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Ovarian reserve and DNA damage in oocytes and granulosa cells of GH deficient Ames Dwarf mice

T.D. Saccon[1], D.N. Garcia[1], R.G. Mondadori[1], M.T. Rovani[3], J. Pradiee[1], C.C. Barros[1], M. Masternak[2], A. Schneider[1]

[1]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil; [2]UCF - University of Central Florida, Orlando, FL, US; [3]IF - Instituto Federal Farroupilha, Santa Maria, RS, Brasil.

Ames Dwarf mice (df/df) have a defective Prop1 (Prophet of Pit1) gene, which impairs anterior pituitary gland development, resulting in deficient growth hormone (GH) secretion (Andersen et al., Reproductive Biology, 172: 494-503, 1995). As a result, df/df mice have very low levels of circulating insulin-like growth factor I (IGF-I), are smaller and live around 30-65% longer than Normal littermates (N) (Chandrashekar, V., Bartke, A., Reproductive Biology, 3: 7-28, 2003). The aim of this study was to evaluate the number of follicles in primordial, primary, secondary and tertiary stages, as well as the level of DNA damage in oocytes and granulosa cells in primordial and primary follicles in 6 month-old df/df and N mice. Ovaries of N (n =5) and df/df mice (n = 5) with 6 months of age were used for the experiments. For histological evaluation, ovarian samples were dehydrated and sectioned with a microtome. Slides were then stained with hematoxylin and eosin. Sections were cut for immunofluorescence staining as well. Ovarian sections were analyzed for numbers of follicle using an optical microscope, at 10x and 40x objectives. For immunofluorescence analysis, the ovarian samples were deparaffinized with xylene and rehydrated with graded alcohols. The primary antibody used was anti-Histone H2A.X (phospho S139) (dilution 1:500; ab81299; Abcam Ltd., Cambridge, USA) and for the secondary antibody Alexa Fluor® 488 (dilution 1:500; ab150077; Abcam Ltd., Cambridge, USA), DNA was stained with DAPI (Sigma-Aldrich, St. Louis, USA). H2AX staining was evaluated according to number of fluorescence foci present in the cell nuclei. Statistical analyzes were performed with GraphPad Prism 7 software (La Jolla, San Diego, USA). The T-Test was used for all analyzes, assuming a significance level of 5%. GH deficient df/df mice had a higher number of primordial follicles (P=0.002) and lower number of primary, secondary and tertiary follicles (P=0.02, P=0.005 and P=0.019, respectively). The total number of follicles was not different between df/df and N mice (P=0.115). Df/df mice had a lower number of H2Ax foci than normal mice in primordial and primary oocytes (P<0.0001 and P=0.004, respectively), suggesting decreased DNA damage. Df/df mice also had fewer H2Ax foci than N mice in granulosa cells of primordial and primary follicles (P=0.004 and P=0.0006, respectively). In conclusion, GH deficient df/df mice were found to have a higher number of primordial follicles compared to N mice, indicating that the deficiency of GH and IGF-I impairs primordial follicle activation and increases the ovarian longevity. In addition, df/df mice have reduced DNA damage foci, suggesting that oocytes from these animals may present higher quality and consequently a better capacity to overcome aging-induced damages.

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Oxidative stress in bovine ovarian tissue cultivated in different in vitro systems

C.B. Silva, L.Z. Bergamo, M.S.N. Hohmann, S.M. González, W.A. Verri Junior, M.M. Seneda

UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

Oxidative stress results from the imbalance between the levels of reactive oxygen species (ROS) and antioxidants. Thus, ROS are important molecules in the context of cell culture. The objective of this work was to quantify the production of ROS in four different methods for *in vitro* culture of preantral follicles of the bovine species. Ovaries (n=12) were collected from a local slaughterhouse, from cyclic Nellore females. After collection, the ovaries were washed with 70% ethanol and PBS. The surrounding ovary tissue was removed, and the ovarian cortex was divided in fragments (n=9) of 5x5x1 mm. One fragment per animal was conditioned at -81°C (control) and the other fragments (n=8) were randomly distributed into four groups: i) standard culture, in culture plate; ii) culture with agarose gel support; iii) culture with agarose gel on the plate; and iv) Millicell culture (Millipore Corp., Bedford, USA). The fragments were individually cultured in minimal essential medium (MEM, Gibco) supplemented with ITS, pyruvate, glutamine, hypoxanthine, BSA (Gibco), penicillin and streptomycin. Culture methods were tested for six (D6) or fourteen (D14) days, with medium exchanges every two days. The levels of ROS and antioxidant capacity of the sample in avoiding the production of ROS were quantified by colorimetric assay of the superoxide anion by the reduction of nitroblue tetrazole (NBT) and 2,2 V azinobis (3-ethylbenzothiazoline 6-sulfonate; ABTS) cation sequestration, respectively. Statistical analysis was performed by one-way ANOVA and Tukey post-test ($P<0.05$). It was observed that all samples cultured by the four methods presented reduction of superoxide anion levels and antioxidant capacity when compared to the control group. In D6, gel culture method on the plate showed higher levels of superoxide anion when compared to the others (mean±SEM = 5.37±0,1in the standard culture, 5.06±0,2 in the culture with gel support, 6.64±0,5 in the culture with gel on the plate and 4.69±0.1 OD/mg protein in Millicell culture). In D14 the same was observed for the gel culture method on the plate, with higher levels of superoxide anion when compared to the standard method (6.42±0.7 and 4.61±0.2 OD/mg protein, respectively). On the other hand, no significant difference was observed in the antioxidant capacity of the culture methods in D6. In D14 it was observed that the gel method on the plate presented lower antioxidant capacity when compared to the standard methods, gel support and Millicell (70.85, 85.17, 47.78 and 86.43 Trolox equivalent/mg protein, respectively). We conclude that the method of agarose gel culture on plaque results in greater oxidative stress on preantral follicles, an aspect that can compromise cell integrity.

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Effect of adding α-lipoic acid in *in vitro* culture of ovarian preantral follicles of *Bos taurus indicus*

D.V. Bonato, C.B. Silva, A.G. Lindquist, T.K. Sanches, M.A. Silva, I. Búfalo, L.Z. Bergamo, M.M. Seneda

UEL - Universidade Estadual de Londrina, Londrina, PR, Brasil.

This study aimed to evaluate the effect of antioxidant α-lipoic acid addition in the media for *in vitro* culture of preantral follicles of *Bos taurus indicus* females. Six pairs of ovaries of cyclic adult Nelore females were collected at the local slaughterhouse, with a body score ranging from 3 to 3.5 (range 0 to 5). Ovaries were washed in 70% ethanol and ovarian cortex was divided into fragments about 3x3x1 mm. One fragment per animal was immediately fixed in Bouin (non-cultured control, D0). The others fragments (n=8) were individually cultured in 24-well culture dishes containing 1 ml of minimum essential medium (MEM, Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) supplemented (MEM+) with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferin, and 6.25 ng/ml selenium; (Sigma, St. Louis, MO, USA)), 0.23 mM pyruvate (Sigma, St. Louis, MO, USA), 2 mM glutamine (Gibco BRL, Rockville, MD, USA), 2 mM hypoxantina (Sigma, St. Louis, MO, USA), 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/ml penicillin (Sigma, St. Louis, MO, USA) and 200 mg/ml streptomycin (Gibco BRL, Rockville, MD, USA). MEM + medium was added to different concentrations (50, 100 and 250 ng/ml) α-lipoic acid (Sigma, St. Louis, MO, USA). The *in vitro* culture medium was tested by six (D6) or twelve (D12) days. Every two days, the culture media were replaced by fresh aliquots. For the analysis of integrity and degree of follicular development, we used histology staining with periodic acid-Schiff (PAS) and hematoxylin. The classification was based on the evaluation of follicular development stage (primordial, primary and secondary) and morphological integrity as normal or degenerated. Data were submitted to ANOVA test ($P \leq 0.05$). We evaluated 1620 preantral follicles (normal or degenerated), being 362 primordial and 1258 in development. After six days of culture, the concentration of 100 ng/ml α-lipoic acid showed a higher proportion (40%; 72/180) of follicles morphologically intact when compared to the other tested concentrations: 16.11% (29/180) to MEM; 19.44% (35/180) to 50ng/ml; 20% (36/180) to 250 ng/ml ($P<0.05$). After twelve days of culture, the concentration of 100 ng/ml α-lipoic acid again showed a higher proportion (31.11%; 56/180) of follicles morphologically intact when compared to the other tested concentrations: 14.44% (26/180) to MEM; 25.55% (46/180) to 50ng/ml; 8.33% (15/180) to 250 ng/ml ($P<0.05$). About the percentage of developing follicles, there was no difference between the treatments, for the cultivation of six or twelve days. Therefore, MEM+ supplemented with 100 ng/ml α-lipoic acid for two or six days of in vitro culture was the most effective treatment to provide the morphological integrity of preantral follicles.

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Nellore females with low antral follicle count show higher conception rates to FTAI

J.M. Gheller[1], A.F. Silva[1], S.S. Oliveira[1], L.R. Zottesso[1], W.A.L. Silva[2], A.C.B. Lima[1], F.A.M. Sterza[1]

[1]UEMS - Universidade Estadual de Mato Grosso do Sul, Aquidauana, MS, Brasil; [2] UFMS - Universidade Federal de Mato Grosso do Sul, Campo Grande, MS, Brasil.

The number of ovarian antral follicles is a determining characteristic in bovine females. The association of this factor with fertility measures is observed in Bos taurus (Ireland et al., Biology of Reproduction, 79: 1219-25, 2008), while in Bos indicus there are still controversies (Morotti et al., Animal Reproduction, 12: 479-86, 2015). The antral follicle count (AFC) has been shown to be a potential tool used to select females in order to optimize results in reproductive programs such as FTAI (Evans et al., Reproduction in Domestic Animals, 47: 31-7, 2012). The objective of the present study was to characterize the ovarian follicular population of Nellore females and to evaluate its effect on the conception rate in FTAI programs and to correlate this characteristic with the size of the dominant follicle and the intensity of estrus. The ovaries of 435 Nellore females were evaluated by ultrasonography using a multifrequency transrectal transducer SonoScape A5 VET® on random days of the estrous cycle. Females were classified according to CFA in low AFC group (B; ≤ 15 follicles), mean AFC (M; 16 to 30 follicles) and high AFC (A; ≥ 31 follicles). All the females evaluated were submitted to FTAI protocol and gestation diagnosis. GLIMIX (SAS) was used for the statistical analysis, when the Tukey-Kramer test was applied (P≤0.05), and then the Pearson correlation test and the logistic regression were performed. AFC, age of females, semen used in crosses, body condition score (BCS) and farm of origin were included in the statistical model, however, only AFC and age had an effect (P≤0.05) on conception rate. In addition, for AFC, no correlation was found with the size of the dominant follicle (r = -0.26, P= 0.096) and the intensity of estrus expression (r = 0.15, P= 0.164), measured by the intensity of the ink on the lumbar region of the females on AI day. In terms of performance of the matrices in the FTAI programs, a higher conception rate was observed for group B, which presented 48.9% (43/88), followed by group M with 42.7% (119/279) and group A with 32.4% (22/68). 64% (279/435) of the animals evaluated in this study had mean AFC (M) and this group had no statistical difference in conception rate with the other groups (B and A), but the difference was evident (P≤ 0.05) between groups A and B. Logistic regression revealed an inverse relationship between AFC and conception rate, that is, the estimated probability of conception rate increases as the CFA becomes lower. It is concluded, therefore, that Nellore matrices with AFC ≤ 15 follicles present the best conception rates to the FTAI and that this characteristic can be used as selection criterion to improve the fertility indexes to FTAI. Acknowledgments: CAPES, Fundect, São Judas Tadeus and Arizona Farms, Real H.

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Effect of melatonin on gene expression of bovine cumulus cells

H. Fernandes, L. Schefer, D.M. Paschoal, F.C. Castro, C.L.V. Leal

FZEA - USP - Department of Veterinary Medicine, Faculty of Animal Science and Food Engineering, Pirassununga, SP, Brazil.

Melatonin (MLT) is synthesized in pineal gland and can act on reproductive competence, has antioxidant and antiapoptotic activities and influences different cell signaling pathways. In a previous study, MLT was able to influence the lipid content in bovine oocytes in the early hours of maturation (9h) coinciding with meiosis resumption. Cumulus cells (CC) may affect lipid content in oocytes and present receptors for MLT. Thus, the aim of this study was to evaluate the effect of the addition of MLT during the *in vitro* maturation (IVM) on the expression of genes related to lipid metabolism, as well as antioxidants, expansion activity and FSH receptor in bovine CC. Pools of 25 to 30 cumulus-oocyte complexes were randomly selected and submitted to IVM in TCM 199 supplemented with 11 µg/mL sodium pyruvate, 10 µg/mL gentamicin and 3 mg/mL BSA (negative control; NC) or added with hormones: 0.5 µg/mL FSH (positive control; FSH) or MLT (10^{-11}, 10^{-9} and 10^{-7}M). After 9 hours, CC were collected and evaluated by real-time quantitative PCR for ATGL, CPT1B and PLIN2 (lipid metabolism); GPX4, SOD1 and SOD2 (antioxidant); HAS1, HAS2 and PTX3 (expansion); FSHR transcripts. To normalize the gene expression data, the geometric mean of ACTB, GAPDH and PPIA was used. Relative expression levels were calculated by the comparative method of $2^{-\Delta\Delta Ct}$ (Livak et al., Methods, 25:402-8, 2001). Statistical analyses were performed by ANOVA, followed by Tukey's test (4 replicates) with 5% significance level (GraphPrism software). For lipid metabolism, only FSH increased ($P<0.0001$) transcripts for PLIN2 when compared to NC and MLT treatments. Antioxidant transcripts were increased ($P<0.05$) also in FSH when compared to NC and MLT groups for GPX4 and SOD2 genes. For expansion-related transcripts HAS1 and HAS2, FSH was about 10 times more abundant ($P<0.0001$) in relation to NC and MTL groups. However, for FSHR transcripts, FSH treatment decreased its abundance ($P<0.05$) in comparison to NC and MLT groups. For the others transcripts evaluated (CPT1B, ATGL, PTX3 and SOD1) there was no difference between treatments ($P> 0.05$). In conclusion, under the conditions studied, MLT was unable to affect the gene expression in CC during the initial 9 hours of IVM. FSH influenced CC expression for transcripts related to expansion and its own receptor, as expected, but also affected those related to lipid metabolism and antioxidants. During IVM, MLT has shown different results in diverse studies and it can be related with culture conditions. Additional studies will be performed at 24h on IVM to investigate the MLT role in CC and oocytes as well. Acknowledgment: FAPESP (HF- 2016/24884-3, LS, DMP, FCC, scholarships; CLVL, financial support 2015/20379-0).

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Efficient *in vitro* culture of mouse granulosa-oocyte complexes

F.H.C. Sugiyama, K.F. Carvalho, C.H. Macabelli, T.S. Machado, M.P. Grejo, M.R. Chiaratti

UFSCar - Universidade Federal de São Carlos, São Carlos, SP, Brasil.

During oogenesis, the oocyte undergoes an increase in volume of more than one hundred times, reaching a diameter larger than 70 µm by the time its growth is ended. Oocyte growing is supported, among others, by proliferation of surrounding granulosa cells, which closely interact with the gamete. Towards studying oogenesis, this work aimed at setting up in our laboratory a system for in vitro culture of mouse Granulosa-Oocyte Complexes (GOCs). In addition, we tested the effect of the supplementation with FSH and cilostamide (phosphodiesterase inhibitor) on GOC development. To this end, mice aged 11-12 days were sacrificed and had their ovaries enzymatically digested with 100 □g/ml collagenase and 10 □g/ml DNase for 30 min. This enabled isolation of GOCs from preantral follicles harboring one or more layers of granulosa cells. Then, GOCs were cultured at 37°C for 7 days in 24-well dishes on 3 µm collagen membranes (Costar Transwell-COL; Corning) and atmosphere of maximum humidity with 5% CO_2. For culture, it was used 750 µl of bicarbonate-buffered □-MEM (Thermo Fisher Scientific) supplemented with 0.01% BSA (Sigma-Aldrich), 10 U/ml penicillin, 10 µg/ml streptomycin and 1x ITS (Sigma-Aldrich). For the treatment, 0.01 U/ml FSH (Sigma-Aldrich) and 0.1 µM cilostamide (Sigma-Aldrich) were added to the medium. On day 0 of culture, a fraction of GOCs was denuded of granulosa cells to have oocyte diameter determined. The remaining GOCs were cultured and assessed on day 7 regarding morphology and oocyte diameter. Groups were compared by T test and regarded as different when $P<0.05$. As a result, mean oocyte diameter on day 0 was equal to 58.2±1.45 µm. On day 7, diameter was equal to 68.6±1.51 µm in the absence of supplementation and 66.8±1.21 µm in the presence of FSH and cilostamide. In spite of the absence of statistical difference between these groups ($P=0.20$), GOCs cultured in the absence of supplementation showed a more typical morphology, with multiple layers of granulosa cells. Moreover, when only oocytes larger than 70 µm were regarded, it was found an increase in oocyte diameter ($P=0.01$) in the absence of supplementation (74.3±0.66 µm vs. 71.2±0.54 µm). In conclusion, we succeeded in establishing the system for in vitro culture of GOCs and this was more efficient when performed in the absence of FSH and cilostamide.

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Use of different ovulation inductors in mares

D.R. Gomes, C.C. Jardim, R.C.L. Morais, G.A. Dutra, G. Stefani, V.L.T. Jesus, J.C.F. Jacob, M.F. Souza

UFRRJ - Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brasil.

Equine species show great variation in the duration of estrus, presenting an obstacl in the prediction of ovulation by rectal palpation and transrectal ultrasonography. Ovulation in mares occurs on average 75 hours after the follicle reaches 35 mm in diameter (Silva et al., Rev. Bras. Med. Vet., 38(Supl.2):45-48, 2016), and can be shortened to 36-48h by ovulation inducers. Human chorionic gonadotrophin (hCG) and gonadotrophin releasing hormone (GnRH) analogs such as deslorelin acetate are the most commonly used hormones for ovulation induction. The aim of this study was to compare the efficiency and precision of different ovulation inducers in mares. A total of 79 estrus cycles of 47 mares (Mangalarga Marchador), between three to 25 years old, weighing between 350 and 500 kg, were used. Ovarian and uterine scans were performed using a digital ultrasound machine, equipped with a linear 5.0 Megahertz transducer (Sonoscape A6). The cycles were distributed in groups; G1 (n = 34) = 1000 IU of hCG (Chorulon®) (IV); G2 (n = 25) = 1mL of histrelin acetate (Strelin®) (IM); G3 (n = 20) = 1000 IU of hCG (IV) + 1 mL of histrelin acetate (IM). The mares were monitored daily, initiating treatment when the largest ovarian follicle reached the diameter ≥ 35mm and endometrial edema compatible with estrus. Twenty four hours after treatment, mares were monitored every 6 hours until ovulation. Data were analyzed using ANOVA. The mean follicular diameter at the time of induction was 35.3 ± 0.7; 35.0 ± 0.5; 35.3 ± 0.8 for G1, G2 and G3, respectively. The percentage of ovulations up to 36 hours was 73.4% (25/34), 4.0% (1/25), and 25.0% (5/20) and up to 48 hours was 100% (34 / 34), 64% (16/25), and 95% (19/20) for G1, G2 and G3, respectively. There was an increase ($P<0.00001$) in ovulation in G1 compared to G2 and G3 in the first 36 hours and within 48 hours there was also a difference ($P<0.05$), but only from G1 to G2. According to the results presented, the best ovulation inducer in the first 36 and 48 hours is the hCG which may favor the reproductive management of the mares with lower cost.

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Effect of intrafollicular BHBA treatment on follicle growth and ovulation rates in Bos taurus cows

J. Ferst[1], R. Ferreira[2], A. Fritzen[2], C.S. Haas[4], F. Oliveira[4], S. Vargas[4], N. Maciel[1], K. Bertolin[1], B. Gasperin[4], P. Gonçalves[3,1]

[1]UFSM - Universidade Federal de Santa Maria, Santa Maria, RS, Brasil; [2]UDESC - Universidade do Estado de Santa Catarina, Florianópolis, SC, Brasil; [3]UNIPAMPA - Universidade Federal do Pampa, Bagé, RS, Brasil; [4]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil.

The postpartum negative energy balance (NEB) is an important risk factor in the establishment of reproductive failure in high producing dairy cows. It is suggested that the increased blood levels of non-esterified fatty acids (NEFAS) and β-hydroxybutyrate (BHBA), resulting from the negative energy balance, may affect follicle development in bovine females. Our previous results have shown that the plasma levels of BHBA affect neither the growth rate of ovarian follicles nor ovulation in cows subjected to artificial insemination protocol. However, little is known about the effect of increased BHBA levels on bovine follicle development. The objective of this study was to evaluate the effects of increased intrafollicular levels of BHBA during follicular deviation on follicle growth and ovulation rates in cows. *Bos taurus* cows had the emergence of a new follicle wave induced by intravaginal progesterone device (1 g progesterone, DIB-Intervet Schering Plough, Brazil) and a single dose of 2 mg of estradiol benzoate by intramuscular injection (Genix, Anápolis, Brazil). At the time of DIB withdrawal (four days later), cows received an i.m. injection of PGF2α analogue (cloprostenol, 250 µg; Schering-Plough Animal Health, Brazil). Ultrasound daily monitoring of the ovaries, by transrectal ultrasonography with 8 MHz linear transducer (Aquila Vet scanner, Pie Medical, Netherlands), allowed the identification of the largest follicle reaching 7 to 8 mm in diameter, establishing the moment of intrafollicular injection. The treated group received 15 mM of BHBA (n = 6 cows) and the control group was injected with PBS (n = 6 cows). BHBA dose was chosen to be approximate to the levels observed in postpartum dairy cows according to Vanholder et al. (Reproduction Domestic Animal, 41, 39-40, 2006). The follicles were injected according to the technique described by Ferreira et al. (Reproduction, 134, 713-9, 2007). Follicular growth was monitored for 72 h and ovulation rate was monitored for 120 h after treatment. The data of follicular diameter after application of the treatments were analyzed using mixed model for repeated data (PROC MIXED). In the control group, the average size of the injected follicles was 8.5±0.3 mm at time 0 h, 8.4±0.6 mm at 24 h, 9.5±0.5 mm at 48 h and 11.5±0.6 mm at 72 h after PBS injection. In BHBA treated group, follicle growth was reduced at 72 h post-treatment in comparison to the control follicle diameter ($P<0.05$), and the average size of the injected follicles was 8.0±0.2 mm at time 0 h, 7.8±0.3 mm at 24 h, 8.7±0.9 mm at 48 h and 7.7±1.6 mm at 72 h. Ovulation rate was 100% in the control group, and 75% of the BHBA treated follicles overcame the growth rate and ovulated 72 h after treatment. In conclusion, increased concentration of BHBA in the dominant follicle by intrafollicular injection at the moment of follicle deviation affected follicle growth but did not prevent ovulation to occur.

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Mutant mitochondrial DNA elimination is not reversed by inhibition of the autophagic pathway in murine oocytes

M.P. Grejo, T.S. Machado, B.M. Garcia, F.H.C. Sugiyama, C.H. Macabelli, M.R. Chiaratti

UFSCar - Universidade Federal de São Carlos, São Carlos, SP, Brasil.

Several human diseases are originated from mutations in the mitochondrial DNA (mtDNA), which is exclusively inherited from the mother. Since hundreds of thousands of mtDNA molecules are present in the oocyte, the transmission of the disease depends on the number of mutant molecules. Also, several evidences support the existence of a mechanism that eliminates mtDNA mutations during oocyte development. The mechanism responsible for this function in the gamete is unknown. However, in somatic cells, a selective form of autophagy (mitophagy) is responsible for the destruction of dysfunctional mitochondria containing mtDNA mutations. Mitofusin 2 (MFN2) is involved in the control of mitochondrial dynamics, which in turn regulates the activity, transport and degradation of mitochondria. The role of MFN2 in the mitophagy pathway seems to be explained by its key role as a target for ubiquitination in dysfunctional mitochondria. In addition, MFN2 regulates autophagosomes formation and its subsequent fusion with lysosomes. In accordance with this information, we have previously observed that the knockout of *Mfn2* in oocytes containing C57BL/6 and NZB/BINJ mtDNA results in accumulation of mutant NZB/BINJ mtDNA in the progeny (unpublished data). That being said, this work aimed to investigate whether the elimination of mtDNA NZB is mediated by autophagy. To this end, autophagy was inhibited by administration of 50 μg of chloroquine per gram of mouse weight, twice a day. Mice aged 11 days with 50-70% of NZB mtDNA were treated with chloroquine for 10 days, corresponding to development of the first follicular wave. As a control, mice were submitted to the same protocol, but using a similar volume of physiological solution (chloroquine diluent). At day 8 of the treatment, mice were stimulated with 5 U.I. of eCG and immature oocytes were collected from antral follicles after 42 h. The level of NZB mtDNA, determined by real-time PCR, was estimated in oocytes and in their respective donor females' ear biopsy. For analysis of the treatment effect, control and treated groups were compared by T test as for ΔNZB, which was calculated by the difference between the percentage of NZB mtDNA in the oocyte and that present in the female's ear. In disagreement with our hypothesis, it was not observed difference between groups regarding the ΔNZB ($P = 0.58$). In conclusion, our current data suggests that elimination of NZB mtDNA cannot be explained by the occurrence of autophagy in oocytes.

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Influence of extracellular matrix replacement during a prolonged in vitro culture of preantral follicles

D.V. Bonato, M.B. Cerezetti, M.A. Silva, A.G. Lindquist, T.K. Sanches, A.M. Almeida, E. Castellani, C.B. Silva, M.M. Seneda

UEL - Universidade Estadual de Londrina, Londrina, Paraná, Brasil.

The *in vitro* culture (CIV) of preantral follicles is an important reproductive biotechnology because it assists in the knowledge of physiology and folliculogenesis. The CIV simulates the events *in vivo* and provides the complete development of preantral follicles. Thus, the objective of this study was to evaluate the replacement of the agarose gel support during *in vitro* culture and its interference in the integrity and development of preantral follicles cultured *in situ*. Five ovarian pairs (n=10) were collected at the local slaughterhouse, from *Bos taurus indicus*, pubescent heifers from the same herd. The ovaries were washed in 70% ethanol and buffered saline (PBS), transported to the laboratory at a temperature of 4°C in minimal essential medium (MEM) supplemented with 200 IU/mL penicillin and 200 mg/mL streptomycin. The ovaries with corpus luteum were discarded, and the respective contralateral of each pair, 2 fragments with approximately 3x3x1mm were removed. Fragments from the same ovary were distributed in two treatments, control treatment and substitution treatment (n=5/treatment). In short, for control treatment, cultivation was carried out for twelve days on the same agarose gel support, while substitution treatment support gel was change on the sixth day of culture. The ovarian fragments were cultured in MEM (Gibco BRL, Rockville, MD, USA) supplemented with ITS, pyruvate, glutamine, hypoxanthine, BSA (Gibco), penicillin and streptomycin. Total replacement of the culture medium was performed every two days. At the end of the cultivation, these fragments were fixed in Bouin for 24 hours and processed into classical histology. For follicular analysis, the slides were stained with periodic acid from Schiff (PAS) and Hematoxylin. Evaluation of the fragments was performed by optical microscopy. The preantral follicles were classified according to the development phase (primordial or developing, primary and secondary) and integrity (intact and degenerate), considering 50 follicles per fragment. The means were compared by analysis of variance ($P < 0.05$). The control and treated groups presented the mean percentage of 25% (3/12) and 75% (9/12) for intact primordial follicles, respectively. This comparison resulted in a value of $P = 0.421$. The mean percentages of healthy follicles developed were, respectively, 41.7% (25/60) and 58.3% (35/60), with $P = 0.909$. The support with the agarose gel allows the preservation of the follicular architecture; consequently, it assists in the development. However, it did not evidence differences when carrying out gel substitution according to the parameters evaluated. Therefore, it is concluded that the replacement of the agarose gel support during the sixth day of culture did not interfere with the integrity and development of cultured preantral follicles.

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Effect of prostaglandin F2 alfa on steroidogenesis of preovulatory follicles in cattle

M.T. Rovani[1], C.A. D'Avila[2], C.S. Haas[2], F.C. Oliveira[2], S.F. Vargas Jr[2], L.M.C. Pegoraro[3], A. Schneider[2], L.F.M. Pfeifer[4], B.G. Gasperin[2], T. Lucia Jr[2], P.B.D. Gonçalves[5]

[1]IFFAR - Instituto Federal Farroupilha, Campus Frederico Westphalen, RS, Brasil; [2]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil; [3]EMBRAPA - Embrapa Clima Temperado, Pelotas, RS, Brasil; [4]EMBRAPA Rondônia - Embrapa Rondônia, Brasil; [5]UFSM - Universidade Federal de Santa Maria, Santa Maria, RS, Brasil.

The present study tested whether an i.m. injection of PGF2 alfa (PGF) affects steroidogenesis and evaluated the effect of a non-steroidal anti-inflammatory drug (NSAID) on GnRH-induced luteinization in cattle. The procedures were approved by UFPel and UFSM Animal Ethics Committee. In the first experiment, the expression of PGF receptor (*PTGFR*) was evaluated in granulosa cells (GC) around follicular deviation and in preovulatory follicles (Rovani et al., Animal Reproduction, 14:383-391 2017). Then, in experiments 2 and 3, 37 Jersey and Holstein non-lactating cyclic cows had the follicular growth synchronized by a hormonal protocol consisting of i.m. injections of estradiol benzoate (2mg) and insertion of an intravaginal device (IVD) containing progesterone (P4; 1g), on D0, and cloprostenol (150µg; D0 and D8). In experiment 2, cows were allocated into three groups at 12h after IVD withdrawal (D9; hour 0): control= saline i.m. (n=6); GnRH= 100µg gonadorelin acetate (GA) i.m. (n=7); and PGF= 500µg cloprostenol i.m. (n=6). In experiment 3, GnRH injections (GA; 100µg i.m.) were performed in all the cows at 20h after IVD withdrawal (hour 0). Then, 17h after GnRH, animals were allocated into three groups and received the following treatments: control= saline i.m. (n=6); NSAID= 2.2mg/kg flunixin meglumine (FM) i.m. (n=6); and NSAID+PGF= 2.2mg/kg FM (n=6) and, 6h later, 25mg dinoprost tromethamine (i.m.). In both experiments, the groups were equalized according to follicular diameter and follicular aspirations were performed 24h after hour 0. Gene expression was evaluated by real-time PCR. The levels of steroids in follicular fluid (FF) were analyzed by chemiluminescence. The effects of treatments were analyzed by ANOVA and $P<0.05$ was considered significant. In experiment 1, it was observed increased *PTGFR* in GC from dominant compared to subordinate follicles after follicular deviation ($P<0.05$). Furthermore, *PTGFR* tended ($P=0.1$) to increase 24h after GnRH treatment. In experiment 2, GnRH-treated cows had significantly lower estradiol (E2) levels in FF compared to the other groups ($P<0.05$; 467±80, 83±24 and 456±94ng/mL, in control, GnRH, and PGF, respectively). However, P4 levels did not differ among groups (227±71, 606±185 and 271±119ng/mL, in control, GnRH, and PGF, respectively). In experiment 3, no differences were observed in FF E2 levels (94±17, 82±24 and 95±12ng/mL, in control, NSAID and NSAID+PGF, respectively), but P4 levels in FF from cows treated with GnRH+NSAID (323.3±19.6ng/mL), regardless of PGF treatment, were lower than those of the control (only GnRH; 477.5±80.1ng/mL; $P<0,05$). Collectively, data suggest that although *PTGFR* was expressed in granulosa cells, PGF treatment did not affect follicular steroidogenesis, whereas NSAID treatment negatively affected luteinization, providing a model to investigate the effect of PGs on ovulation in cattle. The authors are thankful to FAPERGS and CNPq (Edital PRONEX-16/2551-0000494-3) and CAPES for financial support.

A149 Folliculogenesis, Oogenesis and Superovulation

Function of bone morphogenetic protein 15 during follicular growth, ovulation and luteinization in cattle

C.S. Haas[1], F.C. Oliveira[1], V.G.G. Costa[1], M.T. Rovani[2], A.D. Vieira[1], S.F. Vargas Jr[1], R. Duggavathi[4], V. Bordignon[4], R. Ferreira[5], P.B.D. Gonçalves[3], B.G. Gasperin[1]

[1]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil; [2]IFF - Instituto Federal Farroupilha, RS, Brasil; [3]UFSM - Universidade Federal de Santa Maria, Santa Maria, RS, Brasil; [4]McGill - Departamento de Ciências Animais, McGill University, Sainte Anne de Bellevue, QC, Canada; [5]UDESC - Universidade do Estado de Santa Catarina, Chapecó, RS, Brasil.

The oocyte-secreted bone morphogenetic protein 15 (BMP15) is one of the main local regulators of ovarian function and exerts an essential role in the determination of ovulation rate. Although highly relevant, mechanisms of BMP15 in the regulation of ovarian function are not completely established. The objective of the present study was to evaluate the effect of BMP15 treatment on final follicular growth, ovulation and luteinization in cattle. All the procedures were approved by UFPel's Ethics in Animal Experimentation Committee. Follicular growth in Jersey cows (n=18) was synchronized using a hormonal protocol with an intravaginal device (IVD) containing progesterone (P4; 1g; DIB, Zoetis, São Paulo, SP, Brazil), i.m. injections of 2 mg estradiol benzoate (Gonadiol, Zoetis, São Paulo, SP, Brazil) and 150 µg cloprostenol (Tecnopec, São Paulo, SP, Brazil). The IVDs were kept for 7 or 9 days, according to the desired diameter of the follicles in each experiment. In experiment 1, to investigate the effect on follicular growth, intrafollicular injections (IFI) were performed in dominant follicles (7.5 to 9.5 mm diameter) with PBS (control; n=3) or 100 ng/mL of recombinant human BMP15 (BMP15; n=5; Sigma Aldrich, St. Louis, MO, USA). In experiment 2, to evaluate the effect on ovulation and luteinization, IFI were performed in preovulatory follicles (12 to 14 mm diameter) with PBS (control; n=4) or BMP15 (BMP15; n=6), in the final intrafollicular concentration of 500 ng/mL, simultaneously to an i.m. injection of GnRH analogue (buserelin acetate; 21 µg; Gonaxal, Biogénesis Bagó, Vinhedo, SP, Brasil). The effect of treatment on follicular development was performed as repeated measures data using the MIXED procedure (SAS Institute Inc., Cary, NC, USA). Continuous data were tested for normal distribution and normalized if necessary and the significance level was set at 5%. In experiment 1, treatment with BMP15 (D0) tended (P=0.09) to inhibit follicular development. The average follicular diameters were 8.9±0.9 (D0; IFI moment) and 14±2 mm (D2), for control group, and 8.4±0.3 (D0) and 9.1±1.4 mm (D2), for BMP15 group. Ultrasound examinations revealed that BMP15-treated follicles had thicker walls (D2). In experiment 2, all follicles in the control group (4/4) ovulated. However, BMP15-treated follicles did not ovulate (0/6) and became cystic, with an average diameter of 32 mm (D12) and thicker walls, characterizing luteinized cysts. Fluid samples from five out of six cysts had progesterone higher than 500 ng/mL. Serum progesterone levels were 7.1±1.7 for control and 4.1±1.2 ng/mL for BMP15 groups, which confirmed that BMP15 blocked ovulation but did not inhibit luteinization. In conclusion, BMP15 treatment inhibited follicle development and ovulation, inducing luteinization and cysts formation in cattle. The authors are thankful to FAPERGS, CAPES and CNPq for financial support.

A150 Folliculogenesis, Oogenesis and Superovulation

Immune Blockade of Follicular Development in heifers

H.L. Neri[2], C.A. Fernandes[1,2], A.C. Figueiredo[1,2], J.R. Pereira[2], M.P. Palhão[1], G.H.S. Pereira[2], J.H.M. Viana[1,2]

[1]UNIFENAS - Universidade José do Rosário Velano, Alfenas MG, Brasil; [2]BIOTRAN, Alfenas, MG, Barsil.

In order to develop hormonal treatment protocols that allow for the implementation of reproductive biotechnologies to control the estrous cycles of cows, it is necessary to have a precise description on the pharmacodynamics of substances, particularly exogenous gonadotropins. For this purpose, experimental procedures are used to block or limit the action of endogenous hormones, which allows for the real evaluation of the efficacy of the exogenous treatment. This study aims to evaluate the efficiency of the methods of an immune blockade of endogenous FSH (Follicle-Stimulating Hormone). To this purpose, eleven (11) cycling crossbred heifers were allocated into two (2) groups, namely: GBI (n = 6), which received the first dose of immune blocker on D-40 (Bopriva, Zoetis – Brasil), with a booster on D-10; on D-3, an intravaginal progesterone-releasing device (P4) was inserted and a dose of 2 mg of estradiol benzoate was given intramuscularly for follicular wave synchronization; GC (n =5) was the control group that received the same treatment as GBI on D-3, without any hormonal blockade. From D0 to D4, all animals were evaluated daily by transrectal ultrasonography (Mindray™ - M5) to count and measure the follicles which were divided into 4 categories: Up to 4.0; from 4.0 to 7.9; from 8.0 to 12.0; and above 12mm. The heifers were assigned to treatments under a completely randomized design (CRD) and the data analyzed by Anova and compared by the Tukey test at significancy level of 5%. There was no change in the population of follicles up to 4 mm in diameter and in the total of structures between groups from D0 to D4 ($P>0.05$), showing that the blockade does not interfere with follicle recruitment. Over 80% of the follicles did not exceed 4 mm in diameter in the two groups on the different evaluation days, which was expected for the beginning of the wave of follicle development. From the D1, GBI had fewer structures with ranges from 4.0 to 7.9 and from 8.0 to 12.0 mm in diameter than GC group ($P<0.05$). In addition, no follicles greater than 8 mm were observed in GBI. Based on these results, it can be concluded that immune blockade does not affect the folicular recruitment and is effective in blocking follicle development in cows.

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A151 Folliculogenesis, Oogenesis and Superovulation

Different progestagens sources do not affect the follicular population and the morphological quality of oocytes during ovarian stimulation in Santa Inês ewes

G.M. Bragança[1], J.M.G. Souza-Fabjan[1], R.I.T.P. Batista[1], L.S. Ribeiro[1], V.L. Brair[1], L.R. Côrtes[1], C.V. Souza[1], A.R. Taira[1], J.F. Fonseca[2], A. Menchaca[3], F.Z. Brandão[1]

[1]UFF - Universidade Federal Fluminense, Niterói, RJ, Brasil; [2]EMBRAPA - Embrapa Caprinos e Ovinos, Sobral, CE, Brasil; [3]IRAUy - Instituto de Reproducción Animal Uruguay, Montevideo, Uruguai.

In attempt to develop a hormonal protocol more suitable to produce good quality oocytes for use in biotechnologies, an earlier study demonstrated that FSH applied in multiple decreasing doses produced better quality oocytes. However, different progestagens implants used during ovarian stimulation have not yet been tested. The aim of this study was to investigate the effect of different progestagens during ovarian stimulation on follicular population and oocyte morphological quality in Santa Inês ewes. Thirty pluriparous ewes had their estrus synchronized by Day 0 protocol (Balaro et al., Domestic Animal Endocrinology, 54: 10-14, 2016). Day 0 (D0) of the protocol was considered 80 h after sponge removal and ovarian stimulation with pFSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada). All ewes received 80 mg of pFSH distributed in three applications (50, 30 and 20%) every 12 h. At the time of stimulation, the ewes were divided in three groups (n = 10): (1) MAP, received intravaginal sponges containing 60 mg of medroxyprogesterone acetate (Progespon, Zoetis, São Paulo, Brazil); (2) P4 received a silicone device with 0.33 mg progesterone (CIDR, Eazi-Breed, Zoetis); and (3) Control, received no device (luteal P4). The ovarian evaluation was performed by transrectal ultrasonography (SonoScape, Shenzhen, China, 7.5 MHz linear transducer) from D-3 to D2 every 12 h. The follicles were classified as small (<3 mm), medium (3-5 mm) or large (>5 mm). Oocytes were recovered by videolaparoscopy (LOPU) and classified according to morphological quality in grade 1, 2, 3 and 4 (G1 and G2: good, G3: acceptable and G4: poor). Parametric data were analyzed by ANOVA and Tukey's test. For the non-parametric data, the chi-square test was used (significance $P<0.05$). There was no effect ($P>0.05$) of the progesterone source on the follicular population and oocyte morphological quality. On D0, the ewes of all groups had a similar follicular population regarding the number of follicles in each category (small, medium and large), the number of small follicles was higher than the medium follicles, which in turn was higher than the number of large follicles. On D2, the number of follicles in each category was also similar among groups, the number of small and medium follicles was higher than that of large follicles. Likewise, the number of G1 / 2 oocytes (MAP, 4.3 ± 0.9, P4, 5.3 ± 0.8 and Control, 3.7 ± 0.7), G3 (MAP, 1.4 ± 0.5, P4, 2.3 ± 0.8 and Control, 2.0 ± 0.6) and G4 (MAP, 0.2 ± 0.1, P4, 0.5 ± 0.2 and Control, 0.5 ± 0.2), as well as the recovery rate 61% (MAP), 80% (P4) and 67% (Control) was not different among the groups. In conclusion, the source of progestagens used during the ovarian stimulation protocol does not affect the follicular population, nor the oocyte quality. Exogenous progestagens may not be necessary when post-synchronization ovulation is confirmed by ultrasonography.

A152 Folliculogenesis, Oogenesis and Superovulation

Efficiency of *in vivo* embryo production using sorted semen in cattle

A.C.S. Figueiredo[1,2], C.A.C. Fernandes[1,2], H.L.H. Neri[1], G.H.S. Pereira[1], J.R. Pereira[1], J.H.M. Viana[2,4], M.R. Vieira[3]

[1]BIOTRAN LTDA, Alfenas, MG, Brasil; [2]UNIFENAS - Universidade José do Rosário Velano, Alfenas, MG, Brasil; [3]Consulte Embriões, Governadores Valadares, Minas Gerais, Brasil; [4]Embrapa Cenargen - Embrapa Recursos Genéticos, Brasilia, DF, Brasil.

According to IETS statistics (Viana et al., 2017), in Brazil since 2004, *in vitro* embryo production (IVP) is superior to *in vivo* production, formerly called embryo transfer. Even with great evolution, IVP still cannot reach a large number of properties that could be used for genetic multiplication by embryos due to scale and logistics. Mainly some dairy herds do not use IVP for this reason, nor do they use *in vivo* production for the poor results observed with sexed sorted semen. However, in recent years the superovulation protocols were improved, and the sorted semen quality improved significantly. The objective of this study was to determine the technical and economic efficiency of *in vivo* bovine embryo production using sorted sexed semen. Twenty nulliparous donors, aged between 16 and 22 months, girolando breed were used. It was superovulated with 180 mg of Folltropin™ (Vetoquinol) in a decreasing dose protocol. The superovulation protocol was as: D0 - progesterone implant; D1 - 2 mg of estradiol benzoate IM, D5 to D8 - applications of Folltropin every 12 hours. In D7, in the afternoon was applied 0.5mg of Clorprostenol and in D8 the implant was removed in the morning. From D9 the estrus was observed. At the beginning of estrus, 0.05mg of Gonadorelin IM was applied. Two inseminations at 18 and 30 hours after application of the GnRH analogue were made using Holstein sorted semen. Flushings were made seven days after the 1st insemination. The ovaries were evaluated by ultrasonography (Mindray - M5 ™) in D9 for follicle mensuration and on the day of flushing for measurement of corpora lutea (CL). The costs involved in the process were accounted. An average of 8.1±6.8 total structures were collected, with 5.7±3.9 viable embryos, 1.3±1.1 degenerated and 1.1±0.9 non-fertilized structures. The total cost per donor was R$ 758.50, considering hormones (R$ 207.60), medium (R$ 27.50), disposable materials (R$ 67.20), two doses of sexed semen (R$ 176.20) and fees (R$ 280.00). With total cost of R$ 758.50 and production of 6.7 viable embryos/flushing, there is an average cost of of R$ 133.00/viable embryo with sorted semen. Due to the results of the production of embryos obtained it can be affirmed that the genetic multiplication of the herds using *in vivo* embryos produced with sorted semen is technically feasible. In addition, the final cost of each embryo is also competitive when compared to the IVP costs currently practiced in the market. It is concluded that the *in vivo* embryos production with sexed semen is feasible, technically and economically. Supported by: Vetoquinol, Biotran, CNPq e Fapemig.

A153 Folliculogenesis, Oogenesis and Superovulation

Expression of bta-miR-222 in bovine granulosa cells: effect of follicle deviation and regulation by FSH/insulin *in vitro*

P.H. Santos[1], F.F. Franchi[1], L.O. Mendes[2], P.K. Fontes[1], V.G. Pinheiro[1], A.C.S. Castilho[2,3]

[1]UNESP - Universidade Estadual Paulista/Departamento de Farmacologia, Botucatu, SP, Brasil; [2]UNOESTE - Universidade do Oeste Paulista, Presidente Prudente, SP, Brasil; [3]UNESP - Universidade Estadual Paulista/Departamento de Ciências Biológicas, Assis, SP, Brasil.

Antral follicles acquire LHCGR in granulosa cells (GC) after follicle deviation (FD; 2.5 days after ovulation). *In vivo* and *in vitro* studies showed that in GC, the transcriptional regulation of *LHCGR* mRNA is FSH-dependent, on the other hand, several factors regulate negatively the *LHCGR* abundance, including microRNAs (miRNAs), among them, the bta-miR-222. Thus, the aim of present work was to verify the correlation of *LHCGR* and bta-miR-222 abundance in GC of follicles after LHCGR acquisition (after FD) and under *in vitro* effect of FSH and insulin in granulosa cell culture. Experiment 1: Nelore cows (*Bos taurus indicus*, n=5) were hormonally synchronized and slaughtered 3 days after ovulation, and GC from the dominant follicle (DF) and its respective subordinate follicle (SF) were recovered to total RNA extraction. Experiment 2: small follicles (2-5 mm) were dissected from bovine ovaries from abattoir, GC were isolated and cultured in serum-free medium (control group) or treated with FSH (1 ng/ml) combined/or not with insulin (1 or 10 ng/ml) for 6 days with partial medium change (70%) every 2 days. The GC were recovered and submitted to total RNA extraction. The relative expression of *LHCGR* and bta-miR-222 was quantified by RT-qPCR and determined by the Pffafl's equation with *PPIA* as reference gene (exp. 1 and 2). Experiment 3: dominant follicle from bovine ovaries from abattoir was submitted to immunostaining of LHCGR and mature *corpus luteum* was used as positive control. Effect of follicular status (DF or SF) was compared by Student's T-test and effect of FSH+insulin on GC culture was tested by ANOVA and means were compared using the Tukey–test (JMP software, SAS Institute Cary, NC). Significant differences were considered when $P \leq 0.05$. Our findings showed a higher diameter in DF (11.8 ± 0.8 mm) compared to SF (6.5 ± 0.2 mm; P=0.0002). Moreover, the mRNA abundance of *LHCGR* in CG was higher in DF compared to SF (P=0.01). On the other hand, the expression of bta-miR-222 was lower in DF compared to SF (P=0.01). Similarly, in GC culture the association of FSH (1 ng/ml) with insulin (10 ng/ml) demonstrated the highest abundance of *LHCGR* and inversely the lowest abundance of bta-miR-222 ($P<0.05$). Regarding the immunolocalization of LHCGR protein, we demonstrated that LHCGR protein was detected in dominant follicles of adult ovaries in theca cells and GC, corroborating with the mRNA results. In conclusion, the data suggest that the up-regulation of *LHCGR* in GC from dominant follicles could be supported by the down-regulation of bta-mir-222 and that lower levels of bta-miR-222 might be required to improve follicle development in bovine ovary and antral follicle deviation. Furthermore, the data indicates that FSH and insulin are, in parts, the hormones that orchestrated the suppression of bta-miR-222 in cattle. The authors are grateful to grant #2013/11480-3, #2015/04505-5; São Paulo Research Foundation (FAPESP).

A154 Folliculogenesis, Oogenesis and Superovulation

Insulin as a possible modulator of follicular development-related genes in granulosa cells

G.A. Ferronato[1,2], J.A.A. Rincón[1,2], A.S. Maffi[1,2], B. Mion[1], C.C. Brauner[1,2], F.C. Oliveira[1], C.S. Haas[1], E. Schmitt[1,2]

[1]UFPel - Universidade Federal de Pelotas, Pelotas, RS, Brasil; [2]NUPEEC/UFPel - Núcleo de Pesquisa, Ensino e Extensão/Universidade Federal de Pelotas em Pecuária, Pelotas, RS.

Previous studies demonstrated the presence of insulin receptors in pre-ovulatory follicles, indicating an insulin role during this period. The objective of this study was to evaluate insulin ability to modulate the expression of genes related to follicular development in dairy cattle. Ten Jersey cows, multiparous, non-pregnant and non-lactating were submitted to an E2-P4-based protocol to synchronize follicular wave. On day 8, after P4 device removal, the cows were divided in two groups. Two cows failed to respond properly to the follicular wave synchronization protocol and were excluded from the present study. Treatment group (n=4) received a single dose of 0.25 IU/kg s.c. of human insulin (Novolin® N, Novo Nordisk, Bagsvaerd, Denmark) and control group (n=4) received 1 mL of saline solution s.c. (NaCl 0.9%). Dominant follicle of each cow was aspirated 16 hours after treatment (day 9) to evaluate intrafollicular glucose and to collect granulosa cells for gene expression analysis, performed according to previously described (Campos et al., Theriogenology 89:244-249, 2017). The genes evaluated were *IGFBP2 (insulin like growth factor binding protein 2)* and *PAPPA* (*pappalysin 1*), using *HIST1H2AC* (*histone H2A type 1-C)* and *RN18S1* (*18S ribosomal RNA*) as endogenous controls. In addition, blood samples were collected on day 8 and 9 (16 hours after treatment) to evaluate serum glucose through commercial kit (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil). Glucose concentrations were evaluated by repeated measures test in NCSS (2005) software. Gene expression was evaluated by t test in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Serum glucose concentration was lower in the treatment group (41.2±4.2 mg/dL) than control group (64.5±4.2 mg/dL, $P<0.05$), however intrafollicular concentration did not differ between the groups (69.7±3.3 mg/dL and 62.2±3.3 mg/dL, respectively; $P=0.16$). Relative expression of *IGFBP2* mRNA was lower in treatment group than control group ($P<0.05$). A tendency of higher relative expression of *PAPPA* mRNA was observed for treatment group ($P=0.06$). The *PAPPA* is responsible for the proteolytic cleavage of *IGBPs*, which regulate the bioavailability of IGF1 in the ovary. In this way, the lower IGFBP2 expression in the group treated with insulin can be associated to an increase in the availability of IGF1, as there is a negative correlation between free IGF1 concentration and IGFBP-2 levels. This suggests a beneficial potential, since as IGF1 acts as a modulator in gonadotropin action in ovary, stimulating proliferation and differentiation of granulosa and theca cells and preventing follicular apoptosis. In conclusion, insulin can modulate the expression of *PAPPA* and *IGFB2* genes in granulosa cells, which are mediators of follicular development through IGF1 bioavailability.

A155 Folliculogenesis, Oogenesis and Superovulation

Addition of eCG on day 2 of a superovulatory protocol increases embryo production in Aberdeen Angus cows

J. Irouleguy[1], M.A. Silva[2], J.C. Logroño[3], S.P. Wallace[2]

[1]Tres Marias Embriones, Buenos Aires, Argentina; [2]Zoetis Argentina; Buenos Aires, Argentina; [3]FCV/UNLP - Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina.

The aim of this study was to evaluate the effect of the addition of equine chorionic gonadotrophin (eCG) to a FSH-based superovulation protocol on embryo production in Angus cows. The experiment was conducted in Buenos Aires Province (Argentina) where 71 Angus donor cows were synchronized as follows: D0= 5mg 17β estradiol (17 Beta Estradiol®, Río de Janeiro, Argentina) + 10mg injectable P4 (Progesterona®, Rio de Janeiro, Argentina) + 1g P4 intravaginal device insertion (DIB®, Zoetis, Argentina); D4= 100IU FSH (Pluset®, Calier, Argentina) AM and PM; D5= 80IU FSH AM and PM, D6= 70 IU FSH + 500µg sodium cloprostenol (Ciclase DL®, Zoetis, Argentina) AM and PM, D7= P4 device removal AM + 50IU FSH PM; D8 AM= 100ug GnRH (Gonasyn®, Zoetis, Argentina); D8 PM= artificial insemination (AI); D9 AM= AI; D15= embryo collection. On D2 cows were randomly distributed to receive 400IU of eCG (Novormon®, Zoetis, Argentina; Group eCG; n=35) or remained as untreated control (Group Control n=36). Logit regression using the GLIMMIX PROCEDURE of SAS 9.3 version was used to analyze the variables: number of transferable embryos (TRANS), degenerated (DEG), and unfertilized ova (UFO) and all structures (UFO+TRANS+DEG). Unsuccessful collections yielding zero transferable embryos were also analyzed. Treatment with eCG increased the number of TRANS (7.65±5.75 vs 6.69±6.41; mean±SD; P<0.001), DEG (3.82±5.82 vs 2.66±4.36; P<0.001), UFO (3±6.3 vs 2±3.29; P<0.001) and all structures (14.49±9.10 vs 11.36±9.31; P<0.001). The proportion of collections yielding zero transferable embryos did not differ among groups [eCG=8.6% (3/35) vs Control=19.4% (7/36); P=0.2]. In conclusion, eCG treatment on day 2 of a FSH-based superovulatory protocol increases the superovulatory response and the number of transferable embryos in Aberdeen Angus cows.

A156 Folliculogenesis, Oogenesis and Superovulation

Inhibition of meiosis resumption in bovine oocytes to be used in the intrafollicular transfer of immature oocyte (TIFOI)

V.A. Silva[1], O.A.C. Faria[2], L.R.O. Dias[2], F.M.C. Caixeta[2], J.F.W. Sprícigo[3], M.A.N. Dode[4]

[1]ICESP - Faculdades ICESP, Brasília, DF, Brasil; [2]UnB - Universidade de Brasília, Brasília, DF, Brasil; [3]University of Guelph - Department of Animal Bioscience, University of Guelph, ON, Canada; [4]Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brasil.

In the intrafollicular transfer of immature oocytes (TIFOI) the oocytes are aspirated from the donors and transferred to the "ovulators". Considering that removal of the oocyte from the follicular environment causes the spontaneous meiosis resumption, it is necessary to inhibit this process to avoid oocytes aging. The present study aimed to evaluate nuclear maturation kinetics in oocytes kept in different manipulation medium to be used in TIFOI. COCs were obtained from slaughterhouse ovaries and all manipulation and selection was performed in follicular fluid (FF). After selection, a group of COCs was placed directly into the IVM (control), the others were transferred to an eppendorf containing 500µl of Follicular Fluid (T1), Follicular aspiration solution consisting of PBS supplemented with 1% fetal calf serum and 0,02% heparin (T2) and Follicular aspiration solution supplemented with 500Mm of IBMX [(nonspecific phosphodiesterase inhibitor) (T3)]. The COCs remained for three hours in the different medium at 36^{o} C. After that period, oocytes were transferred to IVM medium and kept for 22h. At 0, 9, and 22 h of IVM, sample of oocyte from all groups were removed for meiotic stage evaluation. Only for the control groups, a sample of oocytes was also removed at 12 h, which was used to confirm the efficiency of the treatments, regarding to meiosis retention. At each time point COCs were mechanically denuded, fixed for 48 hours in ethanol and acetic acid and stained with lacmoid (45%). The evaluation of meiotic stage was carried out under a phase contrast microscope (Nikon Eclipse E200, 1000X) and the oocytes were classified as: germinal vesicle (GV), germinal vesicle break down (GVBD); metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII). Nuclear maturation data were analyzed by Chi-square test ($P<0.05$). We evaluated a total of 293 COCs for the control group at 0 (n=63), 9 (n=76), 12 (n=76) and 22h (n=78). For the other groups a total of 587 oocytes were distributed to T1 (n=214), T2 (n=179) and T3 (n=194). At 0h 98.4% of the oocytes were at GV stage. After 9h of IVM, T1 (75.7%) and T3 (82.4%) presented most of oocytes in GVBD, similar to the control group (72.4%). In T2 only 11.8% were in GVBD, which was lower ($P<0.05$) than the other treatments. This group at 9 h had 86% of the oocytes in MI, which was similar to the 72.4% observed for the control group at 12 h. At 22h most of oocytes from all the groups reached the MII stage, with no difference ($P> 0.05$) among them. The results suggest that oocytes can remain for 3 hours in Follicular Fluid or Aspiration Solution with IBMX and proceed with nuclear maturation without affecting the oocytes. Therefore, both are eligible to be used as handling medium before TIFOI procedures.

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A157 Folliculogenesis, Oogenesis and Superovulation

Insulin treatment does not affect follicular growth and steroidogenesis in cattle

J.A.A. Rincón[1], A.S. Maffi[1], B. Mion[2], B.G. Gasperin[2], J. Lazzari[1], C.C. Brauner[1], M.N. Corrêa[1]

[1]NUPEEC - Núcleo de Pesquisa, Ensino e Extensão em Pecuária, Pelotas, RS, Brasil; [2]UFPEL - Universidade Federal de Pelotas, Pelotas, RS, Brasil.

The development of strategies to stimulate follicular development in artificial insemination protocols is important once it is associated with reproductive efficiency of dairy cattle. The aim of this study was to evaluate the effect of insulin administration on follicular growth and estradiol (E2), progesterone (P4) and glucose concentrations in dairy cattle. Eight Jersey cows, multiparous, non-pregnant and non-lactating, with 459±3 Kg, BCS 2.9 and kept under the same management, were used in this study. On day 0 (D0), follicles >9 mm were aspirated and cows received an intravaginal P4-releasing device (1.0 g P4, Sincrogest®, Ouro fino, São Paulo, SP, Brazil), 2 mg of estradiol benzoate i.m. (Sincrodiol®, Ouro fino) and 12.5 mg of PGF2α i.m. (Lutalyse®, Zoetis, São Paulo, SP, Brazil). On Day 6 (D6) and 8 (D8), administrations of 12.5 mg of PGF2α i.m. were performed. On D8, P4 device was removed and cows received the following treatments: insulin group (IG: n=4), received 0.25 IU/kg s.c. of human insulin (Novolin® N, Novo Nordisk, Denmark) and control group (CG: n=4), which received 1 mL s.c. of saline solution (NaCl 0.9%). Follicular diameter was measured throughout transrectal ultrasonography each 24 hours beginning on D6. On D8 and D9, serum samples were collected by puncture of arteriovenous coccygeal complex. Sixteen hours (D9) after treatment, follicular fluid (FF) was collected through follicular aspiration. Estrogen and P4 concentrations were evaluated through chemiluminescence (Beckman Coulter®, Brea, USA) and glucose concentrations were analyzed through colorimetric assay (Labtest Diagnóstica SA, Lagoa Santa, Brazil). Statistical analyses were performed by NCSS (2005) software, using One-way ANOVA to evaluate follicular growth, E2 and P4 concentrations; and repeated measures test was used to evaluate follicular dynamics and glucose, considering $P \leq 0.05$ values as significant and $P \leq 0.10$ as tendency. Insulin decreased serum glucose concentration 16 hours (D9) after treatment (IG: 41.2 ± 4.2 mg/dL and CG: 64.5 ± 4.2 mg/dL, P=0.05); however, no significant effect on intrafollicular glucose concentration was observed (IG: 62.2 ± 3.3 mg/dL and CG: 69.7 ± 3.3 mg/dL; P=0.16). Insulin administration did not affect follicular diameter on day 9 (IG: 12.1 ± 0.6 mm and CG: 11.1 ± 0.6 mm; P=0.88), follicular growth between D8 and D9 (IG: 2.1 ± 0.4 mm and CG: 1.7 ± 0.4 mm; P=0.59) and E2 concentration (IG: 1977.5 ± 327.7 ng/mL, CG: 1732.5 ± 372.7 ng/mL, P=0.65). Although insulin tended to decrease intrafollicular progesterone concentration (IG: 52.7 ±12.7 ng/mL and CG: 93.3 ± 12.7 ng/mL; P=0.08), E2:P4 ratio was not affected (IG: 43.9 ± 9.7 ng/mL and CG: 18.6 ± 9.7 ng/mL; P=0.13). In conclusion, the administration of a single dose of insulin affected serum glucose concentration, however it did not affect intrafollicular glucose, follicular growth and estradiol and progesterone concentration.

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Effects of the overexpression of Mitofusin 1 and Mitofusin 2 during *in vitro* oocyte growth in mice

K.F. Carvalho[1,2], M.R. Chiaratti[1], H. Clarke[2]

[1]UFSCar - Universidade Federal de São Carlos, São Carlos, São Paulo, Brasil; [2]McGill - McGill University, Montreal, Quebec, Canadá.

The determining role of mitochondria in regulating follicular development and oocyte growth is emerging from basic research on model species and clinical studies of woman infertility. Mitochondrial activity depends mostly on the organelle dynamics, which in turn is dependent on events of fusion and fission. Mitochondrial fusion relies on the activity of Mitofusins 1 and 2 (MFN1 and MFN2) and on the Optical Atrophy 1 (OPA1) protein. Recent studies have determined that the conditional knockout (cKO) of *Mfn1* in the oocyte affects folliculogenesis, resulting in the blockage of oocyte development, with the consequent failure of ovulation and female infertility (data not published). In contrast, cKO of both *Mfn1* and *Mfn2* leads to a milder phenotype, where oocytes are capable of growing, but arrest meiotic progression with ovulation, leading the females to infertility (data not published). Moreover, *Mfn2* cKO does not seem to affect folliculogenesis or oocyte growth, impacting ovulation and fertility subtly (data not published). To further investigate whether the balance between MFN1 and MFN2 plays a role in oocyte growth and development, Granulosa cells-Oocyte Complexes (GOC) were collected from 12 days old mice, injected with MFN2 or MFN1+MFN2 cRNAs to promote overexpression and cultured for 7 days. After culture, the oocytes were evaluated regarding chromatin organization, diameter and phosphorylated mTOR (pmTOR) protein quantity. Data were evaluated by ANOVA followed by Duncan posthoc test (P=0.05). The chromatin organization assessment showed no significant differences among the groups concerning the surrounding nucleolus/not surrounding nucleolus ratio. However, the MFN2 overexpressing oocytes showed severe growth impairment, reaching a mean diameter of 67.60 ± 0.60 μm, while the injected control oocytes reached a mean value of 77.25 ± 0.60 μm, and the MFN1+MFN2 overexpressing ones, 74.86 ± 1.02 μm, a statistically intermediate value (P<0,001). Moreover, analysis of protein quantity showed that both MFN2 and MFN1+MFN2 overexpressing oocytes had lower levels of pmTOR, 0.35 ± 0.12 and 0.39 ± 0.14, respectively, in comparison to the control, 1 (P=0.0094). These results show that the excess of MFN2 in relation to MFN1 is detrimental to oocyte growth and is inhibiting important downstream substrates of the PI3K/PTEN pathway, the mTOR complexes. Activation of these pathways is important to oocyte growth and development because they lead to enhanced protein translation, cell growth and secretion of GDF9 and BMP15. In conclusion, this study shows that the overexpression of MFN1 and MFN2 during oocyte development results in a milder effect when compared to the overexpression of only MFN2, which leads to a severe growth blockage, suggesting an important role of MFN1/MFN2 ratio and Mitochondrial Dynamics during oocyte development.

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