A032 Folliculogenesis, Oogenesis and Ovulation

PI3K-Akt signaling pathway association with oocyte competence as indicated by mirna profiling and quality assessment in cattle

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Keywords: microRNA, oocyte competence, PTEN and Akt.

In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes, and therefore, regulate many signaling pathways and cellular process. We hypothesized that intracellular pathways highly regulated by miRNAs in follicular cells are involved in acquisition of oocyte competence. To test this hypothesis slaughterhouse ovaries (3-6mm) were dissected and ruptured for recovery of granulosa cells (GC) and cumulus-oocyte complex (COC), these cells were counted and placed in culture for 96 hours and then used to RNA extraction. A total of 351 miRNAs were profiled in GC and COC in order to identify miRNA-regulated pathways acting into the follicular microenvironment. Were identified 305 miRNAs present in both cell types, GCs and COCs, 8 unique in GCs and 13 exclusive in COCs. Bioinformatic analysis of unique miRNAs for each cell type showed 69 and 85 pathways, respectively, are predicted to be miRNAs regulated. One of the identified pathways, both in GC and COC, was the PI3K-Akt signaling pathway. To test its association with oocyte competence we dissected bovine ovarian follicles ranging from 3-6mm and recovered follicular cells (GC and cumulus cells) and oocytes; the former was stored and the latter were assigned for individually in vitro matured (IVM), parthenogenetically activated and in vitro cultured (IVC) until the blastocyst stage. Individual IVM, activation and IVC were performed in order to track follicular cells, obtained after dissection, with the oocyte fate after activation as follows: non-cleaved oocytes (Ncleav group), mature oocytes that cleaved and did not reach blastocyst stage (Cleav group) or that reached blastocyst stage (Blast group). Follicular cells from these three groups were used to search for the following PI3K-Akt pathway components: phosphorylated-Akt (p-Akt) protein levels (a positive regulator of PI3K-Akt pathway) and PTEN mRNA levels (a negative regulator of PI3K-Akt pathway. Determination of p-Akt levels indicated higher ($p=0.06$) levels of Akt in the Blast group (0.74 ± 0.33) compared to Cleav group (0.45 ± 0.39), while both were equal to Ncleav group (0.59 ± 0.43). PTEN expression in follicular cells from the Blast group (0.60 ± 0.10) was lower ($p<0.05$) than Cleav (2.42 ± 1.55) and Ncleav (2.23 ± 0.47) groups. Taken together these results demonstrated that the miRNA expression profile in follicular cells consists in a useful tool to identify putative molecular pathways involved in oocyte competence acquisition. This principle was given proof by the determination of p-Akt and PTEN levels in follicular cells from ovarian follicles carrying oocytes with distinct developmental competence, which indicate the association of PI3K-Akt signaling pathway activation with oocyte competence.

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Evaluation of glucose and lactate production by canine luteal cells in early cyclic and gestational diestrus

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Keywords: canine, glucose, luteal.

There is great similarity in several aspects between the cyclic and pregnant canine luteal phase. In order to investigate the metabolic pathways of luteal cells in early cyclic and gestational diestrus, we aimed to analyze glucose and lactate production by these cells cultured in vitro. Ovariohysterectomy was performed in 6 females, 3 in early gestational diestrus and 3 in initial cyclic diestrus. Corpora lutea (CL) were enzymatically digested in a solution containing 5 ml DMEM (Dulbecco's Modified Eagle's Medium - high glucose, Sigma -Aldrich, Germany) and 0.0075g collagenase type 1 (Collagenase from Clostridium histolyticum Type I, Sigma -Aldrich, Germany). After one hour at 37.5°C and vortexing every 15 minutes, the cell suspension was filtered (Filter with 70 µm, BD Falcon®, USA), centrifuged 3 times (340G, 220G and 100G respectively at 20°C for 10 min) and resuspended in 12 ml DMEM supplemented with antibiotic, antifungal, L-glutamine (Sigma -Aldrich, USA) and fetal serum bovine (Sigma -Aldrich, EUA). The solution containing luteal cells (experimental solution) was distributed in 24 well plates and incubated in controlled atmosphere (containing 5% CO2 and 95% air). After 24 hours, wells were washed with HBSS (14,175,079; Gibco BRL) and medium replaced. A plate containing only culture medium was used as control (control solution). Experimental and control solution samples were collected 36 (moment 1), 48 (moment 2) and 60 (moment 3) hours after the start of culture, stored in plastic tubes and kept in a freezer at -80°C. Glucose and lactate levels were assessed using VITROS Chemistry Products Calibrator kits (Products Vitro Chemistry, United Kingdom). The statistical analysis was performed using ANOVA ($p < 0.05$) in SAS PROC GLM. We observed that glucose consumption and lactate production increased during in vitro culture in both gestational luteal cells and cyclic diestrus, but glucose consumption and lactate production in gestational CL was greater than cyclic CL at initial (glucose in moment 1, $p=0.0328$ and lactate in moment 2, $p=0.0221$) and final (glucose and lactate in moment 3, $p=0.0085$ and $p=0.0009$, respectively) culture stages. According with these results, we believe that there is no difference in the metabolic pathways used by pregnant and cyclic luteal cells. However, at initial diestrus, energy metabolism appears to be greater in pregnant than cyclic CL.

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Evaluation of estrous cycle of rats submitted to physical activity and growth hormone

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Keywords: growth hormone, physical exercise, vaginal cytology.

Growth hormone (GH) has been used indiscriminately in order to increase muscle mass. Considering that GH can act on different cell types, including reproductive organs, the aim of this study was to assess the effect of GH treatment, with or without physical activity, on estrous cycles of female rats. Forty female rats aging 9 weeks were sorted into 4 groups: CT (control); Ex (submitted to resisted training); GH (treated with GH); and ExGH (submitted to resisted training and GH treatment). Treated animals received 0.2 IU/Kg of GH every two days, while the others received saline solution at the same volume. Resisted training was performed 3 times a week, and consisted in four series of 10 jumps in the water with 1 minute interval between them. Animals had a vest with 50% of their weigh during the exercise. All rats were submitted to vaginal cytology analysis for 7 days before the experiment to confirm cyclicity. Vaginal cytology was performed daily in all rats for 29 days. Vaginal smears were stained with Panótico® and observed in a microscope. Data normality was tested using the Shapiro-Wilk test, and statistical analysis was performed by ANOVA followed by Tukey's test ($p < 0.05$). No statistical differences were observed between experimental groups for the duration of the luteal and follicular phases of the estrous cycle. However, more cycles were observed in the CT group (7.10 ± 0.88) compared to the other groups: GH (6.10 ± 0.99, $p = 0.04$); Ex (6.10 ± 0.57, $p=0.02$) and ExGH (6.00 ± 0.67, $p = 0.01$). Therefore, it was concluded that GH treatment and resisted training decrease the frequency of estrous cycles during a 29 days period.

A035 Folliculogenesis, Oogenesis and Ovulation

Ultrasound evaluation of ovarian dynamics in Indubrasil cows submitted to two nutritional managements

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Keywords: estrous cycle, feeding, reproduction.

The objective of this study was to compare ovarian dynamics of Indubrasil cows submitted to grazing or confinement regimes. Follicular growth was monitored daily starting at estrus during two consecutive estrous cycles in seven adult non-lactating Indubrasil cows with body score 4 (classification 1 to 5), in 2x7 experiment. The first evaluation was in the grazing regime, when animals were kept in pastures of *Brachiaria brizantha* cv Marandu intercropped with *Leucaena leococephala*, with water and mineral salt ad libitum. The second evaluation was performed during the following estrous cycle when animals were submitted to the confinement regime, and fed with corn silage, proteinated mineral salt and water ad libitum. An ultrasound HS 1500 (Honda®, Japan) equipped with a linear 7.5 MHZ probe was used to assess follicle and luteal dynamics. Data were recorded according to the day of the cycle and are presented as means ± standard deviation. Means were compared by T Tests and differences considered significant when $P<0.05$. The duration of the first estrous was 20±1.6 and 21±1.22 days for grazing and confined animals, respectively. The number of follicular waves was 2±0.49 and 3±0.40 and their duration was 9±1.99 and 9±1.65 days, for grazing and confined animals, respectively. The maximum diameter of the ovulatory follicle was larger in confined (15±0.51mm; $p<0.05$) than grazing animals (13±0.87mm). For subordinate follicles, the maximum diameter was 9±1.13mm and 9±0.82mm for grazing and confined animals, respectively. The dominant follicle persisted for 7±1.41 and 6±0.82 days in grazing and confined animals, respectively. Finally, the persistence of the corpus luteum was 14±1.59 and 15±1.06 days for grazing and confined animals, respectively. These data suggest that diet influences growth rate and size of the ovulatory follicle in Indubrasil cows. We speculate that greater dry matter intake in the confinement regime may have promoted an increase in the metabolism of steroid hormones, influencing dominant follicle growth. This abstract describes for the first time follicle dynamics in Indubrasil cows, which was found to be very similar to other *Bos indicus* breeds.

A036 Folliculogenesis, Oogenesis and Ovulation

Characterization of ovarian follicle reserve depletion in Ames dwarf mice

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Keywords: aging, follicles, ovarian reserve.

Ames dwarf mice are deficient in growth hormone (GH) secretion and therefore have reduced levels of insulin-like growth factor type I (IGF-I). Their life expectancy is around 30-50% longer than normal mice (Masoro; Mech Ageing Dev; 2005; 126: 913-22). Studies have shown that the IGF-I signaling pathway is involved in the regulation of follicular growth and development (Ahmed & Farquharson; J Endocrinol, 2010; 206: 249-59). Therefore, the aim of this study was to evaluate the number of follicles in the primordial, primary, secondary and tertiary stages, as well as follicular and oocyte diameters in Ames Dwarf and Normal mice. Ovaries of normal (n=3) and Ames Dwarf (n=4) mice at 12 months of age were used. Ovarian samples were processed, sequentially cut and stained with hematoxylin-eosin. Ovarian sections were assessed in an optical microscope using 10x and 40x objectives for the classification, quantification and measurement of ovarian structures. One of each six histological sections per slide was evaluated. Statistical analyzes was performed using t-tests with the GraphPad Prism 5 software (La Jolla, CA, USA). Differences were considered significant when $P<0.05$. Ames dwarf mice presented ($P=0.001$) more primordial follicles (1548 ± 139) compared to normal mice (378 ± 125). These data indicate that primordial follicles are not progressing to the primary stage, possibly due to reduced serum levels of IGF-I and insulin, indicating that these mice can be a good model to study the relationship between metabolic status and ovarian aging. The number of secondary and tertiary follicles was not different between Ames dwarf and normal mice ($P>0.05$). The total number of follicles tended ($P=0.07$) to be higher in Ames dwarf (2,673 ± 209) than in normal mice (1,668 ± 445). Regarding follicle and oocyte diameters, we only observed a larger diameter ($P=0.02$) for oocytes included in primordial follicles from normal (6.8 ± 0.4 μm) compared to Ames Dwarf mice (5.5 ± 0.3 μm). In conclusion, Ames Dwarf mice have more primordial follicles compared to normal mice, suggesting that GH, IGF-I and insulin deficiency leads to the accumulation of follicles in the primordial stage and increase ovarian longevity.

A037 Folliculogenesis, Oogenesis and Ovulation

Green tea influence on VEGF expression in the rat ovary

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Keywords: *Camellia sinensis*, corpus luteum, reproduction.

Green tea is derived from *Camellia sinensis* and considered a healthy drink as it is associated with prevention of cancer, cardiovascular disease and osteoporosis. It contains catechins, which appear to play antioxidative, antibacterial and antitumoral roles. Despite many potential benefits of green tea consumption, the catechins present in green tea can significantly inhibit proliferation, steroidogenesis and vascular endothelial growth factor (VEGF) production by swine granullosa cells. Therefore, it is important to assess possible reproductive-related consequences. The aim of this work was to assess green tea influence on VEGF expression in the rat corpus luteum. Wistar rats were divided in two groups: the control group (n=30) had access to water ad libitum, and in the treated group (n=30) water was replaced by a commercial green tea (Amor à Vida®, Amor à Vida Produtos Naturais, Brazil) at 2.5% ad libitum as previously performed by Yang et al. (Eur J Cancer Prev, 12:391–395, 2003) and Niwattisaiwong et al. (Drug Metabol Drug Interact., 20:43-56, 2004). Rats were kept in plastic boxes (5 animals per box) with free access to beverage and food. This study was approved by the local ethics committee. The experiment lasted for three months and at the end of each month 10 animals of each group were superovulated with 150IU/Kg of eCG (Folligon®, Intervet Schering-Plough, Brazil) and 150IU/Kg of hCG (Vetecor®, Hertape Calier, Brazil) and killed. Messenger RNA was isolated from corpora lutea with TRIzol® (Life Technologies, Brazil) and reverse transcription was performed with SuperScript III RT Kit (Life Technologies, Brazil) and Oligo (dT) primers (Life Technologies, Brazil). Abundance of VEGF mRNA was assessed by qPCR using TaqMan Ral-Time PCR Master Mix (Life Technologies, Brazil) and HPRT-1 as the housekeeping gene. Relative VEGF mRNA abundance was calculated using the ΔΔCT method and Pfaffl's method for efficiency correction (Nucleic Acids Research, 29:2004-2007, 2001). Statistical analysis was performed with unpaired t tests and the moments for each group were compared by analysis of variance. Differences were considered significant when $p<0.05$. Green tea consumption per rat per day was greater (30.73±0.49) than water consumption (29.13±0.50). No differences were observed between groups for VEGF expression. Mean values and standard errors for VEGF mRNA abundance were: first month (control = 1.07±0.13 and treated = 1.05±0.14); second month (control = 1.09±0.14 and treated = 0.94±0.11); and third month (control = 1.05±0.11 and treated = 0.73±0.10). In conclusion, consumption of green tea for three months did not alter abundance of VEGF mRNA in the rat corpus luteum.

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Estradiol concentration and gene expression of mRNA for CYP19A1, PAPP-A and LHR in dominant and subordinate follicles at follicle deviation in Nelore cows

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Keywords: bovine, follicle selection, gene.

The main objective of this experiment was to investigate expression of mRNA of genes associated with follicle deviation, *CYP19A1* (aromatase), LH receptor (*LHR*) and *PAPPA* (Pappalysin 1), using an *in vivo* model to obtain granulosa cells. Nelore (NEL; n = 10), non-lactating, 3-6 years old females, had follicle wave emergence synchronized by transvaginal aspiration of follicles ≥5 mm in a cross-over design. At the same time, they received an intravaginal progesterone device (CIDR®, Zoetis, Brazil) and 24h later, two doses with 0.5 mg of cloprostenol with 12h interval, with luteolysis monitored by ultrasound. Ovaries were evaluated every 12h to characterize follicular dynamics. A model based on follicular aspiration was used, and cows were distributed among three treatments: group 0h, when the largest follicle reached 6.5 mm, the two largest follicles were aspirated (DF0h and SF0h); group 12h, the two largest follicles (DF12h and SF12h) were aspirated 12h after the largest follicle reached 6.5 mm; and deviation group, the largest follicle (DF0h) was aspirated when it reached 6.5 mm, and the second largest follicle (SF→DF) was aspirated 12h later. Granulosa cells were obtained by washing the follicle cavity by successive aspiration and ejection with 1 mL of sterile saline solution, using an aspiration system with double lumen. The suspension was centrifuged and the supernatant was stored. Follicular fluid estradiol-17β (E2) concentration was measured by ELISA. The cell pellet was suspended in lysis buffer from RNeasy kit (Qiagen, SP, Brazil) and mRNA expression was analyzed by RT-PCR for *LHR*, *CYP19A1* and *PAPPA*. Statistical analysis for follicular dynamics data and gene expression was performed using the PROC MIXED of SAS. Samples contaminated with blood or E2 concentration below 1 ng/mL were removed from the analysis. Follicular fluid E2 concentration (ng/mL) from SF→DF (176.9 ± 48.8; n = 4) was higher (P≤0.02) than from SF0h (34.2 ± 35.8; n = 7) and SF12h (28.9 ± 46.0; n = 4), and did not differ (P>0.05) from DF0h (188.9 ± 30.3; n = 7) and DF12h (244.4 ± 46.0; n = 4). There was no difference (P>0.05) among groups in relative mRNA expression for *PAPPA* (DF0h: 0.14 ± 0.05; SF0h: 0.14 ± 0.07; SF→DF: 0.31 ± 0.08; DF12h: 0.14 ± 0.08, and SF12h: 0.14 ± 0.08) and *CYP19A1* (DF0h: 0.38 ± 0.13; SF0h: 0.27 ± 0.15; SF→DF: 0.49 ± 0.20; DF12h: 0.47 ± 0.19, and SF12h: 0.06 ± 0.19). There was a tendency for greater *LHR* mRNA expression in SF→DF follicles (3.86 ± 1.43) compared to group 0h (DF0h: 0.56 ± 0.86, P=0.07, and SF0h: 0.46 ± 1.08; P=0.06). There were no differences in gene expression between SF→DF and the 12h group (DF12h: 5.71 ± 1.43 and SF12h: 1.04 ± 1.43). DF12h *LHR* expression was highest in groups 0h (DF0h and SF0h; P=0.01) and SF12h (P=0.05). We concluded that increased expression of the LH receptor was the main early mark of dominance in Nelore cows.

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Extracellular vesicles contents isolated from bovine ovarian follicles: association with oocyte competence

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Keywords: bovine, exosomes, follicular fluid.

Intercellular communication is crucial to induce cell proliferation and differentiation during follicle growth. One consequence of follicular growth is production of a viable oocyte capable to generate a pregnancy. Part of the crosstalk occurs in the follicular fluid (FF). Extracellular vesicles (EVs) such as exosomes and microvesicles were identified within the follicular fluid and can mediate cell communication. Extracellular-vesicles can transfer bioactive molecules such as lipids, proteins, mRNA and miRNAs. Our hypothesis is that exosomes from bovine follicular fluid present coding RNA molecules and different lipid composition associated with oocyte competence. In order to test this hypothesis bovine ovarian follicles, between 3-6 mm were individually isolated. Follicle contents were separated under a stereomicroscope to allow the collection of FF and the cumulus-oocyte-complex (COC). Follicular fluid was submitted to differential centrifugation for removal of cellular components and debris before freezing at -80°C. COCs were in vitro matured for 18h and then evaluated for the presence of the 1st polar body. Activation of the denuded oocytes to generate parthenogenetic embryos started 26h after the beginning of maturation. After seven days in culture we accessed the oocyte competence and grouped the follicular fluid according to developmental competence. Developing groups were based on the ability or inability of the oocytes to mature and generate a blastocyst. Based on the competence groups we isolated EVs from pools of 10 follicular fluids. Total RNA and lipids were extracted from the pools of EVs and analyzed by Next Generation Sequencing and tandem mass spectrometry. Transcriptome analysis demonstrated the presence of coding RNA species including HDAC2, HDAC10, EIF4E, EIF2B e BRCA1. Functional annotation analysis of exosomal RNA content demonstrated to be enriched for RNA molecules involved in the regulation of chromatin remodeling or transcription activation. Based on lipids analysis we identified different lipids enriched according to the development competence of the oocytes. We identified six lipids associated with poor oocyte competence; monoalkenyl diacylglycerol (MADAG 52:8+NH4 (-FA 18:1 (NH4) and MADAG 48:8+NH4 (-FA 16:1 (NH4) are examples of the lipids found in extracellular vesicles. Three lipids were associated with oocyte capability to generate a blastocyst, for example Digalactosyldiacylglycerol (DGDG 36:2+NH4 (-DGDG (NH4)), which was identified present in extracellular vesicles. Thus, our results demonstrated that EVs carry coding RNA molecules involved in chromatin regulation. Also, lipids identified are differently expressed in EVs and are associated with oocyte competence. Further experiments are necessary to explore the different lipid molecules present in the EVs and their role during follicle growth and oocyte maturation.

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A040 Folliculogenesis, Oogenesis and Ovulation

Correlation between phenotype, genotype and antral follicle population in *indicus-taurus* heifers

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Keywords: antral follicles count, genetic improvement, heifers.

The objective of this study was to assess the potential correlation between phenotypic and genotypic characteristics with antral follicle count (AFC) in *indicus-taurus* heifers. Braford heifers (Nelore 3/8 x 5/8 Hereford, 18-24 months n = 270) from an genetic improvement program, Conexão Delta G, were evaluated by transvaginal ultrasonography micro convex transducer (7.5 MHz) on a random day of the estrous cycle to determine AFC (follicles > 3 mm diameter), according to Burns et al. Biology of Reproduction (2005). After AFC evaluations (average of 26.81 follicles), the number of antral follicles was correlated with genetic selection parameters using 4 statistical models. In model 1 (n = 270) we consider the effect of contemporary group (CG) and the covariates: age (AG); weight gain from birth to weaning (GW); visual scores for conformation at weaning (CW), precocity at weaning (PW) and musculature at weaning (MW). In Model 2 (n = 270) we considered the effect of CG and covariates: AG; weight gain from weaning to yearling (GY); visual scores for conformation at yearling (GY), precocity at yearling (PY) and musculature at yearling (MY). The effect, variables and covariates of model 1 and 2 were combined to form model 3 (n = 270). Model 4 (n = 270) consisted of the same parameters of model 3 with the inclusion of the paternal effect (sire). Data were analyzed by linear regression using the GLM procedure of SAS and adopting $P < 0.05$. From four models tested, the variables and covariates at yearling (model 2) had no effect on AFC ($P > 0.05$) and the coefficient of determination (R2) was 0.056. The other models also showed low correlation with AFC: 0.072, 0.082 and 0.172 for models 1, 3 and 4, respectively. The model with paternal effect was the correlation with the highest score considering genotypic and phenotypic characteristics and AFC. Models 1, 3 and 4 also showed that AFC of indicus-taurus heifers can be influenced by precocity at weaning ($P < 0.05$). Based on this study, there is no correlation between phenotypic and genotypic characteristics with the antral follicle population from *Bos indicus-taurus* heifers. However, AFC can be affected by precocity at weaning. Additionally, we encourage the use of AFC for in vitro embryo production, since there is a quantitative benefit on the number of embryos produced. However, we highlight the genetic merit as the most important criteria for all reproductive techniques.

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Regional distribution of preantral follicles in equine ovaries

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Keywords: ovary, preantral follicles, region.

Isolation and in vitro culture of preantral follicles in equine ovaries is an emerging technique with several challenges. In addition to the shortage of slaughterhouses for this species, the occurrence of ovarian fragments without preantral follicles is very common. The objective of this study was to define specific ovarian regions in order to increase the probability of a high concentration of follicles from harvested fragments. Ovaries (n=3) of mares in seasonal anestrus were obtained from a local slaughterhouse and transported to the laboratory. Each ovary was divided into two hemiovaries (longitudinal section). Each hemiovary was then sectioned in three parts, each covering the entire length, with the first cut in the region of the small curvature of ovary (C1; near ovulation fossa); the second in the intermediate region between the smaller and larger curvatures (C2; parenchymal region) and the third cut contemplating the greater curvature of the ovary (C3; external region). After that, fragments were immediately fixed in Bouin, kept in 70% ethanol and then processed for histology. A total of 900 slides was prepared with 2.700 histological sections 5 micrometers thick. All preantral follicles containing one oocyte were counted regardless the stage of development (primary, secondary or tertiary) and morphological integrity (normal or degenerated), making the total of 1,514 follicles. Data were submitted to simple logistic regression test ($P \leq 0.05$). All regions showed differences ($P < 0.05$), and the highest proportion of follicles was found close to the small curvature of the ovary, near the ovulatory fossa, with 41.3% of the follicles (625/1514; SD=0.49); the intermediate region had 36.9% of the follicles (559/1514; SD=0.48) and the smallest follicular density was found in the external region, close to the ovarian greater curvature with 21.8% (330/1514; SD= 0.41). This study demonstrates that equine preantral follicles are concentrated in specific regions in the ovary, and a better understanding of this aspect can increase the efficiency of protocols for isolation and culture of preantral follicles in is species.

A042 Folliculogenesis, Oogenesis and Ovulation

Effect of diet on *in vivo* embryo production of non-lactating and non-pregnant Holstein cows

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Keywords: embryo, energy source, superovulation.

The aim of the study was to evaluate the effect of two isocaloric and isonitrogenous diets, a corn-based and a citrus-pulp based diet (both mixed with sugarcane bagasse), on the quality and quantity of embryos produced by 25 non-lactating and non-pregnant Holstein cows. The hypothesis is that a diet generating higher proportion of propionate relative to acetate in the rumen, as citrus pulp, would have positive effects on embryo production. The study was performed in a crossover design with two 70-d long periods each. Two superovulatory (SOV) treatments were performed on each period with a 35-d interval between them, in order to evaluate the acute and chronic effects of energy sources. An adaptation diet was provided for 14 d before the beginning of the experiment and for more 14 d between the two periods. Diet was balanced for maintenance and was offered individually at 1% of body weight in dry matter. Cows were weighed every 14 d. The protocol used for SOVs included eight decreasing doses of FSH (Folltropin-V, Bioniche, Canada), 300 mg i.m in total. Frozen semen of three Holstein bulls, which were balanced between treatments, was used. Seven d after AI the embryos were collected according to the procedure described by Castro Neto et al. (Theriogenology, v.63, p.1249-1255, 2005) and classified according to the stage of development (Souza et al., Animal Reproduction, v.4, p.70-76, 2007) and quality: Grade I and II (freezable), Grade III, degenerate and unfertilized oocytes (UFO). Ultrasonography evaluations were performed at the end of the SOV protocol to count the number of follicles ≥8 mm. Data were evaluated by the ProcMixed of SAS, with a 0.05 significance level. There was no change on body weight throughout the experimental period. No difference was detected on the number of freezable embryos per cow between corn and citrus pulp treatments (2.5 ± 0.79 vs. 3.4 ± 0.77, respectively; $P = 0.24$), Grade III embryos (0.8 ± 0.22 vs. 0.5 ± 0.22; $P = 0.22$), viable embryos (Grades I-III; 3.6 ± 1.01 vs. 4.0 ± 0.99; $P = 0.96$), degenerate (2.3 ± 0.48 vs. 2.4 ± 0.47; $P = 0.85$) and UFO (5.0 ± 1.11 vs. 3.8 ± 1.09; $P = 0.21$). The recovery rate was 60.5 ± 5.86 vs. 56.4 ± 5.77% ($P = 0.53$) and the average embryo development stage was 4.3 ± 0.08 and 4.3 ± 0.08 ($P = 0.85$) for corn and citrus pulp, respectively. Regardless of treatment, the second SOV of each period (chronic effect) resulted in a lower proportion of freezable embryos compared to the first SOV (17.5 ± 5.39 vs. 33.1 ± 5.14%; $P = 0.003$), viable embryos (30.0 ± 6.83 vs. 39.3 ± 6.59%; $P = 0.05$) and UFO (37.4 ± 6.23 vs. 53.1 ± 5.88%; $P = 0.02$). The first period had a greater number of ovulated follicles per cow compared to the second (18.5 ± 1.96 vs. 15.2 ± 1.95; $P = 0.04$). It was concluded that there was no effect of diet on the production and quality of the embryos.

Acknowledgments: FAPESP, CNPq and Carlos A. Rodrigues (SAMVET).

A043 Folliculogenesis, Oogenesis and Ovulation

Effect of different concentrations of EGFon *in vitro* culture of equine ovarian follicles

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Keywords: EGF, equine, *in vitro* culture.

The aim of this study was to evaluate the effect of different concentrations of EGF (Epidermal Growth Factor) on in vitro culture of equine preantral follicles. Ovaries (n=5) obtained at a local slaughterhouse from mares at seasonal anestrus were washed in PBS and alcohol 70 and transported in PBS plus penicillin (200 IU/mL) and streptomycin (200 IU/mL). The inner portion of ovary was divided into 11 fragments of 3x3x1 mm. One fragment of each ovary was immediately fixed in Bouin (control group, D0). The remaining 10 fragments were individually cultured in 24-well culture plates containing 1 mL MEM (Gibco BRL, Rockville, MD, USA) (osmolarity 300 mOsm/L, pH 7.2) supplemented with penicillin (100 IU/mL), streptomycin (100 mg/mL), bovine serum albumin (1.25 mg/mL- Gibco BRL, Rockville, MD, USA), ITS (Insulin- 6.25 g/mL, transferrin - 6.25 g/mL, Selenium - 6.25 ng/mL), pyruvate (0.23 mM), glutamine (2 mM) and hypoxanthine (2mM). This medium was referred as MEM +. Culture was performed for 2 or 6 days with medium change every 2 days. Medium was supplemented with different concentrations of EGF (10, 50, 100 and 200 ng/mL). After culture, fragments were fixed in Bouin and processed for histology. Follicles were classified according to the stage of development (primary or developing) and morphology (normal or degenerated). A total of 825 slides containing 3,300 tissue sections were evaluated. The statistical model used was Proportion test ($P < 0.05$). After two days of culture there was a higher proportion of viable follicles at a concentration of 100 ng/mL EGF (87.5%), while MEM had 44.4%; 10 ng/mL had 22.2%; 50 ng/mL had 46,4% and 200 ng/mL had 64.9%. We observed follicular development in all tested concentrations of EGF after two days of culture. EGF at 100 ng/ml provided the best results, with all follicles in development, while MEM had 50%, 10 ng/mL had 75%, 50 ng/mL had 69.2% and 200 ng/mL had 91.7%. After six days of culture, EGF dose did not alter follicular viability. Regarding the proportion of developing follicles after six days, the best results were obtained with EGF at 10 ng/mL and 50 ng/mL with all follicles classified as developing, and 200 ng/mL with 85.7% of developing follicles. Therefore, EGF at 100 ng/mL promoted the best viability at two days of culture, while there was no difference between treatments for this endpoint after six days of culture. Considering follicular development, EGF at 100 ng/mL was the most effective for two days, whereas the doses of 10, 50 and 200 ng/mL were most effective after 6 days of culture to promote development. We conclude that there is a dynamic demand for EGF in *in vitro* culture of equine preantral follicles.

A044 Folliculogenesis, Oogenesis and Ovulation

Effect of increasing circulating insulin on follicular development of Holstein cows

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Keywords: follicular growth, Holstein, Insulin.

The aim of this study was to test the hypothesis that increased circulating insulin during the pre and post deviation period would increase the initial and final follicular development. Sixteen non-lactating and non pregnant Holstein cows were used. The estrous cycles were synchronized using an intravaginal P4 device of 1.9 g (CIDR®; Zoetis, SP, Brazil) and intramuscular (i.m.) 100 µg of GnRH (Gonadorelin diacetate; Cystorelin®, Merial, Canada) on day -12 of the protocol (random day of the estrous cycle). On day -5, 25 mg were administered i.m. of $PFG_2\alpha$ (Dinoprost tromethamine; Lutalyse®, Zoetis, SP, Brazil). On day -3, the intravaginal P4 device was removed and cows received 25 mg of $PGF_2\alpha$ im. On days 10 and 11 ± 1 of the estrous cycle all follicles > 5 mm were aspirated to synchronize the emergence of a new follicular wave. The second day of aspiration was considered D1 of the experiment, when treatments were initiated. For this, cows were divided into two treatments: water (control; C) or propylene glycol (P) provided orally in four daily doses of 300 mL every 6 hours for 3 consecutive days (D1 to D3, pre follicular deviation period), and another 3 consecutive days (D5 to D7; after follicular deviation period). The experimental design was a Latin square in a 2x2 factorial arrangement Thus, four groups were formed: 1) Group CC = water pre and post follicle deviation (n = 16); 2) Group CP = water pre and propylene glycol post follicle deviation, respectively (n = 16); 3) Group PC = propylene glycol pre and water post follicle deviation, respectively (n = 16); and 4) Group PP = propylene pre and post follicle deviation (n = 16). Blood samples were taken 0 (immediately before), 15, 30, 60 and 120 minutes after propylene glycol for circulating insulin. Ovarian ultrasonography examinations were performed daily for evaluation of follicular dynamics. Statistical analysis was performed by the MIXED procedures of SAS. Plasma insulin concentrations were higher for groups receiving propylene glycol compared to controls (0, 15, 30, 60 and 120 min: 17.5 ± 1.4; 26.3 ± 1.4; 31.2 ± 1.4; 21.8 ± 1.4, 16.9 ± 1.5 vs.12.1 ± 1.5; 11.6 ± 1.5; 11.2 ± 1.5; 10.8 ± 1 5; 11.1 ± 1.5; $P \leq 0.05$). Despite this, there was no effect of circulating insulin increase ($P > 0.05$) on the rate of pre deviation (1.5 ± 0.14; 1.3 ± 0.15; 1.5 ± 0.14; 1.4 ± 0.15 mm/day) and post deviation (1.2 ± 0.13; 1.4 ± 0.14; 1.4 ± 0.13; 1.4 ± 0.13 mm/day) follicular growth and ovulatory follicle size (15.5 ± 0.56; 161 ± 0.55; 15.6 ± 0.52; 15.6 ± 0.54 mm) for CC, CP, PC and PP groups, respectively. We did not confirm the hypothesis that increased circulating insulin during pre and post deviation period interfere on follicle development.

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A045 Folliculogenesis, Oogenesis and Ovulation

Effect of insulin-like growth factor (IGF-1) on the morphology and development of ovine preantral follicles cultured *in situ*

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Keywords: growth, oocyte, sheep.

In vitro studies have demonstrated that insulin-like growth factor-1 (IGF-1) plays important roles in folliculogenesis, such as maintenance of survival after culture of caprine ovarian tissue (MARTINS et al., Animal Reproduction, v.7, n.4, p.349-361, 2010) and growth of isolated follicles in cows (GUTIERREZ et al., Biology of Reproduction, v. 62, p. 1322-1328, 2000). However, there is no report on the effect of different concentrations of IGF-1 on the *in vitro* development of ovine preantral follicles enclosed in ovarian tissue. Thus, the aim of this study was to evaluate the effect of IGF-1 on the morphology and activation *in vitro* of preantral follicles enclosed in ovine ovarian tissue. Ovaries (n=10) from adult mixed-breed sheep were collected at a local slaughterhouse and transported to the laboratory. The ovarian cortex was divided in fragments and one of them was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured *in vitro* for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with insulin, transferrin, selenium, hypoxanthine, glutamine, ascorbic acid and BSA (α-MEM+ - control medium) or in α-MEM$^+$ added by different concentrations of IGF-1 (1, 10, 50,100 or 200 ng/mL). At the end of the culture, morphological analysis of preantral follicles was performed through histology, and they were classified as normal or atretic according to the absence of presence of cytoplasmic shrinkage, nuclear pycnosis and/or disorganization of granulosa cells, as well as classified as primordial or growing follicles (intermediate, primary and secondary follicles). The percentage of normal, primordial and growing follicles were compared by ANOVA and Tukey's test ($P < 0.05$). The results showed that after 7 days of culture, there was a significant reduction in the percentage of morphologically normal follicles in all treatments compared to the fresh control. However, concerning follicle survival, no significant differences ($P > 0.05$) were observed between α-MEM$^+$ and IGF-1 concentrations. In comparison with fresh control, it was observed a significant reduction in primordial follicles and an increase in developing follicles in all treatments. Moreover, treatment with 100 ng/mL IGF-1 promoted higher ($P < 0.05$) follicular activation, compared to α-MEM$^+$. In conclusion, the concentration of 100 ng/mL IGF-1 maintained survival and promoted activation of ovine preantral follicles cultured *in situ*.

A046 Folliculogenesis, Oogenesis and Ovulation

Effect of IGF-1 SnaBI polymorphism on reproductive parameters and metabolic parameters in dairy cows

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Keywords: fertility, IGF, Snp.

Insulin-like growth factor 1 (IGF-1) is associated with increased follicular estradiol production, stimulating the return of postpartum ciclicity (Butler et al., 2004). Therefore, it is possible that genetic variants of the IGF-1 gene can improve reproductive efficiency of postpartum cows. The aim of this study was to evaluate the effect of polymorphisms in the IGF-1 gene on reproductive parameters, and milk production of Holstein dairy cows. Genotyping was performed by electrophoresis of the PCR product after digestion with the enzyme SnaBI. Holstein cows (n=75) from 21 days prepartum up to 210 days in milk (DIM) were used in the study. These cows were submitted to an OvSynch-TAI protocol at 55 DIM, and the protocol was repeated in cows diagnosed as non-pregnant at 30 and 60 days after AI. Milk samples were collected twice per week for determining ovulation. Progesterone levels above 1 ng/mL in two consecutive samples indicated ovulation. Days from calving to first ovulation (CFO) and the calving to conception interval (CCI) were evaluated. Serum concentrations of IGF-1 and β-hydroxybutyrate (BHBA) were measured in samples collected at -21, 0, 7, 21 and 60 DIM. Data were analyzed using the GLM procedure of SAS. Genotype distribution was 14.7% for the TT genotype, 48% for CT and 37.3% for CC. Circulating IGF-1 levels were 79.2 ± 9.9, 66.5 ± 5.2 and 56.6 ± 5.9 ng/ml for TT, TC and CC genotypes, respectively (P=0.05). The CFO interval for TT, TC and CC cows was 19.9 ± 4.2, 30.6 ± 2.3 and 30.4 ± 2.5 days, respectively, indicating a shorter interval (P<0.05) for TT cows, which had the highest levels of IGF-I. A linear effect (P<0.05) was observed among genotypes for the CCI, which was 76.9 ± 12.6, 96.9 ± 6.8 and 111.7 ± 7.8 for TT, CT and CC, respectively. Cows from the TT genotype had a shorter CCI that may be associated with earlier return to postpartum cyclicity and higher serum IGF-I levels. The TT cows had lower serum BHBA values than cows with TC and CC genotypes, 5.0 ± 1.4, 8.2 ± 0.7 and 8.1 ± 0.8 mg/dL (P<0.05), respectively. Milk production was not different between groups (P>0.05). In conclusion, the IGF-1 SnaBI polymorphism (TT) was associated with reduced CFO and CCI in dairy cows.

A047 Folliculogenesis, Oogenesis and Ovulation

Effect of the type of fixative and time of fixation on the morphology of equine preantral follicles

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Keywords: equine, fixatives, ovarian tissue.

The objective of this study was to investigate the efficacy of tissue fixatives Bouin, Carnoy and formaldehyde in equine ovarian fragments. Ovaries (n=4) from mixed breeds mares were obtained at a local slaughterhouse and transported to the laboratory in a thermo-recipient at 20ºC. Immediately after collection ovaries were washed with modified PBS solution (Cultilab®, Campinas-SP, Brazil) and divided into nine fragments with approximately 5x5x1 mm. Ovarian fragments were immersed in Bouin (B), Carnoy (C) or Formalin 10% (F) for 6, 12 or 24 hours. Each fragment was individually immersed 20 mL Falcon tubes containing 20 times the volume of fixative solution. After this period, they were kept in 70% ethanol for 24 hours. Each procedure was performed in four replicates. For histological analysis, samples were dehydrated in increasing concentrations of alcohol, diaphanized in xylene and embedded in paraffin. Five µm thick serial sections were obtaneid with a rotating microtome (Leica® type, Wetzlar, Germany) and stained with periodic acid-Schiff (PAS) and hematoxylin. A total of 540 slides with 1,620 sections were evaluated and 465 preantral follicles were counted and classified as normal or degenerated. Degeneration was determined by the presence of at least one of the following aspects: retraction of the cytoplasm, pyknotic nucleus, cytoplasmic vacuoles, displacement of granulosa cells and disruption of the basal membrane. The statistical model was logistic regression ($p < 0.05$). The Carnoy fixative for 24 hours provided best integrity of ovarian follicles (53.3%; 32/60) compared to the others. Boiun for 24 hours was the worst treatment (19.1%; 9/47). The other treatments showed the following results; C12h 50% (30/60), C6 H 40% (24/60), F24h 37.8% (17/45), F12h 35.1% (13/37), F6h 32% (16/50), B12h 30.5% (18/59) and B6h 24.4% (11/45). Fixation with Carnoy for 24 hours provided best integrity of equine preantral follicles.

A048 Folliculogenesis, Oogenesis and Ovulation

Effects of a high-fat and energy diet on ovarian gene expression in young and aged female mice

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Keywords: aging, AMH, IGF.

Studies indicate that high-fat diets can have adverse effects on ovarian activity and that aging can reduce female fertility due to decreased quality and size of the oocyte ovarian reserve. The PI3K/AKT1 signaling pathway and the FOXO3a transcription factor are important regulators of cell proliferation and survival and are involved in the activation of primordial follicles. The aim of the current study was to determine the effects of a high-fat and energy diet on ovarian gene expression (AMH, IGF-1, AKT1, PI3K, MTOR, BMP15, CAT, SIRT1 e FOXO3) in young and aged female mice. Twenty female mice (C57BL/6) at four and thirteen months of age were used (young: n = 10 and old: n = 10). Animals were divided into four groups: young/control diet (YC); young/high fat diet (YH); old/control diet (OC) and old/high-fat diet (OH). Both diets contained 14.1% crude protein, but the control diet had 75.9% carbohydrate, 10.0% fat and 3,061 kcal/100g of energy, while the high-fat diet contained 54.6%; 32.7% and 4,402/100g kcal, respectively. Females were fed the diets during 55 days, intake was measured every two days and body weight measured weekly. After euthanasia, ovaries were collected, RNA was extracted using the Trizol method (Trizol, Invitrogen, USA), RNA was converted to cDNA (Biorad, , Hercules, CA, EUA) and the expression of target genes measured by qRT-PCR (Applied Biosystems, Foster City, CA, USA) using the β2 - microgobulina as the endogenous control. For statistical analysis data were compared by two-way ANOVA (GraphPad Software Inc., La Jolla, CA, USA) for testing the effect of age, diet and its interaction. AMH was 67% less expressed in old than in young females ($P<0.05$). Furthermore, there was a 46% reduction in the expression of IGF-1 in females fed the high-fat diet ($P<0.05$), as well as lower food intake when compared to mice fed the control diet ($P<0.05$; 2.9 ± 0.1 and 3.9 ± 0.1). Previous studies suggest that IGF-1 may be involved in the premature depletion of the ovarian reserve (Schneider et al., 2015; J Ovarian Res. 7: 120). Females fed the high-fat diet had increased body weight gain compared to controls ($P<0.05$; 28% and 9% gain, respectively). In conclusion, there was a reduction in IGF-1 expression in the ovary of females fed the high fat diet, which may be a factor modulating ovarian aging.

A049 Folliculogenesis, Oogenesis and Ovulation

Effects of fibroblast growth factor 8 on meiosis progression and cumulus expansion of bovine cumulus-oocyte complexes submitted to *in vitro* maturation with amphyregulin

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Keywords: AREG, FGF8, oocyte maturation.

Fibroblast growth factor 8 (FGF8) is expressed by the bovine oocyte and appears to be involved in the mechanisms underlying meiotic arrest in mice via regulation of NPR2 expression in cumulus cells and, consequently, levels of cGMP. Recent studies suggest that replacement of FSH by amphyregulin (AREG) during in vitro maturation (IVM) may improve embryo development. The objective of this study was to assess the effects of FGF8 on cumulus expansion and meiosis progression in bovine cumulus-oocyte complexes (COCs) undergoing IVM with AREG. Bovine COCs were aspirated from slaughterhouse ovaries and cultured (grade 1 and 2) in groups of 20 in 400 µL TCM 199 supplemented with BSA (0.4%), sodium pyruvate (22 µg/mL), amikacin (75 µg/mL), AREG (100 ng/mL) and FGF8 at 0, 10 and 100 ng/mL, at 38.5 °C with 5% CO2 and maximal humidity. The degree of expansion was visually assessed after 22h of IVM (n=5 replicates) using a subjective method (degrees 1: poor expansion, 2: partial expansion and 3: full expansion), while meiosis progression was assessed at 6 (n=4 replicates) and 22h (n=5 replicates) of IVM. In order to assess nuclear maturation, oocytes were mechanically denuded, fixed, stained with Hoechst 33342 and examined under a fluorescence microscope to be classified as at germinal vesicle (GV) or germinal vesicle breakdown (GVBD) at 6h, or at MI (metaphase I) or MII (metaphase II) at 22h of IVM. Data were expressed as percentages and transformed to arcsine before testing effects of treatments by ANOVA followed by the Fisher Protected test. Differences were considered significant when $P < 0.05$. FGF8 at 100 ng/mL increased the percentage of GV oocytes in relation to the control at 6h (37.4 ± 7.9%, 49.8 ± 5.6% and 59.3 ± 4,8% of GV oocytes for 0, 10 and 100 ng/mL FGF8, respectively), but did not change MI (22,7 ± 2,4%; 16±5% and 25,3±8,9%) and MII rates (77.2 ± 2.3%; 83.8 ± 5.03% and 74.5 ± 8.9% for 0, 10 and 100ng/ml FGF8, respectively), nor expansion (63.3 ± 15.8%; 56.6 ± 22.1% and 56.6 ± 27.4% of grade 3 COCs for 0, 10 and 100 ng/mL FGF8, respectively) at 22h. We conclude that addition of FGF8 to the culture medium is capable of retarding germinal vesicle breakdown in oocytes submitted to IVM with AREG without compromising meiosis completion at the end of culture.

A050 Folliculogenesis, Oogenesis and Ovulation

Effects of follicle stimulating hormone (FSH) and amphiregulin (AREG) on meiosis dynamics of oocytes undergoing *in vitro* maturation

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Keywords: AREG, FSH, meiosis.

Oocyte meiosis is arrested at prophase I during early folliculogenesis and is resumed after the ovulatory LH surge, which induces germinal vesicle breakdown (GVBD) and progression of nuclear maturation to metaphase II (MII). Resumption of nuclear maturation is associated with decreases in oocyte transcription/translation and intracellular communication within the cumulus-oocyte complex. Therefore, precocious meiosis resumption may compromise oocyte fertility. The influences of LH in ovulatory follicles and of FSH during in vitro maturation (IVM) are mediated by EGF-like growth factors, including amphiregulin (AREG), which are produced by mural granulosa and cumulus cells. Recent studies suggest that replacement of FSH by AREG in IVM may improve oocyte developmental competence. This study aimed to assess meiosis dynamics in oocytes cultured with FSH or AREG. We tested the hypothesis that the direct stimulation with AREG speeds up nuclear maturation. Cumulus-oocyte complexes (COCs) were aspirated from bovine ovaries obtained at a slaughterhouse, and submitted to IVM for 6 hours in TCM 199 supplemented with BSA (0.4%), amikacin (75µg/mL), pyruvate (22µg/mL) and FSH (1µL/mL; n=81) or AREG (100ηg/mL; n=59). Cultures were performed in 7 replicates. After culture, oocytes were mechanically separated from cumulus cells, fixed with paraformaldehyde 4%, stained with Hoescht 33342 and examined under a fluorescence microscope to assess meiosis. Data were transformed to arcsine and groups compared by the Student t test, considering values of $P<0.05$ as significant. Supplementation with AREG resulted in a lower percentage of germinal vesicle oocytes (39.12% ± 6.6% vs. 61.06 ± 5.12; P=0.04) and a higher percentage of oocytes in MI (23.59% ± 5.5 vs. 9.93 ± 1.04; P = 0.04) in comparison with FSH. The percentage of oocytes at GVBD did not differ between treatments (P = 0.1). The present data indicate that AREG induces nuclear maturation more rapidly than FSH.

Acknowledgments: CAPES, FAPESP, CNPq.

A051 Folliculogenesis, Oogenesis and Ovulation

Expression of LH receptor in bovine *Cumulus oophorus* cells is modulated by follicular diameter and the gonadotropins stimulus

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Keywords: cumulus cells, LH receptor, oocyte.

Reproduction in mammals depends on the pulsatile release of the gonadotropins LH and FSH, which act together to regulate the function of the gonads. In the ovaries, LH influences follicular development until ovulation and this depends on the interaction with its receptor (LHR). However, there is no consensus regarding the use of LH during the in vitro maturation (IVM) of cumulus-oocyte complexes (COC) in cattle. Reports in the literature differ on mRNA expression of LHR in oocytes and cumulus oophorus cells (CC). Thus, the aim of this study was to characterize the expression of the LHR gene in CC and oocytes from bovine follicles at different diameters and after IVM. Ovaries were collected at a commercial abattoir and follicles were dissected and separated into two groups for experiment 1, G1: 3 to 6mm and G2: >6 to 10mm. On a Petri dish, follicles were individually ruptured with 18G needles in order to obtain their respective COC. Pools of 25 COC were used per experimental group from which CC were separated mechanically from the oocytes and stored. In experiment 2, follicles ranging 3-8mm were aspirated and the obtained COC were matured in vitro (groups of 25 COC) in droplets (100 μL in mineral oil) of IVM medium (TCM199, sodium pyruvate, 0.4% BSA and amikacin) at 38.5°C and 5% CO2 in air for 24h. The experimental groups were divided into control (IVM medium); rhLH (IVM+0.01 rhLH); and rhFSH (IVM+0.01 rhFSH) from which CC were separated from oocytes and stored. All samples were subjected to total RNA extraction using Trizol and reversely transcribed with High-capacity kit. For real-time PCR, we used primers targeting a fragment of the LHR gene not subjected to alternative splicing, and PPIA was used as the reference gene. All experiments were performed in triplicate. Data were analyzed by ANOVA followed by Tukey test when necessary (5% significance level). LHR mRNA was not detected in oocyte samples (experiments 1 and 2). Experiment 1 - LHR mRNA was detected in both groups of CCs from follicles at different sizes, and mRNA levels did not differ between these groups ($P > 0.05$). Experiment 2 – After IVM, LHR mRNA was detected in rhLH and rhFSH groups ($P > 0.05$) and was absent in the control group. The data from experiment 1 suggest that follicle growth does not appear to affect abundance of LHR mRNA in CC. In experiment 2, the abundance of LHR mRNA in CC was only observed in groups treated with gonadotropins (rhFSH and rhLH) suggesting that FSH and LH stimulate LH receptor expression in cumulus cells.

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A052 Folliculogenesis, Oogenesis and Ovulation

Expression of activin receptors during follicular deviation and luteolysis in cattle

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Keywords: corpora lutea, follicular deviation, TGFbeta.

Several signaling factors act on theca and granulosa cells and oocytes to promote follicular proliferation and differentiation in the ovary. In this context, the transforming growth factor β (TGFβ) superfamily has been studied. Activin receptors type II were shown to modulate signaling of TGFβ members and betaglycan is a co-receptor that enhances the effect of ligands of type II receptors. The objective of this study was to quantify mRNA levels of activin receptors type I (ACVR1A, ACVR1B), type II (ACVR2A and ACVR2B) and betaglycan during follicular deviation and luteolysis in cattle. Ovaries were collected by colpotomy and granulosa cells from the two largest follicles were isolated before (day 2; F1 and F2; n=4), during (day 3; dominant follicle (DF) and subordinate (SF); n=4) or after (day 4; DF and SF; n=6) follicular deviation. For luteal cells analysis during luteolysis, corpora lutea were dissected from the ovary at 0 (control), 2, 12, 24 and 48 h after treatment with PGF2α, which was injected 10 days after estrus detection (n=4-5 per group). The deviation model was validated by assessing mRNA levels of CYP19A1, which were higher in DFs ($P<0.05$) during and after follicular deviation. To validate the luteolysis model, serum progesterone levels were shown to decrease at 2 h, and reached basal levels at 24 h post-PGF treatment, which confirmed functional luteolysis. Data were tested for normal distribution using the Shapiro-Wilk test, normalized when necessary and submitted to ANOVA. ACVR2A mRNA levels were higher ($P<0.05$) in F1 follicles before deviation (day 2) compared to DFs after deviation (day 4). ACVR1B mRNA abundance decreased in both healthy (F1 and DFs) and atretic (F2 and SFs) follicles from day 2 to day 4. However, ACVR1A and ACVR2B mRNA levels did not change during follicular deviation. Betaglycan mRNA abundance was higher ($P<0.05$) in SFs than DFs at the expected time of deviation (day 3). During luteolysis, ACVR2A mRNA levels increased at 2 h ($P<0.05$) but decreased at 48 h post-PGF treatment. These results indicate that type II activin receptors are regulated during follicular deviation and luteolysis, and betaglycan at the expected time of follicular deviation, which suggests a potential role in the regulation of these ovarian processes.

A053 Folliculogenesis, Oogenesis and Ovulation

Epidermal growth factor (EGF) induces cell proliferation during *in vitro* culture of ovine preantral follicles

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Keywords: activation, mitosis, oocyte.

Efficient *in vitro* culture systems of ovarian preantral follicles can promote oocyte growth and proliferation of somatic cells. After *in vitro* culture of ovine ovarian tissue, it was observed that control medium (α-MEM$^+$) alone or added by 1 ng/mL Epidermal Growth Factor (EGF) can promote the initiation of primordial follicle development, process also called follicular activation (SANTOS et al., 2013, Anais da XXVII Reunião Anual da SBTE). However, there is not information about oocyte growth and proliferation of granulosa cells. Thus, the aim of the present study was to determine the effect of EGF (1 ng/mL) on oocyte diameter and granulosa cell proliferation after *in vitro* culture of ovine ovarian tissue. After collection of ovine ovaries (n=10) in the slaughterhouse, ovarian cortex was fragmented and one fragment was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured *in vitro* for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, 5,0 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumine (BSA) and 50 µg/mL ascorbic acid (α-MEM$^+$ - control medium) or in α-MEM$^+$ added by 1 ng/mL EGF. After culture, the fragments were destined to histology, the oocyte diameter was analyzed and immunohistochemistry was performed to evaluate Proliferating Cell Nuclear Antigen (PCNA; 1:500; Santa Cruz Biotechnology; Santa Cruz, CA, EUA). Proliferating or PCNA-positive cells (brown) were counted in the sections and expressed as percentages. The diameter was analysed by ANOVA and the percentages of PCNA-positive cells were compared by Qui-square test ($P<0.05$). There was no influence of α-MEM$^+$ or EGF on oocyte diameter after *in vitro* culture. Concerning cell proliferation, medium supplementation with EGF significantly increased PCNA-positive cells (55.0%) in relation to the fresh control (13.0%) and control medium (5.0%). In conclusion, 1 ng/mL EGF promotes proliferation of granulosa cells in preantral follicles during *in vitro* culture of ovine ovarian tissue.

A054 Folliculogenesis, Oogenesis and Ovulation

Improvement on the viability of preantral *Bos indicus* follicles cultured *in vitro* by addition of follicle stimulating hormone (FSH)

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Keywords: *Bos indicus*, FSH, *In vitro* culture.

The aim of this study was to evaluate the effect of FSH addition in the medium for *in vitro* culture of preantral follicles of *Bos indicus* females. Ovaries (n=10) were collected at a local slaughterhouse from five adult cycling *Bos indicus* cows (Nelore) with body condition score from 2.5 to 3.5 (range 0-5). After collection, ovaries were washed in 70% ethanol and PBS (Embriolife®, Vitrocell, Brazil). The surrounding tissue was removed and ovaries were sectioned longitudinally. The ovarian cortex was divided in 3x3x1 mm fragments. One fragment per animal was immediately fixed in Bouin (non-cultured control, D0). The other fragments (n=8) were individually cultured in 24-well culture plates containing 1 ml of minimum essential medium (MEM, Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) supplemented (MEM+) with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, and 6.25 ng/ml selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantina, 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/ml penicillin and 200 mg/ml streptomycin. Unspecified reagents were obtained from Sigma (St. Louis, MO, USA). MEM + medium was added to different concentrations (50, 100 and 200 ng/ml) FSH (Folltropin®, Bioniche Canada Inc, Ontario, Canada). The *in vitro* culture medium was tested during two (D2) or six (D6) days. Every two days, the culture media was replaced by fresh aliquots. For the analysis of integrity and degree of follicular development we used histology with periodic acid-Schiff (PAS) and hematoxylin staining. The classification was based on the evaluation of follicular development stage (primordial, primary and secondary) and morphological integrity as normal or degenerated. Data were submitted to ANOVA ($p \leq 0.05$). We evaluated 2,250 preantral follicles (normal or degenerated), being 772 primordial and 1,478 in development. After two days of culture, FSH at 100 ng/ml provided a higher proportion (51.2%; 128/250) of follicles morphologically intact when compared to the other groups: 27.2% (68/250) to MEM; 30.4% (76/250) to 50 ng/ml; 45.2% (113/250) to 200 ng/ml ($p<0.05$). Higher percentage of developing follicles was also obtained with FSH at 100 ng / ml (91.8%, 112/122). After six days of culture, 100 and 200 ng/ml of FSH provided a higher percentage of morphologically intact follicles (40.4%, 101/250 and 36.8%, 92/250, respectively) compared to 50 ng/ml. There was no difference between groups for the rate of development at D6 ($p>0.05$). We conclude that MEM+ supplemented with 100 ng/ml FSH for two or six days of *in vitro* culture was the most effective treatment to provide development and morphological integrity of preantral follicles from *Bos indicus* cows.

A055 Folliculogenesis, Oogenesis and Ovulation

Intrafollicular injection with PPARγ agonist on follicle deviation in cows

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Keywords: cows, follicle deviation, PPAR.

Gamma receptor peroxisome proliferator – activated (PPARγ) is a member of the PPAR nuclear receptors family, and these receptors are expressed in reproductive tissues from different species. PPARs have been shown to control apoptosis and cell cycle and to affect estradiol synthesis and metabolism. In rats, the expression of this receptor increased with follicular growth and decreased after the LH surge. In sheep and buffalos, PPARγ expression is primarily restricted to granulosa cells of developing follicles, and its expression is higher in small follicles. This receptor can also be activated by synthetic ligands such as Thiazolidinediones (TZD), a family of drugs that are insulin-sensitizers and PPARγ agonists. Our hypothesis is that PPARγ signaling is related with follicular atresia and may be involved in follicle deviation in monovulatory species. To test this hypothesis, the aim of this study was to evaluate if intrafollicular injection of a PPARγ agonist (TZD) inhibits dominant follicle growth. *Bos taurus* cows had the emergence of a new follicular wave induced by treatment with a progesterone releasing intravaginal device (1 g of the progesterone, DIB-Intervet Schering Plough, Brazil) and injection of 2 mg estradiol benzoate (i.m.; Genix, Anápolis, Brazil). Four days later, the progesterone device was removed, cows received intramuscularly (i.m.) injections of $PGF_2\alpha$ analogue (cloprostenol, 250 µg; Schering-Plough Animal Health, Brazil) and ovaries were monitored once a day by transrectal ultrasonography using an 8 MHz linear probe (Aquila Vet scanner, Pie Medical, Netherlands). When the largest follicle reached between 7 and 8 mm in diameter (ten cows), tcows were randomly assigned to receive an intrafollicular injection of 50 µM TZD (n=5) or PBS (n=5). The intrafollicular injection volume was adjusted according with follicular size in order to obtain a final concentration of 50 µM TZD in all follicles. The injected follicle was monitored daily by ultrasonography for three days after the injection as described by Ferreira et al. (Reproduction, 134, 713-9, 2007). The average sizes of the PBS injected follicles were 7.5±0.1 mm, 8.3±0.5 mm, 9.9±0.5 mm and 10.9±0.3 mm at 0, 24, 48 and 72h after treatment, respectively. TZD injected follicles stopped growing after injection and the follicular size curve was statistically different in relation to the control group (7.6±0.1 mm, 6.4±0.3 mm, 5.2±0.4 mm and 5.0±0.3 mm at 0, 24, 48 and 72h after treatment, respectively). Treatment with TZD inhibited follicular growth in all cows (5 out 5) and follicles injected with PBS continued growing (5 out 5). In conclusion, the increase in PPARγ signaling inhibited follicular growth and may be involved in the selection of the dominant follicle in cattle.

A056 Folliculogenesis, Oogenesis and Ovulation

Melatonin in *in vitro* maturation and their effect on the expression of antioxidant and apoptosis related genes of murine cumulus-oocyte complexes

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Keywords: IVM, melatonin, mice.

Melatonin (MLT) is a hormone synthesized primarily in the pineal gland and participates in the control of the circadian cycle and reproductive seasonality in some animal species. In addition to these functions, MLT also acts as a powerful antioxidant and antiapoptotic factor. The aim of this study was to assess the effect of the addition of MLT during *in vitro* maturation (IVM) on nuclear maturation and expression of genes related to antioxidant and apoptotic activity in murine *cumulus* cells (CC) and oocytes (OO). Female F1 hybrids (n=20; C57BL/6 x CBA) were subject to intraperitoneal injection of eCG (5IU/0.1 mL/animal) and after 48h, the *cumulus*-oocyte complexes (COCs) were collected. COCs (n=25 per group/treatment) were randomly selected and *in vitro* matured with MLT (10^{-9}, 10^{-6} and 10^{-3} M) or 0.5 µg/mL FSH (control) for 17h in an incubator at 37ºC and 5% CO_2 in air. Only MLT and not its association with FSH was used in order to evaluate their individual action on IVM and gene expression. Maturation rate was assessed according to the presence of the first polar body under an inverted microscope. Gene expression was evaluated by real-time quantitative PCR (4 replicates) for BAX and BCL2L1 (apoptosis) and GPX1, SOD1 and SOD2 (antioxidant) in CC and OO. As endogenous control, the geometric mean of H2AFZ and HPRT1 genes was used. Statistical analyses were performed by ANOVA followed by Tukey's test (4 replicates) with 5% significance level. No differences were detected (P>0.05) between groups matured with MLT at different concentrations (56.0%, 56/100; 53.7%, 51/95; and 48.9%, 43/88; for 10^{-9}, 10^{-6} and 10^{-3} M MLT, respectively) compared with the control group (57.3%, 55/96; FSH). The expression of GPX1 and SOD1 in CC was increased by MLT at 10^{-9} and 10^{-6} M (P=0.0006 e 0.0045, respectively). For OO, the group treated with 10^{-6} M MLT showed increased expression (P=0.0208) of the BAX pro-apoptotic gene. For the other genes, there was no difference between treatments (P>0.05). In conclusion, under the conditions studied, MLT was unable to improve the IVM rate, but alone was as efficient as FSH in promoting maturation of murine oocytes, indicating its potential effect on stimulating meiosis. In the real-time quantitative PCR analysis, the group treated with melatonin at 10^{-9} M presented increased expression of an antioxidant enzyme, suggesting the ability to enhance antioxidant action in CC.

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A057 Folliculogenesis, Oogenesis and Ovulation

Fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 15 (BMP15) inhibit apoptosis in cumulus cells from bovine cumulus-oocyte complexes undergoing *in vitro* maturation

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Keywords: apoptosis, cumulus cells, FGF10.

Oocyte secreted factors (OSFs) regulate apoptosis in cumulus cells (CC). FGF10 and BMP15 are OSFs that enhance cumulus expansion and embryo development when added to the IVM medium. Progesterone signaling is anti-apoptotic in CC and essential for oocyte developmental competence. The aim of this study was to assess the influence of BMP15 and FGF10 alone or combined on CC apoptosis, as well as on expression of apoptosis related genes (FAS, PUMA, BAX, BCL2, BIRC4, MDM2 and nPR) and progesterone production in bovine COCs undergoing IVM. Groups of 20 COCs (grades 1 and 2) from 3-8mm follicles of abattoir ovaries were cultured for 22 hours in 450µL of maturation medium (TCM 199 containing Earle's salts supplemented with 0.4% BSA; 1µl/mL FSH; 22µg/ml sodium pyruvate, 75µg/ml amicacin) without growth factors (control group) or supplemented with FGF10 (R&D Systems, 10ng/mL), BMP15 (R&D Systems, 100ng/mL) or FGF10 and BMP15 combined (5 replicates for gene expression and progesterone; 8 replicates for apoptosis). After IVM, CC and oocytes were mechanically separated and CC were stained with propidium iodide (PI) and Annexin V- APC (BD Biosciences Pharmingen), and counted in a flow cytometer to quantify viable (PI-/A-), early apoptotic (PI-/A+), apoptotic (PI+/A+) and necrotic (PI+/A-) cells. Progesterone concentrations were measured in the culture medium by radioimmunoassay (Kodalmedical, IgAc). Abundance of mRNA encoding pro and anti-apoptotic factors was assessed by real time qPCR normalized to CYC-A. Effects of treatments were tested by ANOVA and means were compared by the Fisher protected test. FGF10 increased the percentage of viable cells (66±2.4, 81.9±3.2, 72±3.2 and 71.9±3.5 for control, FGF10, BMP15 and FGF10+BMP15, respectively), decreased the percentage of apoptotic cells (22.7±2.1, 7.2±1.1, 19.7±2.9 and 21.9±2.7) and increased the BCL2/BAX ratio (0.7±0.1, 1.2±0.1, 0.5±0.1 and 0.7±0.1). The combination FGF10+BMP15 decreased the percentage of early apoptotic cells (6.9±1.3, 7.7±2.3, 4.4±0.8 and 2.7±0.4 for control, FGF10, BMP15 and FGF10+BMP15, respectively), decreased FAS (1.3±0.21, 1.3±0.1, 0.98±0.22 and 0.81±0.17) and increased nPR (0.41±0.05, 0.64±0.15, 0.8±0.27 and 0.99±0.16) mRNA abundance. Progesterone production and expression of PUMA, BIRC4 and MDM2 were not affected by growth factors. The present data suggest an antiapototic role for FGF10 in the bovine COC by mechanisms involving regulation of BLC2/BAX ratio. Moreover FGF10 appears to interact with BMP15 to prevent early apoptosis during IVM by mechanisms involving suppression of FAS and increased nPR expression. The anti-apoptotic action of these OSFs on CC may account for their positive impact on oocyte developmental competence.

A058 Folliculogenesis, Oogenesis and Ovulation

The chemokine receptor-2 (CCR2) plays a critical role on the follicular activation and preantral folliculogenesis and CCR2 deficiency leads in reduced fecundity

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Keywords: CCL2, CCR2, folliculogenesis.

Chemokines are cytokines with low molecular weight classically characterized by controlling the recruitment and activation of leukocytes during inflammation. The PI3K (Phosphoinositide-3-kinase) pathway is important for follicular activation and CCL2 chemokine (MCP-1) and its main receptor (CCR2), both highly expressed in mice ovaries, are modulated by PI3K levels. The study of the role of CCR2 in the reproductive system has clinical significance because CCR2 antagonists are under clinical trials for the treatment of autoimmune diseases. In recent studies performed by our group, we detected expression of CCR2 in mouse and human testicles, and observed that CCR2 deficiency caused a drastic reduction in daily sperm production. However, the role of this chemokine-receptor interaction in ovarian homeostasis is not known. The aim of the present study was to investigate CCR2 expression in wild-type mice ovaries (WT), and to quantify and compare the ovarian follicular population, recruitment rates, and follicular atresia in WT and CCR2-/ mice. Ovaries were collected from WT (n=10) and CCR2-/- (n=10) female (60 days-old) mice and processed for morphometric analysis, follicular quantification, immunohistochemistry, and western blotting. The mean number of litters per bred female and the number of pups per litter (during 1 year) were also recorded. All data were compared using t tests. The CCR2 protein was observed in the WT ovarian lysate and was not observed in the CCR2-/-, while β-actin expression (control) was observed in both groups. Immunohistochemical analysis revealed that CCR2 was not present in primordial follicles. However, this receptor was immunolocalized to oocytes included in activated follicles (primary, secondary, antral, and atretic). Although body and ovarian weight and gonadal-somatic index were not different between groups ($P>0.05$), CCR2 deficiency affected ovarian follicular population. CCR2-/- mice had an ovarian follicular reserve ~40% larger than WT ($P<0.01$). However, there was a reduction (~50%) in the number of preantral follicles ($P<0.01$), while the number of antral follicles was not different ($P>0.05$). Corroborating these findings, lower activation and follicular atresia index and fewer litters/bred female were observed in CCR2-/- ($P<0.01$). Overall, this study demonstrates for the first time that (i) CCR2 is a phenotypic marker and a potential regulator of follicular activation, (ii) CCR2 regulates mainly early stages of folliculogenesis, and (iii) CCR2 deficiency can lead to a significant reduction in fertility. Finally, this study indicates that the effects of CCR2 antagonists on folliculogenesis should be carefully studied before commercialization. CEUA 27/2014.

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A059 Folliculogenesis, Oogenesis and Ovulation

Is the NPPC/NPR2 system, described for murine model, applied to bovine antral follicles?

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Keywords: *In vitro* maturation, meiotic arrest, NPPC.

Recent studies in mice have demonstrated that the precursor of C-type natriuretic peptide (NPPC) and its receptor type 2 (NPR2) are essential for maintaining meiotic arrest in the oocyte and that estradiol, present in follicular fluid (FF), promotes and maintain expression of NPR2 in cumulus cells (CC), acting on the system NPPC/NPR2. The bovine FF influences nuclear maturation when added to the maturation media. In addition, extracellular vesicles (ECV), present in the FF, might contribute in the communication between ovarian somatic cells and oocyte. However, the presence of NPPC-NPR2 has not yet been identified in bovine ovary. Therefore, the objectives of this study are to elucidate the NPPC-NPR2 system in the bovine ovary and assess the influence of exogenous NPPC, estradiol and ECV on mRNA expression of PDE3 and NPR2, levels of cAMP in the oocyte and cGMP in CC and progression of meiosis I in bovine cumulus-oocyte complexes (COC). Five experiments were performed: 1) Immunoassay (ELISA) was used to detect and measure the concentration of NPPC in FF, lysate granulosa cells and ECV from groups with different follicular diameters (3-6mm;.>6-8mm and >8mm); 2) Immunolocalization of NPR2 receptor in bovine antral follicles; 3) COCs were cultured with exogenous NPPC, estradiol or ECV a) for 6 hours to assess mRNA expression of PDE3 in the oocyte and NPR2 in CC by RT-PCR and b) for 9 hours to evaluate meiosis progression by orcein staining and 4) Levels of cAMP and cGMP in COCs matured for 6 hours with exogenous NPPC or ECV were measured using commercial EIA kits (cAMP and cGMP, Format A PLUS, Biomol). Data were tested by ANOVA and means compared by Dunnett test at JMP 7.0 software (SAS Institute). Differences were considered significant when $P < 0.05$ and trend when $0.05 < P < 0.1$. NPPC was detected in higher concentrations in ECV, regardless follicular diameter. NPR2 was preferably located in mural granulosa cells. Estradiol decreased mRNA abundance of PDE3 in the oocyte and treatments did not influence the expression of NPR2 mRNA in CC. ECV and NPPC decreased degradation of cAMP (in the oocyte) and cGMP (in CC), respectively. All three treatments increased the percentage of COCs in GV stage. We conclude that NPPC, exogenously or contained in the ECV of bovine FF, regulates meiotic arrest via inhibition of oocyte PDE3 expression, increasing cGMP in the CC and cAMP in the oocyte, respectively. Estradiol also acts in the meiotic arrest by inhibiting PDE3 expression. However, current data did not show any effect on NPR2 mRNA expression.

A060 Folliculogenesis, Oogenesis and Ovulation

The role of INOS/NO/CGMP pathway on *in vitro* maturation of bovine oocytes-cumulus complexes in presence of follicular wall hemisections

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Keywords: *in vitro* maturation, nitric oxide, nucleotides.

The role of nitric oxide (NO) in the mammalian oocyte maturation and the mechanisms involved in this process has not been completely understood. NO is produced by nitric oxide synthases (NOS) and stimulates the soluble guanylate cyclase enzyme (sGC) to produce cGMP. This nucleotide controls the action of phosphodiesterase 3A, which has the function of metabolizing cAMP to the inactive form, leading to the resumption of meiosis. Aiming to better understand these events, we used two active substances in this pathway: aminoguanidine (AG), which inhibits the inducible NOS isoform (iNOS); and 1H- [1,2,4] oxadiazol [4,3-a] quinoxalin-1-one (ODQ), which acts by inhibiting the sGC. Groups of 20 COC (120 COC/treatment) were cultured with eight follicular wall hemisections (HS) in an incubator at 38.5°C and 5% CO_2 in 200 µl of maturation medium (TCM-199/BSA) supplemented with AG (1 and 100 mM) and ODQ (10^{-3} M). The controls consisted of COCs cultured in the presence (C -) and absence of HS (C +). Oocyte nuclear maturation state was assessed by staining with 0.2% acetic orcein after 22h of IVM. The intracellular concentrations of cGMP (30 COC/treatment) and cAMP (10 COC/treatment and 50 oocytes/treatment) were measured at 0, 1, 3, 6, 9h for COCs and 0, 3, 6, 9h in the oocytes with an enzyme immunoassay. The results were analyzed with the Tukey test ($P<0.05$). The presence of HS (C -) decreased the percentage of oocytes that reached metaphase II (MII) (41.0 ± 4.0%) compared to the C + (78.5 ± 3.9%; $P<0.05$). Addition of 1 mM AG stimulated meiosis resumption compared to C - ($P<0.05$) and was the same as in C + ($P>0.05$), while 100 mM AG inhibited resumption of meiosis and progression to MII. The addition of ODQ stimulated meiosis resumption compared to C -. However, it inhibited the progression to MII (21.9 ± 3.5%; $P<0.05$). cGMP concentrations decreased over time in all experimental groups. The results observed in group with 1 mM AG do not differ from those observed in group C -. However, the addition of 100 mM AG and 10^{-3} M of ODQ decreased the concentration of cGMP, except at 3 and 6h. The group treated with ODQ had the lowest concentrations ($P<0.05$). The concentration of cAMP in COCs increased over time in all treatments ($P<0.05$). For this reason, this nucleotide was only measured in oocytes, where its concentration was descending. The C+ group had the lowest ($P<0.05$) concentration of cAMP, while groups treated with 100 mM AG and 10^{-3} M of ODQ had higher concentrations of cAMP ($P<0.05$). The results suggest that the activity of the iNOS/NO/cGMP pathway is important for maintaining the COC in the germinal vesicle stage (GV) of meiosis, and that progression to the MII is modulated by cGMP and cAMP concentrations in the COC/oocyte.

A061 Folliculogenesis, Oogenesis and Ovulation

Protein profile of follicular fluid during folliculogenesis of the mare

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UFRGS.

Keywords: follicular fluid, folliculogenesis, proteomics.

Follicular fluid (FF) is the only environment in which the avascular compartment of the follicle (granulosa cells and oocyte) are exposed and provides the microenvironment within which the cumulus-oocyte complex (COC) matures and granulosa cells differentiate. This experiment aims to compare the FF protein profile of the largest follicle (F1) and compare the largest and second largest follicle (F2) during different stages of follicular development (emergency, deviation, dominance and pre-ovulatory). Ovaries from 20 non-pregnant and cyclic mares were collected in an abattoir. Before slaughter, the mares were examined by transrectal palpation and ultrasound examination of the genital tract in order to evaluate ovaries and uterus. Blood samples were collected by jugular venipuncture. The diameter of the two largest follicles (F1 and F2) and the corpus luteum (CL) were obtained from each mare. Echotexture of the endometrium (EE) was evaluated and scored from 1 to 4. Mares were classified in the following experimental groups: G 15 (n = 4) F1 ≤ 15 mm, EE ≤ 2.5, CL > 27 mm; G 20 (n = 8) F1 20 to 26 mm, EE 2.5 to 3, CL 17 to 26 mm; G 30 (n = 4) F1 30 to 38 mm, EE > 3, CL < 16 mm; G 40 (n = 4) F1 > 40 mm, EE > 3, CL < 16 mm. Plasma progesterone concentrations were assayed by chemilluminescence. After slaughter, the FF of F1 and F2 was aspirated and submitted to 2D-PAGE for protein separation and identification by mass spectrometry. For statistical analysis a one-way analysis of variance (GLM procedure of SAS) was performed to evaluate the relative optical density of each protein spot as the dependent factor and the experimental groups, F1 and F2 and their interactions as independent variables. From the 20 mares studied, four constituted G15, eight G20, four G30 and four G40. Plasma progesterone concentrations varied from 8.1 to 12.7 in G15, 6.7 to 12.6 in G20, 0.6 to 1.3 in G30 and 0.6 to 0.7 in G40. A total of 43 spots was observed in gels (38 from F1 and 35 from F2). Nine spots presenting significant differences between treatments were submitted to mass spectrometry. Albumin, apolipoprotein A-I, gelsolin, serotransferrin and alpha-1-antiproteinase 2 were detected in the fluid of F1 and differed in abundance ($P < 0.05$) between the experimental groups. POM121 and ZP3 fusion protein (POMZP3) differed ($P = 0.02$) in abundance since deviation (G20), and alpha-1-antiproteinase 2 showed interaction ($P = 0.05$) between F1 and F2. The majority of the proteins identified in FF are present in blood plasma. It was not possible to correlate a specific protein with a particular stage of follicular development. However, serotransferrin and alpha-1-antiproteinase 2 had greater abundance during dominance and apolipoprotein A-1 and gelsolin during the pre-ovulatory stage. POMZP3 showed higher abundance in the dominant follicle compared to the subordinate one.

A062 Folliculogenesis, Oogenesis and Ovulation

Blood perfusion in preovulatory follicle in Nelore cows under FTAI protocols

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Keywords: Doppler, estradiol benzoate, ovulation.

The objective of this study was to characterize changes in blood flow in the wall of the pre-ovulatory follicle during the latest wave of follicular growth since the withdrawal of progesterone implant until ovulation using an ovulation-inducing protocol in Nelore cows. Twenty eight cows were divided randomly into two protocols, the protocol BED9 (n=14) consisted of placing an intravaginal progesterone implant (Sincrogest®, Ourofino, Cravinhos-SP) and 2mg IM estradiol benzoate (BE, Benzoato HC, Hertape Calier, Juatuba-MG) in D0; withdrawal of progesterone implant and 0.150mg of PGF2α (Veteglan®, Hertape Calier, Juatuba-MG) IM in D8 and 1mg of BE IM after 24 h. The protocol BED8 (n=14) consisted of placing an intravaginal progesterone implant and 2mg IM estradiol benzoate on D0; withdrawal of progesterone implant, injection of 0.150mg de PGF2α and 1mg of BE in D8, in both cases IM. Animals were evaluated by color doppler ultrasound (MyLab™30Gold, Esaote) every 6 hours for 90 hours or until ovulation of the pre ovulatory follicle. Hour zero corresponded to the time of progesterone implant removal. Of the 28 cows, 11 (39.3%) did not ovulate before 90 hours, 4 of BED9 and 7 of BED8. Follicle vascularization was subjectively assessed using a score system in which follicles with from 0 to 20% of its circumference irrigated were classified as grade 1, from 20 to 40% as grade 2, from 40 to 60% as grade 3, from 60 to 80% as grade 4 and from 80 to 100% as grade 5. For statistical analyzes of the ovulation time and size of the pre-ovulatory follicle, t-tests were used for normal distribution and the Mann-Whitney test for non-normal distribution ($P < 0.05$). For the total number of follicles in each time evaluated ranked at different grades of irrigation the Fisher's test was used ($p < 0.05$). The average size of the ovulated follicles was 12.83 ± 1.31 and 11.85 ± 1.71mm ($p = 0.20$) in BED9 and BED8, respectively. A statistical difference was observed for the time of of ovulation; 74.4 ± 3.9 in BED9 and 61.71±11.33 hours in BED8 ($p = 0.01$). Regarding the vasculature, it was observed that in BED9 the first 24 h most of the follicles remained at grade 2, in the range from 24 to 30 h it was observed a change of vascularization from grade 2 to 3, grade 3 to 4 in the range from 36 to 42 hours and grade 4 to grade 5 in the range from 66 to 72 hours. In BED8, it was observed that from 0 to 30 hours, most follicles remained at grade 3, from 30 to 36 h there was an increase in the percentage of follicles at grade 4, and from 54 to 60 hours an increase of follicles at grade 4 to 5. The results suggest that the use of BE at the moment of P4 withdrawal hastens ovulation compared to injection 24 h later, and that near ovulation there is an increase in the vascularization of the wall of the pre-ovulatory follicle, in which Color Doppler can distinguish the pre-ovulatory follicle by its irrigation and ovulation proximity.

A063 Folliculogenesis, Oogenesis and Ovulation

Regulation of TGFβ family members around follicular deviation and final growth in bovine

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Keywords: follicular growth, GDF9, growth factors.

The family members of the transforming growth factor β (TGFβ) have been studied since the description of the naturally occurring BMP15 and GDF9 mutations in sheep, which are associated with infertility in homozygous or increased ovulation rate in heterozygous. However, the regulation and function of TGFβ family members around follicular deviation in bovine have not been completely characterized. Therefore, the objective of this study was to investigate the expression of inhibin (Alpha, BetaA and BetaB) and BMPs (1, 2 and 4) during follicular deviation, and the effect of intrafollicular injection (IFI) of GDF9 on growth of dominant follicles in cattle. Ovaries were collected by colpotomy and granulosa cells were isolated from the two largest follicles before (day 2; F1 and F2; n=4), during (day 3; dominant follicle (DF) and subordinate (SF); n=4) or after (day 4; DF and SF; n=6) deviation (Evans and Fortune, 1997. Endocrinology, v.138, p.2963–2971). In order to investigate the effect of GDF9 on follicular growth, cows were synchronized (n=11) and IFI of either vehicle (PBS; control group, n=5) or 100 ng/mL recombinant human GDF9 (GDF9) as final intrafollicular concentration (n=6; starting dilution of 1000 ng/mL) was performed when the DF reached 9 to 10mm. Follicular growth was monitored every 24h after treatment. All data were tested for normal distribution using Shapiro-Wilk test, normalized by log transformation when necessary and submitted to ANOVA. The effect of GDF9 on follicular development was evaluated as repeated measures data using the MIXED procedure. The deviation model was validated by assessing the transcript levels of CYP19A1, which was higher in the DF ($P<0.05$) during and after deviation. Moreover, mRNA levels of inhibin Alpha (INHA), BetaA (INHBA) and BetaB (INHBB) were consistently higher in the DF. INHBB mRNA was more abundant before (day 2), during (day 3) and after (day 4), while INHBA transcripts were higher during and after dominance. INHA mRNA levels were higher in the DF after follicular dominance. The relative levels of BMP1 mRNA were significantly higher ($P<0.05$) in F2 on day 2 of the follicular wave, but similar between DF and SF on days 3 and 4. BMP2 mRNA abundance did not differ between the two largest follicles, but BMP4 mRNA was more abundant in the DF on days 3 and 4. The IFI of GDF9 did not affect follicular growth and ovulation, as all the cows in the control group (n=5) and five out of six cows in the GDF9 group ovulated three to four days after treatment. These results revealed that BMP1 is expressed in the bovine ovary, and its expression pattern suggests a potential inhibitory role on the growth of the future SF. Transcript levels suggest that BMP2 is likely not involved in the regulation of follicular deviation while BMP4 may be associated with follicular dominance. Finally, GDF9 seems to not alter the development of large follicles and ovulation, however, other concentrations of the peptide should be tested.

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A064 Folliculogenesis, Oogenesis and Ovulation

Resveratrol mantains survival and promotes cell proliferation in ovine preantral follicles cultured *in situ*

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Keywords: antioxidant, oocyte, sheep.

Some studies have demonstrated that resveratrol, a fitoalexin produced by several plants and present in grapes, reduces follicular atresia (KONG et al., J Endocrinol Invest., v. 34, p. 374-81, 2011) and reactive oxygen species (KWAK, et al., Theriogenology, v. 78, p. 86-101, 2012) after rat ovarian follicle culture. However, there are no reports about the effect of resveratrol on the in vitro culture of ovine ovarian preantral follicles. Thus, the aim of this study is to evaluate the effect of resveratrol on the morphology, activation and cell proliferation of ovine preantral follicles cultured in situ. After collection of ovine ovaries (n=10) in the slaughterhouse, ovarian cortex was fragmented and one fragment was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured in vitro for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with insulin, transferrin, selenium, hypoxanthine, glutamine, ascorbic acid and BSA (α-MEM+ - control medium) or in α-MEM+ added by different concentrations of resveratrol (2; 10 or 30 μM). After culture, preantral follicles were morphologically analysed and classified as normal or atretic according to the absence or presence of cytoplasmic shrinkage, nuclear pycnosis and/or disorganization of granulosa cells, as well as classified as primordial or growing follicles (intermediate, primary and secondary follicles). Immunohistochemical analysis was also perfomed for detection of Proliferation Cell Nuclear Antigen (PCNA; 1:500; Santa Cruz Biotechnology; Santa Cruz, CA, EUA). The PCNA positive cells (brown) were counted in the sections and expressed as percentage. The percentage of normal, primordial and growing follicles were compared by ANOVA and Tukey's test ($P<0.05$). After 7 days of culture, there was a significant reduction in the percentage of morphologically normal follicles compared to the fresh control. The concentration of 2 μM resveratrol showed percentages of normal follicles similar to the control medium and significantly higher than other resveratrol concentrations. In addition, there was a significant reduction in the percentage of primordial follicles and an increase in the percentage of growing follicles (follicular activation) in all treatments compared to the fresh control. Moreover, it was possible to observe a significant increase in the percentage of PCNA positive cells in follicles cultured in 2 μM resveratrol (50.7%) in comparison to fresh control (34.0%) and α-MEM+ (4.3%). In conclusion, resveratrol at 2μM maintains survival, promotes activation and cell proliferation of ovine preantral follicles cultured in situ.

A065 Folliculogenesis, Oogenesis and Ovulation

Omega 3 supplementation to prepubertal gilts increases leptin and its receptor in preantral follicles

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Keywords: gilts, leptin, Omega 3.

The main approach to anticipate puberty is providing high energy diet to gilts to reach satisfactory body condition score (AMARAL; Animal Reproduction Science; 121:139-144, 2010). Diets containing high levels of omega 3 fatty acids increase serum leptin regulating its synthesis and secretion. The objectives of the present study were to evaluate body weight and backfat depth in prepubertal gilts supplemented with omega 3 source as well as the effect of omega 3 supplementation on reproductive organs. Prepubertal gilts were daily supplemented with 9 mL soybean oil (control group; n=13) or 9 mL fish oil with 5 g omega 3 containing 33% DHA and 22% EPA (omega 3 group; n=12) during 45 days. After slaughter, uteri were weighed and ovaries evaluated to verify the presence of corpus luteum (CL). For immunohistochemistry (IHC), ovarian sections (n=4/group) were incubated with primary polyclonal antibodies anti-leptin (Ob; 1:2000) and anti-leptin receptor (ObRb; 1:100). Oocytes were classified as included in primordial/primary follicles (OIPF); secondary follicles (OISF) or tertiary follicles (OITF). Protein quantification was done by software image analysis (ImageJ® software) to obtain the most common value (the mode) for each area (MOREIRA; Animal Reproduction Science; 139: 89-94, 2013). Analysis of productive parameters was performed as repeated measures data and analyzed using the MIXED procedure (SAS®). ANOVA was used to test for effects of omega 3 on uterus weight and immunostaining intensity and differences between means were determined with Tukey test using Statistix® software (2008). Body weight did not differ ($P>0.05$) between groups at any time. However, a significant interaction between group and moment ($P<0.05$) was observed, being observed higher backfat deposition in omega 3 gilts 45 days after the beginning of supplementation ($P=0.06$). CLs were not detected whereas uterus weight tended to be higher in omega 3 gilts ($P=0.09$) after slaughter. In omega 3 gilts, immunostaining for leptin was higher in OIPF and OISF ($P<0.05$) compared to control gilts, but no differences were observed in OITF ($P>0.05$). For leptin receptor, it was observed higher immunostaining ($P<0.05$) in OISF from omega 3 gilts and no differences were observed in other follicular phases ($P>0.05$). Collectively, data suggest that omega 3 supplementation increases the chance of reaching satisfactory body condition score at puberty and that increased leptin levels induced through omega 3 supplementation may positively influence oocyte/follicle and reproductive tract development.

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A066 Folliculogenesis, Oogenesis and Ovulation

Validation of molecular markers for oocyte competence in bovine cumulus cells

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Keywords: individual culture, markers, oocyte competence.

Considering that cumulus cells (CC) have a bi-directional communication with the oocyte and that they play an important role in the growth and maturation, they can be used to indicate oocyte quality in a noninvasive way. Several studies have identified candidate genes in CCs, which expression is associated with oocyte competence and can be used as markers. However, to prove the efficiency of those as markers, it is necessary to follow the development of each cumulus-oocyte-complex (COC) individually until the blastocyst formation. The present study aimed to quantify the expression of candidate genes in CC from COC with high and low potential to develop in vitro up to the blastocyst stage. The different culture systems were used for IMV, IVF and IVC. Initially, the effect of the individual culture system and biopsy on embryo development was evaluated and COCs were distributed into 3 groups: control (COCs were cultured in groups); WOW (COCs were cultured individually in the WOW system); and micro droplet (COCs were individually cultured in micro droplet of 20 μL). Then, embryo production was compared between the control and the individual system (micro droplet), in which the COCs were submitted or not to biopsy. Expression levels of GPC4, IGFBP4, FSHR, GHR, EGFR, FGF11, SLC2A1, SLC2A3, SPRY1, VCAN and KRT8 genes were quantified by real time PCR (RT-qPCR) in 5 pools with 7 CC biopsies obtained from immature COC. Each biopsied COC was individually tracked by culturing them in a micro droplet, and categorized based on his fate: embryo at expanded blastocyst stage at D7, cleaved and arrested and not cleaved. Blastocyst rates were lower in individual culture systems (WOW = 17.9% n=95; microdrop = 26.3% n=95) than in the control group (45.0 %, n=209). However, no effect of the biopsy was observed for both groups (P>0.05). From the 11 genes evaluated, 3 showed differential expression. Higher expression of GHR (P=0.09) and VCAN (P=0.06) was observed in CCs that formed embryos compared to those that did not cleave. The GPC4 gene was overexpressed (P=0.007) in CC from formed embryos compared to cleaved and arrested ones. It was concluded that individual culture reduced blastocyst production, but biopsy did not affect embryo development. The expression of GHR, VCAN and GPC4 genes can be used as markers to distinguish COCs associated with embryo development from COCs with limited developmental potential.

A067E Folliculogenesis, Oogenesis and Ovulation

Osmotic challenge of bovine early pre-antral follicles with different cryoprotectant agents

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Keywords: bovine preantral follicle, cryoprotectant agent, DMSO, EG, follicle permeability, osmotic challenge.

Significant advances in cancer diagnosis and treatments have stimulated interest in fertility preservation strategies as chemical or ionizing radiotherapy often threatens future reproduction. Ovarian tissue cryopreservation currently is the only option for preserving the reproduction potential of pre-pubertal girls and women whom cannot undergo hormonal stimulation, ovarian tissue cryopreservation currently is the only option. However, there is a huge concern regarding the possible presence of malignant cells in the retrieved ovarian tissue, which could lead to cancer reintroduction after reimplantation of the frozen-thawed tissue strip. Cryopreservation of isolated early preantral follicles (PAFs) (and subsequent *in vitro* culture (IVC), maturation (IVM) and fertilisation (IVF)) might therefore represent an interesting alternative. Existing protocols are based on protocols for freezing embryos and oocytes . However, in order to improve follicular survival after cryopreservation, it is essential to develop a protocol for follicles specifically. Indeed, follicles are quite different from both embryos and oocytes, if only because they are composed of two different cell types (namely the oocyte and the surrounding (pre-)granulosa cells). In order to provide a biophysical base for choosing optimal cryoprotectant agents (CPAs) that avoid severe volume changes and formation of intracellular ice crystals, in this experiment, two-day-old isolated bovine PAFs were osmotically challenged by exposing them to different concentrations of cryoprotectant agents (CPAs). Briefly, isolated bovine early PAFs were exposed to either ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in different final concentrations: 1 M, 2 M, 3 M, 4 M and 5 M at room temperature, and photographed at different time points (every half minute between 0 and 5 minutes) after the onset of exposure to calculate their volume over time (5 - 10 follicles per CPA and per concentration). Although there was a high variability in the individual response of the follicles to this CPA challenge, all follicles showed a typical ‘shrink/swell’ curve. Analysis with two-way ANOVA showed no interaction between the type of CPA and the respective concentrations. This means that volume differences in time between the minimum and maximum for both EG and DMSO were uniform across concentrations. Across all concentrations, time until the post stimulus maximum (i.e. the maximum volume to which follicles re-expand after shrinkage) appeared significantly longer in the EG group (P = 0.04), indicating that bovine early PAFs are less permeable to EG than DMSO. To our knowledge, this is the first time that isolated bovine early PAFs were osmotically challenged by exposing them to different concentrations of penetrating CPAs. This has provided us with some basic insights in follicular permeability to CPAs. These insights are a first step in the design of cryopreservation protocols for isolated early PAFs specifically.

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A068E Folliculogenesis, Oogenesis and Ovulation

Developmental competence of porcine oocytes that have finished growth phase from follicles of different diameter

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Keywords: Brilliant cresyl blue, diameter, follicular development, pig oocytes.

Numerous factors determined developmental competence of the oocytes. Brilliant cresyl blue (BCB) staining has been used for selection of oocytes from several mammalian species, including pigs (Ericsson S. et al, 1993 Theriogenology, 39(1): p.214). The aim of the present study was to evaluate the developmental competence of porcine oocytes that have finished growth phase (BCB^+) depending on diameter (d) of follicles (d < 3 mm, 3 - 6 mm, >6 mm) and to detect expression of estrogen receptor (ER) in cumulus cells of BCB^+ and BCB^- oocytes. Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 60 minutes. Treated oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored cytoplasm). Only BCB^+ oocytes were used in the experiments. The medium used for oocyte maturation was NCSU 23 supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG. FF was collected from follicles with 3 - 6 mm in diameter, COC cultured in maturation medium with pieces of wall (600-900 μm in length) from non atretic healthy follicles (d 3-6 mm). After 20-22 h of culture, COC and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for another 20-22 h of culture. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols (Stokes P. et al., Developmental Biology, 284, p.62 – 71, 2005). All chemicals used in this study were purchased from Sigma-**Aldrich (Moscow, Russia).** The question was: have BCB^+ oocytes from follicles of different diameters the same developmental competence? We did not find significant differences between the level of cleavage and blastocyst in all groups of experiments. Percentages of cleavage and blastocyst in groups were: follicles d < 3 mm - 43% (27/63) and 29% (18/63); follicles d 3 - 6 mm - 46% (45/98) and 35% (34/98); follicles d > 6 - 48% (28/58) and 28% (16/58) (χ^2 test).Immunocytochemical analysis was used for detection of *estrogen receptor* expression (ER) in cumulus cells of 53 BCB^+ and 33 BCB^- oocytes. Immunocytochemical staining was performed using the first rabbit polyclonal anti-human ER antibodies (NCL-ERp, Novocastra, **OOO BMS**, St.Petersburg, Russia). The visualization system (ABC-universal kit) consists of avidin–biotinilated peroxidase (DakoCytomation) was applied. 3,3'-diaminobenzidine was used as it was recommended from manufacture Novocastra (**OOO BMS**, St.Petersburg, Russia). Hematoxylin *(Abrisplus***,** St.Petersburg, Russia) was used to stain cells. Antigen optical density was measured using morphometric VideoTest (Russia) computer program. Positive immunocytochemical reaction was mainly observed in the nuclei membrane and slightly on the cytoplasmic membrane of cumulus cells (probably as non-specific background). It was shown that cumulus cells of BCB^+ oocytes had ~~a~~ significantly more pronounced expression of the ER than the cumulus cells of BCB^- oocytes ($p<0,001$, Mann-Whitney test).

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A069E Folliculogenesis, Oogenesis and Ovulation

Determining intrafollicular concentrations of cortisol and progesterone in horses and the effects of cortisol on *in vitro* maturation of equine oocytes

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Keywords: cortisol, follicles, horse, progesterone, IVM, oocytes.

Increased glucocorticoid release and synthesis in response to acute or chronic stress has been shown to impair reproductive function in a variety of species and therefore may affect fertility. The aims of this study were 1) to determine cortisol and progesterone concentrations in equine follicular fluid and serum and 2) to assess the effects of cortisone supplementation to the maturation medium on IVM rates of equine oocytes. We hypothesized that challenging equine oocytes during IVM with higher doses of cortisone than physiological levels does not affect IVM rates. Light horse mares (n=9) used in this study were reproductively sound and cycling. Follicular fluid samples were collected by ultrasound-guided transvaginal follicle aspiration from the following follicle classes: G1: 5-9 mm, G2: 10-14 mm, G3: 15-19 mm, G4: 20-24 mm and G5≥25 mm. Blood samples were collected from each animal at the beginning and at the end of the aspiration period, respectively. Hormone determinations for cortisol (DE1887, Demeditec, Kiel-Wellsee, Germany) and for progesterone (ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) were performed by ELISA. Cumulus oocyte complexes (COCs) were collected by OPU only from healthy, growing follicles, in the absence of a preovulatory follicle. Compact COCs (n=84) were randomly assessed either to control group, or to one of the treatment groups, in which hydrocortisone (H4001, Sigma Chemical, St. Louis, MO, USA) was added to the standard maturation medium in the following concentrations: 0.1 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml. After 30h, oocytes were denuded, stained with Hoechst (33342, Sigma) and IVM rates were assessed. Statistical analysis was done with the SPSS Statistics 22 software. As all data were normally distributed (Kolmogorov–Smirnov test, $p > 0.05$ for all parameters), one way ANOVA, Post-Hoc-Test and Pearson's correlation were applied for the hormones, whereas Chi-Square Test was used to analyse IVM rates. In follicular fluid from G5 follicles, concentrations of cortisol and progesterone were significantly higher ($p<0.05$) than in all other groups. Concentrations of cortisol and progesterone were positively correlated ($r=0.8$; $p<0.001$). In contrast, serum concentrations of progesterone and cortisol in mares did not differ at the beginning and the end of the aspiration period. There was no significant difference in the percentage of matured oocytes between groups, regardless of the concentration of cortisone added to the medium. Our results demonstrate a significant increase of cortisol in preovulatory follicles in vivo, suggesting its importance for oocyte maturation. Moreover, challenging equine oocytes in vitro with up to 100 times more cortisol than physiologically existent in follicles larger than 25 mm did not significantly affect IVM rates, suggesting that the equine oocyte is able to modulate cortisol levels and therefore to adapt to stress situation.