A027 Folliculogenesis, Oogenesis and Superovulation

Antral follicle counts in Nellore females with different reproductive parameters and body condition score

A.S. Rodrigues, S.N. Oliveira, P.A. Ferraz, M.V.G. Loiola, T.P. Coutinho, M.M. Santos, B.H.A. Andrade, C.H.C. Oliveira, T.C.B.S.C. Bittencourt, R.F. Bittencourt, M. Chalhoulb, A.L. Ribeiro Filho

Universidade Federal da Bahia, Salvador, BA, Brasil.

Keywords: antral follicular population, *Bos taurus indicus*, physiological status.

This study aimed to determine the effect of some reproductive parameters and Body Condition Score (BCS) on Antral Follicular Counts (AFC) in Nellore females. For this purpose, 481 Nellore females submitted to a protocol for follicular wave synchronization (Ferraz et al. 2010, Acta scientiae veterinariae 38, 728) were used. On D4, females were examined by transrectal ultrasonography using a 8MHz linear transducer (Pie-Medical, 100 Falco, Maastricht, Holanda) to determine the population of antral follicles ≥3mm and the Ovarian Diameter (OVD). In this study, the parameters evaluated were animal category (CAT), postpartum interval (PPI), OVD and BCS. Within CAT, animals were classified as lactating cows (LC, n = 313), dry cows (DC, n = 70) and heifers (HEI, n = 98). Within PPI, DC were divided into PPI1 (n=28), PPI2 (n=101) and PPI3 (n=160), for animals at ≤63, between 63 and 105 and ≥105 days postpartum, respectively. Ovaries of 216 females were measured and those with diameters ≤1.90, between 1.90 and 2.50 and ≥2.50cm were grouped into OVD1 (n = 50), OVD2 (n = 73) and OVD3 (n = 93), respectively. Finally, BCS of 311 animals was evaluated considering the scale from 1 to 5, and those with BCS ≤2, between 2 and 3 and ≥3 were classified as low (n=42), intermediate (n=125) and high BCS (n=144). The data were analyzed by the ANOVA and Pearson correlation, with $P<0.05$. The overall mean AFC was 46.52±22.47 follicles (FOL), showing a high variability for this trait, ranging from 7 to 145 FOL/animal. AFC did not vary between DC (47.00±23.50 FOL), LC (46.55±23.08 FOL) and HEI (45.17±19.37 FOL). AFC did not vary between PPI1 (41.39±16.14 FOL), PPI2 (49.06±22.94 FOL) and PPI3 (48.66±25.31 FOL) either. BCS did not alter AFC, which were 45.17±27.35; 44.60±21.95 and 45.54±22.48 FOL for animals with low, intermediate and high BCS, respectively. However, OVD had a significant impact on AFC, which were 32.24±13.99; 44.53±17.66 and 57.70±23.96 FOL for animals at OVD1, OVD2 and OVD3, respectively. In addition, there was a high positive correlation (r=0.53; P=0.0003) between OVD and AFC. The present results suggest that reproductive status, postpartum interval and body condition do not seem to affect AFC. The positive relationship between OVD and AFC may allow the use of OVD as a parameter to estimate AFC in Nellore females.

A028 Folliculogenesis, Oogenesis and Superovulation

Effects of levels of sulfur and cobalt on the diet on biochemical parameters, gene expression, and viability of bovine oocytes

A.R. Ferreira[1], R. Sartori[1], B.D.M. Silva[2], J.F.W. Sprícigo[3], L.O. Leme[3], E.L. Silveira[2], V.O. Miranda[3], M.N. Diógenes[3], D.B. Araújo[4], R. Guimaraes Junior[5], M.A.N. Dode[2], M.M. Franco[2]

[1]UNESP Botucatu, São Paulo, SP, Brasil; [2]EMBRAPA Recursos Genéticos e Biotecnologia, Brasília, DF, Brasil; [3]Universidade de Brasília, Brasília, DF, Brasil; [4]KEMIN, Indaiatuba, SP, Brasil; [5]EMBRAPA Cerrados, Brasília, DF, Brasil.

Keywords: epigenetic reprogramming, nutrition, s-adenosylmethionine.

S-adenosylmethionine, produced from methionine, is a precursor of methyl groups for the establishment of DNA methylation. Methionine comes from the diet and remethylation of homocysteine or betaine, which derives from choline. It is necessary to reprogram DNA methylation to form oocytes and embryos, and methylation is susceptible to nutrition changes. We evaluated if sulphur and cobalt deficiency would affect the levels of blood biochemical components of the methionine cycle and metabolism of glucose, gene expression, quality of oocytes and embryo production rate. Thirty Nellore pubertal heifers (10/treatment) submitted to ad libitum diet offered twice a day for 5 months were divided in 3 groups: sulphur+cobalt-deficient group: sugar cane and urea - sulphur (1%) and mineral premix - cobalt and sulphur (100g/animal), control group: sugar cane and urea (1%) + sulphur (9:1) and complete mineral premix (100g/animal); the methionine+choline group: the same diet of the control group, + salt (100g/animal) encapsulated (Kemin®) methionine (50%; 20g/animal) and choline (50%; 15g/animal). B9 and B12 vitamins, homocysteine, insulin, IGF-I and glucose levels were measured in blood plasma each 15 days during the dietary period. Animals were submitted to OPU weekly since the third month of the diet. Oocytes in stages I, II and III were considered viable. Part of the oocytes was denuded, and expression of MAT2B, mSHMT2, SAHH, DHFR, DNMT1 and MTR and the constitutive genes GAPDH and βACTINA was evaluated in cumulus cells. Remaining oocytes were used for *in vitro* embryo production. Data were evaluated by Student's t test or Mann-Whitney test. The average consumption of salt, choline and methionine was 19.5 kg 93.1 g, 18.6 g and 14 g; 21 kg, 97 g, 0.0 g and 0.0 g; 20 kg, 95.5 g 0,0 g and 0.0 g for the methionine+choline group, control and the sulphur+cobalt-deficient group, respectively. The sulphur+cobalt-deficient group differed from the control group for homocysteine (13.8±5.69 vs 10.6±3.19; P=0.0001), B9 (29.9±11.94 vs 26±10.52; P=0.0156); B12 (143.8±30.07 vs 165.6±48.7; P=0.0001) IGF-I (375.3±95.6 vs 410.2±121; P=0.024) and glucose (82.2±18.5 vs 76.2±13.9; P=0.0142). Among the genes evaluated, MAT2B (βactina P=0.0636; GAPDH: P=0.0565) and DNMT1 (βactina P=0.0105; GAPDH: P=0.032) expression was reduced in the sulphur+cobalt-deficient group in relation to the control group. The methionine+choline group (46.0±8.78%) compared to the control group (60.5±8.76%), presented a lower rate of viable oocytes (P=0.0285), with no difference for blastocyst (D7) and hatching rates (D8). The alteration of dietary compounds affected the level of metabolites, the pattern of gene expression and morphological quality of the oocytes. Whether there were alterations in epigenetic reprogramming of the oocyte capable of affecting the viability of the embryos without changing embryo production rates remains to be investigated.

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A029 Folliculogenesis, Oogenesis and Superovulation

Effect of enalapril on pregnancy rate in goats submitted to fixed-time artificial insemination

A.P.R. Costa, J.A.T. Souza, A. Sousa Junior, M.N. Bueno, I.O.T. Sousa, S.R.S. Sousa, L.C.C. Nunes

UFPI, Teresina, PI, Brasil.

Keywords: angiotensin-converting enzyme, caprine, ovulation.

Enalapril, an angiotensin-converting enzyme inhibitor, increased the efficiency of a protocol for fixed-time artificial insemination (TAI) in goats, when applied during the eleven days of the estrus synchronization protocol. However, there is a need to simplify the protocol, to provide convenience to its use in farm conditions. The aim of this study was to evaluate the effect of ACE inhibition on pregnancy rates in goats subjected to TAI associated with the use of a single dose of enalapril in a slow-release vehicle by two routes of administration. A total of 94 goats were subjected to estrus synchronization with intravaginal sponges (Progespon ®, Syntex, Argentina) impregnated with 60 mg of medroxyprogesterone acetate-MAP inserted on day zero (D0) for a period of 12 days followed by intramuscular injection of 300 IU of Equine Chorionic Gonadotropin (Novormon ®, Sintex, Argentina) and 75 µg of cloprostenol (Prolise ®, Tecnopec, Brazil) on the tenth day of treatment (D10). Three groups were formed: group 1 (G1; n = 34) control, G2 (n = 30) received vaginal ovules containing 60 mg of enalapril maleate on D10, and G3 (n = 30) received 3 ml of an oily enalapril maleate suspension at 20 mg/mL subcutaneously on D10. There were two inseminations with fresh semen, from males of proven fertility, diluted in coconut water the first was done 36 hours after sponge removal (dose = 0.5 mL) and the second 12 hours after the first insemination (dose = 0.25 ml). Pregnancy diagnosis was performed by transrectal ultrasonography 35 days later. Data were analyzed by $\chi 2$ test ($p < 0.05$). The overall pregnancy rate was 60.63% (57/94), while it was 61.76% (21/34) in the control group (G1), 63.33% (19/30) in the intravaginal enalapril group (G2) and 56.66% (17/30) in subcutaneous enalapril group (G3). There was no significant difference between groups. The results show that treatment with enalapril in a single dose was not sufficient to increase pregnancy rates. Other experiments are required to determine the minimum number of days necessary to obtain the increase previously observed in the treatment of eleven days.

A030 Folliculogenesis, Oogenesis and Superovulation

Estimate of number of antral follicles in Nelore females in reproductive age

A.C.S. Sanches, N.A. Luz, D.P.L. Giudicissi, F.A.M. Oliveira, W. Blaschi, T.R.R. Barreiros

Laboratório de Reprodução Animal, Universidade Estadual do Norte do Paraná, Bandeirantes, PR, Brasil.

Keywords: *Bos indicus*, follicles, population.

The estimate of the number of antral follicles in the ovary has been investigated by many research groups in the world. The relation between number of follicles and hormone levels was demonstrated as a possible parameter to represent the reproductive potential in *Bos Taurus* females (Ireland et al., 2011, Reprod. Fertil. Dev. 23, 1–14). The objective of this study was to determine the population of antral follicles in Nelore females. Were used, during the month of October of 2012, 204 Nelore females with body condition score between 2.25 and 3.5 (scale 1-5), including prepubertal heifers (n = 62), pubertal heifers (n= 36), primiparous cows (n = 32) and multiparous cows (n = 73) with 30 to 45 days postpartum. The ages of the animals ranged from 22 to 24, 22 to 26, 36 to 40 and from 48 60 to 98 months, respectively, in the four categories mentioned above. Animals were kept in pasture of *Brachiaria brizantha* with mineral supplementation *ad libtum* containing 9% phosphorus. All animals were submitted to two transrectal ultrasonographic examinations (7.5 MHz transducer, Mindray, China), with an interval of 10 days, to assess cyclicity by identifying a corpus luteum in one of two exams and to count all antral follicles (AFC). Data were submitted to nonparametric analysis of variance (P <.05). Mean AFC values were similar in all groups (p> 0.05), and were 16, 18, 20 and 18 for prepubertal heifers, pubertal heifers, primiparous cows and pluriparous cows, respectively. Data were grouped, and females with AFC up to 18 follicles were classified as intermediate AFC and with more than 18 follicles were classified as high AFC. The distribution of females with high AFC was 40% (25/62), 55.5% (20/36) for pre-pubertal and pubertal heifers and 56.2% (18/32) and 45.2% (33 / 73) for primiparous and pluriparous cows, respectively. There were differences (P <0.001) in AFC values in each category (Intermediate AFC: 14, 16, 14:14 vs. High AFC: 24, 22, 23, 24), and similarity (p> 0.05) in intermediate and high AFC values between categories, respectively for pre-pubertal and pubertal heifers and primiparous and pluriparous cows. This study suggests that AFC in the ovaries of Nelore females in reproductive age is similar, and lower numbers (20-25) compared to the results previously published in Nellore females were observed, possibly because animals from commercial herds and evaluated by transrectal ultrasonography were used.

A031 Folliculogenesis, Oogenesis and Superovulation

Estimate of ovarian follicular population in mules: preliminary results

A.K. Souza, S.M. Gonzalez, R.G. Gomes, M.M. Seneda

Universidade Estadual de Londrina, Londrina, PR, Brasil.

Keywords: follicles, mule, ovarian.

Female mules are classically considered infertile, although some mules are able to manifest estrus at regular intervals. Due to the shortage of embryo recipients in the equine and asinine species, as well as to the increased number of mules in our country, this paper proposes an investigation of one reproductive aspect of mules, the ovarian follicular population. During the months of March and April of 2013, 3 ovaries of mules were collected, with no information regarding age or the respective pairs of ovaries, being randomly selected. The ovaries were collected from a slaughterhouse, which did not allow further information on these animals. For an hour, the ovaries were transported to the laboratory at 20 ° C, where they were measured and weighed. Each ovary was fragmented according to its size, being one ovary divided into 8 fragments and the other 2 ovaries divided into 2 fragments. Then, the ovaries were fixed in a Bouin solution for 24 hours and kept in 70% ethanol. Later, they were dehydrated in increasing concentrations of alcohol, diaphanized in xylene and infiltrated in paraffin. With the inclusion of paraffin, serial sections of 0.5 micrometers were performed, with a rotary microtome (Leica ®, Wetzlar - Germany). For each 50^{th} section, a histological slide was assembled. The staining was performed with periodic acid-Schiff (PAS) and hematoxylin for structural analysis in microscopy. We analyzed 260 blades, from which only 6 disposed follicles located in small clusters. Until now, mixed results were found regarding the parameters analyzed: in one ovary, with the weight / volume of 9.17 g / 9 mL, 22 follicles were observed, of which 21 were primary and 1 was antral. In the second ovary (2.3 g / 2 ml), no follicles were found; and in the third ovary (1.61 g/2mL), only 3 primary follicles were present. The ovarian parenchyma was quite distinct from the equine and asinine species; with significant vascularization, macroscopically visible. Considerable variation in weight and size were noted, as well as in the number of follicles. The variation can be explained by the great reproductive variability, including follicular reserve by mules, because the species of origin (equine and asinine) have different numbers of chromosomes. There is a big gap in the morpho-physiological characteristics of mule ovaries, due to the extreme difficulty in obtaining ovaries of these animals. By the initial data obtained in this study, quite unique particularities were found regarding the organization of the parenchyma and vascular distribution, in addition to follicular population. These findings may be related to variations in the fertility found in female mules.

A032 Folliculogenesis, Oogenesis and Superovulation

Fibroblast growth factor 10 (FGF10) inhibits expression of genes that regulate steroidogenic and ovulatory capacity in bovine granulosa cells

A.C.S. Castilho[1], F.M. Dalanezi[1], C. Price[2], J. Buratini[1]

[1]UNESP, Botucatu, SP, Brasil; [2]Universidade de Montreal, St. Hyacinthe, Canadá.

Keywords: cattle, FGF10, follicle.

Fibroblast growth factors are grouped into subfamilies in accordance with structural and binding properties. FGF10 belongs to the FGF7 subfamily and activates receptors FGFR2B and FGFR1B. In bovine antral follicles, FGF10 expression was detected in oocytes and theca cells, where mRNA levels were negatively correlated with estradiol intrafollicular concentrations (Buratini et al., 2007, Mol Reprod and Dev, 77, 743-750). FGF10 inhibited estradiol production in cultured granulosa cells (GC) and blocked follicle development when administered via intrafollicular injections (Buratini et al., 2007, Mol Reprod and Dev, 77, 743-750; Gasperin *et al*., 2012, Reproduction, 143, 815-823.). Morever, FGF10 and FGFR2B mRNA expression was lower in dominant compared to subordinate follicles around follicle deviation. To gain more insight into the mechanisms by which FGF10 inhibits follicle development, the effects of FGF10 on mRNA abundance of CYP19A1, HSD3B1, HSD17B, CYP11A1, STAR, LHR, MVK (mevalonate kinase), FSHR, IGFBP4, IGFR1, IGFR2, PAPPA, AGTR2 and GADD45 in cultured bovine GC were assessed. Small antral follicles (2-5mm) were dissected from abattoir ovaries, GC were separated and cultured in serum-free medium with graded doses of recombinant FGF10 (0, 1, 10 and 100 ng/ml; R&D Systems®) for six days with partial medium change (70%) every two days. GC were recovered and submitted to total RNA extraction. Effects of FGF10 on gene expression were assessed by real time RT-qPCR using bovine-specific primers and PPIA as the endogenous control. Relative expression was determined by the Pffafl's equation. Effects of FGF10 treatment on mRNA expression was tested by ANOVA and means were compared using the Tukey-Kramer HSD test. Significant differences were considered when $P<0.05$. FGF10 decreased mRNA abundance of CYP19 ($P=0.02$; at 100 ng/ml), FSHR ($P=0.0001$; at 1 ng/ml), IGFR1 ($P=0.04$; at 1 ng/ml), AGTR2 ($P=0.02$; at 10 ng/ml) and MVK ($P=0.03$; at 10 ng/ml). In conclusion, the present data suggest that suppression of aromatization capacity and FSH, IGF and AngII signaling are included in the mechanisms by which FGF10 inhibits antral follicle development.

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A033 Folliculogenesis, Oogenesis and Superovulation

Ovarian response following the use of estradiol benzoate or estradiol cypionate as inductors of ovulation in TAI synchronization protocols in Holstein cows

B.G. Freitas[1], R.V. Sala[1], L.M. Vieira[1], G.G. Macedo[1], M. Santos de Miranda[2], P.S. Baruselli[1]

[1]FMVZ/USP, São Paulo, SP, Brasil;[2]IZ, Nova Odessa, SP, Brasil.

Keywords: estrogen, inductor, ovulation.

The objective of the present study was to evaluate the use of Estradiol Benzoate (EB) or Estradiol Cypionate (EC) as inductors of ovulation in Timed Artificial Insemination (TAI) protocol in Holstein females. A total of 50 females (31 cows and 19 heifers) that presented a body condition score of 3.04±0.04 (1 to 5 scale) at the first day of the synchronization protocol were used. At the first day (D0), they received an intravaginal progesterone device [P4; PRIMER®, Agener União; new device for cows and; used device for heifers (8 days)] associated with a 2mg i.m. injection of EB (Sincrodiol®, Ourofino Saúde Animal). At D8, the devices were removed and it was administrated i.m. 500µg of sodium cloprostenol (Ciosin®, MSD Saúde Animal). At this time, animals were randomly allocated into one of two different treatments, according to the inductor of ovulation utilized: EC at D8 (1,0mg i.m. of ECP®, Zoetis Brasil; n=25) and EB at D9 (n=25). Ultrassonographic exams (IBEX PRO, E.I. Medical Imaging, EUA) were realized every 24 hours between D8 and D10 and every 12 hours between D10 and D12. At D17, the volume of the formed corpus luteum was also measured. The statistical analysis was performed by the GLIMMIX procedure of SAS. There was no difference between the inductors of ovulation (EB vs. EC) according to the diameter of the follicle at D8 (10.9±0.6 vs. 12.2±0.6; P = 0.13), estrus occurrence (84% vs. 88%; P = 0.98) and time of estrus between D8 and D10 (72.5±1.2 vs. 71.5±2.1; P = 0.29), diameter of the ovulatory follicle (13.9±0.5 vs. 15.0±0.5; P = 0.10), ovulation rate (92% vs. 92%; P = 0.94), time of ovulation related to D8 (72.5±1.2 vs. 71.5±2.1; P = 0.29) and volume of CL at D17 (7,199.29±678.39 vs. 7,441.68±672.21; P = 0.95). However, it was observed that the EC determined more dispersed ovulations (P = 0.04) than females treated with EB. Such greater variation in timing of ovulation was mainly related to the higher (P = 0.06) occurrence of early ovulation (≤ 60 hours) observed in those females treated with EC (28%) than in females treated with EB (8%). Thus, although the higher dispersion of the timing of ovulation observed in the EC treated animals than those treated with EB, both inductors of ovulations resulted in a satisfactory ovarian response in synchronization of ovulation protocols for TAI in Holstein females.

A034 Folliculogenesis, Oogenesis and Superovulation

Plasma and intrafollicular testosterone concentration in Nelore and Angus heifers with high and low follicle counts

B. Loureiro, R.L. Ereno, M.G. Favoreto, A.G. Pupulim, J. Buratini, C.M. Barros

Universidade Estadual Paulista Julio de Mesquita Filho, Botucatu, SP, Brasil.

Keywords: bovine, follicle, testosterone.

During follicle development androgens are synthesized by theca cells under the influence of LH through the conversion of androgen precursors. In monkeys and mice, androgens act in the follicle promoting the expression of growth factors (IGF1, GDF9, TGFB) and FSH receptor (Vendola et al., 1999, Biol Reprod 61, 353-7; Dumesic e Abbott, 2008, Semin Reprod Med 26, 53-61). In previous studies with these same animals we have shown that follicles from Angus heifers had higher expression of *IGF1R*, *IGF2* and *FSHR* (Favoreto et al., 2013, Reprod Fert Dev 25, 237 e Ereno et al., 2013, Reprod Fert Dev 25, 236). This experiment was designed to evaluate plasma and intra-follicular testosterone concentrations in Nelore and Angus heifers with high (HFC) and low (LFC) follicle counts. Sixteen Nelore and nineteen Angus heifers (24 months approximately) were kept in *Brachiaria bizantus* grass, animals also received a mix of grains and had access to salt and water ad libitum. Estrus was synchronized with two doses of PGF2α 11 days apart. To determine the number of follicles for each animal three ultrasound (US) exams were performed on day 1 of subsequent cycles. Animals were slaughtered 24 h after ovulation. Blood was collected before slaughter. Follicular fluid was collected from three follicles with 2-4 mm diameter. Plasma and intrafollicular testosterone concentrations were determined using the enzyme-linked immunosorbent assay specific for bovine testosterone (USCN Life Sciences, Wuhan, China). Data were analyzed using the PROC GLM and PDIFF from SAS (SAS 9.2), considering the effects of breed and group (HFC and LFC). Plasma concentration of testosterone was higher ($P<0.001$) in Angus heifers (6.8 ± 0.8 ng/ml) when compared with Nelore heifers (1.5 ± 0.9 ng/ml), however there was no difference between groups within breed. Intrafollicular concentration of testosterone was also higher ($P<0.02$) in Angus heifers (46.0 ± 4.6 ng/ml) when compared with Nelore heifers (29.2 ± 4.9 ng/ml), but no difference between groups within breeds was found. In conclusion, higher plasma and intrafollicular testosterone concentration in Angus heifers might account for higher expression of growth factors and *FSHR* in follicles from these animals. However, testosterone is not involved in follicle recruitment.

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A035 Folliculogenesis, Oogenesis and Superovulation

Influence of ascorbic acid on in vitro culture of equine preantral follicles: preliminary results

C.B. Silva[1], R.G. Gomes[2], L.A. Lisboa[2], A.K. Souza[2], R.L. Oliveira[2], L.S.R. Marinho[2], M.M. Seneda[2]

[1]UNOPAR, Londrina, PR, Brasil; [2]UEL, Londrina, PR, Brasil.

Keywords: ascorbic acid, equine preantral follicles, *in vitro* culture.

The equine ovary has important features that hinder its handling, as the differentiated disposal of the cortical (internal) and medullar (external) areas and the sparse population of preantral follicles. Moreover, the difficulty in obtaining ovaries has led to a lack of information regarding folliculogenesis in this species. The aim of this study was to evaluate the effect of adding different concentrations of ascorbic acid in the in vitro culture of preantral follicles in horses. All ovaries were obtained from a local slaughterhouse. The ovaries (n = 10) of five mares were collected, washed in 70% ethanol and PBS. The internal region (cortex) was divided into nine fragments of approximately 6x6x2 mm. One fragment was immediately fixed in Bouin (control) and the others were transported to the laboratory in PBS with antibiotics at 4°C. In the laboratory, they were cultured at 39°C in atmosphere with 5% CO_2 for 2 to 6 days, with 2 ml of Minimum Essential Medium (MEM) supplemented with ITS (Insulin-Transferrin-Selenium), pyruvate, glutamine, hypoxanthine, bovine serum albumin and antibiotics (MEM+) or MEM+ added with ascorbic acid (25, 50 or 100 µg/ml). The medium exchange was performed every two days. After the culture period, the ovarian fragments were fixed in Bouin and processed for histology. Preantral follicles were classified aDCording to the stage of development as primordial and developing follicles (primary and secondary), and aDCording to viability in normal or degenerated. One hundred and sixty-two follicles were evaluated in the control group, 79 (D2) and 33 (D6) in MEM, 89 (D2) and 33 (D6) in 25 µg/ml, 143 (D2) and 9 (D6) in 50 µg/ml, and 96 (D2) and 4 (D6) in 100 µg/ml, totalizing 649 follicles. The results showed that, compared to non-cultivated tissue (control), the culture of preantral follicles *in situ* reduced the percentage of normal follicles in all the tested media ($P < 0.05$). In all treatments, there was a reduction in the percentage of primordial follicles, with a concomitant increase in the percentage of developing follicles when compared with control (88.9% primary, 11.1% developing follicles), and the highest percentage of developing follicles (77.1%) was obtained with the concentration of 50 µg/ml of ascorbic acid. In conclusion, 50 µg/ml of ascorbic acid was effective in promoting the activation of primordial follicles in horses.

A036 Folliculogenesis, Oogenesis and Superovulation

Influence of the ovarian reserve on the numbers of antral follicles in *Bos indicus* and *Bos taurus* purebred cows

C.O. Rosa[1], K.C. Silva-Santos[1], G.M.G. Santos[1], L.S. Siloto[1], J.T. Santos[2], F.Z. Machado[1], E.R. Oliveira[1], M.M. Seneda[1]

[1]UEL, Londrina, PR, Brasil; [2]UFSM, Santa Maria, RS, Brasil.

Keywords: antral follicular count; bovine, preantral follicles.

The aim of the present study was to compare the population of preantral ovarian follicles among *Bos indicus* and *Bos taurus* purebred females with high and low numbers of antral follicles during follicular waves. Ovaries from Nelore (*Bos indicus,* n = 100) and Aberdeen Angus (*Bos taurus,* n = 100) cows (72-96 months-old) were collected at abattoirs and transported to the laboratory. After collection, ovaries were evaluated by ultrasonography and antral follicles ≥ 3 mm were counted using a microconvex array. Cows were assigned into groups with high (G-High) or low (G-Low) antral follicle count (AFC) based on the mean number of antral follicles ± SD (Nelore, mean = 39 follicles; Angus, mean = 29 follicles) as follows: *Bos indicus* cows with high- (≥ 57 follicles, n = 8) or low-AFC (≤ 21 follicles, n = 8) and *Bos taurus* with high- (≥ 45 follicles, n = 10) or low-AFC (≤ 13 follicles, n = 10). Ovaries were processed for histological evaluation and the number of preantral follicles was estimated using a correction factor (Gougeon e Chainy, 1987. J Reprod Fertil, 81:433-442). Comparisons between groups were made with ANOVA ($P \leq 0.05$). The mean numbers of antral follicles (mean ± SD) in *Bos indicus* cows was 63 ± 8 (G-High) and 15 ± 5 follicles (G-Low), and 59 ± 23 (G-High) and 11 ± 3 follicles (G-Low) for *Bos taurus* females. A large variation in the number of preantral follicles was observed among animals. There was no difference between the average number of preantral follicles of *Bos indicus* of G-High (48,349 ± 30,149 follicles) or G-Low (33,037 ± 31,710 follicles), or between *Bos taurus* of G-High (35,050 ± 36,060 follicles) or G-Low (30,481 ± 43,360 follicles). It is concluded that the population of preantral follicles did not influence the population of antral follicles in *Bos indicus* and *Bos taurus* purebred cattle with high and low AFC.

A037 Folliculogenesis, Oogenesis and Superovulation

Local blood flow changes in the preovulatory follicle of Santa Ines ewes

C.V. Muterlle[1], J.F.W. Sprícigo[1], E. Melo[2], J.P. Neves[1], B.D.M. Silva[2]

[1]Universidade de Brasília, Brasília, DF, Brasil; [2]EMBRAPA Cenargen, Brasília, DF, Brasil.

Keywords: colour Doppler, ovulation, sheep.

An extensive vascular formation (angiogenesis) and cell differentiation (luteinization) oDCur in the follicular wall between LH peak and early development of corpus luteum. Recent studies indicate the colour doppler ultrasound as a non-invasive and useful tool for evaluation of ovarian vascular function,allowing real-time observation of blood flow in an enclosed area, such as preovulatory follicles wall. The aim of this study was to characterize the blood flow changes in the preovulatory follicle wall in the last phase of follicular growth before ovulation in Santa Ines ewes. Nine ewes were used in this experiment, synchronized with two PGF2α doses (37.5 mg, IM; Croniben, Biogenesis), with an interval of 7 days. For the estrus observation, 48 hours after the second dose of PGF2α, a marked thug was introduced to the ewes flock and every 4 hours the labeled ewes or those aDCepting mounts were considered in estrus (0 h). The ewes were evaluated using colour Doppler ultrasound (MyLab ™ 30Gold Cardiovascular, Esaote) with a 5 MHz trans rectal multifrequency probe. The evaluation after estrus detection was performed with 4 hours intervals between assessments until preovulatory follicles ovulation. A total of 17 preovulatory follicles (greater than 4.0 mm) were evaluated. The average size of the follicle at the ovulation time was 5.85 ± 0.45 mm and the average period between estrus and ovulation was 18.24 ± 4.05 h. For follicular vascularization evaluation a subjective classification was used: follicles with intense vascularization were graded as 1; intermediate vascularization 2 and follicles without irrigation 3. For the comparison was used the total follicles number in each time after estrus (0, 4, 8, 12, 16 h), and classified with different degrees of irrigation (1, 2 or 3). To compare the follicles groups chi-square test ($P < 0.05$) was performed. Regarding follicles with moderate irrigation (2), no significant difference ($P > 0.05$) was observed at 0 (58%), 4 (76%), 8 (80%), 12 (50%) and 16 h (50%). At 12h there was an increase ($P < 0.05$) in the number of follicles with intense irrigation (50%), at 16h this pattern was maintained. Before 12 h very few follicles had intense irrigation, showing no significant differences ($p > 0.05$) in 0 (0%), 4 (5.8%) and 8h (13.3%). It was observed that at 8h, follicles without irrigation (6%) was significantly lower ($P < 0.05$) compared to that observed at 0 (41%) and 4 h (17%). At 12h there was no pre ovulatory follicle without irrigation. The results suggest that 12 hours after estrus detection oDCurs an increase in the vascularization of preovulatory follicle wall. This difference in blood flow can be useful to determine the proximity of ovulation, and differentiate preovulatory follicles from anovulatory follicles, even when the diameter is similar between them.

A038 Folliculogenesis, Oogenesis and Superovulation

Effect of the alterations in L-arginine/Nitric Oxide synthase/Nitric Oxide pathway on nuclear maturation and intracellular concentration of CAMP and CGMP in bovine COC's

D.F. Dubeibe[1], M.C. Caldas-Bussiere[1], W.V. Sampaio[1], M.P. Cesaro[2], P.B.D. Gonçalves[2], N.F. Torres[1]

[1]UENF, Campos dos Goytacazes, RJ, Brasil; [2]UFSM, Santa Maria, RS, Brasil.

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

L-arginine (L-arg) is the precursor for the Synthesis of Nitric Oxide (NO) by the activity of the enzyme Nitric Oxide Synthase (NOS). The main pathway of NO action is through the cGMP pathway. Changes in the production of NO / cGMP have been related with the events of the resumption and progression of meiosis in oocytes. The follicular wall hemi-sections (HS) in maturation medium partially inhibit nuclear maturation of oocytes in culture. The aim of this study was to evaluate the effect of changes in L-arginine/NOS/NO pathway in the nuclear maturation of bovine oocytes and their relation with the production of intracellular nucleotides cAMP and cGMP in presence of HS. Groups of 20 COCs (140 COCs/treatment) were cultured for 22 h with 8 HS of follicular wall in incubator at 38.5°C and 5% CO_2 in 200 µL of maturation medium (TCM 199/BSA) supplemented with 5 mM of L-arg, 1 mM of Nω-nitro-L-arginine methylester (L-NAME) NOS inhibitor, or any supplementation (control). Oocyte nuclear maturation state was assessed by staining with 2% acetic orcein and the intracellular concentrations of cAMP and cGMP in COCs were determined at 0, 1, 3 and 6 h of culture by enzyme immunoassay technique. The results were evaluated by analysis of variance and means were compared by SNK test at 5% probability. Compared to the control, the addition of L-arg or L-NAME decreased ($P<0.05$) the percentage of oocytes at germinal vesicle state (VG) (5.9 ± 7.6 and 5.9 ± 5 vs 33.9 ± 23, 8 and increased ($P<0.05$) the percentage of oocytes that reached the MII state (27.1 ± 6.4 and 72.9 ± 14.6 vs. 8.4 ± 6.5) ($P <0, 05$). L-arg did not affect ($P> 0.05$) cAMP concentration in DCOs, but attenuated ($P <0.05$) the cGMP decrease during the first hour of culture compared with L-NAME and the control (0.87 ± 0.13 vs 0.25 ± 0.26, 0.33 ± 0.20). L-NAME increased ($P <0.05$) cAMP concentration in the first hour when compared with the control (0.92 ± 0.26 vs 0.43 ± 0.15) and decreased ($P <0.05$) the concentration of cGMP at 3 h of culture in relation to the control and L-arg (0.07 ± 0.50 vs 0.55 ± 0.05 and 0.53 ± 0.05). In presence of HS of the follicular wall, changes on the L-arg/NOS/NO pathway affected the resumption and progression of oocytes meiosis. These results suggest that the events of nuclear maturation after addition of L-arg cannot be related to changes in the concentration of cAMP and cGMP. However, partial inhibition of NOS activity with the addition of L-NAME in the culture medium, change the concentration of nucleotides (cAMP and cGMP) suggesting a possible stimulatory pathway on nuclear maturation of bovine oocytes in a inhibitory *in vitro* culture system.

A039 Folliculogenesis, Oogenesis and Superovulation

Gene expression related to ovulatory capacity in superovulated or non-superovulated Angus cows

E. Lucacin, A.G. Pupulim, P.K. Fontes, A.C.S. Castilho, B. Loureiro, R.L. Ereno, M.F. Machado, E.M. Razza, R.A. Satrapa, C.M. Barros

UNESP, Botucatu, SP, Brasil.

Keywords: bovine, MOET, ovulatory capacity.

Embryo transfer (MOET) has contributed significantly for genetic improvement of Brazilian cattle and the superestimulatory treatment P-36 has been successfully used in MOET programs. Recently, increased mRNA levels of LH receptor (LHR) and angiotensin II receptor type 2 (AGTR2) have been reported in granulosa cells of superovulated Nelore cows. The aim of this study was to evaluate the effects of the P-36 protocol on the gene expression of AGTR2 and LHR, related to ovulatory capacity, in granulosa cells of superovulated Angus cows. Multiparous Angus cows (n=17), were randomly assigned to two experimental groups: control and P-36. The control group (non-superovulated cows, n=7) received in a random day of the estrous cycle (D0) an intravaginal device containing progesterone (1.0g; Primer®; Agener Animal Health, SP, Brazil) and 2.5 mg of estradiol benzoate (EB; RIC-BE®; Agener Animal Health SP, Brazil; IM). After 8 days (D8) cows were treated with PGF2α (150 mg dcloprostenol; Prolise®; Agener Animal Health, SP, Brazil; IM; 07:00am) and the intravaginal device was removed (07:00pm). Animals in the P-36 group (n=10) received, at the beginning of the protocol (D0), Primer® and 2.5 mg of EB. Five days after (D5), FSH treatment was started (pFSH, 200 mg, Folltropin®; Bioniche Animal Health, Ontario, Canada, IM) and consisted of twice daily IM injections for four days, on D7, PGF2α was administered and the intravaginal device was removed 36h later (D8, 07:00pm). Animals in both groups were slaughtered 12h (D9) after device removal and the ovaries were transported to the laboratory immediately after slaughter. Control dominant follicles (n=7, control group) and superovulated follicles (n=20) were dissected to obtain the granulosa cells and subsequent extraction of total mRNA. Amplification of housekeeping (cyclophilin; PPIA) and target genes was performed by real time RT-PCR according with the Sybr Green protocol. Relative gene expression valeus were determined by the Pfaffl method. The means of follicular diameter (mm) and the abundance of mRNA was compared by ANOVA and t test, respectively, and significance level was $P<0.05$. There was no significant difference in follicular diameter of the control group (13,85±0,68) when compared to the P36 group (13,13±0,42). The mRNA levels of LHR and AGTR2 were higher ($P<0.05$) in the control group (1.02±0.24 and 3.76±1.07, respectively) when compared to P-36 group (0.33±0.04 and 1.02±0.21, respectively). Therefore, our results suggest that the gene expression related to ovulatory capacity is negatively influenced by the P-36 protocol, suggesting the need for adjustments in the superestimulatory protocol when used in *Bos taurus* cattle.

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A040 Folliculogenesis, Oogenesis and Superovulation

Expression of genes related to oocyte competence in bovine cumulus-oocyte complexes morphologically divided in different grades

E.M. Razza, M.F. Machado, E.M. Pioltine, L.V. Saura, L.R. Chica, M.F.G. Nogueira

UNESP, Botucatu, SP, Brasil.

Keywords: cumulus-oocyte complex,gene expression, in vitro maturation.

Traditional methods for the evaluation of oocyte quality are based on morphological classification of the immature *cumulus*–oocyte complex (COCs) because they resemble the atresia grade of its follicle source. Although imprecise and controversial, the use of a morphological classification system is crucial for pre-selection of COCs with high development potential and able to maximize the production of more competent embryos. However, little is known about the expression of competence-related genes in morphologically different oocytes. The objective of this study was to evaluate the expression of genes related to competence in immature and IVM oocytes derived from COCs classified into grades I, II and III (n=3 replicates/group). COCs from follicles ranging 3-8 mm obtained from abattoir ovaries were separated into three groups according to the morphological classification: grade I (GI, oocytes with homogeneous cytoplasm, uniformly granular and surrounded by three layers of compact *cumulus* cells); grade II (GII, oocytes with homogeneous cytoplasm, uniformly granular and surrounded by less than three layers of *cumulus* cells) and grade III (GIII, partially denuded oocytes). Groups of 20 COCs were denuded by repeated pipetting before (group Immature) and after IVM (24 h) in TCM 199 bicarbonate supplemented with 6% BSA, pyruvate (11 µg/µL), amikacin (16.67 mg/uL), FSH (0.1 mg/mL, Pluset®, Serovet, Rome, Italy), LH (50 mg/mL, Lutropin®, Bioniche, Belleville, Ontario, Canada) and estradiol (1 ug/uL), total RNA was extracted by RNeasy® kit (Qiagen) and RNA pools of 20 oocytes were reverse transcribed by SuperScript III® enzyme (Life Technologies). Expression of growth and differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and the oocyte-secreted protein 1 (OOSP1) was investigated by real-time PCR (StepOnePlus®: Life Technologies) using PowerSybrGreen® (Life Technologies). The relative quantification method ΔΔCt was used with cyclophilin (PPIA) as the endogenous gene. The effects of the grade of the COC and the maturation period were tested by ANOVA and the groups were compared by the Tukey-Kramer HSD test. Differences were considered significant when $P<0.05$. The expression of GDF9 was reduced following IVM, and inversely the OOSP1 expression was increased after IVM, while BMP15 expression did not differ. There was no effect of morphological status on the expression of the genes analyzed. Results suggest that IVM may influence the expression of genes related to oocyte competence, but the morphological discrimination did not indicate any difference in the expression of competence-related genes.

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A041 Folliculogenesis, Oogenesis and Superovulation

Optimization of lipofection conditions in bovine granulosa cells: preliminary results

F.C. Castro, L.G. Mesquita, M.C.R.V. Cunha, H. Fernandes, K.L. Schwarz, R.C. Botigelli, M.H.C. Cruz, F.G. Zaffalon, C.L.V. Leal

Faculdade de Zootecnia e Engenharia de Alimentos - FZEA/USP, Pirassununga, SP, Brasil.

Keywords: bovine, granulosa cells, lipofection.

Granulosa cells (GC) are important constituents of the follicular environment for oocyte competence acquisition, performing functions such as steroidogenesis, expression of LH receptors (LHR), and the synthesis of many essential proteins (Richard *et al*. 1996, Biol Reprod, 54, 22-28). Lipofection, which is used as a means to introduce interfering RNAs for gene silencing is an efficient and less aggressive transfection method, being an important tool for to investigate the role of cellular genes and proteins (Oliveira *et al*. 2,005, Genetics and Molecular Research, 4, 185-196). The aim of this work was to establish ideal conditions for lipofection in bovine GC. To obtain GC, slaughterhouse bovine ovaries had their follicles of 2-6mm aspirated and after removal of *cumullus*-oocyte complexes the cells were cultured in DMEM with 44 mM $NaHCO_3$, 50μg/mL ciprofloxacin, 2.5 mg/mL amphotericin B and 5% FCS in 100mm petri dishes at 38.5°C and 5% CO_2 in air. After confluency, the GC were separated with 0.05% trypsin and 0.02% EDTA in PBS and seeded at 5 x 10^4 cell / well in 4-well dishes. After 48 hours of culture, the medium it was replaced with lipofection agents Lipofectamine® RNAiMAX (1, 2 and 3μL; Invitrogen, São Paulo, Brazil) or Lipofectamine™ 2000 (1, 2 and 3μL; Invitrogen, São Paulo, Brazil) and the transfection indicators siGLO® (30, 50, 75 and 100 nM; Thermo Scientific Dharmacon®, São Paulo, Brazil) or FUGW transgenic plasmid prepared in the laboratory (100, 200, 300, 400, 600 and 900 nM), in 5 replicates per treatment. As control, GC groups were not exposed to drugs and cultured in conventional DMEM. The lipofection efficiency was visually verified in a subjective manner considering the proportion fluorescent GC (indicative of penetration of the lipofector with agent SiGLO® or FUGW) at 24 and 48 h of culture. The highest efficiency was observed at 24 h of culture, once at 48 h the GC were already fairly degenerate. Regarding lipofection agents, groups with Lipofectamine® RNAiMAX + siGLO® or FUGW, at all concentrations, showed a low proportion of transfected GC (20%); Lipofectamine™ 2000 + FUGW groups, for all concentrations, also showed low percentage of transfected GC (30-40%), but with good fluorescence intensity. Lipofectamine™ 2000 + siGLO® groups showed the highest efficiency of lipofection, both in intensity and in percentage (80-90%). Concentrations were optimized with 2μL and 100 nM, respectively. From the best conditions found at 24 h of culture, with 2μL Lipofectamine™ 2000 + 100nM siGLO®, it is possible to establish a methodology for gene silencing by lipofection in GC and to use such strategy as a tool for functional analysis of genes of interest. Evaluation of the inclusion of the silencing agent (siRNA) by lipofection in the conditions defined in this experiment are underway.

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A042 Folliculogenesis, Oogenesis and Superovulation

Relation between antral follicle count and size of the uterus in heifers

G. Conde, A.C.S. Sanches, H.G.R. Mendonça, F.A.M. Oliveira, D.P.L. Giudicissi, W. Blaschi, T.R.R. Barreiros

Laboratório de Reprodução Animal, Universidade Estadual do Norte do Paraná, Bandeirantes, PR, Brasil.

Keywords: antral follicle, *Bos indicus*, uterus.

The relations between the number of follicles and hormone dosage were demonstrated as possible parameters to represent the reproductive potential in Bos Taurus females (Ireland et al., 2011, Reprod. Fertil. Dev. 23, 1–14). The objective of this study was to compare the relation between antral follicles population and uterine diameter in Nelore heifers. Were used during october 2012, 98 Nelore heifers with body score condition between 3,0 to 3,5 (scale 1 to 5), managed in pasture of Brachiaria brizantha and mineral supplementation ad libitum containing 9% phosphorus. The animals were submitted to two transrectal ultrasonography exams (7.5 mHz transducer, Mindray, China) with 10 days of interval, for evaluation of cyclicity, viewing CL in one of the two tests, counting all the visualized antral follicles (AFC) and measurements of uterine diameter, after obtaining three transversal evaluations and averaging between dimensions. The results were submitted to analysis of variance and correlation (P<0,05) and heifers were classified into low AFC (10.7±2.1, n = 22), medium AFC (15.0± 1.0, n = 29) and high AFC (21±3.2, n = 47) ; (P<0.05). The uterine diameter was greater (P <0.05) for heifers with high AFC (10,7 ± 1,6 mm) and medium AFC (10.2 ± 1.6 mm) than the low AFC (9.7±1.8 mm). Prepubertal (n=62) and pubertal (n=36) heifers had similar values (P>0.05) for AFC, resulting in 17.1 ± 5.1 vs 16.7 ± 4.6, respectively. The uterine diameter was greater (P=0.003) in pubertal (12±1.1 mm) than in prepubertal heifers (10.2±1.8 mm). The AFC was positively correlated with the size of the uterus (CR = 0.23, p=0.01). It was observed in prepubertal and pubertal heirfers CR = 0.34, p = 0.006 and CR = 0.01, p = 0.9, respectively. In conclusion, the results suggest that the AFC may influence the uterine development of prepubertal heifers.

A043 Folliculogenesis, Oogenesis and Superovulation

Kisspeptin stimulates LH release, is enhanced by estradiol and induces ovulation in bovine females

G.G. Macedo[1], J.R.G. Maio, B.M. Monteiro[1], E.O.S. Batista[1], R.V. Sala[1], L.M. Vieira[1], J.G. Soares[1], M.F. Sá Filho[1], G.M.G. Santos[2], M.F. Mendanha[1], G.P. Nogueira[3], P.S. Baruselli[1]

[1]Universidade de São Paulo, São Paulo, SP, Brasil; [2]Universidade Estadual de Londrina, Londrina, PR, Brasil; [3]UNESP, Araçatuba, SP, Brasil.

Keywords: Kiss1, neuroendocrinology, synchronization of ovulation.

The aim of this study was to evaluate the viability of the use of Kisspeptin (KP) in the pharmacological control of LH release and ovulation in bovine females. *EXP 1* investigated the capacity and profile of LH release of prepubertal females (absence of CL and P4<1ng/mL) of different breeds [24 Gir calves (6 - 9 m.o.) and 24 Holstein calves (4 - 8 m.o.)] after intramuscular (i.m.) administration of KP. Females were randomly distributed in two treatment-groups receiving i.m. KP or GnRH (buserelin acetate; positive control). Thirteen blood samples were collected 20 min apart to measure circulating LH. Holstein females showed higher LH released than Gir (AUC, 457.8±91.7 and 172.1±27.9 ng/mL/min, respectively; P=0.001), where KP provided similar LH response in relation to GnRH (P=0.27). KP provided maximum LH release amplitude in 20 min after treatment. *EXP 2* tested the hypothesis that LH release in response to KP stimulus is enhanced by previous E2 exposition. Eight ovariectomized Nelore females were allocated in four treatment-groups (crossover, four replicates, n=8/group): SAL (saline solution); KP (i.m. injection of KP); EB+KP [i.m. injection of KP + estradiol benzoate (EB)]; and EB+KP12 (EB injection 12 h before KP adm). The SAL group did not show LH surge. The AUC of LH was greater (P=0.02) for EB+KP12 (448.2 ± 139.2 ng/mL/min) than for KP (189.1 ± 39.1 ng/mL/min). EB+KP group presented same (P>0.05) AUC (253.6 ± 41.9 ng/mL/min) compared to KP and EB+KP12. The LH surge started very quick after KP administration, at 9.5 ± 5.8 min; 4.0 ± 6.3 min e 18.5 ± 2.7 min for KP, EB+KP and EB+KP12 groups, repectively (P>0.05). After treatments, the LH peak was observed 28.8 ± 8.8 min; 37.5 ± 20.5 min e 63.7 ± 15.8 min after treatment for KP, EB+KP and EB+KP12, respectively (P=0.22). *EXP 3* studied the KP capacity as an ovulation inducer. Holstein females (n=47) were synchronized and during the initial diestrus (seven days after AI day), females were grouped according with the presence and size of the CL and dominant follicle (DF) of the first follicular wave growth in one of the three treatments: SAL (n=12); KP (n=19); or GnRH (buserelin acetate; n=16). Blood was collected from six animals per group at -20, 0, 40, 80, 120, 160, 210, 270 min after treatments to measure circulating LH and P4. At the moment of treatment, follicles showed same size (P=0.15) as well as CL (P=0.13) and circulating P4 (P=0.16) among groups. GnRH provided higher LH release (AUC 237.60±39.43; P<0.0001) than KP (AUC = 61.43±7.16). The LH peak occurs 20 min after KP i.m. injection, which was missed in this experiment. However, KP provided the same ovulation rate (50.0%) in comparison with GnRH (37.5%; P=0.14). There was no ovulation or LH surge in SAL. Thus, i.m. application of KP was effective to induce a LH pre-ovulatory surge; it was enhanced by previous exposition of E2 and induced ovulation in bovine females.

A044 Folliculogenesis, Oogenesis and Superovulation

Comparison of follicular irrigation of mares supplemented and non-supplemented with L-arginine - partial results

H.L. Resende[1], H.S. Canesin[1], C. Ramires Neto[1], F.L.V. Pinaffi[2], L.A. Silva[2], M.A. Alvarenga[1]

[1]Faculdade de Medicina Veterinária e Zootecnia, UNESP, Botucatu, SP, Brasil; [2]Faculdade de Zootecnia e Engenharia de Alimentos (FZEA), USP – Universidade de São Paulo, Pirassununga, SP, Brasil.

Keywords: Doppler ultassonography, L-arginine, mare.

The vascularization of the preovulatory follicle has been positively correlated with pregnancy rates in mares[1]. In a previous study[2], the intake of the amino acid L-Arginine (Arg) caused an increase in uterine blood flow and resulted in acceleration of postpartum uterine involution in mares. Therefore, the present study aimed to evaluate vascular perfusion of ovaries and follicles at the time of induction of ovulation in mares supplemented or not with Arg. Sixteen mares between 3 and 8 years old were randomly divided in two groups: mares supplemented with Arg (n = 8) and mares non-supplemented with Arg (n = 8). The animals received 100 g Arg (Ajinomoto Amino Science LLC, Raleigh, NC, USA) with 3 kg of feed from the eighth day after ovulation in portions twice a day. B mode ultrasound examination was performed to assess the follicular dynamics. At the time when a follicle ≥ 35 mm in diameter was detected, the ovarian and follicular vascular perfusion was assessed by spectral and color Doppler ultrasonography mode. In color Doppler mode, follicles vascularization were subjectively estimated and graded in percentages (0 to 100) of the follicular wall with signs of vascularization. In spectral Doppler mode, the ovarian artery ipsilateral to the preovulatory follicle was examined and the ovarian vascular perfusion evaluated by the resistance (RI) and pulsatility index (PI). To obtain these indexes, the cursor was positioned in an artery of the ovarian pedicle and three identical spectral graphics of subsequent cardiac cycles were generated to obtain the RI and PI values. After the evaluations, ovulation was induced with 1 mg of deslorelin acetate and detected between 24 and 48 hours after induction. Statistical analysis was performed with SAS (Release 9.2, SAS Institute, Inc., Cary, NC, USA). The distribution of variable responses was analyzed by the Shapiro-Wilk test, and differences between groups were evaluated by the LSD test (Least Significant Different test) and non-paired t-test. The level of statistical significance was set as 0.05. No statistical differences were found for the variables PI (P = 0.42), RI (P = 0.60) and subjective follicular vascularization (P = 0.65) between Arg supplemented and non-supplemented mares. The indexes represented inverse relationship with vascular perfusion of the target tissue. In young mares, Arg was not efficient to increase vascular perfusion of the preovulatory follicle.

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A045 Folliculogenesis, Oogenesis and Superovulation

Ovarian antral follicular population in cows of Nelore and Girolando breeds

J.S. Oliveira Junior, M.B. Souza, E.R. Andrade, H. Kischel, C.J. Tavares Cardoso, W.A. Leite da Silva, F.A. Melo-Sterza

UEMS, Aquidauana, MS, Brasil.

Keywords: antral follicles, Girolando, Nelore.

The ovarian antral follicular population (OAFP) is positively associated with fertility in cattle (Ireland et al., 2008, Biology of Reproduction, 79, 1219–1225). In previous studies, dairy cows with low OAFP had lower reproductive performance when compared to cows with higher OAFP (Mossa et al., 2012, J. Dairy Sci., 95, 2355–2361). The growing interest in dairy breeds adapted to the tropics demands more studies on this topic. This study aimed to evaluate the OAFP in Nelore (n = 13; *Bos taurus indicus*) and Girolando cows (n = 19; *Bos taurus indicus* ¼ x *Bos taurus taurus* ¾). All animals received an intravaginal progesterone device (CIDR®, InterAg, Hamilton, New Zealand), 2 mg IM of estradiol benzoate (Estrogin®, Biofarm, Jaboticabal, São Paulo, Brazil) and 25 mg IM of Dinoprost (Lutalyse ®, Pfizer, Guarulhos, São Paulo, Brazil) on a random day (D0) to synchronize the emergence of a new follicular wave. After seven days (D7) the devices were removed and on day 11 (D11) antral follicles were counted using an ultrasound machine equipped with a 7.5 MHz transducer (Aquila ®, Pie Medical, Maastricht, The Netherlands). All follicles ≥ 3 mm in diameter were counted in both ovaries. The counting procedure was repeated three times with intervals of 35 days between consecutive measurements. To evaluate the effect of breed on OAFP counts, ANOVA followed by Tukey test was used considering $P<0.05$ for significant differences (ASISTAT 7 6-beta; 2013). No significant difference was observed between OAFP in Nelore and Girolando females. In Nelore, the average OAFP was 31.48 ± 9.83, with no significant variation between the three counts (35.15, 33.61 and 25.69). In Girolando females, the average OAFP was 27.01 ± 11.25, and similarly there was no significant variation between the OAFP counts (30.26, 27.47 and 23.31). It was concluded that OAFP of adult Nelore and Girolando females is similar and remains constant during consecutive estrous cycles when animals are maintained under the same experimental conditions.

A046 Folliculogenesis, Oogenesis and Superovulation

Establishment of a model for the study of follicle dominance in cows based on follicle aspiration in vivo

J.N. Drum[1], R.S. Surjus[1], A.P. Lemes[1], R.V. Valle[1], R.T. Souza[1], M.C. Wiltbank[2], R. Sartori[1]

[1]ESALQ - USP, Piracicaba, SP, Brasil; [2]University of Wisconsin, Madison, USA.

Keywords: dominance, follicle deviation, in vivo aspiration.

One of the main challenges for the study of follicular dominance in cattle is to determine the exact moment that follicle deviation occurs. Studies show that European breeds have follicular deviation at around 8.5 to 9.0 mm, whereas in Nelore females, deviation occurs between 5.5 and 7.0 mm (reviewed by Sartori et al., Repr. Dom. Anim., v.7, p.357-375, 2010). However, an individual variation does not allow us to precisely define when deviation will occur. The objective was to establish a model for the study of follicular dominance, identifying more precisely the exact time of occurrence of follicular dominance. Thus, 11 Nelore (NEL) and 10 Holstein cows (HOL) were used in a crossover design. All cows had the emergence of a new wave synchronized by OPU of follicles > 7 mm. Cows were then implanted with norgestomet and 24 h later, two injections of PGF2α were given 12 h apart. CL regression was monitored by ultrasound. Ovarian ultrasound evaluation was also performed every 12 h for follicle measurements. The biggest growing follicle (F1) reached the expected deviation for each breed (NEL = 6.5 mm, HOL = 8.5 mm) at 72 ± 12 h after the onset of the study. At this time, cows were randomly allocated into two groups (C: control, T: Treatment). The cows in group T had the F1 aspirated, and in group C there was no aspiration. Ultrasound evaluation of the control and the other follicles (F2 and F3) were performed until 72 h after deviation. The data were analyzed by generalized linear models (SAS® 9.2) and presented as least squares means ± SE. In group C, 12 h after F1 reached its expected size at deviation, it was bigger than the others (F2 and F3), featuring dominance. No difference was observed between the diameter of F1 at the time of aspiration and F2 12 h later in the T group for both NEL (F1: 6.9±0.20 vs F2: 7.0±0.15 mm; P=0.0003) and HOL (F1: 8.8±0.35 vs F2: 8.5±0.29 mm; P=0.0003). Likewise, there was no difference in diameter between F1 of group C and F2 of group T after aspiration of F1 of group T over the time of evaluation. However, F2 of group T had a bigger diameter (P<0.05) as compared to F2 of group C from 24 h after the time of deviation for NEL and HOL. It is concluded that the second largest follicle is able to become morphologically dominant when the largest follicle is aspirated, allowing for a more precise estimation of the exact moment of dominance, established by another follicle.

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A047 Folliculogenesis, Oogenesis and Superovulation

Hormonal evaluation during the ovulatory cycle of Holstein, Gir and Buffalo heifers at the same environment and nutritional management

J.M. Baldrighi, A.F. Siqueira, P.M. Assis, M.F. Sá Filho, J.A. Visintin, G.P. Nogueira, P.S. Baruselli, M.E.O.D. Assumpção

FMVZ, São Paulo, SP, Brasil.

Keywords: heifers,hormones, interovulatory interval.

The objective of the present study was to evaluate during the same interovulatory interval, plasma concentrations of P4, testosterone, insulin and IGF-I in Holstein (n=14), Gir (n=5) and Buffalo (Murrah cross; n=15) heifers, maintained under the same nutritional management. All heifers were kept at the Department of Animal Reproduction (CBRA/FMVZ/USP), Pirassununga Campus. They were pre-synchronized with two doses of prostaglandin F2α (D-cloprostenol, 150μg, IM; Prolise® Tecnopec, São Paulo, SP), fourteen days apart (D0 = day of first ovulation). From D0, daily ultrasound scan of the ovaries and blood collection from their jugular vein were performed until the detection of the following ovulation. The CL volume was calculated based on the equation for volume of a sphere (3/4)*(pi)*(radius)^3. The CL cavity volume was disregarded when present. P4 analysis (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic®, Los Angeles, CA) was performed every day during the interval whereas testosterone analysis (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic®, Los Angeles, CA) was performed from D0 to D13 of the interval. Insulin (RIA kit, Coat-A-Count, Siemens Medical Solution Diagnostic®, Los Angeles, CA) and total IGF-I (Elisa kit Quantikine® ELISA, Human IGF-I, R&D Systems®, Minneapolis, MN) were measured in two phases: the follicular phase (D0 and D1) and the luteal phase (D9 to D13). The response variables were analyzed by PROC GLIMMIX for repeated measures of SAS software (SAS System for Windows, 9.3; SAS Institute Inc., Cary, NC). P4 concentrations were affected by day ($P<0.0001$) and by interaction breed*day ($P=0.001$). These effects were observed for testosterone concentrations [breed, ($P<0.0001$); day ($P<0.0001$), interaction ($P=0.001$)]. The results showed that Holstein heifers presented similar concentrations of P4 and lower concentrations of testosterone than Gir heifers. The bubaline heifers presented the lowest concentration of both steroids. Regarding CL volume, it was observed effect of breed ($P<0.0001$), day ($P<0.0001$) and their interaction ($P<0.0001$). CL volume was greater in taurine heifers. Buffalo heifers showed the smallest CL volume during the entire interval. IGF-I ($P<0.0001$) and insulin ($P<0.0001$) concentrations were higher in the follicular (129.5±4.5ng/ml and 7.1±0.7UI/ml, respectively) than luteal phase (102.8±4.5ng/ml and 4.7±0.7UI/ml, respectively) for the three breeds. Moreover, insulin concentration was affected by breed ($P=0.025$). Gir heifers (9±2.1UI/ml) presented higher levels of insulin when compared to the Holstein heifers (4.1±0.6UI/ml). However, IGF-I levels did not differ among breeds ($P=0.054$). Results presented herein may indicate that breed differences in steroid production or liver metabolism could have an effect on circulating P4 and testosterone concentrations.

A048 Folliculogenesis, Oogenesis and Superovulation

A role for exosomes in regulation of TGF-β family members during equine ovarian follicular development

J.C. Silveira[1], E.M. Carnevale[1], J.B. Rossini[1], J.S. Rodriguez[1], W.G. Glanzner[2], Q.A. Winger[1], G.J. Bouma[1]

[1]Colorado State University, Fort Collins, USA; [2]Universidade Federal de Santa Maria, Santa Maria, RS, Brazil.

Keywords: exosomes, follicular fluid, miRNAs.

During ovarian follicular development, cell communication is a crucial and well-regulated event, culminating with follicular ovulation or atresia. These events are dependent on endocrine, paracrine and autocrine signaling. TGFβ signaling is key in follicular development and consequently ovulation and oocyte competence (Knight, 2006, Reproduction, 132, 191-206). Exosomes are cell-secreted vesicles between 40-100 nm in size, and contain bioactive materials such as miRNAs and proteins (Raposo, 2013, Journal of Cell Biology, 200, 4, 373-383). Exosomes can be taken up by target cells through different endocytotic pathways and mediate the delivery of mRNA, protein and miRNAs. Recently, we described the presence of exosomes in follicular fluid that can be taken up by granulosa cells (da Silveira, 2012, Biology of Reproduction 86, 71). Our hypothesis is that regulation of TGFβ signaling members in granulosa cells during follicle development is mediated by exosomes secreted in ovarian follicular fluid. In order to test this hypothesis, granulosa cells and follicular fluid were collected from ovarian follicles (35mm size; immature, n=4) and 34h after GnRH/LH stimulation (mature, n=4). Real-time PCR was used to investigate 18 members of the TGFβ family in freshly isolated granulosa cells before culture and granulosa cells in culture exposed for 24h to exosomes (EXO), student's t-test was used to compare the treatments. *ACVR1* ($P<0.05$) and *ACVR2B* ($P<0.05$) levels were decreased in granulosa cells following EXO treatments compared to no treatment. For SMAD target genes, *CDKN2B* ($P<0.03$) levels in granulosa cells were increased following EXO treatments, while both *ID1* ($P<0.02$) and *ID2* levels were decreased ($P<0.02$) in granulosa cells by treatment with EXO from immature follicles. Therefore, treatment with exosomes originating from immature follicles leads to altered gene expression of selected TGFβ family members in granulosa cells from mature follicles. Interestingly, we identified high levels of ACVR1 and miR-27b (a predicted regulator of *ID2*) in exosomes isolated from mid-estrous follicles. We are currently investigating the presence of miRNAs in EXO isolations in order to identify miRNAs involved in regulating TGFβ family members.

A049 Folliculogenesis, Oogenesis and Superovulation

Differences in lipid profiles of oocytes recovered by ovum pick-up from *Bos indicus* and 1/2 *indicus-taurus* with high versus low oocyte yield

K.C. Silva-Santos[1], C.R. Ferreira[2], G.M.G. Santos[1], M.N. Eberlin[2], L.S. Siloto[1], C.O. Rosa[1], T.N. Marcantonio[1], M.M. Seneda[1]

[1]UEL, Londrina, PR, Brasil; [2]Thomson Laboratório de Espectrometria de Massas, Campinas, SP, Brasil.

Keywords: antral follicle count, lipid content, oocyte yied.

The objective of this study was to compare the overall lipid profile of oocytes recovered from *Bos indicus* (Nelore) and *indicus-taurus* (1/2 Nelore x Angus) cows with high versus low oocyte yield and to correlate such profiles with the mechanisms involved in oocyte competence. Nelore (n = 20) and 1/2 Nelore X Angus (n = 20) cows were randomly subjected to follicular aspiration during their estrous cycle (D0) to withdraw all follicles ≥ 3 mm and to induce the growth of a new follicular wave. Ovaries were examined by ultrasonography during days 4, 19, 34, 49 and 64, and antral follicles ≥ 3 mm were counted. The last ultrasound (D64) evaluation was performed after the animals were slaughtered. Cows were then assigned to one of two groups: a) high antral follicle count (AFC) / oocyte yield (mean ≥ 30 follicles; Nelore NH-group, n = 3; 1/2 Nelore x Angus, AH-group, n = 5) and b) low AFC (≤ 15 antral follicles; Nelore, NL-group, n = 4; 1/2 Nelore x Angus, AL-group, n = 4). A new OPU procedure was performed on D49 to obtain the oocytes used in this study. Following OPU recovery, the lipid profiles of the oocytes were obtained via matrix-assisted desorption/ ionization – mass spectrometry (MALDI-MS). Profiles constituted of ions corresponding to sphingommielyn (SM), phosphatidylcholine (PC) and triacylglycerol (TAG) were observed. Data was submitted to partial least squares-discriminant analysis (PLS-DA). COCs from preantral follicles and expanded oocytes presented differences in the abundance of membrane structural lipids scompared to GI, GII and GIII quality oocytes. Considering just GI, GII and GIII oocytes, Nelore samples tend to cluster separately from 1/2 Nelore x Angus samples, specially the NH-group. There were no differences among oocytes from 1/2 Nelore x Angus (AH- and AL-groups). The lipid ions $[PC (P-38:5) + H]^+$ and/or $[PC (P-36:2) + Na]^+$, $[PC (38:2) + H]^+$, $[PC (38:5) + Na]^+$ and $[TAG (60:8) + NH^4]^+$ were more abundant in the Nelore oocytes (NH- and NL-group) compared to 1/2 Nelore x Angus (AH- and AL-groups). There are therefore membrane structural differences, and also in the lipid droplets, between Nelore and 1/2 Nelore x Angus oocytes recovered from cattle with high versus low oocyte yield. The greater abundance of some PC in Nelore oocytes may be related to lipidic metabolic rate differences and contribute to oocyte competence and embryo development.

A050 Folliculogenesis, Oogenesis and Superovulation

Temporal expression of BMPRII and EGFR genes in bovine cumulus cells and oocytes during maturation *in vitro*-preliminary results

L.G. Mesquita, F.C. Castro, P.R.L. Pires, F.G. Zaffalon, E.T. Pansani, M.C.R.V. Cunha, K.L. Schwarz, H. Fernandes, R.C. Botigelli, M.H.C. Cruz, C.L.V. Leal

Faculdade de Zootecnia e Engenharia de Alimentos-USP, Pirassununga, SP, Brasil.

Keywords: BMPRII, cumulus cells, EGFR.

GDF-9 and BMP15 are important oocyte-derived paracrine factors that control cumulus cells function (Gilchrist et al. 2008, Human Reproduction Update, 14, 159–177). These factors use a combination of signaling systems including the TGFb superfamily, BMPRII receptor. EGFR is essential for LH signaling in COCs to induce maturation and assist paracrine factors GDF-9 and BMP15 in cumulus cells expansion (Su et al. 2010, Molecular Endocrinology, 24, 1230-1239). Gene silencing by lipofection in cumulus cells may be used to study the interaction between cumulus cells and oocyte and to evaluate the functions of cumulus genes on oocyte maturation and competence. The aim of this study was to evaluate EGFR and BMPRII expression to determine their temporal expression pattern during *in vitro* maturation in bovine oocytes and cumulus cells and determine whether they could be candidate-genes for gene silencing studies. For this purpose, genes must have stable or increased expression during culture. Cumulus-oocyte complexes were aspirated from abattoir ovaries, transferred in groups of 23 to 90µL drops of TCM 199 supplemented with FSH (0.5µg/ml), LH (5µg/ml), cysteine (100mM), sodium pyruvate (0,25mM), 10% FCS and gentamicin (25 µg/ml) and cultured for 24 hours at 38.5°C and 5% CO_2 in air. For transcripts detection, every 3 hours, cumulus cells and oocytes samples were separated by pipetting COCs (pools of 23) and then frozen in PBS solution containing 0.1% polyvinyl alcohol and 100 IU/mL RNaseOUT inhibitor (Invitrogen ™ - S.P./Br.), plunged into liquid N_2, and stored at -80°C for later analysis. RNA isolation was performed with TRIzol reagent (Invitrogen ™- S.P./Br.) and reverse transcription with the enzyme MultiScribe ™ Reverse Transcriptase (Applied Biosystems® - S.P./Br.), following the manufacturers recommendations. Relative quantification of gene transcripts was performed by real-time PCR using SYBR green PCR kit master mix (Applied Biosystems® - Brazil) with 3 endogenous controls (PPIA, GAPDH and β actin). Time effects were tested by ANOVA and means were compared by Student's t test (3 replicates). In oocytes, EGFR and BMPRII expression was stable throughout maturation ($P>0.05$). In cumulus cells, EGFR expression was increased at 3, 6, 9 hours compared to other periods ($P<0.05$). BMPRII expression increased after 12 hours and decreased after 18 hours. At 6 hours, there was an inverse pattern between EGFR and BMPRII expression, as EGFR decreased BMPRII expression increased at this time point. In conclusion, this study suggests that both selected genes may be suitable candidates for gene silencing studies in oocytes. In cumulus cells, they may be candidates as long as silenced for up to 6 h for EGFR and after 6 h for BMPRII. Also, the results indicate a temporal relationship between genes, with sequential expression of EGFR followed by BMPRII, which must be investigated and compared with previous studies like Caixeta et al. 2012.

A051 Folliculogenesis, Oogenesis and Superovulation

Effects of supplementation with detoxified castor meal on gene expression in goat oocytes

L.M. Silva[1], C.R. Lazzarotto[2], K.C.S. Tavares[2], L.H. Aguiar[2], L.T. Martins[2], S. Gaudêncio Neto[2], L.R. Bertolini[2], M. Bertolini[2], C.M.G. Silva[1], D. Rondina[1]

[1]UECE, Fortaleza, CE, Brasil; 2.UNIFOR, Fortaleza, CE, Brasil.

Keywords: byoproducts of biodiesel, goats, oocyte maturation.

Currently, biodiesel production from oilseed plants (*Ricinus communis*) in the northeast of Brazil receives great incentives, mainly in the semi-arid region. However, this process results in great environmental concerns regarding the residues, due to the presence of ricin, a potentially toxic compound. Despite the presence of this compound in castor meal, previous studies demonstrated this product as having a high protein content (41%), making it attractive to be used as an alternative dietary source for ruminants (Diniz et al., 2011, Livestock Science, 135, 153-167). To evaluate the effect of feeding detoxified castor meal on oocyte viability and quality, the expression of BMP15 and GDF9 genes were measured in oocytes from 25 mixed breed adult goats allocated into two balanced dietary groups: diet I: Tifton hay and concentrate (n=12); and diet II: Tifton hay and concentrate with 15% detoxified castor meal substituting soybean meal (n=13). After sixteen months of feeding, estrus was synchronized and all animals were subsequently slaughtered. Following ovarian recovery, only grade I, II and III *cumulus*-oocyte complexes (COCs) were selected for IVM for 22 h, being subsequently analyzed by multiplex qPCR. The expression of GDF9 and BMP15 genes was evaluated using pools (4 to 5 oocytes/pool) of denuded oocytes at grades I (n = 3), II and III (n = 4) with and without the presence of the first polar body. RNA extraction, reverse transcription and qPCR were done with the *CellsDirect™ One-Step qRT-PCR* kit (Invitrogen, Paisley, UK), using 400 µM of each primer and 80 µM of a specific TaqMan® probe (Invitrogen, Paisley, UK). Results were normalized using the $2^{-\Delta\Delta Ct}$ method with ribosomal protein S9 (RPS9) as the housekeeping gene. Relative mean values were compared by the Fisher test ($P<0.05$). No dietary effect was observed on BMP15 and GDF9 mRNA expression (BMP15 – 1.925 ± 0.218 diet I e 2.474 ± 0.248 diet II; GDF9 – 4.602 ± 0.779 diet I and 3.604 ± 0.557 diet II) between goat oocyte categories. However, irrespective of oocyte grade and diet, expression of BMP15 was higher in oocytes without polar body (3.466 ± 0.452 without polar body and 1.190 ± 0.087 with polar body; $P<0.05$); and expression of GDF9 was higher in matured oocytes (6.198 ± 0.934 with polar body and 1.489 ± 0.270 without polar body; $P<0.05$). In conclusion, expression of GDF9 and BMP15 genes was not affected by diet, suggesting that feeding detoxified castor meal did not change oocyte quality in goats.

A052 Folliculogenesis, Oogenesis and Superovulation

Evaluation of the luteal function in sheep using color doppler ultrasonography

L.M. Figueira[1], F.Z. Brandao[1], A.L.R. Rodrigues[1], A.C. Sarzedas[1], E. Oba[2], E.K.N. Arashiro[3], J.F. Fonseca[4], J.H.M. Viana[3]

[1]Faculdade de Veterinária da UFF, Niteroi, RJ, Brasil; [2]FMVZ - UNESP, Botucatu, SP, Brasil; [3]EMBRAPA Gado de Leite, Juiz de Fora, MG, Brasil; [4]EMBRAPA Caprinos e Ovinos, Coronel Pacheco, MG, Brasil.

Keywords: color Doppler, luteal dynamic, sheep.

The aim of the present study was to evaluate the use of color Doppler ultrasonography in the study of luteal function in sheep. Estrus was synchronized in nulliparous Santa Ines ewes (n=18) with short-term protocol of 6 days (Cavalcanti, R. Bras. Zootec., v. 41, p.1412-1418, 2012). After estrus detection, ovulation (D0) and subsequent corpus luteum (CL) development was assessed every 24 hours by ultrasonographic exams using a portable ultrasound device with color Doppler (Sonoscape® S6, Shenzhen, China), equipped with a 7.5 MHz linear transducer. Ultrasonography exams were performed until detection of a subsequent ovulation. In each sonogram, the ovaries were scanned with both B and color flow Doppler modes and a video was recorded. Luteal tissue and vascularization areas were also measured in a single 2D image (at CL largest diameter). Blood samples were collected daily for plasma progesterone (P4) determination by radioimmunoassay. Results were evaluated by ANOVA and differences between means were determined by Tukey's test. Correlations were analyzed using Pearson's correlation method. Results are shown as means±SD. Color Doppler ultrasonography allowed an early examination of the developing CL, which was first visualized at day 0.77±0.62 with an average area of 29.68±13.21 mm^2. As expected, luteal dynamics was characterized by a luteogenesis period, in which a progressive increase of the luteal tissue area and plasma P4 concentration was observed (maximum 124.0±38.0 mm^2 and 11.23±4.89 ng/mL, respectively), a plateau phase in which no significant increase was detected, and a luteolysis period in which plasma P4 concentration decreased abruptly while luteal tissue area decreased gradually. Similarly, vascularization area progressively increased during luteogenesis (maximum 52.78±24.08 mm^2) and gradually decreased during luteolysis. These results demonstrate the importance of a functional vascular structure for CL development. Also, variations observed with color Doppler ultrasonography were directly related to luteal function. A positive correlation between CL vascularization area and plasma P4 concentration during luteogenesis ($r=0.22$, $P<0.05$) and luteolysis ($r=0.48$, $P<0.05$) was observed. The low correlation value observed during luteogenesis period was likely due to high vascularization of the early CL (corpus hemorrhagicum). When CL was first visualized, mean vascularization area was 12.26±6.9 mm^2, which represented 44.3% of the luteal tissue area. In conclusion, luteal dynamics in Santa Ines sheep was similar to the patterns observed in other sheep breeds and domestics ruminant species (cows and goats). Color Doppler ultrasonography allowed an early visualization of the CL, but its use to quantitatively assess luteal function remains a challenge.

A053 Folliculogenesis, Oogenesis and Superovulation

Effects of epidermal growth factor (EGF) on the morphology and activation of ovine primordial follicles *in situ*

L.P. Santos, A.Y. Cavalcante, T.L. Lins, R.S. Barberino, B.B. Gouveia, J.M. Silva Santos, R.J.S. Gonçalves, M.H.T. Matos

UNIVASF, Petrolina, PE, Brasil.

Keywords: ewe, oocyte, ovary.

Epidermal Growth Factor (EGF) promotes the transition of primordial to primary follicles in goats (Celestino et al., 2009, Reprod. Sci., v.16, p.239-246). However, few studies were performed in sheep. The aim of this study was to evaluate the effects of EGF on the morphology and *in vitro* development of ovine preantral follicles. After collection of ovine ovaries (n=10) in the slaughterhouse, the cortex was divided and one fragment was used for histology (fresh control), while the remaining fragments were *in vitro* cultured for 7 days in supplemented α-Minimal Essential Medium (α-MEM- GIBCO-Invitrogen, St Louis, EUA), in the absence (control medium) or presence of EGF (1, 10, 50, 100 or 200 ng/mL). After culture, morphological analysis of preantral follicles was performed, and the follicles were classified as normal or atretic, as well as primordial or developing follicles (intermediate, primary and secondary follicles). The percentage of normal, primordial and developing follicles were compared by ANOVA and Tukey test ($P<0.05$). After 7 days of culture, all treatments significantly reduced the percentage of morphologically normal follicles compared with the fresh control, where follicles had a centralized oocyte without pyknotic bodies and cytoplasmic retraction, and layers of granulosa cells well organized. However, no differences ($P>0.05$) were observed between MEM and different concentrations of EGF regarding this parameter. In comparison with the fresh control, a significant decrease of primordial follicles and an increase of developing follicles were observed in all treatments, except in cultures with 200 ng/ml of EGF. In addition, α-MEM and 1 ng/ml of EGF promoted higher ($P<0.05$) follicular activation than the other treatments. In conclusion, the α-MEM culture medium alone or supplemented with 1 ng/ml of EGF promotes ovine primordial follicles activation *in vitro*.

A054 Folliculogenesis, Oogenesis and Superovulation

Estimate of preantral follicles population in prepubertal and adult bitches

N.T. Lunardon, K.C. Silva-Santos, R.C. Justino, G.T. Dessunti, M.M. Seneda, M.I.M. Martins

Universidade Estadual de Londrina, Londrina, PR, Brasil.

Keywords: canine, preantral follicles, prepubertal.

Information on the distribution and quantification of preantral ovarian follicles in bitches are scarce in the literature, especially in prepubertal bitches. The comparison between prepubertal and adult bitches will contribute for the use of reproductive biotechnologies. The objectives of this study were to estimate the population of preantral follicles in small and medium-sized bitches at prepubertal and adult ages, and to compare the population between the right and left ovaries. Eighty ovaries obtained by elective ovariohysterectomy from 40 bitches were divided into 4 groups (prepubertal small size, n = 10; prepubertal medium size, n=10; adult small size, n = 10; adult medium size, n=10). After surgery, ovaries were immersed in Bouin's fixative for histological processing. Subsequently, the ovaries were dehydrated in alcohol, fixed in xylene, embedded in paraffin and serially sectioned every 5 µm. Every 70 histological sections were made with a blade tissue and stained with periodic acid-Schiff (PAS) and hematoxylin. The number of preantral follicles was estimated by counting the follicles in each section using the oocyte nucleus as a marker and a correction factor (Gougen and Chainy, 1987). Preantral follicles were classified according to the developmental stage. The Kruskal-Wallis test followed by the Dunn's test was used to compare groups, and $P \leq 0.05$ was considered significant. Prepubertal medium bitches (± 12.65 kg) had more preantral follicles compared to the other groups. The population of preantral follicles was variable between individuals of the same age and between groups (mean ± standard error; 51611 ± 18577 for small prepubertal females, 143562 ± 21718 for medium prepubertal bitches, 49546 ± 10951 for small adult bitches, and 28090 ± 15705 for medium adult bitches). The estimates of the population of preantral follicles were different for the right and left ovaries. In conclusion, the use of the ovaries of prepubertal bitches will provide a greater number of preantral follicles for assisted reproduction techniques and he use of only one of the ovaries to estimate the population of preantral follicles in bitches may overestimate or underestimate the results.

A055 Folliculogenesis, Oogenesis and Superovulation

Effects of kit ligand (KL) on Cyclin B1 and Y box Binding Protein 2 mRNA expression in bovine oocytes submitted to *in vitro* maturation

P.F. Lima[1], C.M. Ormond[1], E.S. Caixeta[1], M.F. Machado[1], D.J. Sartor[1], F.F. Franchi[1], C. Price[2], J. Buratini[1]

[1]UNESP, Botucatu, SP, Brasil; [2]University of Montreal, Montreal, ZZ, Canadá.

Keywords: IVM, Kit ligand, oocyte.

The oocyte and surrounding cumulus cells (DC) secrete paracrine factors that regulate cumulus-oocyte complex (COC) differentiation. We have recently reported that oocyte-derived bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 10 (FGF10) cooperate to increase kit ligand (KL) mRNA expression in cumulus cells, which stimulates meiosis progression during *in vitro* maturation (IVM) in cattle (Lima *et al*. in SSR 2012 Annual Meeting, abstract 295). Cyclin B1, a subunit of maturation promoting factor (MPF; Liu *et al*., 2012 Theriogenology 78, 1171 -1181), and Y box binding protein 2 (YBX2), a regulator of mRNA stability and spindle formation (Medvedev *et al*. Biology of Reproduction 85, 575-583), are expressed by the oocyte and are required for meiosis resumption. The aims of this study were to assess the expression patterns of Cyclin B1 and YBX2 mRNA during IVM (Experiment 1), and to test the effects of KL on cyclin B1 and YBX2 mRNA expression in bovine oocytes submitted to IVM (Experiment 2). Groups of 20 immature COCs (grades 1 and 2) from 3-8mm follicles of abattoir ovaries were used throughout the study. In Exp. 1, COCs were cultured for 1, 4, 8, 12, 16 and 22 hours (n = 4 per time point; immature COCs were used to represent 0h) in 100µl drops of maturation medium (TCM 199 containing Earle's salts supplemented with 0.4% BSA; 1µg/mL FSH; 10UI/mL LH; 22µg/mL sodium pyruvate and 75µg/mL amicacin). In Exp. 2, COCs were cultured for 22 hours as described above with graded doses of KL (0, 10, 50 and 100ng/ml, n=4 per dose). In Exps. 1 and 2, cumulus cells and oocytes were separated mechanically after culture, total RNA was extracted from oocytes using RNeasy (Qiagen), and 100ng of RNA was treated with DNase and submitted to reverse transcription using OligoDT and Sensiscript (Qiagen). Messenger RNA abundance of cyclin B1 and YBX2 was assessed by real time RT-PCR (normalized by cyclophilin) in oocytes from Exps. 1 and 2. Treatment effects were tested by ANOVA and means were compared by the Tukey-Kramer HSD test. In Exp. 1, cyclin B1 mRNA abundance did not change during the first 12h of culture in the oocyte, but increased from 12 to 22h of culture. In contrast, oocyte YBX2 mRNA levels decreased gradually during IVM and were four times lower at 22h compared with 0h. In Exp. 2, KL did not affect cyclin B1 mRNA expression, but caused a three fold increase in oocyte YBX2 mRNA abundance at all doses tested (P=0.02). In conclusion, the present data suggest that the mechanisms by which KL stimulates meiosis progression during IVM in cattle appear to include up-regulation of YBX2 expression in the oocyte.

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A056 Folliculogenesis, Oogenesis and Superovulation

Expression of genes associated with steroidogenic and ovulatory capacity in Angus cows undergoing fixed-time artificial insemination protocol

R.A. Satrapa[1], E. Lucacin[1], E.M. Razza[1], A.G. Pupulim[1], A.C.S. Castilho[1], B. Loureiro[1], M.F. Machado[1], P.K. Fontes[1], R.L. Ereno[1], C.M. Barros[1]

[1]Departamento de Farmacologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brasil.

Keywords: gene expression,ovulation, TAI.

In fixed-time artificial insemination (FTAI) protocols the diameter of the preovulatory follicle is modulated also by progesterone (P4) exposure length. Exposure to P4 may affect the future ovulatory follicle, which reflects in the quality of the oocyte and the subsequent embryo. Thus, the objective was to study the effects of P4 on the expression of genes related to ovulatory and steroidogenic capacity (LHR, AGTR2, FSHR e CYP19A1) in granulosa cells derived from Angus cows submitted to FTAI protocol. Multiparous Angus cows (n=14) were randomly assigned to two experimental groups: P4/12h and P4/24h. The animals from group P4/12h (cows slaughtered 12 h after implant removal P4; n = 7) received in a random day of the estrous cycle (D0) an intravaginal device containing progesterone (1.0 g; Primer®; Agener Animal Health, SP, Brazil) and 2.5 mg of estradiol benzoate (EB; RIC-BE®; Agener Animal Health SP, Brazil; IM). After 8 days (D8) cows were treated with $PGF_2\alpha$ (150 mg d-cloprostenol; Prolise®; Agener Animal Health, SP, Brazil; IM) and Primer® was removed (19:00pm). The animals from P4/24h (cows slaughtered 24 h after implant removal P4, n = 7) were treated with a protocol similar to that described above, except that the P4 was withdrawn 12 h before (D8; 07h00min). All animals were slaughtered at D9 (07h00min) and the ovaries were transported to the laboratory immediately. Only the dominant follicle from each animal was dissected to obtain granulosa cells, which were used for extraction of total mRNA. Expression of target genes and of the constitutive gene cyclophilin A (PPIA) was assessed by real time RT-PCR with the Sybr Green protocol. Relative gene expression values were determined by the Pfaffl method. Messenger RNA levels (mean ± SEM) were compared by the t test and the significance level was $P<0.05$. There was no significant difference between mRNA levels of AGTR2, FSHR e CYP19A1 in granulosa cells from groups P4/12 (3.76±1.07, 0.91±0.16 and 0.96±0.13, respectively) and P4/24h (3.84±1.42, 1.06±0.05 and 1.58±0.31, respectively). However, mRNA expression of LHR was higher in granulosa cells in group P4/12h (1.02±0.24, $P<0.05$) compared to P4/24h (0.40±0.19). Thus, it is suggested that the exposure length to the P4 intravaginal device in FTAI protocols might affect the expression of genes related to steroidogenic and ovulatory capacity in follicles from *Bos taurus* cattle.

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A057 Folliculogenesis, Oogenesis and Superovulation

Luteinizing hormone receptor gene expression during follicular divergence in *B. taurus* vs *B. indicus* dairy breeds

S. Wohlres-Viana[1], E.K.N. Arashiro[2], A.P. Sudré[3], L.G.B. Siqueira[2], M.P. Palhão[4], L.S.A. Camargo[2], C.A.C. Fernandes[4], M.A. Machado[2], J.H.M. Viana[2]

[1]UFJF, Juiz de Fora, MG, Brasil; [2]EMBRAPA Gado de Leite, Juiz de Fora, MG, Brasil; [3]UFF, Noterói, RJ, Brasil; [4]UNIFENAS, Alfenas, MG, Brasil.

Keywords: follicular dominance, follicular dynamic, steroidogenisis.

The establishment of follicular dominance is a key phenomenon to determine the specie-specific ovulation rate, and involves the transition from FSH to LH-dependence by the dominant follicle. This physiological event occurs at different moments in *B. taurus* and *B. indicus* cattle. Previous studies of our group analyzed the relationship between the diameter at deviation and the progression of the intrafollicular steroidogenesis in dairy breeds (Arashiro et al., Reprod Fertil Dev 25:235, 2013). The objective of the current study was to evaluate the expression of the LH receptor (LHR) gene during follicular deviation in two dairy breeds. Mural granulosa cells (GC) were recovered from Holstein (*B. taurus*; n=10) and Gir (*B. indicus*; n=10) heifers, as described before (Arashiro et al., Reprod Fertil Dev 24:175, 2012). GC were collected by ultrasound-guided follicular aspiration of follicles at 6, 8, 10, and 12 mm in diameter from Holstein heifers, and 4, 6, 8 and 10 mm from Gir heifers. The recovered follicular fluid was centrifuged and the cells were washed with NaCl 0.9% saline and kept in RNA Later (Ambion, Austin, TX, USA). Total RNA extraction was performed using the RNeasy Micro Kit (Qiagen, Hilden, Germany), quantified in spectrophotometer (Nanodrop), and cDNA was synthesized using the Superscript III kit (Invitrogen, Carlsbad, CA, USA). The obtained cDNA underwent real-time PCR, using LHR specific primers in a region without occurrence of isoforms and thus producing a single fragment, a primer pair for the CYP17A1 gene as a marker of thecal cell contamination, and a primer pair for the GAPDH gene as an endogenous control. Samples with thecal cell contamination were discarded. Results were analyzed by the software REST© and are presented as means±SEM. In both breeds, LHR expression was identified in follicles of all size categories. The expression of LRH in 4 and 6 mm follicles (for Gir and Holstein, respectively) was used as a reference value (=1). A peak in LHR expression (11.0±5.8 and 10.7±8.0 -fold the reference value) was observed in 10 mm (Holstein) and 8 mm (Gir) follicles, i.e., diameters only reached after deviation in these breeds (8.6±0.4 and 6.3±0.2; Holstein and Gir, respectively). The increase in LHR expression occurred in parallel to the previously described increase in intrafollicular estradiol concentrations at this same interval of follicular growth (Arashiro et al., 2013). Results of the present study demonstrate that LHR gene is expressed even in follicles smaller than the expected diameter at deviation in both *Bos taurus* and *Bos indicus* females. Also, relative LHR expression increases during the establishment of dominance, consistently with the progression of steroidogenesis. Further studies will evaluate whether and in which proportion LHR isoforms are present in these follicles.

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A058 Folliculogenesis, Oogenesis and Superovulation

The role of insulin-like growth factor-I on heat-induced microtubule changes and meiotic progression in bovine oocytes

T.A. Rodrigues[1], J. Ispada[1], P.H.B. Risolia[2], F.F. Paula Lopes[1]

[1]Universidade Federal de São Paulo, Diadema, SP, Brasil; [2]Universidade Estadual de São Paulo, Pirassununga, SP, Brasil.

Keywords: IGF-I, meiotic progression, microtubule.

Heat stress promotes, among several physiological and cellular alterations, changes in the reproductive tract microenvironment, compromising oocyte nuclear maturation, fertilization and embryonic development. It has been demonstrated that insulin-like growth factor-I (IGF-I) plays a thermoprotective role in the bovine oocyte. Therefore, the objective of this study was to: 1) determine the effect of heat shock on bovine oocyte microtubule and meiotic progression and 2) evaluate the thermoprotective role of IGF-I in this context. Cumulus-oocyte complexes (COCs) collected from slaughterhouse ovaries were subjected to control (38.5°C for 22 hours) and heat shock (41°C for 14 hours followed by 38.5°C for 8 hours) treatments in the presence of 0 or 100 ng/ml IGF-I during *in vitro* maturation (IVM). COCs were denuded, fixed in 3.7% formaldehyde and permeabilized in 0,5% Triton-X 100. Oocytes were incubated in bovine anti α-tubulin mouse IgG monoclonal primary antibody (2µg/mL) followed by Alexa Fluor® 488 goat anti-mouse IgG (10µg/mL) and Hoechst 33342 (5µg/mL) to evaluate microtubules and meiotic progression, respectively. Microtubule fluorescence intensity was quantified in the metaphase plate, polar body and total oocyte using the software Image J 1.45s. Parametric data were subjected to least-squares analysis of variance and non-parametric data were analyzed by Wilcoxon's test of SAS. Heat shock reduced (59 ± 0.11% to 29 ± 0.11%; $P< 0.05$) the percentage of metaphase II oocytes and reduced ($P<0.05$) metaphase plate microtubule organization (1.2 ± 0.06, and 1.4 ± 0.06 arbitrary units to control and heat shock, respectively. Addition of 100 ng/mL IGF-I to IVM medium minimized ($P<0.05$) the deleterious effect of temperature on oocyte meiotic progression increasing the percentage of MII of 29 ± 0.11% to 35 ± 0.11%. However, IGF-I did not affect microtubule organization in heat-shocked oocytes. There was no temperature or IGF-I effect on polar body microtubules organization. In conclusion, IGF-I played a thermoprotective role on meiotic progression. However, the same effect was not observed on microtubule organization. It has been shown that oocyte meiotic division is intimately linked to microfilament. Therefore, the positive effect of IGF-I on meiotic progression could be mediated by microfilament structure. Our previous studies demonstrated the thermoprotective effect of IGF-I on microfilament organization in bovine oocytes exposed to heat shock.

A059 Folliculogenesis, Oogenesis and Superovulation

Influence of melatonin and FSH in a sequential culture medium on *in vitro* development of isolated caprine preantral follicles

V.R.P. Barros[1], A.Y. Cavalcante[1], T.J.S.E. Macedo[1], R.S. Barberino[1], T.L. Lins[1], B.B. Gouveia[1], V.G. Menezes[1], M.Á. Queiroz[1], V.R. Araújo[2], R.C. Palheta-Júnior[1], M.C. Leite[3], M.H.T. Matos[1]

[1]Universidade Federal do Vale do São Francisco, Petrolina, PE, Brasil; [2]Universidade Estadual do Ceará, Fortaleza, CE, Brasil; [3]Universidade Federal do Recõncavo da Bahia, Cruz das Almas, BA, Brasil.

Keywords: cultive, immunohistochemistry, ovary.

Studies have demonstrated that melatonin is a potent antioxidant (Gao et al., 2012, J. Pineal. Res., 52, 305-311) and its receptors were expressed in granulosa cells of secondary and antral follicles in goats (Menezes et al., 2011, VI Jornada de Iniciação Científica, Univasf). One gonadotropin that has been widely used in *in vitro* studies for maintenance of viability and to promote follicular growth is FSH (Saraiva et al., 2010, Reprod. Sci. 17, 1135-1143). Recently, the use of Melatonin associated with FSH in the *in vitro* culture medium promoted an increase in the diameter of preantral follicles after culturing goat ovarian tissue for 7 days (Rocha *et al.,* 2013, Domest. Anim. Endocrinol., 44, 1-9). However, the effect of melatonin, alone or associated with FSH, on the *in vitro* growth of preantral follicles isolated from caprine ovaries is not known. The aim of this study is to evaluate the effect of melatonin and FSH on the *in vitro* development of isolated goat preantral follicles. Goat ovaries (n=60) were collected in slaughterhouse and after mechanical isolation, preantral follicles (diameter ≤ 200 μm) were individually cultured in 100 μL droplets, during 12 days, at 39°C, in α-Minimum Essential Medium (α-MEM$^+$; GIBCO-Invitrogen, St Louis, EUA; control medium) alone or added by fixed concentrations of Melatonin (100 or 1000 pg/mL) or in sequential medium containing Melatonin (100 pg/mL: from day 0 to 6; 1000 pg/mL: from day 6 to 12), corresponding to Experiment 1. For the Experiment 2, follicles were cultured in control medium or sequential FSH medium (100 ng/mL: from day 0 to 6; 500 ng/mL: from day 6 to 12) or sequential melatonin associated with sequential FSH. Parameters such follicular survival and extrusion rate, antrum formation, recovery rate of *in vitro* grown oocytes (diameter ≥110 μm) were analyzed at each 6 days and data were submitted to Qui-square test. Follicular diameter and growth rate of Experiment 1 were submitted to Shapiro-Wilk and Tukey tests, and those from Experiment 2, to Kruskal-Wallis test ($P<0.05$). Results from Experiment 1 showed that, after 12 days of culture, sequential melatonin medium increased ($P<0.05$) the percentage of normal follicles and the recovery rate of *in vitro* grown oocytes, compared with control and 1000 pg/ml of melatonin. In Experiment 2, all treatments increased ($P<0.05$) the percentage of normal follicles, antrum formation and recovery rate of oocytes, compared with the control, however no differences were observed among treatments ($P>0.05$). In conclusion, this study demonstrated that the use of defined concentrations of Melatonin and/or FSH, added at specific periods of culture and in a progressive way, can be successfully used to preserve follicular survival and to promote goat oocyte growth.

A060 Folliculogenesis, Oogenesis and Superovulation

Extract of amburana cearensis has cytotoxic effect on sheep preantral follicles preserved *in vitro*

V.G. Menezes, B.B. Gouveia, R.S. Barberino, R.J.S. Gonçalves, V.R.P. Barros, T.J.S.E. Macedo, J.R.G.S. Almeida, M.H.T. Matos

UNIVASF, Petrolina, PE, Brasil.

Keywords: amburana cearensis, apoptosis, ovary.

Amburana cearensis is a native medicinal plant from the semi-arid region in the northeast of Brazil (Caatinga biome) (Albuquerque e Oliveira, 2007, Journal of Ethnopharmacology, 113, 156–170), which has antibacterial and antioxidant actions (Bravo et al., 1999, Phytochemistry, 50, 71–74). However, there are no reports about this plant and ovine folliculogenesis. The aim of this study was to assess survival and apoptosis rates of ovine preantral follicles preserved *in vitro* in the extract of *A. cearensis*. Ovine ovaries (n=08) were collected in a slaughterhouse and divided into fragments (3x3x1 mm), one of them was fixed and used for histology (fresh control). The remaining fragments were preserved in Minimal Essential Medium (MEM – Sigma 87 Chemical Co, St Louis, USA; control medium) or different concentrations of the extract of *A. cearensis* (0,1; 0,2 or 0,4 mg/mL), at 4ºC for 6 h. After conservation, the fragments were fixed and submitted to morphological analysis of the preantral follicles, which were classified as normal or atretic. Analysis of oocyte and granulosa cell apoptosis was performed using TUNEL, with the *in situ* cell death detection Kit (Roche Diagnostics Ltd., 158 Indianapolis, USA). The rates of follicular survival and apoptosis were analysed by Tukey and qui-square tests, respectively ($P<0.05$). After preservation in MEM, rates of follicular survival and apoptotic cells were similar ($P>0.05$) to those found in fresh control. However, preservation of ovarian tissue in different concentrations of the *A. cearensis* extract reduced ($P<0.05$) the percentage of normal follicles and increased ($P<0.05$) rates of follicular apoptosis, compared with fresh control and MEM. In conclusion, preservation of ovine ovarian tissue at 4ºC for 6 h in the extract of *A. cearensis* increased apoptosis rate in preantral follicles, thus showing a cytotoxic effect.