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Are miRNAs related to sperm cryotolerance in boars

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The present study aimed to evaluate whether the miRNAs profile present in sperm and extracellular vesicles (EVs) from seminal plasma differ between boars ejaculates with high and low freezability, to understand if miRNAs can be biomarkers of sperm cryotolerance. It is known that these molecules have post-transcriptional action in several metabolic processes and are very important during spermatogenesis and sperm maturation. For this purpose, 27 high-quality seminal ejaculates were used (Total motility-TM and plasmatic and acrosomal integrity-MIAI were both higher than 85%). Sperm and seminal plasma samples were separated and centrifuged in aliquots still in the fresh state, both of sperm and seminal plasma. Ejaculates were later cryopreserved and divided after thawing into two groups: High freezability (HF; N=4) and low freezability (LF; N=4), according to the MIAI (HF>40%; LF<25%) and TM (HF>30% and LF<20%), obtained through flow cytometry and computer-assisted sperm analysis, respectively. Once the groups were determined, miRNA was extracted from both sperm and seminal plasma EVs, subsequently cDNA synthesis and real-time polymerase chain reaction (RT-PCR). The data obtained from the RT-PCR were compared by the unpaired Student's T-test considering 10% significance using the JMP8 SAS software. Bioinformatics analyzes were performed using the mirPath v.3 software on the DIANA TOOLS platform with the microT-CDS v5.0 and TarBase v7.0 databases. Among the 383 miRNAs evaluated, only one miRNA was detected differently (p<0.1) in spermatozoa cell samples, identified as ssc-miR-503. In seminal plasma EVs, two differently abundant miRNAs (p<0.1), identified as ssc-miR-130a and scc-miR-9, were detected. All of the miRNAs were more abundant in LF than the HF group. The miR-503 was, through this study, unprecedentedly detected in sperm. The bioinformatics analyzes performed showed that it could act in different pathways related to the pluripotency of stem cells as TGF-β, PI3K, and WNT signaling pathways that are related which cell proliferation, grown, survival and in their differentiation in various cell types including spermatozoa. MiR-130a and miR-9 have also not been reported in seminal plasma. Our results showed that miR-130a acts inhibiting mainly on fatty acid biosynthesis pathways, it is known that these are essential for the fluidity of the plasma membrane of the sperm, this factor is directly related to the resistance of this structure to cryopreservation. It has also been shown that miR-9 acts on the adhesion complex pathways, modulating the expression of cadherin, a glycoprotein found throughout the entire epididymis, and that has a direct action on fertilization. Based on these results, we were able to conclude that the miRNAs profile present in spermatozoa cell and seminal plasma EVs differ between high and low freezability boars ejaculates, demonstrating that miRNAs can be biomarkers of boars sperm cryotolerance.

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Molecular effects of TUDCA addition during in vitro maturation of bovine oocytes

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Tauroursodeoxycholic acid (TUDCA), a bile acid that acts as a powerful chemical chaperone to inhibit endoplasmic reticulum (ER) stress, has been shown to be beneficial duringstages of the embryos from *in vitro* production (IVP) in some species. This study aimed to evaluate the effect of three concentrations of TUDCA (50 µM, 200 µM and 1,000 µM) during *in vitro* maturation (IVM) on target-transcripts abundance in bovine oocytes. After 24 hours of the IVM with the treatments, denuded oocytes were submitted to RNA extraction and cDNA reverse transcription. The Biomark HD platform was used to relatively quantify the mRNA of interest. This experiment was replicated five times using a total of 100 oocytes/treatment. For statistical analysis, it was calculated the ΔCq values relative to the geometric mean of the best reference genes - *i.e.* GAPDH, PPIA and HPRT1 - among the 96-gene set. Fold-changes were calculated as $2^{-\Delta Cq}$. Transcript abundance from 6 genes was significantly affected in oocyte after TUDCA treatment. When compared to Control group, upregulation was observed in the groups with 50 µM (CAT and GPX1), 200 µM (CAT, GPX1 and HMOX1) and 1,000 µM (HSPA5, CASP3 and CD40) of TUDCA. Of the differentially expressed genes there were transcripts related to oxidative stress and response to cellular stress (50 and 200 µM TUDCA), and endoplasmic reticulum stress and apoptosis (1,000 µM TUDCA). There is an intimate connection between ER stress and oxidative stress. Knowing this relationship, this study demonstrated that supplementing the IVM medium with 50 or 200 µM of TUDCA seems to relieve ER stress by increasing the transcripts abundance related to antioxidant activity in bovine oocytes. On the other hand, treatment with 1,000 µM TUDCA appeared to be detrimental to the oocyte, increasing the expression of the pro-apoptotic genes. This study was financed in part by the Coordenação de AperfeiçoamentoPessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 and São Paulo Research Foundation (FAPESP, grant #2012/50533-2).

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Vitrification technique improves more alterations on gene expression of in vivo-derived sheep blastocysts than slow freezing

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Transfer of fresh sheep embryos has a higher pregnancy rate compared to cryopreserved embryos, suggesting that cryopreservation compromises embryonic signaling during implantation and establishment of pregnancy. Thus, this study assessed the effect of cryopreservation techniques (slow freezing or vitrification) on the expression of genes related to implantation (*CDX2*), maintenance of pluripotency(*NANOG*), cell proliferation (*TGFB1*),mitochondrial activity(*NRF1*) and regulator apoptosis (*BAX* and *BCL2*) of *in vivo*-derived sheep embryos. After superovulation of 32 ewes, and non-surgical embryo recovery, a total of 100 grade I and II embryos were retrieved and uniformly allocated into the groups: slow freezing (SF; *n*=42), vitrification (VT; *n*=43) and control for gene expression (CTL; *n*=15; stored in liquid N_2 in cryotubes RNase/DNase free until RNA extraction). After thawing/warming, three pools of five blastocysts per group (SF: *n*=15 and VT: *n*=15) were dry frozen as described above for later RNA extraction. The remaining 55 (SF: *n*=27 and VT: *n*=28) embryos were cultured *in vitro* (24/48 h) in SOFaa medium at 38.5 °C and 5% CO_2. After RNA extraction, the relative quantification was performed in triplicate by RT-qPCR and evaluated by the comparative Ct method *($2^{-\Delta\Delta Ct}$)*. Survival rate was analyzed by Fisher's Exact Test. The re-expanded embryos results, after *in vitro* culture (survival rate) of SF and VT was, respectively, at 24 h: 29.6% (8/27) and 14.2% (4/28); and at 48 h: 48.1% (13/27) and 32.1% (9/28) (P>0.05). The expression of transcripts was unaffected in embryos submitted to SF compared to the CTL, except for the up-regulated *CDX2* in the SF (P<0.05). On the other hand, VT had an increased (P<0.05) expression of all genes (*CDX2, TGFB1, BAX* and *BCL2*), except for *NANOG* and *NRF1*, when compared to the CTL. Comparing SF with VT was observed difference only in *BAX* gene (P<0.05), that was up-regulated in VT group. In conclusion, *in vivo*-derived embryos submitted to either SF or VT have similar ability to survive *in vitro* and VT led to increased changes in blastocysts gene expression compared to CTL and SF. Acknowledgments: Embrapa (22.13.06.026.00.05) and CNPq (434302/2018-0).Keywords:cryopreservation, implantation, embryo, ovine.

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Effects of hCG administered 7.5 days after synchronous estrus induction during the non-breeding season in Morada Nova ewes

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This study was designed to assess the effects of human chorionic gonadotropin (hCG) administration 7.5 days after the end of synchronous estrus induction protocol in Morada Nova ewes in the non-breeding season on biometrics and vascularization of the luteal tissue, progesterone (P4) concentrations and reproductive performance. Morada Nova ewes (n=113) were submitted to synchronous estrus induction, during October and November, with 60 mg medroxyprogesterone intravaginal sponge for sex days plus 200 IU of eCG i.m. and 30 µg of d-cloprostenol i.m. 36 h before sponge removal. Then submitted to natural mating for three days after the end of induction. Ewes were equally assigned to receive either 1 mL of saline solution (G-Control; n = 56) or 300 IU of hCG (G-hCG; n = 57) i.m. on Day 7.5 after sponge removal. Ovarian ultrasound evaluation and blood collection were performed on Days 7.5, 13.5, 17.5, 21.5 and 30.5 after sponge removal, to quantify and qualify the structures present in the ovary and the serum concentration of P4. The data was analyzed using Chi-Square for reproductive performance, ANOVA for follicular populations and repeated measures over time for biometric and vascularization of luteal tissue and P4 concentration. The number of small antral follicles (< 3.5 mm), large follicles (> 4.5 mm) and the total number of follicles (≥ 2 mm) was greater (P < 0.05) for G-Control, 3.9 ± 0.6; 0.6 ± 0.1; 5.7 ± 0.2 respectively, against 2.8 ± 0.2; 0.4 ± 0.1; 4.5 ± 0.2 for G-hCG. Accessory corpora lutea (aCL) was noted in 0.0% (0/56, G-Control) and 80.7% (46/57, G-hCG ewes) (P = 0.0001). Diameter, area and volume of luteal tissue were greater (P < 0.05) in G-hCG compared with G-Control ewes from Day 13.5 to 30.5. Concentrations of P4 were greater (P < 0.05) on Days 13.5, 21.5 and 30.5 for G-hCG against G-Control (5.33, 4.24 and 5.45 ng/ml x 2.7, 1.74, 2.35 ng/ml respectively). Pregnancy rate was similar (P = 0.15) between groups 46.4% (26/56, G-Control) and 61.4% (35/57, G-hCG), however, when considering only the ewes that had at least one aCL from G-hCG, the pregnancy rate was greater (P < 0.017) for G-hCG ewes 71.7% (33/46) x 46.4% (26/56) G-Control. In addition, the rate of total number of lambs born by the total number of synchronized ewes was greater (P = 0.005) in G-hCG 89.4% (51/57), compared with G-Control 66.1% (37/56). The G-hCG could have better results for aCL formation considering the disadvantage in the number of follicles against G-Control. In conclusion the use of hCG 7.5 days after sponge removal is efficient to induce aCL formation, improving luteal tissue biometry, P4 concentrations, pregnancy rate when successfully induce at least one aCL and improve the total number of born lambs.Financial support: Embrapa (Project 22.13.06.026.00.06)

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Enhancer binding protein gamma gene expression profile in gametes and during bovine early embryo development

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Transcription factors (TFs) and long noncoding RNA (lncRNAs) are crucial for gene expression regulation in a stage and tissue-specific manner. In bovine, the enhancer binding protein gamma (CEBPG) was recently classified in both categories, highlighting the fact of little knowledge on its biological role. To investigate the role of CEBPG on bovine embryo development, we used public RNA-seq data from 21 experiments with a total of 299 samples from cumulus and ganulosa cells, gametes (sperm and oocytes) and embryos up to day 19 (D19) of development the NCBI GEO (Gene Expression Omnibus) and Embryogene Profiler (EP). We confirmed the CEBPG coding potential on human and bovine with ENSEMBL and UNIPROT reference, followed by CNIT and CPC2 tolls. Moreover, we used SRA Toolkit to download and convert data, fastQC for quality check; Trim Galore for read trimming. RSUBREAD was used to do the read alignment and gene count. We used DESEQ2 for differential gene expression with adjusted p value < 0.05 and absolute value of log2 fold change > 2, and finally clVALID and CeTF (in association with WebGestalt) packages for co-expression and enrichment analysis. In humans, we found 3 coding transcripts while in bovines there was only one transcript classified as lncRNA by ENSEMBL and confirmed with CPC2 and CNIT. On UNIPROT database we found cebpg protein in both human and cattle, suggesting that, in bovine, there is at least two transcripts in this gene. EP revealed that CEBPG is highly expressed in oocyte stages with a decreasing expression tendency to blastocyst stage. NCBI GEO data showed that CEBPG is highly expressed on granulosa cells, oocytes (GV and MII), and embryos at 16 cells and Morula stage. Therewithal, the lowest expression values were found on cumulus cells, Sperm, and embryos at day 10. We were able to identify 11 lncRNAs, 21 TFs, and 1012 mRNAs that are corelated to CEBPG expression. We also identified 12 enriched process on Biological Process, Cellular component and Molecular function, including ncRNA metabolic process, chromatin organization, methylation, mitochondrion organization, RNA modification, cellular response to DNA damage stimulus, mitochondrial transport, mitochondrial gene expression, isomerase activity, transferase activity, transferring one-carbon groups, mitochondrial matrix, and nucleolus. In conclusion, our analysis is an indicative that bovine CEBPG is a maternally inherited transcript with active function from embryo genome activation to morula stage. Further studies are on course to better understand CEBPG functions. Keywords: CEBPG, transcription regulation, genome activation. Acknowledgments: the project is part of CNPQ/FAPESP - INCT Células-Tronco e TerapiaCelular no Cancer (465539/2014-9), FAPESP- CEPID/CTC - Centro de TerapiaCelular (13/08135-2) and FAPESP Postdoctoral fellowship (19/04738-0).

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Study of the gene expression of histone methyltransferase g9a in response to different inhibition methods

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H3K9 methylation has been shown to be a barrier in nuclear reprogramming processes, both in cloning and in the production of iPS cells (Santos et al. Current Biology, 13: 1116–1121, 2003; Chen et al., Nature genetics, 45: 34–42, 2013). Decreased H3K9me2 (Histone 3 Lysine 9 dimethylation) levels in cells subjected to cloning reprogramming and iPS generation pointed to a direct relationship between H3K9me2 levels of nucleus donor cells and embryo methylation levels at the beginning of their development (Sampaio, Doctoral Thesis. University of São Paulo, single volume: 54-88, 2015) G9a, also known as *EHMT2* (Euchromatic Histone-lysine N-Methyl-Transferase2), is one of the major enzymes that generate H3K9me2 (Tachibana et al., Journal of Biological Chemistry, 276: 25309-25317, 2001). Our hypothesis is that different *EHMT2* inhibition methods lead to different responses in chromatin and/or gene expression patterns. Therefore, this study aimed to investigate the gene expression of the histone methyltransferase enzyme *G9a* or *EHMT2* during and after its inhibition via siRNA knockdown and inhibition of its catalytic activity through UNC0638. In addition to gene expression, the levels of H3K9me2, an epigenetic mark regulated by action of *G9a*/*EHMT2.* Bovine fibroblasts, from a slaughterhouse, were cultured in vitro. The cells were pooled according to the Control Group treatment (regular culture medium), iEHMT2 Group (cells transfected with siRNA anti-G9a), DMSO Group (96h [48h + 48h] with 0.1% DMSO) and UNC0638 GROUP (96h [48 + 48h] with 250 nM of UNC0638). Each one of the groups was cultured in quadruplicate in a 24-well plate of mm (Corning®). Then it was followed by an immunofluorescence protocol (culture with coverslips) with antibodies anti-H3K9me2 and anti- H3K9me3. The captured images were analyzed with ImageJ software. The levels of gene expression of epigenetic enzymes were also evaluated by RT-qPCR. The genes of interest were: *EHMT1*, *EHMT2*, *SUV39H1* and *H2A* (reference gene). Samples were taken at 0h and 48h post-treatment. The inhibition of EHMT2 by knockdown presented an inverse result than the expected, since the treated group showed higher levels of H3K9me2. On the other hand, the treatment with the chemical probe efficiently reduced H3K9me2 levels ($p > 0.0001$, Student's Test). In addition, their action proved to be specific, since H3K9me3 levels were increased. Nonetheless, in the RT-qPCR, the knockdown treatment more effectively reduced ($p > 0.05$) the transcriptional *EHMT2* levels than in UNC0638 treatment. Our results indicate that the method of inhibition of *EHMT2* by UNC0638 was more efficient than the knockdown method to decrease H3K9me2. Nevertheless, in the transcriptional levels of *EHMT2*, knockdown proved to be the most efficient method here. Extra analysis will be necessary to better support the central hypothesis.

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Quantification of interferon-tau (IFN-τ) stimulated genes in blood leukocytes for pregnancy diagnosis 15 days after insemination using ddPCR

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Development of techniques to accurately diagnosis pregnancy in early gestation is desired to decrease the interval between inseminations in nonpregnant cows and allow better characterization of pregnancy in cattle for research purposes. Quantification of interferon-tau (IFNT) stimulated genes (ISGs) in peripheral blood leukocytes (PBL) have been used as a method to indirectly detect conceptus presence using RT-PCR relative expression as early as days 18 – 20 post insemination (Green et al., Animal Reproduction Science, 121:24-33, 2010). Droplet digital PCR (ddPCR) is the third generation of PCR, widely used for diagnosis of infectious diseases due to its ability to provide absolute quantification of gene copies without the use of a housekeeping reference gene. The objective was to test the efficacy of ISGs quantification in PBL using ddPCR to diagnose pregnancy on day 15 post insemination. Based on previous literature, ISG15 and Mx2 were selected because it is known to be increased in pregnant cows earlier in gestation. Our hypothesis was that pregnant cows would have higher abundance of ISGs (ISG15 and Mx2) compared to non-pregnant cows. Multiparous, non-lactating beef cows (n = 12) were synchronized using 7-day CO–SYNCH + CIDR and artificially inseminated on day 0. Buffy coat from jugular sample was collected on day 0 (baseline) and day 15 for comparison. All cows were confirmed for ovulation and had a functional CL on day 15. Leukocyte RNA was extracted using Trizol (Thermo Fisher Scientific, USA) associated with the DirectZol-RNA kit (Zymo Research, USA). Total leukocyte RNA was transcribed into cDNA by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Absolute quantification of *ISG15* and *Mx2* DNA was performed using QX100^{TM} Droplet Digital^{TM} PCR System (Bio-Rad Laboratories, USA) according to manufacturer's recommendations using EvaGreen ddPCR Supermix (Bio-Rad Laboratories, USA) and previously validated primers. Pregnancy diagnosis was performed on day 26 using transrectal ultrasonography. Data was analyzed using PROC MIXED and PROC LOGISTIC on SAS 9.4. Pregnant cows had similar (P = 0.71) *ISG15* baseline copy numbers (17.5 vs 15.1 copies/µl) but increased (P = 0.04) copy numbers at day 15 compared to open cows (151.8 vs 73.5 copies/µl), respectively. There was no difference (P = 0.43) in day 15 *Mx2* copy number between pregnant and open cows (42.3 vs 33.8 copies/µl), respectively. The addition of baseline concentration to calculate delta or ratio change did not increase the sensitivity of diagnosis. A ROC curve for *ISG15* raw copy number values at day 15 had an R^2 of 0.89 while *Mx2* had an R^2 of 0.75. In conclusion, ddPCR is a suitable approach to diagnose pregnancy using single ISGs measurement at day 15. Further studies are needed with greater number of animals to establish diagnostic cut off values and to determine repeatability in different animal categories.

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Cellular and apoptotic status monitoring during embryo post-cryopreservation development

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A better understanding of embryonic metabolism and the mechanisms that define the survival capacity of cryopreserved embryos are crucial points for the optimization and success of in vitro production and embryo vitrification techniques. In this study, oocytes containing homogeneous cytoplasm and more than three layers of cumulus cells were in vitro matured and fertilized (Day 0). Presumptive zygotes were cultured in SOFaaci containing 2.5% FCS until D7.Blastocysts of excellent quality were cryopreserved by the Cryotop method (n = 355). After warming, the embryos were cultured and distributed in four different groups according its resumption of development: Embryos not re-expanded at 12h (NE12); embryos re-expanded at 12h and hatched at 24h (E12H24); embryos re-expanded at 12h and hatched at 48h (E12H48); embryos re-expanded at 12h and not hatched at 48h (E12NE48). Afterward, the embryos were subjected to TdT-mediated dUTP nick-end labelling (TUNEL) and Hoechst staining to determine the percentage of apoptotic cells. In addition, the apoptotic percentage of cryopreserved embryos was compared with that of fresh PIV embryos (n = 70). The count of the total number of Hoechst-labeled blastomeres and the total number of positive Tunel cells was performed using the Image J. software.The apoptotic index was obtained by dividing the number of apoptotic cells by the total number of cells in each embryo. The results obtained in the different groups were analyzed by analysis of variance (ANOVA) using the GraphPad software. If ANOVA was significant, the pair analysis was performed using the Tukey test. To compare the apoptotic index of fresh x vitrified embryos, the results obtained with the evaluation of all cryopreserved embryos were compared to the results of a similar number of fresh embryos. For analyses, a significance level of 5% ($P < 0.05$) was used. The results showed that the blastocoel ability to re-expand was negatively affected by the higher percentage ($P < 0.05$) of apoptotic cells found in the NE12 group compared to the others. Vitrified and warmed embryos showed a higher apoptotic index ($P < 0.05$) than fresh embryos. Finally, a greater total number of blastomeres ($P < 0.05$), found in groups E12H24 and E12H48, seems to have a positive influence on the ability of embryos to hatch after cryopreservation. In conclusion, we identified that the total number of cells and the apoptotic percentage are decisive factors for the survival of cryopreserved embryos.

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MALDI-MS characterization of sperm membrane lipid profile of young Nellore bulls receiving long-term dietary supplementation with rumen-protected polyunsaturated fatty acids

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Fertile mammalian spermatozoa are characterized by a higher proportion of polyunsaturated fatty acids (PUFA), which are obtained through the diet. The ingested PUFA are then transferred to the sperm membranes, whose lipid composition will directly affect sperm viability. The aim of this study was to examine the effects of long-term dietary supplementation of young Nellore bulls with PUFA on sperm lipid profile by using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Twelve Nellore young bulls (*Bos taurus indicus*) were supplemented from 14 to 24 months of age with base diet (control group; n = 6) or with rumen-protected PUFA-enriched diet (FA group = base diet and 200 g Megalac-E® per bull per day; n = 6). The semen was collected and frozen when the animals reached 24 months old. Samples of frozen/thawed semen were analyzed in a MALDI-TOF-TOF Autoflex III (BrukerDaltonics), equipped with a laser at λ=337 nm. The analysis was performed in Reflector positive mode in a mass ranging from 500-900Da. Normalized data were subjected to principal component analysis (PCA) and statistically significant features was determined according to false discovery rate (FDR<0.05). The lipid attribution of the significant ions was performed using LIPID MAPS and LipidomeDB databases and was based on previous publications using the same MALDI-MS approach for semen lipid profiling. Although PCA revealed no evident separation between treatments on the membrane lipid profile of sperm (P>0.05), a total of 10 ions (lipid species) were selected (FDR<0.05). The semen of the animals in the FA group showed an increase in the abundance of lipid of m/z 578, tentatively attributed as Lyso-SM(+K), that is a sperm degradation product that can occur as a result of the damage to the plasma membrane resulting from the freezing and thawing cycle (Schiller et al., Chemistry and Physics of Lipids, 106:145–156, 2000). Moreover, the abundance of lipids of m/z 750, 751 and 765 (attributed as [PE(38:6)Alkyl–Acyl+H]+, [PE(38:5)Alkyl-Acyl+H]+ and [PE(38:6)Acyl-Acyl+H]+, respectively) were decreased in semen of animals from FA group, whereas the abundance of ions of m/z 757 and 781 (attributed as [PC(32:0)+Na]+ and [PC(34:2)+Na]+, respectively) were increased. As it is well established that PC levels increase during capacitation due to methylation of PE (Jones et al., FertilSteril, 31:531-537, 1979), these results may suggest that the semen of animals from FA group may have undergone an early capacitation process. Taken together, the results of the present study demonstrated changes in the lipid profile of sperm membrane resulting from the dietary supplementation of bulls with rumen-protected PUFA and suggest that such modifications may make sperm less resistant to cryopreservation and more susceptible to an early occurrence of capacitation. However, further studies are needed to evaluate this assumption.Financial support: Fapesp (2015/06733-5)

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Exploratory metabolomic study in lactation milk induced in Holstein cows

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In the occurrence of reproductive failures, subsequent lactation will not occur, causing a decline in milk production. Alternatively, there are artificial lactation induction protocols composed of hormonal combinations that alter the physiology of the mammary gland. Currently trereisn´t any study who metabolically characterized this type of induced milk. Therefore, this study performed an exploratory analysis of metabolites in induced lactation milk comparing them with physiological lactation milk. The exploratory research analyzed 50 milk samples: milk induced lactation (n=20); physiological lactation milk (n=20); cooling tank milk (n=10). Four high-performance Holstein cows were selected: 2 cows in physiological lactation and 2 cows submitted to a lactation induction protocol. The lactation induction protocol started 20 days (day -20) before the first day of milking (day 0). Bovine somatotropin - bST (500mg - Boostin®) was administered every 7 days. From the -20 to the -13 day, 30 mg of estradiol benzoate were administered daily, together with 2 mg of progesterone. From the -12 until the -7 day, animals only received daily doses of 20 mg estradiol benzoate. On -5 day, 2 mg of clopostrenol (Ciosin®) was administered and on -2, -1 and 0, animals received 40 mg of dexamethasone. After the beginning of lactation (day 0), cows received bST (500 mg) on days 1, 8 and 23. Samples were collected on days 0 (start of milking), 1, 7, 10 and 24, with 2 samples per day (morning and afternoon). For analysis, the indicrectmetabolomic method with high performance liquid chromatography was used. The qualitative data of the metabolites were analyzed descriptively and compared with the spectra of the spectral libraries Milk Composition Database (MCDB), Human Metabolome Database (HMDB), ReSpect, Massbank and NIST14. The ion fragmentation spectra that were similar to the libraries spectra were manually compared with the spectra fragmentation of the proposed compounds, and their mass errors were calculated and only accepted if less than 5 ppm. Qualitative omic analysis identified 39 metabolites, most of which were endogenous origin derived from lipids, carnitine and amino acids, and only 7 exogenous metabolites. Of the 31 metabolites exclusive to induced milk samples, 21 were also detected in the cooling tank, being 14 metabolites of endogenous origin and 7 of exogenous origin, possibly from the drugs metabolism, not being detected from D7 onwards. The artificial lactation induction altered the dynamics and metabolites type found in the cows milk submitted to the hormonal protocol and in the presente milk in the cooling tank during the lactation period, configuring the presence of distinct metabolites from physiological milk. Therefore, the results provide comprehensive information on the metabolomic profile, indicating an indirect influence of induced milk even in the cooling tank, suggesting a possible chemical signature.

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Bovine placenta as biomaterial for bioengineering: proteomic analysis

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Bioengineered biomaterial produced by decellularization of whole organs have been studied in the recent years. Placenta is promising as alternative source of extracellular matrix (ECM), such as bovine placenta with rich ECM and well-developed vasculature that easily build anastomoses to host's organ. Our group was able to produce cell culture viable bovine placenta biomaterials; however, their protein content was unknown (DOI: 10.1002/term.2618). Then, non-decellularized (n=3) and SDS-decellularized (n=3) 4-month old bovine cotyledon sampled around umbilical cord were washed, lysed, urea reduced, acetone precipitated, DTT reduced, iodoacetamide alkylated, trypsin digested, and C-18 column purified. Finally, 3 µg protein were loaded in OrbitrapFusionLumos spectrometer (ThermoScientific). Spectra were exported to MaxQuant software (v1.6.10.43) to protein list production of each sample, and Label-Free Quantification (LFQ) intensity were statistically (P > 0.05) analyzed by Inferno software (v.1.1.6970). After, proteins related to ECM and cellular junction onthologies were filtered and manually annotated using DAVID Bioinformatics Resources 6.8. From 2,650 identified protein sequences by MaxQuant software, 194 (7.3%) were filtered by selected onthologies, and 146 (75.2%) unique proteins were enriched. From this, 110 (75.3%) proteins were overexpressed in non-decellularized condition, whereas 19 (13.0%) were overexpressed in bovine decellularized placenta. At the end, 16 (10.9%) proteins were equally expressed in both samples. Indeed, two (1.4%) proteins were only expressed in natural condition, whereas 25 (17.2%) were only expressed in biomaterial, and 118 (81,4%) proteins were commonly expressed in both. The enrichment of the 25 proteins in the biomaterial sample, may be due protein with low expression that could not be detected in the non-decellularized condition sample.

In conclusion, most of the proteins related to ECM, such as collagens, and other fibrous and adhesive proteins, and cellular junction were maintained after decellularization, suggesting bovine placenta as a source of biomaterial with rich and functional environment.Support: FAPESP (2014/50844-3 and 2015/14535-9) and CNPq (467476/2014-4).

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

Mice placenta a small size biomaterial for bioengineering: proteomic analysis

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Three-dimensional (3D) biological microenvironments produced by decellularization, are examples of biomaterial. Therefore, mice placenta that are discarded and have a rich ECM could be an interesting alternative source for 3D biomaterial. We previously presented a viable mouse whole placenta biomaterial, that has biocompatible environment for cell culture, however their protein content still unknown. For this, non-decellularized (n=3) and SDS-decellularized (n=3) 18.5-day old mouse placenta were washed, lysed, urea reduced, acetone precipitated, DTT reduced, iodoacetamide alkylated, trypsin digested, and C-18 column purified (DOI: 10.1002/9780470559277.ch140272). At the end, 3 μg protein were loaded in OrbitrapFusionLumos spectrometer (Thermo Scientific). Generated spectra were exported to MaxQuant software (v1.6.10.43) to produce the protein list of each sample, and the LFQ intensity were statistically (P > 0.05) analyzed by Inferno software (v.1.1.6970). After this, proteins related to ECM and cellular junction onthologies were filtered and manually annotated using DAVID Bioinformatics Resources 6.8. From 2.316 identified protein sequences by MaxQuant software, 198 (8.5%) were filtered by selected onthologies, and 156 (78.8%) unique proteins were enriched. From this, 119 (76.3%) proteins were overexpressed in non-decellularized, whereas 24 (15.4%) were overexpressed in mice placenta biomaterial. At the end, 14 (8.9%) proteins were equally expressed in both samples. Indeed, 36 (23.1%) proteins were only expressed in non-decellularized, whereas 38 (24.4%) were only expressed in biomaterial, and 82 (52.6%) proteins were commonly expressed in both. The enrichment of the 38 proteins in the biomaterial sample, may be due protein with low expression that could not be detected in the non-decellularized sample due interference of other intracellular overexpressed proteins. In conclusion, around moiety of the proteins related to ECM, such as collagens, and other fibrous and adhesive proteins, and cellular junction onthologies were maintained after decellularization suggesting that this biomaterial still keep some functional environment. Support: FAPESP (2014/50844-3 and 2015/14535-9) and CNPq (467476/2014-4).*This abstract only counts the descriptive analysis of protein group. Differential protein analysis is in progress by the group.

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

Canine chorionic girdle as biomaterial for bioengineering: proteomic analysis

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Currently, the high need and scarcity for tissues for transplantation leads to the emergence of new technologies in regenerative medicine. Thus, the decellularized placental extracellular matrix (ECM) has emerged as a new tool to produce biomaterials. Previously, we established a viable canine placenta biomaterial for cell culture; however, its protein content has not yet been described.

For this, non-decellularized (n=3) and SDS-decellularized (n=3) 35-day old fetal part of canine placenta sample were washed, lysed, urea reduced, acetone precipitated, DTT reduced, iodoacetamide alkylated, trypsin digested, and C-18 column purified (DOI: 10.1002/9780470559277.ch140272). Finally, 3 μg protein were loaded in OrbitrapFusionLumos spectrometer (Thermo Scientific). Spectra were exported to MaxQuant software (v1.6.10.43) to produce the protein list of each sample, and the LFQ intensity were statistically ($p > 0.05$) analyzed by Inferno software (v.1.1.6970). After this, proteins related to ECM and cellular junction were filtered and manually annotated using DAVID Bioinformatics Resources 6.8. From 1.306 identified protein sequences by MaxQuant software, 91 (6.9%) were filtered by selected ontologies, and 66 (75.2%) unique proteins were enriched. From this, 33 (50%) proteins were overexpressed in non-decellularized, whereas 18 (27.3%) were overexpressed in canine placenta biomaterial. At the end, 15 (22.7%) proteins were equally expressed in both samples. Indeed, 8 (12.1%) proteins were only expressed in non-decellularized, whereas 6 (9.1%) were only expressed in biomaterial, and 52 (78.8%) proteins were commonly expressed in both. The enrichment of the 6 proteins in the biomaterial sample, may be due protein with low expression that could not be detected in the non-decellularized sample. In conclusion, mostly the proteins related to ECM, such as collagens, and other fibrous and adhesive proteins, and cellular junction ontologies were maintained after decellularization, suggesting canine placenta as a source of biomaterial with rich and functional environment. Support: FAPESP (2014/50844-3 and 2015/14535-9) and CNPq (467476/2014-4).*This abstract only counts the descriptive analysis of protein group. Differential protein analysis is in progress by the group.

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

Using placenta morphological diversity as advantage in tissue bioengineering

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The search for an alternative non-synthetical extracellular matrix (ECM) scaffold for tissue engineering have been increasing due to advances in bioengineering techniques to supply regenerative medicine grafts. As a disposable material with well-structured and rich ECM, placenta may be an adequate source. Also, morphological placental diversity can reach several approaches. Canine (day 35), mice (day 18.5) and bovine (4 month) placentas were SDS decellularized and were validated as biomaterial by means of morphology (standard histology), structure (electron microscopy) and content (immunohistochemistry for major ECM proteins and genomic DNA). The three different models were sterilized firstly by complete dehydration, and then by 70% alcohol bath associated with UV light, to evaluate cytocompatibility by cell culture (fibroblast, mesenchymal stem cell, and/or hematopoietic precursor). Withal, 3 non-decellularized and3 decellularized samples were analyzed by OrbitrapFusionLumos mass-spectrometer (Thermo Scientific), and protein list were generated (MaxQuant software, v1.6.10.43). The LFQ intensity were statistically (P > 0.05 Inferno software, v.1.1.6970), and ECM and cellular junction-related proteins were manually annotated (DAVID Bioinformatics Resources 6.8). Overall, all three models were validated as biomaterials, because decellularization maintained natural gross morphology and structure and major ECM protein, also decreased genomic DNA to safe levels for regenerative medicine. All models where cytocompatibility and allowed different cell lineages adhesion. Bovine and canine placenta-derived biomaterials maintained around 80% of ECM and cellular junction related proteins, however mice were around 50%. Bovine placenta as parenchymatous and villous-shaped may be used for profound and deep grafts. Canine placenta, however, as thin and membranous-shaped could be elected for superficial grafts. And finally, mice placenta, due their small size and lower protein content maintenance could be designated for delicate cell-driven grafts. In conclusion, those three placentas produced adequate biomaterial models aiming different suggest purposes in tissue bioengineering.Support: FAPESP (2014/50844-3 and 2015/14535-9), CNPq (467476/2014-4 and 406022/2016-0) and CAPES.