/2500 µm2) decreased ($P<0.05$) in all grafted groups and were similar ($P>0.05$) between transplantation sites within the same period. A positive correlation ($R2=0.10$; $r=0.32$; $P<0.01$) of stromal cell density with the proportion of normal follicles was observed by linear regression analysis. Also, a negative association ($r=-0.65$; $P<0.01$) between stromal cell density and days post-transplantation was verified. In summary, both SC and IM sites promoted similar follicular growth after transplantation with a greater follicular density being maintained in the SC group at 7 days post-transplantation. Moreover, a close association between stromal cell density and maintenance of the follicular viability was observed and could be considered as a quality marker in ovarian fragments subjected to transplantation. Finally, Doppler sonographic may be used to monitoring the function of grafted ovarian tissues.

A273 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

GPX4 expression levels in cumulus cells as a possible implantation potential predictor

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Introduction: Embryo selection is a major challenge in assisted reproduction techniques. Cumulus Oophorus cells (CCs) surround the oocyte and have great participation in gamete maturation and support processes. These cells are separated from the oocytes and routinely discarded in assisted reproduction laboratories, allowing intracitoplasmic sperm injection to generate embryos that are transferred to the maternal uterus. Knowing that CCs protect the oocyte from oxidative stress, our group analyzed the gene expression and activity of the enzyme glutathione peroxidase (GPx) in human CCs as a potential biomarker of oocyte quality and correlated the observed results with the implantation potential of the embryos. GPX4 mRNA encodes isoform 4 of GPx protein that acts preferably reducing lipid hydroperoxides and protects cells against membrane lipid peroxidation and cell death. During maturation from germinal vesicle to MII state, oocytes do not produce mRNAs and therefore depend directly of stored mRNAs and CCs mRNAs, which are transferred through GAP junctions. It is possible that oocytes protected by CCs with better defense mechanisms against oxidative stress during oocyte maturation present greater chances of becoming a good quality oocyte. Objectives: To analyze and identify possible correlations of GPX4 mRNA expression levels and GPx enzyme activity with oocyte quality and embryo potential. Material and Methods: 74 CCs samples were analyzed for GPx enzyme activity. Of these, 14 were analyzed for *GPX4* isoform expression by the quantitative rt-PCR technique. Samples were divided accordingly to the result of the corresponding embryo transfer and the determination of b-HCG hormone levels in the patient's blood, indicating if the embryo implantation after transfer was successful. Data were analyzed by one-way ANOVA and regression models. Results: *GPX4* was overexpressed in Positive pregnancy group (P=0.010541996), and this significance is independent of the clinical variables of each patient. On the other hand, activity levels of GPx enzyme were not significantly different between the two groups. For validation, this result needs to be tested in a larger cohort, analyzing individually collected CCs samples from patients submitted to single embryo transfer. Conclusion: CCs gene expression is a reflection of the follicular microenvironment which they are part of and that also contains the oocyte. However, mRNA expression levels do not necessarily reflect the corresponding protein levels, or even the activity of the enzyme in question. In this study, we observed that *GPX4* gene expression level, but not the enzyme's activity, is a potential biomarker of oocyte quality. Our study revealed that *GPX4* mRNA expression levels are related to embryo implantation potential, and therefore is a promising candidate to identify good quality oocytes independently of patient's clinical profile.

A274 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Association of polymorphisms in the promoter region of paraoxonase 1 (PON1) gene with reproductive performance, health and milk production of Holstein cows

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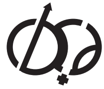
The aim of this study was to evaluate the association of single nucleotide polymorphisms (SNPs) in the paraoxonase 1 (PON1) promoter region with plasma PON1 activity, fertility, energy status, feed intake, occurrence of peripartum diseases and milk production of Holstein dairy cows. Eighty-four Holstein cows were used in this study. Cows were pre-synchronized with two injections of prostaglandin F2 alfa followed by timed AI after an Ovsynch program. The pregnancy was confirmed after rectal palpation and reproductive performance data was recorded until 210 DIM. Blood samples were collected weekly before calving, twice a week in the first two weeks of lactation and once a week thereafter for Beta-hidroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and plasma PON1 activity analysis. Daily dietary intake of each cow was measured from 40 days prepartum up to 60 days in milk (DIM) and clinical data and milk production were evaluated up to 60 DIM. DNA was extracted from the whole blood samples for the PCR reaction and a fragment of 828 bp from the PON1 gene promoter was sent for sequencing. Also, the SNP -221 genotyping was validated by amplification refractory mutation system (ARMS-PCR) and restriction fragment length polymorphism (RFLP) using the*Bsl*I enzyme. Analyses involving repeated measures over time (e.g., plasma PON1 activity, NEFA, BHBA, milk production) were compared by analysis of variance for repeated measures using the MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA). In addition, the average PON1 activity was calculated and along with the CCI and feed intake were evaluated using polynomial models for the linear or quadratic effects. Pregnancy rates were evaluated by Kaplan-Meier survival analysis. Seven SNPs were identified in the promoter region of the PON1 gene, located at positions -22, -105, -176, -221, -392, -611 and -676, considering 1 as the first nucleotide of PON1 gene first exon, and six of them were associated with plasma PON1 activity. The SNPs -221 and -392 were associated with the calving to conception interval (CCI, P<0.05), and the genotypes associated with higher serum PON1 activity were also associated with shorter CCI. There was no association of the SNPs on energy status, feed intake, diseases and milk production (P>0.05). It was possible to identify the three SNP-221 genotypes by ARMS-PCR and by digestion with the *Bsl*Ienzyme. Therefore, the SNPs -105, -176, -221, -392, -611 and -676 were associated with serum PON1 activity. In conclusion, the genotypes associated with higher serum PON1 activity for the SNPs -221 and -392 were also associated with shorter CCI, which may serve as novel genetic markers for improved fertility in dairy cows.



A275 Support Biotechnologies: Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of melatonin in culture medium and blastocoel collapse on viability of vitrified bovine embryos

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Melatonin treatment and blastocoel collapse (BC) had been suggested as potential alternatives to enhance embryo development and viability after cryopreservation of bovine embryos. We investigated the effects of BC before vitrification of embryos produced *in vitro* in the presence of melatonin. The re-expansion rates after 2 and 24 hours, and hatching rates after 24, 48 and 72 hours from heating were evaluated in three treatments: 1- Control (embryos produced without melatonin and vitrified), 2- CIV+M (embryos cultured with 10^{-9}M melatonin and vitrified), 3- CIV + M10^{-9} (removal of the blastocoel fluid from embryos cultured with 10^{-9}M melatonin). Total number of cells, number of apoptotic cells and expression of genes related to cellular metabolism, oxidative stress, cell repair, placentation and implantation were evaluated in expanded blastocysts from all treatments. Independent of BC, melatonin supplementation during embryo culture at 10^{-9} M improved re-expansion rate (P <0.05). Hatching rate from BC in association with melatonin treatment at 24, 48 and 72 hours was higher than the others groups (P <0.05). Embryos from control group needed more than 24 h of culture for total re-expansion in relation to the groups supplemented with melatonin (P <0.05). The number of apoptotic cells was similar in the groups supplemented with melatonin, independent of blastocoel fluid removal (P> 0.05), but lower than those without melatonin. The BC procedure did not affect messenger RNA expression of genes related to cellular metabolism (SLC2A1, SLC2A3), oxidative stress (HSPB1, HSPA5, HSP1A1, SOD2), cell repair (MSH6), placentation (KRT8) and implantation (FOSL1) (P> 0.05). This research demonstrated a beneficial effect of melatonin supplementation at 10^{-9} M to the culture medium on embryo quality, but its association with BC was important to increase the hatching rate of vitrified and thawed bovine embryos.

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