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Evaluation of protein profile in uterine secretion in pregnant bitches during peri-implantation period

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Keywords: bitches, gestation, protein profile.

During peri-implantation period canine embryos are nourished by secretions into the uterine lumen, in which several proteins are present. The aim of this study was to compare the protein profile in uterine secretion in pre and post implantation period, using two-dimensional electrophoresis technique. For that, we used 10 canine females artificially inseminated, being five females submitted to OHE on the 16th day after the preovulatory LH surge (group A), and with the presence of blastocysts and five females were castrated on 25th day after the preovulatory LH surge (Group B), observing the presence of embryonic vesicles. The samples were obtained by uterine flushing using PBS solution and resulting washed was stored at -20oC. We prepared pools with these samples, each one containing five samples of the respective groups. Two-dimensional electrophoresis were performed with 138.40 µg/uL of protein per strip (7 cm) and four replicates per group. The gels were stained using Coomassie G-250, scanned and interpreted in a specific software (Image Master version 7.0, GE HealthCare, Upsalla, SE). Fifty spots were expressed in group A and 43 spots in group B. Two proteins were expressed exclusively in group A and 24 proteins exclusively in group B. Based on that, the protein production in uterus post implantation is higher than uterus in pre- implantation phase, when the embryos are at migratory state. This fact can be attributed the presence of proteins involved in intimate connection in embryo-endometrium, which are important in the decidualization process and a better understanding of the molecular participation in implantation process will allow the knowledge of the failures on blastocyst nidation in the canine endometrium.

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Achievement of a pregnancy rate of 40% after direct transfer of cryopreserved IVP bovine embryos

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Keywords: bovine, direct transfer, IVF embryo.

After decades of research seeking for an efficient protocol for the cryopreservation of in vitro-produced (IVP) bovine embryos, recently it has become possible to achieve commercially viable pregnancy results using the vitrification method. However, the complex process of rewarming these embryos after vitrification remains an obstacle to the commercial use of this technique, with low applicability under field conditions. The aim of this study was to assess the pregnancy rates obtained after the transfer of IVP bovine embryos frozen by the slow method and transferred directly to the recipients. Oocytes recovered by ovum pick up (OPU) of Nelore females were selected and subjected to in vitro maturation (IVM) for 24 h at 38.5 °C with 5% CO2 and saturated humidity. In vitro fertilization (IVF) was performed with a previously tested frozen-thawed semen from an Angus bull. The oocytes remained in coculture with spermatozoa for 22 h under the same conditions of IVM. After this period, presumptive zygotes were denuded and cultured for seven days under the same temperature and humidity conditions of IVM and IVF, but with 5% CO2 and 5% O2 in air. Grade I embryos in BL or BX stages were exposed to ethylene glycol (EG) 1.5 M for 10 min and were placed in the freezing machine TK-1000®, previously stabilized at -6 °C. After 5 min of exposure to EG 1.5, the embryos were loaded into 0.25 mL straws. The straws contained 5 columns of media separated by air bubbles, the central column being composed of 1.5 M EG and the embryo, and the 4 lateral columns being composed of 0.5 M EG (thawing medium). Two minutes after placing the embryos in the freezing machine, the crystallization of the straws was carried out in the two center columns that were closest to the embryo (above and below). After crystallization, a freezing curve of 0.5 °C per minute was carried out until the temperature of -32 °C was reached. When the freezing curve was completed, the straws were placed in appropriate racks and stored in liquid nitrogen until the day of the embryo transfer. For thawing, the embryos were removed from liquid nitrogen, exposed to air for 10 seconds and immersed in water at 35 °C for 30 seconds. The embryos were transferred to selected recipients seven days after natural estrus. The pregnancy rate obtained was 40% (62/155). In the same property and in the same period, vitrified IVP embryos and fresh IVP embryos of several breeds were transferred. The transfer of vitrified embryos resulted in a pregnancy rate of 37.5% (162/432) and the transfer of fresh IVP embryos resulted in a pregnancy rate of 49.6% (576/1162). The rates obtained with the direct transfer of frozen IVP embryos indicate that this protocol provides results similar to those obtained after the transfer of vitrified IVP embryos. We highlight the positive aspects of establishing a technique that enabled the cryopreservation of IVP embryos with the convenience of the direct transfer.

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The use of doppler ultrasonography (power-flow mode) for the evaluation of luteal blood perfusion and its correlation with the diagnosis of pregnancy in mares

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Keywords: corpus luteum, doppler, ultrasonography.

The color Doppler ultrasonography, an effective and practical real time procedure for the non-invasive evaluation of blood vessels and tissues, is considered one of the most appropriate techniques to study the hemodynamics of the reproductive tract of large animals (GINTHER, OJ Animal Reproduction Science, v. 99, p 213-220, 2007). The present study aimed to evaluate the perfusion of the primary CL and its correlation with the positive diagnosis of pregnancy in mares. A total of 16 healthy crossbred mares aged from 4 to 15 years old was used. They were divided into two groups, one group of pregnant mares (n = 8) and other of non-pregnant mares (n = 8). Both groups were evaluated on blood perfusion of CL and plasma progesterone profile (RIA). All parameters we reassessed seven and fourteen days after ovulation (D7 and D14). The mares were examined by B-mode and Power-flow mode ultrasonography. The blood perfusion of CL was estimated in real time, taking into account the percentage (0-100%) of luteal tissue with colorful signs displayed during continuous Doppler ultrasonographic examination of at least one minute. The assess mentor blood perfusion was divided into three classifications: below 50% (poorly vascularized); between 50 and 75% (vascularized) and between 75 and 100% (highly vascularized). It was used the Chi-square test at 5% probability to analyze blood perfusion in both groups. The amounts of progesterone in the two different groups were subjected to statistical analysis of variance at 5% probability. In the group of non-pregnant mares, at the moment D7, 12.50% (n = 1) of mares showed poorly vascularized CL; 37.50% (n = 3) of mares showed vascularized CL and 50% (n = 4) of mares showed very vascularized CL. At moment D14, 12.50% (n = 1) of mares showed poorly vascularized CL; 62.50% (n = 5) of mares showed vascularized CL and 25.00% (n = 2) of mares showed very vascularized CL. In the group of pregnant mares, at moment D7, 0% (n = 0) of mares present poorly vascularized CL; 0% (n = 0) of mares presented a vascularized CL and 100% (n = 8) of mares present every vascularized CL. At moment D14, 25.00% (n = 2) of mares showed poorly vascularized CL; 75.00% (n = 6) of mares showed vascularized CL and 0% (n = 0) of mares had very vascularized CL. It was observed that the distribution among the blood perfusion classes had a significant effect in either mares, pregnant or not. In assessing the circulating concentrations of progesterone it was not observed differences between two groups ($P > 0.05$), being 10.25 ng / ml vs 14.10 ± 5.92 ng / mL ± 2.39 and 11.67 ng / mL ± 8.74 vs 10.39 ng / mL ± 2.61 at moments D7 and D14, respectively for non-pregnant and pregnant mares. In summary, the color Doppler ultrasonography may suggest a positive pregnancy diagnosis, since the pregnant mares showed a primary CL more vascularized than non-pregnant mares.

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Use of three-dimensional models as an alternative to evaluate body condition in cattle: preliminary results

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Keywords: 3D modeling, body condition score, bovine.

Body condition in cattle is closely related to reproductive performance and it is widely used as a parameter to select embryo recipients. The aim of the present study was to evaluate the potential of using three-dimensional models (3D) to assess body condition in bovine. Cross-bred (Holstein-Zebu) cows were used (n=70). In the first experiment, 3D models of the posterior region of the animals were generated using an infrared sensor connected to a laptop computer equipped with custom-developed software (ReconstructMe). Another software (Rhinoceros) was used for measurements. At the base of the tail, depth was measured from a perpendicular line between the skin surface and the reference line traced between both ischium. In rump region, the reference line was traced between ilium and ischium. In lumbar region, reference line was traced between transverse and spinous processes of the lumbar vertebra near the 13th rib. As reference parameters were used, as follows: visual evaluation of the body condition score (BSC, Edmonson et al., 1989), and measurement of fat thickness by ultrasonography at insertion of biceps femoris muscle (region 1) and the region of longissimus dorsi muscle between 12th and 13th ribs (region 2), both performed by the same technician immediately after 3D model construction. The association between variables was evaluated by Pearson's correlation method. The values obtained by objective measurements using 3D models were highly and significantly correlated with BCS and fat thickness in regions 1 and 2, both at the base of the tail (R=0.80, R=0.62 and R=0.62; respectively, $P<0.0001$) and rump regions (R=0.72, R=0.71 e R=0.72; respectively, $P<0.0001$). On the other hand, the measurements performed at dorsal region had lower correlations with the reference parameters (R=0.47, R=0.28 and R=0.35; respectively, $P<0.05$). In the second experiment, three experienced BCS evaluators (A1, A2 and A3) received colored images and 3D models of the posterior region of the animals used in the first experiment. Each evaluator assigned BCS values to the colored images and thereafter to the 3D models, without knowing the relation between them. Correlations between the evaluators tended to be higher for BCS values assigned to 3D models (A1 x A2, R=0.84; A1 x A3, R=0.91; A2 x A3, R=0.81; $P<0.0001$) than for BCS values assigned to colored images (A1 x A2, R=0.79; A1 x A3, R=0.85; A2 x A3, R=0.79; $P<0.0001$). These results demonstrate that objective assessment of body condition using 3D models is consistent with the conventional evaluation by visual score or by subcutaneous fat thickness using ultrasonography; and 3D models can also be used to subjective evaluation of BCS.

Acknowledgments: Fapemig and CNPq.

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Ethylene glycol monomethyl ether: a potential cryoprotectant for vitrification of bovine embryos produced *in vitro*

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Keywords: cryopreservation, ethylene glycol, toxicity.

Vitrification in an efficient method for cryopreservation of in vitro-produced bovine embryos. Ethylene glycol (EG) is commonly used as cryoprotectant in vitrification solutions due to its high permeability. However, vitrification requires high concentration of EG, which increases potential harmful effects on the embryos due to toxicity. The addition of a methyl group to the structure of EG generates ethylene glycol monomethyl ether (EGMME), with improved vitrification capability that is yet to be tested for in vitro production (IVP) of bovine embryos. The objective of this study is to evaluate the re-expansion and hatching rates of bovine embryos produced in vitro after exposure to different concentrations of EGMME. Oocytes from ovaries collected in an abattoir were used for IVP of bovine embryos using a commercial medium (Biotecnologia Animal®, Brazilia, DF, Brazil). The 704 blastocysts and expanded blastocysts obtained after twelve replications, were cultured in 199 medium at 35°C and divided in four treatments (n = 176 each): control: without cryoprotectant; T: 10% EGMME; T2: 15% EGMME; and T3: 20% EG. All embryos were kept in the medium for 5 min, and then washed in sucrose gradients (0.4, 0.26, and 0.16 M), for 5 min each. After culturing the embryos in SOFaa during 24 h, the rates of re-expansion (after 12 h) and hatching (after 24 h) were determined. Such rates were compared among treatments through the Kruskal-Wallis analysis of variance for non parametric data, using the Statistix® software. Mean re-expansion rates did not differ for control, T1, T2 and T3 ($P > 0.05$): 23.8%; 17.7%; 10.5%; and 15.2%, respectively. Likewise, hatching rates were similar among treatments ($P > 0.05$): 74.4% for the control; 74.3% for T1; 43.8% for T2; and 66.5% for T3. Therefore, the inclusion of EGMME in vitrification solutions, at the concentrations tested, did not incur in toxic effects for bovine embryos produced in vitro. Further studies will be conducted to evaluate the effect of EGMME in association with dimethyl sulfoxide, which is the most commonly used cryoprotectant in vitrification of bovine embryos produced in vitro.

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Effect of estradiol benzoate and progesterone administration on endometrial gene expression of estrogen and progesterone receptors in non-cyclic mares

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Keywords: endometrium, non-cyclic mares, steroidal receptors.

The aim of the present study was to evaluate the effect of an estradiol benzoate and progesterone protocol administration, for preparing non-cyclic embryo recipient mares, on endometrial estrogen and progesterone receptors gene expression. Twelve mares were used during anestrus (Anestrus group), transition (Transition group) and subsequently during cyclic phase (Control group). Mares from Anestrus and Transition groups were treated with a single dose of 2.5mg of estradiol benzoate (EB; Estrogin™, Farmavet, Brazil) and 48 hours after they received 1500mg of long action progesterone (LA P4; Sincrogest Injetável™, Ourofino, Brazil). Control group mares did not receive hormonal treatment. Uterine biopsies were performed using an equine uterine biopsy instrument (Botupharma, Botucatu, Brazil) immediately before the onset of EB treatment (M1), 48 hours after EB administration (M2) and five days after LA P4 administration (M3) in Anestrus and Transition groups. In the Control group, samples were collected during estrus, when uterine edema and at least a 35mm follicle were detected by ultrasonography, and during diestrus, five days after ovulation. The biopsies were preserved in liquid nitrogen inside sterile cryovials until further processing. The relative gene expression analysis of estrogen receptor alpha (ERα) and beta (ERβ) and progesterone receptor (PR) was performed by RT-qPCR, using the GAPDH as a reference gene. The Wilcoxon signed-rank test for paired data was used to compare relative gene expression between the studied moments. The administration of EB did not affect ERα and PR gene expression ($p>0.05$) in Anestrus and Transition groups, although a 2.6-fold reduction of ERβ expression was observed in Anestrus group ($p<0.01$). The administration of LA P4 in Anestrus group did not affect ERα expression in M3 ($p>0.05$), although a 2.5-fold reduction in ERβ ($p<0.01$) and 1.2-fold reduction in PR ($p<0.05$) were found. In Transition group, only ERβ expression was reduced (2.3-fold; $p<0.05$) in M3. There were no differences detected on the three receptors gene expression between moments M2 and M3 in Anestrus and Transition groups, as opposed to Control group, in which ERα, ERβ and PR expression were lesser during diestrus than estrus (2.9-fold, 3.8-fold and 3.2-fold, respectively; $p<0.05$). In conclusion, the administration of 2.5mg of EB and 1500mg of LA P4 did not affect ERα and PR gene expression dynamics, as found in the corresponding phases in cyclic mares. However, the administration of EB reduced the ERβ expression in anestrous mares, although this finding was not observed in transitional and cyclic mares. Therefore, the evaluation of different hormonal protocols becomes necessary and the role of ERβ in non-cyclic and cyclic mares needs to be elucidated.

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Study of triple methylation of H3K4 in bovine embryos in vivo produced through superovulation cryopreserved or not

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Keywords: cryopreservation, epigenetics, superovulation.

The cryopreservation of in vivo produced embryos is well established and widely used, but the same is not observed with embryos in vitro produced. The study of epigenetics in such embryos should help to identify differences between in vivo and in vitro produced embryos that could explain the differences of their viability after cryopreservation. Methylation at lysine 4 of histone 3 (H3K4), in general, is related to the active state of chromatin, allowing access of the transcription machinery. Accordingly, the objective of this study was to evaluate the epigenetic modifications in bovine embryos produced in vivo, fresh or cryopreserved, by identifying the trimethylation of H3K4. Girolando cows were submitted to superovulation protocol (SOV) with FSH and embryo were collected seven days after artificial insemination by uterine flushing. Thirty-seven SOV procedures were performed 60 days apart with semen from the same bull. Only embryos of excellent quality, according to the IETS standards were used for immunofluorescence. Fresh embryos (n=15) were fixed in paraformaldehyde (3%) supplemented with sacarose (2%) and stored at -20°C for later analysis. The embryos (n=15) were cryopreserved by slow freezing method and after few days they were thawed and stored in paraformaldehyde (3%) supplemented with sacarose (2%). After blocking and permeabilization, the embryos were exposed to polyclonal antibody against tri-methylated H3K4 (CellSignaling®) at a concentration of 1:1000, overnight at 4°C. They were then exposed to the secondary antibody (anti-Rabbit Alexa 647; Invitrogen A21245) at a concentration of 1:200, and then subjected to staining of the nucleus (SYBR Green, Molecular Probes, Goettingen, Germany, 1:500). Embryos were individually evaluated under immunofluorescence and confocal microscopy. The same pattern of staining was observed in all groups, indicating that embryos produced in vivo, fresh and cryopreserved present an intense and well distributed patter of H3K4 in the trimethylated form.

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Artificial intelligence meets the same challenges as humans in morphological classification of bovine blastocysts

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Keywords: artificial neural networks, classification, embryology.

Embryonic morphological classification has great importance for several laboratory techniques (from basic to applied research in assisted reproduction). However, the current method to perform the classification of embryos in various degrees of quality has always been based on the subjectivity of the evaluator, and besides a great variety of descriptions of morphological characteristics that categorize an embryo in each grade, there is no accurate method that can generate consistent and reliable results for morphological classification. This paper presents partial results in the development of a software able to perform the classification of morphological quality in bovine embryos. Digital Image Processing and Artificial Intelligence Techniques (specifically Artificial Neural Networks - ANN) were used as a base of implementation. Test data of 56 embryos were used for verification of the results (15% of the total database). The data were randomized and submitted to ANN only after completion of training. Thus, it is possible to ascertain whether the training was really effective, and learning from RNA occurred correctly. For blastocysts Grade 1 (Excellent), the ANN currently has an 84.2% accuracy in classification. For Grade 3 blastocysts a hit of 88.9%. The classification of Grade 2 blastocysts still has a hit of only 20%. Analyzing the database as a whole, the accuracy of the ANN is 75%. Partial results achieved demonstrate that it is possible to obtain a fully automated information extraction process and a classification system based on Artificial Neural Networks to obtain a correct evaluation of morphological quality of a bovine blastocyst, and the current system is satisfactory (accuracy greater than 80%) for the classification of blastocysts in grades 1 and 3 (according to the classification recommended by the IETS). Currently, the prototype uses an average of 2.5 seconds to perform an in an automated way all the necessary steps for the classification of a bovine blastocyst, from importing the image, standardization, segmentation, information extraction and classification by ANN process. An intriguing fact for partial achievements is that, as embryologists, the artificial intelligence system has difficulties distinguishing similar degrees of quality. Thus, the classification of grade 2 blastocysts was not good enough to validate effectiveness. Would then be possible to develop an artificial intelligence system that overcomes human accuracy?

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Identification of lipid subclasses by MALDI-MS in blood and follicular fluid of cows supplemented with different vegetable oils

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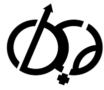
Keywords: bovine, lipids, MALDI-MS.

Nutrition has major effects on reproductive processes in cattle. Vegetable oils are major sources of polyunsaturated (PUFA) fatty acids, whose effects are related to the ability to modulate the metabolism of arachidonic acid and prostaglandin, to increase circulating levels of insulin -like growth factor (IGF-I) and to promote the increase of plasma cholesterol which is a substrate for the synthesis of steroid hormones such as progesterone (P4) and estrogen (E2). The aim of this study was to identify different lipid classes in blood and follicular fluid of animals subjected to supplementation nutritional with sunflower oil, soybean oil or canola through the technique of direct analysis assisted laser desorption matrix (MALDI - MS). The animals were fed their respective diets for 70 days. The control group received the same diet of others, without the addition of vegetable oil. Weekly blood samples and follicular fluid were collected. The identification of the lipids in the samples was performed using MALDI -TOF MS. In females supplemented with canola oil lipid subclasses conjugated steroids (ST) and phosphatidylserines (PS) have been identified in follicular fluid. The animals supplemented with sunflower group showed conjugated steroid (ST) in blood serum and phosphatidylinositol conjugated to ceramides (Cer - PI) and phosphatidylserines (PS) in follicular fluid. Finally, the group receiving soybean oil as a supplement, the compounds phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were identified in the blood serum and no lipid subclass was identified in follicular fluid of these animals in the control group. The separation between the control and the treatments was observed and identified substances, mostly accounted for constituent membrane lipids (phosphatidylserine - PS, phosphatidylethanolamine - PE), intracellular second messengers (phosphatidylinositol - PI) or conjugated steroids (ST), which are precursors of steroid hormones such as progesterone and estradiol. It is concluded that canola and sunflower oils were capable of altering the lipid composition of intra - follicular environment which may influence the quality of the oocytes formed. Changes in steroid conjugates observed in the blood serum of animals supplemented with sunflower oil may contribute to an increased production of steroid hormones by increasing further the steroidogenic process. Soybean oil induced changes in the blood profile, but these were not reflected in changes in intra- follicular environment.



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Effects of artificial collapse of blastocoele prior to vitrification on survival of in vitro produced bovine embryos

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Keywords: bovine, embryo, vitrification.

Bovine embryos produced in vitro have lower cryotolerance than those obtained in vivo. To improve the rates of embryo surviving after cryopreservation, an alternative might be to carry out an artificial blastocoele collapse before vitrification, which can be a mechanic or non-mechanic stimulus, for example by using needles and sucrose hyperosmotic solutions, respectively. The aim of this study was to evaluate the effects of artificial shrinkage of blastocoele of bovine IVP embryos on embryonic survival and quality after vitrification. Oocytes (n=993) were IVM during 24h in TCM-199 medium with bicarbonate, hormones and 10% FCS. After IVF, zygotes were IVC in SOF medium supplemented with 5% BSA + 2.5% FCS. The cleavage was evaluated at 48 hpi and blastocysts (Bl) at 168 hpi, when they were divided into three groups: 1) embryos without treatment (CONTR; n=72); 2) embryos were placed successively for 5 min in two different sucrose solutions: 0.125 and 0.25M (SAC; n=64); and 3) embryos were subjected to needle puncturing with the help from a micromanipulator (COL; n=76). Subsequently, Bl were vitrified by the Vitri-Ingá® protocol (Ingámed®, Maringá-PR, Brazil) and later they were thawed (n=212) to evaluate the re-expansion rates (embryo survival) after 3 and 24h of IVC. Re-expanded embryos were underwent to staining with TUNEL (Roche Applied Science) and Mito Tracker Red® (Molecular Probes, Invitrogen) and after they were evaluated on an inverted epifluorescence microscope (excitation of 579/510nm and emission of 599/590nm, respectively). The images of embryos stained with Mito Tracker Red® were analyzed by Q-Capture Pro Image Software, to determine the intensity of fluorescence. The difference between groups were compared by ANOVA followed by Tukey's test and the rates of re-expansion by $\chi 2$ ($P<0.05$). Experiments were repeated five times. Cleavage rate was 81.2%±2.6 and Bl rate was 42.6%±3.7. Rates of re-expansion after 3h of IVC were higher ($P<0.05$) for CONTR (91.7%) than for SAC (56.3%) and COL (40.8%). After 24h of IVC, the rates differ among the three treatments ($P<0.05$), being 86.1% (CONTR), 51.6% (SAC) and 27.6% (COL). The number of total cells were similar ($P>0.05$) among treatments (80.8±4.1 to 69.5±6.8) and the percentage of apoptotic cells were higher ($P<0.05$) in CONTR (79.7±8.1) than SAC (31.9±13.4) and COL (35.8±10.0). The fluorescence intensity was similar ($P>0.05$) among the treatments (1.0±0.1 to 0.8±0.1). Although apoptosis has decreased in those embryos that underwent the artificial blastocoele shrinkage, both methods tested in this work reduced embryonic survival after vitrification. However, the mitochondrial membrane potential and the number of total cells were unaffected in the surviving blastocysts. Results indicate that the decrease in the volume of water can be beneficial to improve the quality of the surviving embryos to cryopreservation. However, it is clear that the methodology must be improved.

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Oocyte viability associated with lipid profile of bovine follicular fluid by mass spectrometry (LC-MS/MS)

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Keywords: bovine follicular fluid, lipidomic, oocyte competence.

In vitro production of bovine embryos is an important technique among animal reproduction biotechnologies. The major challenge is to obtain similar embryo production and survival between in vitro and naturally produced embryos. Follicular microenvironment is composed by different somatic cells, follicular fluid and oocyte. These components form a functional unit, which is essential for hormones production, follicle growth, and production of oocyte with development potential. Ovarian follicle is isolated by a basement membrane that provides a single intrafollicular environment containing nutrients and signaling molecules. Lipids are signaling molecules in follicular fluid capable of influence embryo quality, and induce changes in chromatin. This study aimed to characterize the lipid profile of follicular fluid and its effect on oocytes viability. Lipids identification was performed by liquid chromatography with mass spectrometry in tandem (LC-MS/MS) and retrospective analysis of oocyte development through individual embryo development after parthenogenetic activation. We performed analysis in 30 samples of follicular fluid from routines with a blastocyst/cleaved rate of at least 35%. Experimental groups were determined according to the stage of oocyte development in non-cleaved (NCLIV), cleaved (CLIV) and blastocysts (BLAST). Lipids identified were submitted to lipid profile study by principal component analysis (PCA) and analysis of variance (ANOVA) of the principal lipids for the chemometric separation of experimental groups. LC-MS/MS identified 842 lipids in positive mode and 136 in negative mode, with significant participation in positive mode of triacylglycerols, phosphatidylcholines, sphingomyelins, mono and diacylglycerols. In the negative mode of ionization were characterized mitochondrial lipids as cardiolipins. Besides these, important cytosolic lipids and phosphatidylinositol (4,5) bisphosphate (PIP2) and phosphatidylinositol (3,4,5) triphosphate (PIP3) were identified. These lipids are glyceroglycolipids and were not previously described in follicular fluid. We also identified unique lipids between the groups, 15 lipids in NCLIV group, 7 in CLIV and 8 in BLAST. Comparative analysis, in both ionization modes, identified significant different lipids ($p<0.05$), demonstrating their potential as biological markers of oocyte quality, as cardiolipins: CL 64:6 (16:0 FA) m/z 669.4 and CL 68:6 (18:0 FA) of 697.5 m/z, monoalkyldiacylglycerol (MADAG) 50:8 NH4 + (m/z 822.8), sphingomyelin (SM) 34:2 (m/z 733.6) and sphyngolipid (GT2) 26:4 (m/z 904.9). Oocyte competence acquisition is a multifactorial process that depends on internal and external follicle factors. Our results indicate that the statistically differential lipids may be biomarkers of oocyte competence.

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Cryopreservation of bovine embryos produced in vitro using protocol with trehalose

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Keywords: cryopreservation, in vitro embryo, Trehalose.

The objective of this study was to evaluate the rates of re-expansion and hatching of bovine IVP embryos after cryopreservation with trehalose. Two-stage embryos, blastocysts and expanded blastocysts, were divided into two groups: control group, using fresh embryos (n = 191), and trehalose group, with cryopreserved embryos in D7 of the IVC (n = 192). COCs grade 1 and 2, obtained from slaughterhouse ovaries were used. The freezing step comprises cryoprotectant solution 1 - Holding medium 0.4% BSA (IMV Technologies®), 20% FBS (Gibco BRL, Burlington, Canada) and 5% glycerol (Sigma- Aldrich, St. Louis, USA) and cryoprotectant solution 2 - Holding medium 0.4% BSA (IMV Technologies®), 20% FBS (Gibco BRL, Burlington, Canada), 9% glycerol and 0.2M trehalose (Sigma - Aldrich, St. Louis, USA). Embryos were immersed for 8 minutes in each solution, loaded in 0.25-mL straws, and cryopreserved by slow-freezing method in a freezing machine (TK reprodução®), with a reduction of 1°C/minute, from 20°C to -7°C, completion of seeding, with a decrease of 0.3°C/minute, from -7°C to -32,5°C and submerged into liquid nitrogen. Thawing was performed in 35°C water bath for 20 seconds, with three solutions: 1 - Holding medium 0.4% BSA (IMV Technologies®), 20 % FBS (Gibco BRL, Burlington, Canada) and Sucrose 0.5M (Sigma-Aldrich, St. Louis, USA), 2 - Holding medium 0.4% BSA (IMV Technologies®), 20% FBS (Gibco BRL, Burlington, Canada) and 0.2 M Sucrose (Sigma- Aldrich, St. Louis, USA), 3 - Holding medium 0.4% BSA (IMV Technologies®) and 20% FBS (Gibco BRL, Burlington, Canada). After thawing, embryos were incubated at 38.7°C and 5% CO_2 in medium CR2, in the same way the embryos in the control group were kept. The evaluation was through the re-expansion and hatching at 48 and 72 hours rates, using the chi-square test to compare the control and trehalose groups in each embryonic stage. Analyzing the re-expansion rate (24h), there was statistical difference ($p < 0.01$) between the control group (n = 97/97) and trehalose group (n = 92/58) with, respectively, 100% and 63.04% of re-expansion rates of blastocysts. For expanded blastocysts, there was no statistical difference ($p > 0.05$) between the control (n = 94/93) and trehalose group (n = 100/90), with 98.94% and 90% of re-expansion rates, respectively. Hatching rates at 48 and 72 hours did not differ ($p > 0.05$) in any of the embryonic stages. The blastocysts hatching rate for the trehalose group was 69.57% (n = 92/64) and 72.83% (n = 92/67) for 48 and 72 hours, respectively; for the control group, was 78.35% (n = 97/76) and 88.66% (n = 97/86). To expanded blastocysts, hatching rates at 48 and 72 hours were 74% (n = 100/74) and 79% (n = 100/79) for the trehalose group, and 79.79% (n = 94/75) and 88.3% (n = 94/83) for the control group. We conclude that cryopreservation with trehalose was efficient, allowing a similar development of post-thawed bovine IVP embryos in comparison to fresh IVP embryos.

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Heat shock effect on expression of genes related to apoptosis, cell organization and oxidative stress in cumulus cells from *Bos taurus indicus* and *Bos taurus taurus*

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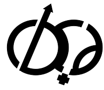
Keywords: cumulus cells, gene expression, heat shock.

Exposure of Cumulus Oocyte Complexes (COCs) to heat stress affects the necessary events for successful oocyte maturation, fertilization and pre implantation embryonic development. The bovine oocyte is direct associated to numerous layers of cumulus cells (CCs), which are important for role for growth, metabolism and oocyte maturation. Heat shock apoptosis induction has been demonstrated (Molecular Reproduction and Development, 79: 31-41, 2012) and it does affect the CCs transcriptome (Reproduction, 146: 193-207, 2013). The objective of this study was to evaluate the genotype role and temperature on expression of genes related to apoptosis (BIRC3 and DAP), cell organization (CLDN11, CCT4, DICER1 and KIF3A) and oxidative stress (MT1E) in CCs. For this, COCs were collected from *Bos taurus indicus* (Nelore- NEL) and *Bos taurus taurus* (Holstein- HPB) cows were submitted to control treatment (38.5 °C for 22 hours) and heat shock (41 °C for 12 hours, followed by 38.5 °C for10 hours) during IVM. COCs were mechanically denudated by repeated pipetting within Calcium – Magnesium free PBS 100 µl drops to CCs removal. The CCs (n = 5 replicates) were stored at -80 °C until RT-PCR. Total RNA was extracted by RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) and all CCs samples were standardized at 100 ng of RNA. Reverse transcript reaction (RT) was performed by SuperScript III kit. Target genes amplification was performed by Power Sybr®Green PCR Master Mix. Ribosomal protein L30 (RPL30) expression was used as endogenous control. Data was submitted to Analysis of Variance on SAS and considering significant differences when $P < 0.05$. Logarithmic transformation or square root was applied to obtain normal distribution when necessary. Relative abundance of mRNA of tight junctions claudin 11 gene (CLDN11) was increased at NEL CCs in relationship to HPB ($P = 0.06$). The metallothionein antioxidant molecule 1E (MT1E) expression was affected by breed ($P < 0.05$) and temperature ($P < 0.05$). Thus, the relative abundance of MT1E was greater in HPB CCs in relation to NEL. In addition, COCs exposure to heat shock reduced ($P < 0.05$) the MT1E expression at CCs in relation to control. There was no effect of breed, temperature and temperature x breed interaction for the remaining genes evaluated. In conclusion, the increased CLDN11 expression at NEL CCs suggests a role for this gene on apoptosis regulation in atretic follicles, cell migration and proliferation during antral follicles formation. In the other hand, the reduction of MT1E expression caused by elevated temperature can be associated to increasing use of mRNA for antioxidant protein synthesis.



A215 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Quantitative and qualitative lipidomic analysis of in vitro produced (IVP) bovine embryos with different developmental kinetics

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Keywords: bovine embryo, lipids, mass spectrometry.

Lipid accumulation in in vitro produced (IVP) embryos has been related to lower cryopreservation efficiency and developmental potential, being indicative of inadequate in vitro culture conditions when compared with in vivo system. The hypothesis of this work is that embryos with different developmental kinetics metabolize and pile up lipids differentially, which may be reflected in the embryo viability. Therefore, our objective was to gather quantitative, by SUDAN BLACK B staining, and qualitative, by matrix assisted laser desorption Ionization – mass spectrometry (MALDI-MS), data about the lipid composition of embryos of fast (4 cells 40hpi) and slow (2 cells 40hpi) development. The IVP bovine embryos were produced and cultured individually, classified as fast and slow according to the number cells of the first cleavages (40hpi) and finally analyzed by staining and mass spectrometry at cleavage (40hpi), morulae (96hpi) and blastocyst (168hpi) stages. For staining, we calculated the number of pixels obtained from each image and converted into arbitrary units by a script created in the development environment Matlab using the Image Processing toolbox. For staining analysis, conventional group cultured used as control. The MS data were acquired directly on single embryos, without extraction, in the positive ion mode using an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) using 2,5-dihydroxybenzoic acid (DHB) matrix to favor the ionization of the lipids. For MALDI-MS analysis, in vivo blastocysts were included as control group. Both the qualitative and quantitative data were submitted to ANOVA with Tukey post test (SUDAN BLACK) and MetaboAnalyst 2.0 (MALDI-MS) for statistical analysis. No difference in the amount of lipids between cleavage (Fast: 93884±4331; Slow: 68911±7180; Fast Control: 74622±21180; Slow Control: 70763±20046) and morulae (Fast: 65803±13774; Slow: 43511±4097; Control: 4610±12298), groups has been observed by staining, although slow blastocyst presented lower amount of lipids when compared to the fast and control groups (Fast: 122626±30378; Slow: 38617±3379; Control: 95658±15138). These data were corroborated by MALDI-MS, where the highest variability of phospolipid species (of m/z 725.5, 730.5, 732.5, 752.5, 758.5, 780.6, 782.6) was observed in fast blastocysts, while phospholipid ions of m/z 704.5, 746.5, 756.5, 788.5, 810.5 look characteristic of slow blastocysts. Based on these data, we can conclude that embryo kinetic has an important role on lipid metabolism of IVP embryos.

Acknowledgments: FAPESP, CAPES and UFABC.

A216 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Epigenetic profile - triple methylation of H3K4 - bovine embryos produced in vivo with sexed semen: preliminary results

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Keywords: epigenetics, sexed semen, superovulation.

Methylation at lysine 4 of histone 3 (H3K4), in general, is related to the active state of chromatin, allowing the access of the transcription machinery (Mellor, J. Trends in Genetics, 22:320-329, 2006). Accordingly, the objective of this study was to evaluate the epigenetic modifications of bovine embryos produced in vivo with sexed semen through the identification of trimethylation of H3K4. Girolando Cows (n=10) underwent superovulation protocol (SOV) with FSH and embryo collection was performed seven days after artificial insemination by uterine flushing. Thirty-seven procedures were carried out at intervals of 60 days, and half of them were performed with sexed semen and the other half with conventional semen from the same bull. Only embryos of excellent quality, according to the IETS standards, were used for evaluation by immunofluorescence. For this conventional (n=15) and sexed (n=15) embryos were fixed in paraformaldehyde (3%) supplemented with sucrose (2%) and stored in the refrigerator for later analysis. After blocking and permeabilization, the embryos were exposed to polyclonal antibody against trimethylated H3K4 (CellSignaling ®) at a concentration of 1:100, overnight at 4 ° C. After, the embryos were exposed to the secondary antibody (anti-Rabbit Alexa 647; Invitrogen A21245) in a concentration of 1:200, and then subjected to nuclear staining (SYBR Green, Molecular Probes, Goettingen, Germany, 1:500). Embryos were individually evaluated under confocal microscopy. The same labeling pattern was observed in all groups, indicating that embryos produced in vivo with sexed semen have the same pattern of H3K4 trimethylation as those produced with conventional semen.

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Cryopreservation of buffaloes (*Bubalus bubalis*) sperm in Tes-Tris diluter containing low-density lipoproteins in replacement of egg yolk

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Keywords: buffaloes, cryopreservation, Tes-Tris.

The objective of this research was to test different concentrations of low density lipoproteins (LDL) in replacement of egg yolk in Tes-Tris extender for cryopreservation of buffalo spermatozoa. The experiment was conducted at the Bufalloes, Experimental Farm on the UFMG, in Pedro Leopoldo, MG. Six Murrah bulls, aged 29-36 months, were used to collect semen (through artificial vagina) 3 ejaculates from each animal, performing a total of 18 ejaculates were collected. Total motility was analyzed by a Sperm Class Analyzer-SCA® v.4.0. Sperm concentration was evaluated by Neubauer chamber, sperm morphology analysis (phase contrast microscope) and the hypo-osmotic test (Host) was performed in fresh semen samples. The ejaculates were fractioned and each fraction was diluted in different extender. Dilution was performed to reach a final concentration of 50x106 spermatozoa mL-1. Treatments were: control (20% egg yolk) and 2, 4, 8 and 14% (v/v) LDL. Extender semen was packaged into 0.25 mL straws starting there after the cooling process using -0.25oC/min cooling rate (computer assisted cooling machine TK 4000). Samples were maintained in equilibrium at 5°C for 4 hours. Straws were frozen in liquid nitrogen vapor, 5 cm above the liquid surface, for 20 minutes. There after straws were immersed in liquid nitrogen and stored for a minimum of one week. After thawing (37oC for 30 seconds) straws contents were submitted to a thermal resistance test (TRT/120 minutes) evaluated by a CASA. Sperm cells were evaluated by a hypo-osmotic swelling test and integrity of the membranes by fluorescent probes (CFDA/Pi). Results were compared by the Tukey test ($P<0.05$). Pre-cooling sperm motility was less in samples diluted in the control extender containing egg yolk than in 2 and 4% LDL extenders. Post-thaw sperm motility was higher (56.53±9.73%) in the control extender than in all extender containing LDL (47.16±11.06%, 45.64±7.46%, 45.71±6.56 %, 43.33±5.97%, for 2, 4, 8 and 14% LDL extenders, respectively). However, during the TRT sperm motilities were similar between all extenders. Values of LDL, VCL, VSL, VAP, LIN and BCF were lower in sperm diluted in the control extender than in extenders containing LDL, during the pre-cooling phase, post-thaw and throughout the TRT. There was no difference between treatments regarding the integrity of sperm membranes (38.75 to 43.14% intact) and the reaction to the Host (49.39 to 55.39% reacted). It can be concluded that 2, 4 and 8% LDL concentrations in Tes-Tris extender, were more effective than Tes-Tris containing 20% of egg yolk for increase the kinetics characteristics of buffalo sperm cells.

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Ovulation influence on oviductal genomic profile in Nelore heifer (*Bos taurus indicus*) and angus (*Bos taurus taurus*)

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Keywords: antimere of ovulation, follicular count, oviduct.

The oviduct plays a key role to promoting a favorable microenvironment to gametes transport, fertilization and early embryo development. Numerous differences in reproductive physiology are known among animals of Zebu and European breeds. Reports indicate that female Zebu cattle have a higher number of follicles per wave than female European cattle and individual distinctions in the number of follicles recruited are present in both breeds, namely animals with high follicular count (HFC) and low (LFC). Furthermore, the follicular count is related to animal fertility and is greatly influenced by the activity of FSH, estradiol and androgens. However, little is known about the effects of follicular count differences between Zebu and European cattle, and between breeds in the oviduct molecular profile. Based in these information, we hypothesized that differences in bovine breed (Nelore and Aberdeen Angus), differences in the follicular count (FC) and differences in the antimere related to ovulation (ipsilateral and contralateral) alter the molecular profile of genes involved in oviductal functions during the initial period after ovulation. To do so, oviduct from Nelore heifer (HFC, n=4; LFC, n=4) and oviduct from Aberdeen Angus heifer (HFC, n=4; LFC, n=4) were isolated and oviductal segments were divided (infundibulum, ampulla, isthmus) from ipsilateral and contrateral antimere. Total RNA was extracted using Illustra TriplePrep Kit (GE Healthcare, USA) and then Reverse Transcription was performance using High Capacity cDNA kit (life Technologies, USA), according to the manufacturer's protocols. Relative RT-qPCR analysis was performed with TaqMan® Low Density Array (TLDA, Life Technologies, USA). The mRNA abundance of the target was tested by ANOVA analysis, using PROC GLM procedure of SAS (SAS, 9.2, SAS Inst., Cary, NC, USA). Individual differences were analyzed through pair-wise comparisons (SAS). All the comparisons were performed in each segment (ampulla, infundibulum and isthmus), no comparisons were performed between segments. The differences were considered significant when $P<0.05$. In the ampulla, the mRNA abundance of COX2, OVGP1, GPR78, FUCA1 and ANXA4 showed higher levels in ipsilateral antimere compared to contralateral. Similarly, in the infundibulum the mRNA abundance of GRP78, PGTER4, FUCA2 and FUCA1 was higher in ipsilateral antimere. No difference was found in the isthmus. In conclusion, the breed and the follicular count have no effect on the molecular profile of bovine oviduct, suggesting the ovulation has the main effect in gene expression related to gametes transport, fertilization and early embryo development.

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A219 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Lipid profile of bovine oocytes and embryos by MALDI imaging

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Keywords: embryo, MALDI imaging, oocyte.

In the field of "single cell analysis", many classical strategies like immunoflurescence and electron microscopy are the fundamental choices. However, these methodologies are time consuming and do not permit direct identification of specific molecular classes such as lipids. This study was conducted to determine a new mass spectrometry based analytical approach to bovine oocytes and embryos in vitro produced. Images and mass spectra were acquired in a MALDI-LTQ-XL instrument with imaging feature (Thermo Scientific, California, USA). It was equipped with a UV laser (Nd:YAG, 355 nm) at "minimum" focus. Imaging data were analyzed in quadruplicates using ImageQuest software (Thermo Scientific, California, USA). A new metabolomics based application uses Mass Spectrometry Imaging (MSI), efficient data processing and multivariate data analysis. Metabolic fingerprinting (MF) was applied to the analysis of bovine occytes and blastocysts. A semi quantitative strategy for sphingomyelin [SM (16:0) + Na]+ (m/z 725) and phosphatidylcholine [PC (32:0) + Na]+ (m/z 756) was developed, showing that lipid concentration was useful for selecting the best biomarkers. This information furthers our understanding of fertilization and pre implantation events during bovine embryo development.

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Altered expression of GDF9 and FGF16 suggests a role in the low developmental competence of repeat breeder oocytes during summer

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Keywords: heat stress, oocyte maturation, repeat breeder.

The aim of this study was to investigate whether the poor oocyte competence verified for repeat breeder (RB) cows during summer (Ferreira et al., JDS 94:2383-92, 2011) might be associated to altered expression of genes related to oocyte maturation (GDF9, BMP15, FGF8, FGF10, FGF16 and FGF17). These genes encode local factors secreted by the oocyte that are essential to control an adequate maturation of the gamete. The oocytes were collected by OPU from 74 heifers (H), 73 peak lactation (PL) and 71 RB Holstein cows from two commercial dairy farms during the summer and winter. Ten to fifteen percent of the viable oocytes of each animal (total of 100 oocytes) was used to study the expression of selected genes, and the rest was sent to IVP (total of 1,493). Ten reference genes were analyzed with the geNorm application and three of them (RPL15, PPIA and GUSB) were determined as the most stable (Macabelli et al. 2014) and were used herein as references for normalization of target genes. Quantification of gene-specific mRNA transcripts was carried out by real-time reverse transcription (RT)-PCR using TaqMan assays. Data were tested for normality of residuals and homogeneity of variances followed by ANOVA using the GLIMMIX procedure of SAS. Amplification of FGF17 failed in all samples, leading to its exclusion from further analysis. An increased expression of BMP15 ($P < 0.0001$) and FGF8 ($P < 0.0001$) was observed during summer, regardless of category, when expression of targets was normalized by the reference genes. On other hand, in the absence of normalization, expression of BMP15 ($P = 0.006$) and FGF10 ($P = 0.004$) decreased from winter to summer, regardless of animal category. Furthermore, expression of FGF16 decreased during winter in RB compared to H ($P = 0.01$) and PL ($P = 0.03$), as well as it decreased from winter to summer in H ($P < 0.0001$) and PL ($P = 0.0003$). Yet, expression of GDF9 increased during summer in RB compared to H ($P = 0.04$) and PL ($P = 0.01$), and decreased from winter to summer in H ($P < 0.0001$) and PL ($P < 0.0001$). Together, these data provide evidence that GDF9 and FGF16 were differentially expressed in RB oocytes during summer and winter, respectively, suggesting a possible role of them in the low developmental competence of RB oocytes. This is further supported by the finding that GDF9 ($P < 0.05$) and FGF16 ($P < 0.05$) correlated positively with the number of total oocytes ($r = 0.27$ and 0.24, respectively), number of viable oocytes ($r = 0.27$ and 0.23) and blastocyst rate ($r = 0.24$ and 0.21). Thus, altered expression of GDF9 and FGF16 suggests that the low development competence of RB breeder oocytes during summer may be related to disrupted maturation of these gametes.

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A221 Supporting Biotechnologies: Cryopreservation and Cryobiology, Image Analysis and Diagnosis, Molecular Biology and "Omics"

Expression of LH receptor isoforms during follicle deviation in cattle

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Keywords: alternative splicing, LHR, transcript.

The aim of this study was to evaluate the changes in the expression of LHR isoforms associated with follicle divergence in cattle, using dairy breeds with different dominant follicle sizes at deviation as models. Granulosa cells (GC) were collected using an adapted ultrasound-guided follicular aspiration system (Arashiro et al., Reprod Biol Endocrinol, 11:73, 2013) from follicles of 6, 8, 10 or 12 mm in diameter of Holstein, and of 4, 6, 8 or 10 mm of Gir heifers. The recovered follicular fluid was centrifuged and the cells were washed with NaCl 0.9% saline and kept in RNA Later (Ambion). Total RNA extraction was performed using a commercial RNeasy Micro Kit (Qiagen), quantified in spectrophotometer (Nanodrop), and reverse transcribed using the commercial Superscript III kit (Invitrogen). The samples were tested for theca cell contamination using a primer to detect the CYP17A1 gene, and those showing contamination were excluded from analysis. The cDNAs were PCR amplified to screen for the presence of isoforms. Our previous results showed that follicles from all diameters evaluated and from both breeds presented the full-length form and/or the isoforms screened (Wohlres-Viana et al., Reprod Fertil Dev 26:176, 2014). In the present study, the relative expression of these isoforms during follicular development was evaluated. The cDNAs were submitted to Real Time PCR using three primers specific for each isoform (S10, S11, and S10+11 primers). The beta-actin gene was used as an endogenous control, and the results were analyzed using the REST software. Expression values are shown as mean±SEM. The expression results in samples from 10 mm in Holstein and from 8 mm follicles in Gir heifers were standardized as 1.00 and this value was used for further comparisons within breed. For Holstein, the S11 isoform was under-expressed ($P<0.05$) in 10 mm follicles, when compared to 8 mm (11.69±5.11) or 12 mm follicles (21.59±8.76). The same pattern was observed for Gir samples, in which the S11 isoform was under-expressed ($P<0.01$) in 8 mm follicles, when compared to 6 mm (230.05±213.16) or 10 mm follicles (5,474.99±5,560.00). Although not significant, the same trend was observed for S10 and S10+11 isoforms. In both breeds, this reduction in isoform expression occurred in follicles sampled after deviation, when the transition from FSH to LH dependence is expected to take place. Our previous results demonstrated that the expression of the LHR was maximum at 10 mm and 8 mm follicles in Holstein and Gir, respectively (Wohlres-Viana et al., Anim Reprod 10:425, 2013), suggesting a predominance of the full-length form of the LHR at this moment. Altogether, these results suggest a possible role of the modulation of LHR isoforms expression in the fine regulation of LHR function during follicle deviation in cattle.

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