



A155 Folliculogenesis, oogenesis, and superovulation

Xenograft of fresh and vitrified ovarian tissue from agouti (*Dasyprocta leporina*) to scid mice

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The vitrification followed by xenograft of gonadal tissue in the agouti is a useful tool to promote the conservation, multiplication, and formation of germplasm banks, especially when adapted for endangered rodents as the *Dasyprocta mexicana*. The objective was to evaluate the development of fresh and vitrified agoutis' ovarian tissue after xenograft to C57Bl/6 SCID female mice. Ovaries were obtained from five female agoutis and divided into 16 fragments. Five fragments were immediately transplanted to SCID mice (xeno-control group) and the others were vitrified (xeno-vitrified group) on a solid surface using solution consisting of MEM plus fetal bovine serum, 0.25M sucrose and association of 3 M dimethylsulfoxide with 3 M ethylene glycol, being transplanted only after rewarming. Transplantation was performed under the renal capsule of the recipients. Recipients' ovarian activity return was monitored by observation of external estrus signs and vaginal cytology during 40 days. At the end of this period, the collection of blood destined for estrogen dosage by electrochemiluminescence was conducted. Comparisons between treatments for morphologically normal proportions of ovarian follicles were performed by the Fisher PLSD test ($P < 0.05$). We verified that 80% (4/5) of the mice that received fresh ovarian tissue of agoutis and 16.7% (1/6) who received vitrified tissue returned to ovarian activity at 20.6 ± 8.6 days after xenograft, evidencing proestrus signs such as vulvar edema and presence of mucus, as well as evident increase in the proportion of cornified epithelial cells in the vaginal cytology. At 40 days, the same females showed increase in estrogen levels, related to estrus occurrence. Histological analysis of xenotransplanted tissues showed a predominance of primordial and primary follicles in all treatments. In two individuals (40%) of the xeno-control group, luteal bodies were identified, as well as the presence of a hemorrhagic corporea in one of the females of the same group which was in cytological metaestrus, possibly indicating the occurrence of ovulation. In 80% (4/5 - xeno-control group) and 16.7% (1/6 xeno-vitrified group) of the recipients, there was revascularization of the xenotransplanted tissue through the presence of blood vessels connected to the graft. In conclusion, it has been demonstrated that ovarian tissue xenotransplantation of *D. leporina*, fresh or vitrified, is able to promote the return of ovarian activity in SCID mice. It should be noted that this is the first study to describe the process of vitrification of agoutis' ovarian tissue followed by *in vivo* culture.

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Characterization of cystic ovarian condition in Nelore (*Bos Indicus*) cows used as oocyte donors

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Repeated ultrasound-guided follicle aspiration (OPU) may alter follicular dynamics in oocyte donors (Viana et al. 2010), probably due to changes in LH/FSH releasing patterns. Moreover, donors cows usually undergo frequent hormonal treatments to synchronize follicular wave emergence and are prone to become obese, both risk factors for the development of endocrine misbalance and, consequently, cystic ovarian disease (COD). Thus, the aim of this study was to characterize COD in Nelore (*Bos indicus*) cows previously used as oocyte donors. Cows (n=16) were selected based on records indicating recurrent occurrence of COD and lack of response to conventional treatments. The average weight and body condition score were 615.9±11.5 Kg and 4.0±0.1, respectively. Weekly transrectal ultrasonography was performed to evaluate the number of follicles and distribution among size classes, endometrial thickness, and clinical presence of mucometra (scored in a scale of 0 to 3). Non-pregnant, cyclic Nelore cows from the same herd (Controls) were used as a reference of physiological distribution of follicle population among size classes. Results are shown as mean±SEM. The average diameter of the largest follicle in cows with COD was 20.6±0.6 mm, larger than the usual maximum diameter of ovulatory follicles in Nelore (17.0 mm, Sartori et al. 2011), but smaller than the classic reference value to determine follicular cysts in *Bos taurus* (25 mm). However, 37.5 % (6/16) of donor cows did not have a follicle larger than 17 mm in at least one exam. When compared to controls, donor cows with COD had a greater number (3.6±0.2 vs 0.9±0.1, P<0.001) of follicles larger than the maximum diameter reported for the dominant follicle at deviation in Nelore (8 mm) and a lesser number (16.4±1.1 vs 23.5±2.6, P<0.01) of small follicles (≤4.9mm). Follicular population was negatively correlated both to the size of the largest follicle and the number of follicles ≥7.9 mm (R= -0.44 and -0.36, respectively; P<0.01). There was no difference in endometrial thickness between groups (4.4±0.2 vs 4.1±0.2 mm; P>0.05). In cows with follicular cysts, however, endometrial thickness was negatively correlated to the amount of mucus in the uterine lumen (R= -0.71). In summary, our results suggest that: 1) classification of follicular cysts must consider the expected range of follicular diameter in each breed; 2) COD in oocyte donors is characterized not only by ovulation failure and persistent dominant follicles, but also by an abnormal distribution of follicle population of distinct size classes; 3) COD decreases the average number of follicles on the ovaries of oocyte donors and, consequently, may compromise the outcome of OPU/IVF.

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Ovarian superstimulation increases oviductal estradiol and upregulates genes involved with gamete interaction

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P-36 protocol for ovarian superstimulation (OVS) using FSH was designed and later adapted for P-36/eCG to increase embryo viability due to the dual action of eCG on FSH and LH receptors (FSHR and LHCGR). Several oocytes are transported into oviduct during OVS, where they are fertilized and take the initial steps towards embryo development until transfer. The present study aimed to analyze the effect of OVS on steroids concentration in the oviduct and on the abundance of mRNA encoding genes related to gamete interaction and hormonal actions. Fifteen Nelore cows (n=5/group) were divided in three experimental groups: non-superstimulated (control) or submitted to two OVS protocols (P-36 or P-36/eCG). At 12h before endogenous LH surge, the ipsilateral oviduct (control cows) and a random oviduct (OVS cows) were collected to quantify the local concentration of E₂ and P₄. Total RNA was extracted and reverse transcribed from each segment (infundibulum, ampulla and isthmus). Relative RT-qPCR was performed using Power Sybr Green system with bovine-specific primers. The mRNA abundance of genes described as modulators for gamete interaction: Alfa-L-Fucosidase (*FUCA1* and *FUCA2*), Oviductal Glicoprotein 1 (*OVGP1*) and Heat Shock Protein Family A Member 5 (*HSPA5*); and also genes involved with endocrine control (*ESR1*, *ESR2*, *PGR*, *FSHR*, *LHCGR* and *CYP19A1*) was quantified. ANOVA was performed to assess the effect of OVS on mRNA abundance and concentration of hormones in oviduct. Mean values were compared by Tukey-Kramer test using JMP software (SAS Institute Cary, NC). Differences were considered significant when P≤0.05. In summary, oviductal E₂ concentration was higher in cows from P-36/eCG group (464±153 pg/mL) compared with control group (249±32 pg/mL) and showed intermediary values in P-36 group (326±24 pg/mL); however, the oviductal P₄ levels were similar among groups. The mRNA abundance of *FUCA1*, *FUCA2*, *OVGP1* and *HSPA5* was higher in infundibulum and ampulla of cows from P-36/eCG group compared to control group; however, no differences were observed in the isthmus. The expression of estrogen receptors (*ESR1* and *ESR2*) and progesterone receptor (*PGR*) were identified in all oviduct and showed higher abundance in ampulla (*ESR1* and *PGR*) and infundibulum (only *PGR*) in P-36/eCG cows compared to control group. The expression of *CYP19A*, *FSHR* and *LHCGR* were not detected in any oviductal segment. In conclusion, OVS increases E₂ concentration in the bovine oviduct, which may up-regulate genes involved with gamete interaction through activation of *ESR1* and *ESR2*, maybe setting the oviduct to receive more oocytes and embryos upon OVS treatment. Additionally, the findings presented here suggest no potential interaction of FSH and LH directly into the bovine oviduct during the preovulatory period. Supported by FAPESP #12/09498-9, 13/08629-5, 12/50514-8, 13/11480-3).



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Prematuration of bovine oocytes with forskolin and IBMX: Cumulus cells transcriptomics and oocyte meiotic status

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Prematuration (Pre-IVM) systems use cyclic adenosine monophosphate (cAMP) modulators such as forskolin (FSK) and 3-Isobutyl-1-methylxanthine (IBMX) for artificially blocking meiotic resumption. To verify Pre-IVM effects in cumulus-oocyte complexes (COCs) we have sequenced cumulus cells transcripts, validated some key genes involved in the regulation of meiotic arrest/resumption and assessed the meiotic status of oocytes. COCs were cultured for 2h in M199 with FSK (100 μ M) and IBMX (500 μ M) followed by in vitro maturation (IVM) in M199 with rhFSH (0.1 IU/mL) for 24h. Control COCs were only matured for 24h in M199 with rhFSH and were used as the reference group in all comparisons. After 2h (Pre-IVM) and 26h (Pre-IVM + IVM) cumulus cells were recovered and total RNA was extracted. Sequencing was performed on HISEQ2500 Illumina platform and mapped to the *Bos t. taurus* reference genome UCSC bTau8. Gene expression was estimated with the R/BioConductor and tested with a quasi-likelihood F-test. Enrichment test indicated 80 significant categories of biological processes, 22 for cellular compartment and 25 for molecular function. Also, 7 pathways were significantly enriched: 3 stimulated and 4 suppressed in Pre-IVM. After 2h culture, 727 genes were differentially expressed, 334 upregulated (UR) and 393 downregulated (DR) by Pre-IVM. There were 292 differentially expressed genes after IVM: 185 UR and 107 DR by Pre-IVM. qPCR validated the downregulation of phosphodiesterases (PDE5A, PDE1B, and PDE4B) and NOS3, which may act in the oocyte with the involvement of gap junctions to maintain higher levels of cGMP and cAMP, sustaining meiotic arrest. Those observations were reinforced by lucifer-yellow staining where gap junctions were substantially more open in Pre-IVM COCs ($P < 0.05$) and also by orcein staining indicating higher amount of germinal vesicle oocytes assessed in Pre-IVM (91.0%) when compared to Control (59.3%). Genes related to cumulus expansion (PTX3, HAS2 and TNFAIP6) were also DR by Pre-IVM, however PTX3 became UR after IVM corroborating that Pre-IVM oocytes have the ability to overcome the arresting effects of Pre-IVM. Indeed, Hoechst 33342 staining proved the meiotic progression to be equivalent in both groups after IVM (62.1% of MII oocytes). After 2h we also validated the DR of genes related to lipid metabolism (PLIN2 and LIPE) in cumulus cells, which agrees with the lipolytic action of FSK, although we have observed an increased lipid content in matured oocytes treated with Pre-IVM after Sudan-Black B staining. In conclusion, RNA sequencing data showed that Pre-IVM deeply modulates the transcriptional profile of cumulus cells through the regulation of important genes that might be responsible for arresting meiosis in a reversible manner, since oocytes were able to be fertilized and sustain embryonic development similarly to conventionally matured oocytes. FAPESP grants 12/50533-2, 13/05083-1, 12/10737-8 and 12/23409-9.



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Effects of different melatonin concentrations on *in vitro* maturation of bovine oocytes: Preliminary results

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Melatonin, a molecule derived from tryptophan and synthesized mainly by the pineal gland, mediates several processes in animal reproduction and has drawn attention for its potent antioxidant, anti-apoptotic, anti-inflammatory action and, more recently, for its benefits on oocyte maturation and embryo development *in vitro*. The aim of this study was to determine the concentration of melatonin that provides greater benefits during the *in vitro* maturation of bovine oocytes. For this, bovine cumulus-oocyte complexes (COCs) were obtained by aspiration of follicles (2-6 mm) from slaughterhouse ovaries, selected (grades I and II) and transferred to 4 well plates (25-30 COCs/well) containing *in vitro* maturation (IVM) medium [TCM199 supplemented with sodium bicarbonate (26 mM), sodium pyruvate (0.25 mM), FSH (0.5 µg/mL), LH (5.0 µg/mL), 0.3% Bovine Serum Albumin (BSA) and gentamicin (50 µg/ml)] with 0, 10^{-5} , 10^{-7} , 10^{-9} or 10^{-11} M of melatonin and cultured for 24 hours at 38.5°C and 5% CO₂ in air. At the end of IVM, oocytes were denuded, stained with Hoechst 33342 (10µg/ml) and evaluated for nuclear maturation rate. Cumulus cells (CC) were evaluated for the expression of antioxidant genes (SOD1, SOD2 and GPX4). For transcripts detection in CC, RNA isolation was performed with TRIzol[®] Reagent (Invitrogen[™]) and reverse transcription with “High Capacity cDNA Reverse Transcription” kit (Applied Biosystems[™]; Invitrogen[™]), following the manufacturer’s recommendations. Relative quantification of SOD1, SOD2 and GPX4 transcripts was performed by Real Time qPCR using Power SybrGreen[®] PCR Master Mix (Applied Biosystems[®]) with 3 endogenous controls (β-Actin, GAPDH and PPIA). Nuclear maturation rate and gene expression were tested by ANOVA and means were compared by Tukey’s test (4 replicates) (p<0.05). In CC, the different concentrations of melatonin used, did not significantly alter the expression of the investigated antioxidant genes (p>0.05), however, the concentration of 10^{-7} M provided a numerical increase in the expression of SOD1, SOD2 and GPX4. In oocytes, the rate of nuclear maturation was not statistically different (p>0.05) among the tested treatments, but was numerically higher in the 10^{-7} M melatonin treated group. In conclusion, under the studied conditions, melatonin was unable to improve maturation rate and expression of antioxidant genes, but was able to improve numerically all the parameters evaluated at an intermediate concentration (10^{-7} M), indicating its beneficial effect on the IVM of bovine oocytes. The next step will involve performing more replicates of this experiment, to investigate further the potential cytoprotector role of melatonin and its benefits on oocyte maturation and developmental competence *in vitro*.
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Changes in MicroRNAs expression in granulosa cells from superstimulated cows

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Ovarian superstimulation (OVS) is widely used in cattle to obtain multiple ovulations and can impact the follicular microenvironment. MicroRNAs (miRNAs) play a key role on ovarian follicle development and are expressed in bovine theca and granulosa cells. Particularly, the mir-222 shows a higher expression in theca and granulosa cells from bovine atretic follicles and mir-122 is described as a negative modulator of progesterone production. On the other hand, mir-144, mir-202 and mir-873 are up regulated in granulosa cells of bovine dominant follicle compared to subordinate follicles and are suggested as markers of steroidogenic capacity. To gain insight into the effects of OVS on regulation of miRNAs abundance in granulosa cells, the present study assessed the expression of mir-222, mir-122, miR-144, mir-202 and mir-873 in granulosa cells of preovulatory follicles from cows submitted to P-36 (only by FSH; n=10) or to P-36/eCG protocol (replacement of FSH by eCG administration on the last day of treatment; n=10). To obtain non-superstimulated preovulatory follicles, cows were only submitted to estrous synchronization (n=10). At 12h before endogenous LH surge, preovulatory follicles were obtained, and granulosa cells were harvested and submitted to miRNA extraction using MirVana™ miRNA Isolation Kit (LifeTechnologies®, São Paulo, Brazil). The relative abundance of miRNAs was measured by real time RT-PCR using TaqMan® Universal PCR Master Mix (Applied Biosystems, São Paulo, Brazil) and RNU43 plus has-miR-191 were used as reference. The effect of OVS on abundance of target miRNAs was tested by ANOVA and the mean values were compared with orthogonal contrast. Differences were considered significant when $p \leq 0.05$. In summary, the OVS did not alter the relative abundance of mir-144, mir-202 and mir-873 in bovine granulosa cells. On the other hand, the relative abundance of mir-122 was lower in cows submitted to P-36 protocol ($0,89 \pm 0,36$) compared to control group ($4,72 \pm 2,02$). In the same way, the mir-222 showed a higher relative abundance in animals from control group ($3,36 \pm 1,04$) compared to P-36 ($0,68 \pm 0,29$) and P-36/eCG ($0,84 \pm 0,25$) groups. In conclusion, the lower levels of mir-222 and mir-122 in superstimulated granulosa cells reinforce that these miRNAs should be suppressed to allow the antral follicle development. Moreover, the absence on regulation of mir-144, mir-202 and mir-873 suggests that the superstimulatory protocols affect specific pathways to promote the final maturation of preovulatory follicles and corroborates with previous data which indicates that miR-144, mir-202 and mir-873 may exert a special role during follicle deviation in cattle. Financial support: FAPESP (grant #2013/11480-3 and grant #2011/50593-2).



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Effects of eCG administration 4 versus 2 days prior to timed AI on *Nellore* cows

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The objective of this study was to evaluate the growth of the dominant follicle (DF) in multiparous *Nellore* cows treated with equine chorionic gonadotropin (eCG) either 4 or 2 d prior to timed AI (TAI). We hypothesized that early eCG administration improves follicular growth and increases DF diameter at TAI. Cows (n = 137) had their estrous cycle synchronized with an intravaginal device containing 1.9 g of progesterone (CIDR) and 2.0 mg of estradiol benzoate (d 0), 12.5 mg of PGF2 α (d 7), CIDR withdrawal and 0.3 mg of estradiol cypionate (d 9), and TAI 48 h later (d 11). On d 7, ovaries were evaluated by ultrasonography and cows were blocked based on DF diameter (< 9 mm vs. \geq 9 mm). Within block, cows were randomly assigned to receive 300 IU of eCG either on d 7 (eCGD7; n = 64) or on d 9 (eCGD9; n = 73). Diameter of the DF was evaluated by transrectal ultrasonography on d 7, 9, and 11. Follicular growth from d 7 to 9, 7 to 11, and 9 to 11 was analyzed by ANOVA using the GLIMMIX procedure of SAS. Follicular diameter was analyzed by ANOVA for repeated measures and cow was considered a random effect. Follicular growth from d 7 to 11 tended to be greater (P = 0.08) for eCGD7 compared with eCGD9 (4.9 \pm 0.2 vs. 4.4 \pm 0.2 mm). The overall effect of treatment was explained mostly by DF growth from d 7 to 9 (eCGD7 = 2.6 \pm 0.2 vs. eCGD9 = 2.0 \pm 0.2 mm; P < 0.01), as growth from d 9 to 11 did not differ (P = 0.44) between treatments (eCGD7 = 2.3 \pm 0.2 vs. eCGD9 = 2.4 \pm 0.1 mm). Follicular diameter was affected (P < 0.01) by the interaction between treatment and day. Although no difference was observed on d 7 (eCGD7 = 9.7 \pm 0.2 vs. eCGD9 = 9.6 \pm 0.2 mm), DF in eCGD7 cows was larger (P < 0.01) on d 9 (12.3 \pm 0.2 vs. 11.5 \pm 0.2 mm). In addition, DF on d 11 did not differ (P = 0.12) between treatments (eCGD7 = 14.5 \pm 0.3 vs. eCGD9 = 14.0 \pm 0.2 mm). Treatment with 300 IU of eCG 4 d prior to TAI improved follicular growth but did not increase overall mean of DF diameter at TAI compared with eCG administration 2 d before TAI in *Nellore* cows.



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Glycogen synthase kinase-3 (GSK-3) inhibition during bovine oocytes *in vitro* maturation negatively affects embryo *in vitro* production

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Glycogen synthase kinase-3 (GSK-3) acts on several signaling pathways as cell cycle, protein synthesis and microtubule dynamics. This enzyme was identified in bovine oocytes and cumulus cells at different stages of development. It is known that its unspecific inhibition negatively affects oocytes *in vitro* maturation (IVM), but there is no research that demonstrates its role on oocyte viability, cytoplasmic maturation and the impact of GSK3 inhibition on embryo development. Therefore, the aim of this study was to evaluate the impact of GSK-3 inhibition using a specific inhibitor, CHIR99021, on bovine oocytes IVM and its effects on embryos *in vitro* production (IVP). COCs (grade I and II) were *in vitro* matured in TCM 199 supplemented with 10% FCS, FSH 10 g/mL, LH 5 µg/mL and 1% penicillin/streptomycin, supplemented with different concentrations of CHIR99021 [Control group (CG), 1.5 µM(G1), 3.0 µM(G2) e 6.0 µM(G3)]. IVM was performed in 100 µL drop (20 COCs/drop), submerged in mineral oil and maintained in a humidified atmosphere containing 5% CO₂ in air, at 38.5°C, for 24 hours. After this period were evaluated the cumulus cells expansion, cellular viability by Calcein AM and Propidium iodide staining, nuclear and cytoplasmic maturation by acetic orcein and Lens culinaris-FITC (LCA) staining, respectively. COCs submitted to same conditions were also fertilized and *in vitro* cultured to determine cleavage (D3) and blastocysts (D7) rate. The results were analyzed by ANOVA and submitted to Tukey test ($p \leq 0.05$). The highest CHIR99021 concentrations (3 e 6 µM) reduced cumulus cells expansion. The inhibitor use also significantly reduced in a dose-dependent manner the oocyte viability (CG: $81.62 \pm 11.15a$; G1: $66.67 \pm 8.47ab$; G2: $52.02 \pm 12.60bc$; G3: $34.86 \pm 10.43c$) and nuclear maturation (CG: $92.71 \pm 8.58a$; G1: $54.67 \pm 5.58b$; G2: $44.27 \pm 7.96b$; G3: $28.47 \pm 1.65c$). Cortical granules distribution were also reduced when 3 and 6 µM were used (C: $75.06 \pm 5.97a$; G1: $60.41 \pm 8.46a$; G2: $39.11 \pm 4.78b$; G3: $28.31 \pm 9.84b$). This conditions resulted to lower cleavage (CG: $83.63 \pm 9.39a$; G1: $80.72 \pm 9.06a$; G2: $65.44 \pm 16.94ab$; G3: $39.58 \pm 12.39b$) and blastocyst rates (CG: $40.50 \pm 3.90a$; G1: $34.11 \pm 5.35ab$; G2: $25.34 \pm 3.78b$; G3: $13.41 \pm 4.43c$). Thus, it is possible to conclude that GSK-3 is essential for bovine oocyte *in vitro* maturation and its inhibition negatively affects IVP. To our knowledge, this is the first work that shows the negative effect of GSK3 inhibition during IVM on *in vitro* embryos production.



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Analysis of metabolism and hormonal production by canine luteal cells in cyclic and gestational diestrus cultured *in vitro*

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Despite the knowledge about reproductive physiology of the canine species, the similarity between cyclic and gestational luteal phase is still an intriguing and unknown factor. Our objective was to measure glucose and lactate production and P4 and E2 levels in culture medium of canine cyclic and gestational luteal cells. The CLs were collected (n = 40) after OSH on different days after LH preovulatory surge (D10, D20, D40 and D60) during cyclic (n = 20) and gestational (n = 20) diestrus. The CLs were digested enzymatically by collagenase type 1 (1mg / ml, Sigma Aldrich, Saint Louis, USA) plus high glucose DMEN (Sigma Aldrich, Saint Louis, USA) and the contents were filtered and centrifuged (3x, for 10 minutes at 20 ° C). The cell concentration used was 2x10⁵ cells/ml (500 ul / well) and the medium was collected and stored in cryotubes (-80°C) at 3 moments: M1 (36 hours), M2 (48 hours) and M3 (60 hours). A plate containing only culture medium was used as control. For glucose and lactate measurement were used Calibrator 1 VITROS (Ortho Clinicals, Rochester, USA) and for P4 and E2 measurement were used Imunotech and ultra-sensitive DSL-4800 kits (Beckman Counter, Indianapolis, USA). For statistical analysis were used ANOVA and PROC GLIMMIX of SAS, with significance when p <0.05. There was glucose consumption by cyclic (p = 0.032) and gestational (p = 0.0134) luteal cells in D20 and D60 only by cyclic luteal cells (p = 0.015). Probably this higher consumption is due to high energy requirement during this exponential cell growth phase (Zagari F., et al., N Biotechnol., 238-45,2013) and the differentiated consumption in D60 may be associated with a differentiated metabolism during luteolysis. Lactate production was higher in D10 and D40, in M3 (p <0.05) in both cyclic and gestational diestrus, and low in D60, although gestational CL production was higher (p = 0.02), suggesting a reduction in metabolic enzymes activity at the time of luteolysis. In relation to P4, there was no difference in production between cyclic and gestational CLs in all days studied. Only in the cyclic C.L, the concentration in D10 was similar to D40, both higher than D20 and D60 in M3 (p <0.05). In relation to E2, there was no variation between the cyclic and gestational luteal cells in D10, D20 and D60, but in D40, in cyclic diestrus, the estradiol production was high (342 pg / ml), 9 times more than gestational phase (51.51 pg / ml, p = 0.042). In bitches, the concentrations of P4 in vitro culture showed the same pattern observed in vivo. E2 values were high in D40 cyclic diestrus, a fact also evidenced by Concannon et al, 2002 (Reprod Domest Anim, page 3-15), but never reported in cell culture when compared to gestational diestrus. This differentiated pattern may indicate a distinct role for the action of E2 on the cyclic CL Luteal cells from cyclic and gestational diestrus, cultured in vitro, presented metabolic and hormonal differences, mainly in phases close to luteolysis moment.



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Evaluation of oocyte meiotic arrest after the combined use of natriuretic peptide precursor C (NPPC) and rhFSH during *in vitro* prematuration of bovine cumulus-oocyte complexes

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Studies have shown that natriuretic peptide C (NPPC) and its type 2-receptor (NPR2) are essential for the maintenance of oocyte meiotic arrest. Further details are still lacking, especially in the bovine species, regarding the NPPC-NPR2 signaling and the possible effects of their association with other factors used during *in vitro* maturation (IVM). Among those, FSH is widely used to stimulate cumulus cells expansion and meiosis progression. Since the active NPPC-NPR2 system can delay the spontaneous resumption of meiosis *in vitro* and FSH has an antagonistic action, the combined use of these two drugs is avoided when designing systems for IVM or pre-IVM. The objective of this study was to evaluate the effect of NPPC, associated or not to recombinant human (rh)-FSH, in a 6h pre-IVM system on the progression of meiosis in bovine oocytes. Cumulus-oocyte complexes (COCs) were matured according to the following groups: I) Laboratory control: 24h IVM with control medium (M199, pFSH, pLH, E2, FCS, pyruvate and amikacin); II) Experimental control: 6h pre-IVM with basic medium (M199, BSA, pyruvate and amikacin); III) Pre-IVM-NPPC: 6h pre-IVM with basic medium plus NPPC (100 nM); IV) Pre-IVM-FSH: 6h pre-IVM with basic medium plus rhFSH (0.1 µg/ml); V) Pre-IVM-NPPC/FSH: 6h pre-IVM with basic medium plus NPPC and rhFSH (at same concentrations of groups III or IV). In all groups, except I, pre-IVM was followed by 24h IVM with the control medium. COCs were collected at 9h and 15h of culture time, i.e., COCs from groups II, III, IV and V were collected after 3h and 9h of IVM. Collected COCs were stained with orcein to assess the percentage of oocytes still arrested in meiosis at the germinal vesicle stage (GV). Five replicates were performed with 15 to 20 COCs per group. The effect of treatments was tested by one way-ANOVA after data normalization and the means were compared with the post-hoc test by Fisher LSD method. Significance was considered with $P \leq 0.05$. After 9h IVM, the percentage of GV oocytes was higher in groups III and V (79,1% and 51,7%, respectively) when compared to groups I, II and IV (38,1%, 40,3% and 33,2%, respectively). After 15h IVM, the percentage of GV oocytes of group III (28,8%) was increased when compared to groups IV and V (12,4% and 14,1%, respectively), as well as of group IV with group II. We can conclude that after 9h there was a partial inhibition of the effect of NPPC due to the combined use of rhFSH, indicating that the use of drugs with opposed effects was not fully detrimental. However, this inhibition was complete after 15h IVM.



A165 Folliculogenesis, oogenesis, and superovulation

Morphological and morphometric evaluation of preantral multi-oocyte ovarian follicles of *Bos indicus* fetuses

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The objective of this study was to evaluate the morphology and morphometry of preantral multi-oocyte follicles of bovine fetuses in initial, middle and final third of gestational development. Thirty two ovaries (16 pairs) of *Bos indicus* fetuses in three distinct gestational phases: initial (6 pairs), middle (3 pairs) and final third (7 pairs) were removed after the slaughter of females. The pregnant uterus were packed in boxes and sent to the lab, where the foetuses were dissected for the obtaining of the ovaries. These were immediately fixed in Bouin for 18 hours, subsequently placed under running water for 12 hours and packed in 70% alcohol to histological processing. To make the blades, the paraffin blocks were cut with a rotary microtome (Leika®, Germany) in cuts 5 µm thick serials and interval of 6 histological sections. The fragments were stained with periodic acid Schiff (PAS) and were analyzed under an optical microscope (Axio, Scope. A1-Zeiss, Switzerland). The total number of multi-oocyte follicles was evaluated and these were classified morphologically into primordial, primary and secondary. Morphometry was performed with the aid of the Image Pro Plus Version 4.5 Program by image capture of the slides, calculating the mean area of each follicle. The results were submitted to analysis of variance ($P < 0.05$). Seven and forty six preantral multi-oocyte follicles were found from 16 pairs of ovaries. There was a higher proportion of primordial follicles in thirds and end multi-oocyte [70.3% (154/219) and 69.4% (245/353)] respectively, compared to the initial third [28.7% (50/174)]. The primary follicles showed similar proportion ($P > 0.05$) in the initial third [50.5% (88/174)], middle third [28.3% (62/219)] and final third [22.6% (80/353)]. The secondary follicles presented a higher proportion ($P < 0.05$) in initial thirds [20.6% (36/174)] and end [7.9% (28/353)] in relation to the middle third [1.3% (3/219)]. The morphometry resulted in different sizes for the primordial follicles multioócitos in three-thirds of pregnancy (third: 246.8 µm; middle third: 388.0 µm; final third: 409.0 µm) in relation to primary follicles (third: 797.2 µm; middle third: 851.1 µm; final third: 569.1 µm) and secondary (third: 713.3 µm; middle third: 1141.6 µm; final third: 699.6 µm). The primordial follicles were lower in the initial third of gestation (246.8 µm) in relation to the middle third (388.0 µm) and final (409.0 µm). Only the primordial follicles, initial third pregnancy showed lower size ($P < 0.05$) compared to medium and final thirds. The secondary follicles were similar ($P > 0.05$) in three-thirds of pregnancy (third: 713.3 µm; middle third: 1141.6 µm; final third: 699.6 µm). We conclude that primordial multioocyte follicles were predominantly found in ovaries of *Bos indicus* fetuses in the middle and late thirds, and this follicular class presented a smaller diameter in the fetuses in the initial third of gestation by morphometric evaluation.

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A166 Folliculogenesis, oogenesis, and superovulation

Varian morphological characteristics influencing population of anthropological folliculos in femesas *Bos indicus* of different ages - preliminary results

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The antral follicular population has been associated with fertility traits in *Bos taurus* (Ireland et al., *Reprod Fertil Develop*, v.23, p.1-14, 2011; Jimenez-Krassel et al., *Biol Reprod*, v.80, p.1272-81, 2009), but not in *Bos indicus* (Rodrigues et al., *Pesq agropec bras*, v.48, p.801-804, 2013). However, in both species there is positive relation of the antral follicular population (PFA) with the number of embryos produced in vitro (Ireland et al., *Reprod Fertil Develop*, v.23, p.1-14, 2011; Rodrigues et al., *Pesq agropec bras*, v.48, p.801-804, 2013), which has made this feature interesting for selection (Oliveira Jr. et al., *Semina*, v.36, p. 3741-50, 2015). The best understanding regarding the relation of ovary morphology and PFA can optimize the selection method based on this characteristic. The objective of the present study was to evaluate the relationship between ovary weight and diameter and the number of antral follicles ≥ 1 mm in bovine females of different ages. The ovaries of 398 *Bos indicus* females were individually collected at a local slaughterhouse. The age of the animals was determined by analysis of the dentition as recommended by McManus et al. (*Serie técnica: Genética. UnB- DF*, 2010). After that, the ovaries were sent to the laboratory and the weight, diameter and count of the antral follicles were measured. The obtained data were submitted to analysis of variance and Pearson correlation by the program R. Large variations in ovarian weight and PFA were observed among the animals. Significant positive correlations were found between ovarian diameter and weight ($r \geq 0.66$) and between ovary diameter and PFA ($r \geq 0.30$). There was a difference ($P < 0.05$) in PFA of the animals presenting CL in one of the ovaries, when compared to those who did not have CL (32.8 ± 2.04 ; 27.1 ± 0.95 respectively). There was no difference ($P > 0.05$) in PFA between null females of 15 (n=41), 24 (n=38), 36 (n=31), 48 (n=45) and >60 (n=43) months of age. There was a correlation between age and ovarian weight ($r = 0.33$, $P < 0.05$) and corpus luteum diameter ($r = 0.18$, $P < 0.05$). The same did not occur when the age was correlated with the weight and diameter of the FD ($P > 0.05$). It was concluded, therefore, that PFA varied according to the diameter, ovary weight and presence of CL, but no influence of FD was observed on *Bos indicus* females. And although ovary weight increased with age, PFA did not vary in females between 15 and 60 months of age.



A167 Folliculogenesis, oogenesis, and superovulation

Comparison among different systems of *in vitro* culture of preantral follicles in cattle

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The aim of this study was to evaluate the efficiency of four methods for *in vitro* culture method of preantral follicles in the bovine species. Ovaries (n = 10) were collected from local slaughterhouse, from cyclic Nellore cows (n = 5). After collection, the ovaries were washed in 70% ethanol and PBS. The surrounding ovarian tissue was removed and the ovary cortex was dissected into fragments of approximately 3x3x1 mm. One fragment per animal was immediately fixed in Bouin and the remaining fragments (n = 8) were individually randomly distributed into four groups: i) standard culture in plastic plate, ii) culture on the agarose gel; iii), agarose gel culture on the plate; and iv) Millicell culture. Fragments were grown with supplemented minimal essential medium (MEM, Gibco) with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, and 6.25 ng/ml selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantina, 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/ml penicillin and 200 mg/ml streptomycin. Culture methods were tested for six (D6) or fourteen (D14) days. The culture media were replaced with fresh aliquots every two days. For the analysis of the integrity and degree of development of the follicles, the classical histology with Schiff Periodic Acid (PAS) and Hematoxylin staining, and the morphometric evaluation of the follicles and oocytes was performed. Classification was based on evaluation of the stage of follicular development (primordial, primary or secondary) and morphological integrity in intact or degenerate. For the analysis of morphometry, 5 healthy follicles were observed per treatment. For the morphometric analysis, images were used to measure oocyte and follicle diameter, calculating the arithmetic mean of two perpendicular measurements, using the program Motic Plus 2.0. Data were analyzed by ANOVA tests ($p \leq 0.05$). We evaluated 1,330 preantral follicles, of which 326 were primordial follicles and 1,004 developing follicles. After six days of culture, the fragments grown on the agarose gel support presented a higher proportion of intact follicles (75.3%; 113/150) compared to the other methodologies tested: 58.7% (88/150) for the standard culture; 62.6% (94/150) for culture with agarose gel on the plate; 54% (81/150) for the Millicell culture ($p < 0.05$). As for the percentage of developing follicles in D6, there were no differences between the culture methods tested ($p < 0.05$). After fourteen days of culture, the standard cultures on the agarose gel and agarose gel on the plate allowed a higher percentage of morphologically intact follicles: 54.7% (82/150), 58% (87/150) and 54.7% (82/150), respectively, when compared to the millicell culture (35.3%, 53/150). For the rate of development in D14 the treatments also did not differ among themselves ($p > 0.05$). Morphometric evaluation at six days of culture presented similar results among the four groups. However, after fourteen days, the follicles cultured on the agarose gel presented a greater average diameter in comparison to the other treatments. We concluded that the culture method on the agarose gel was more effective in maintaining the morphological integrity and allowing bovine preantral follicles to reach greater diameters.



A168 Folliculogenesis, oogenesis, and superovulation

Progesterone dosage with Sincrogest Injetável® on the induction of cyclicity

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The induction of cyclicity (IC) with intravaginal progesterone device associated with estradiol IM treatment increases cyclicity and conception rate at TAI in *Bos indicus* heifers (Sá Filho, et al. Anim.Reprod. Sci., v.160, 2015). Furthermore, similar results are obtained when 150mg of long-action injectable P4 was used (P4LA; Sincrogest Injetável®, Ourofino Saúde Animal; Neto, et al., Anual da SBTE, Foz do Iguaçu, 2016). However, the period between the increase of blood P4 concentration after treatment with P4LA in prepubertal Nelore heifers remains unknown. Therefore, the objective of the present study was to evaluate plasma concentration of P4, the diameter of the dominant follicle (\emptyset FolDom) and ovulation rate (RtOv) in prepubertal Nelore heifers treated with 150mg of P4LA. The experiment was conducted in a commercial farm in Mato Grosso state in Brazil, where 21 prepubertal heifers were selected [absence of CL in two US with a 6-day interval (D-18 and D-12)] aged 27.0±0.3 months and BCS = 3.01±0.03. On D-12, concomitantly with the US, blood collection was performed and heifers were separated in four experimental groups: 1) Control Group: did not receive hormonal treatment; 2) InjD10 Group: received 1.0ml (150mg) of P4LA on D-10; 3) InjD12: received 1.0ml (150mg) of P4LA on D-12; 4) DispD12: received an intravaginal progesterone device previously used for 24 days. Blood collection and US was performed promptly on D-12 and daily from D-2 to D2. The P4 device was removed on D0, at the same time the heifers of the InjD10, InjD12 and DispD12 groups received 0.5ml of estradiol cypionate (SincroCP®, Ourofino Saúde Animal). Data were analyzed using SAS (v9.4), the "Fisher's Exact Test" was used for binomial variables and the PROC GLIMIX for continuous variables. Treatment effect was observed for RtOv [Control = 20%b (1/5); InjD10 = 20%b (1/5); DispD12 = 50%b (3/6) and InjD12 = 100%a (5/5); P = 0.02]. Treatment effect was observed (P = 0.07) for \emptyset FolDom (Control = 10.4±0.83b; InjD10 = 13.2±1.1a; DispD12 = 9.5±2.5a and InjD12 = 12.2±0.85a). Time*treatment interaction was observed (P = 0.08) for [P4]. Time effect was observed (P < 0.001). Treatment effect for [P4] was observed (Control = 0.25±0.04b ng/mL; InjD10 = 0.59±0.1a ng/mL; DispD12 = 0.71±0.14a and InjD12 = 0.71±0.12a; P = 0.03). Punctually evaluating the D-1 time, Sincrogest Injetável®, presented [P4] near 1ng / ml, not observing difference when compared to the P4 device (DispD12 = 1.728 ± 0.38a ng/mL; InjD10 = 1.160 ± 0.28a ng/mL; InjD12 = 1.207 ± 0.5a ng/mL) as well as remaining superior to the Control Group (0.56 ± 0.2b ng/mL). However, Sincrogest Injetável® showed intermediate values to DispD12 and the control group observed in other times. Therefore, it was concluded that Sincrogest Injetável® promoted intermediate levels of [P4] compared to the Sincrogest Dispositivo®, maintaining [P4] above 1ng/ml for 11 days. Injectable Sincrogest® may be an alternative to induction of cyclicity in zebu heifers.



A169 Folliculogenesis, oogenesis, and superovulation

Effect of overfeeding with rehydrated grain corn silage on ovulation of crossbred Santa Inês Ewes

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Overfeeding (flushing) during the breeding season is an usual practice to increase the number of ovulations, once the greater energy intake stimulates folliculogenesis. Regarding the better ruminal digestibility of the starch on the rehydrated grain corn silage (RGCS), the objective of this study was to evaluate the effect of flushing with RGCS on ovulation of ewes. Fifteen crossbred Santa Inês ewes were randomized allocated to treatments: diet with ground corn (GC, n=7) or with RGCS (RGCS, n=8). Diets contained corn silage (40% of the dry matter of the diet), soybean meal, ground or rehydrated corn, and mineral salt were offered for 30 days before the breeding season. Estrus behavior was synchronized with progestagen impregnated implant (Progespon®, Zoetis, Campinas, Brazil) for 11 days, i.m. administration of 37.5 mg of d-cloprostenol (Prolise®, Arsa, Buenos Aires, Argentina) and 300 UI of eCG (Novormon®, Zoetis, São Paulo, Brazil) two days before the implant removal (Day 9). Estrus behavior was observed twice a day. Daily from the day 9, the number and diameter of all follicles ≥ 3 mm were observed through ultrasonography (Aloka SSD 500, linear transducer of 7.5 MHz) until the ovulation or until 8 days after the implant removal. The ovulation time was estimated as the mean interval between the last follicle ultrasound image and its disappearance on the following examination. Daily dry matter intake (DMI) was evaluated. Blood samples were collected every six days for glucose dosage. Percentages of estrus and ovulation, estrus length, interval from implant removal to the beginning of estrus and interval from estrus to the ovulation were analyzed by generalized linear models, using SAS® software (v 9.3, SAS Institute, Cary, USA). Variables such as DMI, serum glucose, number, growth rate and diameter of the ovulatory follicles were analyzed by variance analysis. Initial body weight was used as covariate in all analyses. The DMI (1.2 ± 0.1 and 1.1 ± 0.0 kg/day) and the serum glucose concentration (73.0 ± 8.7 and 82.2 ± 8.0 mg/dL) did not differ ($P > 0.05$) between the treatments GC and RGCS, respectively. The percentage of estrus behavior (57 vs 63%) and the estrus length (43.6 ± 10.6 vs 37.1 ± 9.5 h) did not differ ($P > 0.05$) between treatments, but the implant removal-estrus interval was shorter ($P < 0.05$) in the treatment GC (26.5 ± 8.5 vs 62.8 ± 8.3 h). The estrus-ovulation interval (41.2 ± 11.2 vs 36.6 ± 10.0 h), the percentage of ewes that ovulated (86 vs 75%), the number (2.7 ± 0.5 vs 1.7 ± 0.2 follicles) and growth rate of the ovulatory follicles (0.5 ± 0.1 vs 0.7 ± 0.1 mm/day) did not differ ($P > 0.05$) between treatments. However, the ovulatory follicles diameter was greater ($P < 0.05$) in the RGCS treatment (6.2 ± 0.5 mm vs 8.2 ± 0.7 mm). Flushing with RGCS did not promoted increase in the ovulation number, but delayed the estrus beginning and resulted in the ovulation of greater follicles. Acknowledgments: FAPEMIG.



A170 Folliculogenesis, oogenesis, and superovulation

Effect of different melatonin concentration on meiosis resumption and lipid content of bovine oocytes matured *in vitro*: Preliminary results

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Melatonin (MLT) is synthesized in pineal gland and can act on reproductive competence, has antioxidant and antiapoptotic activities and influences different cell signaling pathways. The objective of this study was to evaluate the effect of the addition of different concentrations of MLT during the *in vitro* maturation (IVM) on the meiosis resumption rate and lipid content of bovine oocytes. Pools of 25 to 30 cumulus-oocyte complexes (COCs) were randomly selected and submitted to IVM in TCM 199 medium supplemented with 11 µg/mL sodium pyruvate, 10 µg/mL gentamicin and 3 mg/mL BSA (negative control; NC) or added with hormones: 1 µg/mL FSH (positive control; PC) or different concentrations of MLT (10^{-11} , 10^{-9} and 10^{-7} M), in order to evaluate the individual action of each treatment on IVM and lipid content. Only melatonin was used and not its association with FSH in order to evaluate its individual action on IVM and lipid content. After 9 hours, the oocytes were denuded and stained with Hoechst 33342 and Nile Red, and evaluated by epifluorescence microscope to determine the nuclear maturation stage and lipid content, respectively (emission 445-450nm and 475-490nm excitation and emission 590nm and 516-560nm excitation, respectively). The fluorescence intensity (FI) was measured by ImageJ software. The statistical analyses were realized by ANOVA, followed by Tukey (3 replicates/group), with 5% of significance (GraphPrism software). There was an increase ($P < 0.05$) on the meiosis resumption rate (metaphase I) for MLT 10^{-9} M group (39.0%, 32/82) when compared to NC group (5.6%, 5/89). For the other treatments, there was no difference in the meiosis resumption (13.3%, 10/75; 8.3%, 6/72 and 35.8%, 29/81 to MLT 10^{-11} , 10^{-7} M and FSH, respectively) in relation to NC group. For MLT 10^{-11} M group was observed an FI of 8.43 ± 7.61 , characterized by a higher lipid content ($P < 0.0001$) in relation to the others treatments with MLT 10^{-9} M (5.6 ± 4.13), MLT 10^{-7} M (4.8 ± 3.21), FSH (5.34 ± 3.66) and NC (4.27 ± 3.28). In conclusion, under the conditions studied, MLT 10^{-9} M was able to influence meiosis resumption rate during the initial 9 hours of IVM. In the same way, MLT 10^{-11} M stimulated the increasing intraoocyte lipid content. Further studies are necessary to improve the knowledge of MLT role on the lipid synthesis and if it is associated with meiosis resumption in bovine oocyte. Acknowledgment: FAPESP (HF-2016/24884-3, LS, FCC, DMP, scholarships; CLVL, financial support 2015/20379-0).



A171 Folliculogenesis, oogenesis, and superovulation

Effect of epidermal growth factor on the development of preantral follicles of *Bos taurus indicus* females cultured *in vitro*

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Epidermal Growth Factor (EGF) is considered a potent mitogenic factor for follicular cells. Produced by the oocyte and granulosa cells, EGF plays an important role in the modulation of ovarian folliculogenesis. The objective of the present work was to test the addition of different concentrations of EGF to the *in vitro* culture medium for 5 or 10 days on the development of preantral follicles included in bovine ovarian fragments. Ovaries (n=10) of *Bos taurus indicus* females (n=5) were collected at a local slaughterhouse. Ovarian tissue fragments (n=9) with approximately 3x3x1 mm were obtained from each animal. One fragment was immediately fixed (Bouin's fixative) and processed for histological analysis (control group: Day 0), the remainder was placed in MEM® (Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/L, pH 7,2) supplemented with penicillin (200 IU/mL) and streptomycin (200 mg/mL) at 20°C, remaining for 1 hour (period of transport to the laboratory). The other fragments were *in vitro* cultured for 5 (D5) or 10 days (D10) in MEM+ (cultured control) or MEM+ plus different concentrations of EGF (50, 100 or 200 ng/mL), as the following groups: control (D0); MEM+ (D5); MEM+ + 50 ng/mL de EGF (D5); MEM+ + 100 ng/mL de EGF (D5); MEM+ + 200 ng/mL de EGF (D5); MEM+ (D10); MEM+ + 50 ng/mL de EGF (D10); MEM+ + 100 ng/mL de EGF (D10); MEM+ + 200 ng/mL de EGF (D10). The preantral follicles were evaluated by light microscopy and classified according to the stage of developmental (primordial or developing follicles) and morphology (intact or degenerate). The averages were compared by the Tukey's test ($P \leq 0.05$). In this experiment, 2,203 follicles in 720 histological sections were evaluated, totalizing 240 slides and 45 ovarian fragments. The non-cultivated control treatment (D0) predominantly presented preantral follicles at the primordial stage (82.8%), some primary follicles and rarely secondary follicles (17.2%). There was an increase in the number of developing follicles when 100 ng/mL EGF was added to the culture medium for 10 days (48.4%), compared to the control treatment (17.2%). In this way, we conclude that the addition of 100 ng/mL of EGF for 10 days to the *in vitro* culture medium of bovine preantral follicles was efficient in promoting follicles development and maintaining follicular integrity.



A172 Folliculogenesis, oogenesis, and superovulation

Study on nuclear maturation kinetics of bovine oocytes with different degrees of competence

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Oocyte nuclear maturation is a complex process involving modification of chromatin from the germinal vesicle stage to metaphase II. Studies have reported that oocytes from large follicles have greater developmental capacity than oocytes derived from small follicles, resulting in higher rates of in vitro embryo production. The aim of this study was to evaluate the chromatin condensation and nuclear maturation kinetics in oocytes with different degrees of competence. For this, small follicles were used (SF= 1.0-2.9 mm diameter follicles with less competent oocytes; n=120) or large (LF= 6.0-8.0 mm with more competent oocytes; n=130) dissected from the ovarian cortex were (Caixeta ES et al., *Reprod. Fertil.* 21,p.655–664, 2009). The control group (Con; n=151) was recovered by aspiration from 3 to 8 mm follicles. The COCs obtained from each treatment were fixed at 0 and 24 hours of maturation and then stained with lacmoid to identify meiotic stage: GV (GV0, GV1, GV2, GV3; Lodde et al., *Mol. Reprod. Dev.* 74, p.740–749, 2007), GVBD, MI, AI, TI, MII and abnormal. The data were analyzed by Chi-square ($P < 0,05$). The results showed that at 0 hour of maturation, a higher percentage ($P < 0,05$) of SF (98.33%; 59/60) were at GV stage than the Con (89.77%; 79/88). At the 24 h of culture, the Con and LF groups did not present any other oocyte in GV, and the LF group presented 93.4% (57/61) of MII. The SF group still had 10% (6/60) oocytes in GV ($P < 0,05$) and only 81.7% (49/60) in MII. Regarding to the degrees of chromatin condensation at GV stage, the SF (20%; 12/60) showed a higher percentage of GV0 than the Con (2.27%; 2/88) group. However, the more competent and the less competent groups did not differ ($P < 0,05$) for the percentage of oocytes in GV1, GV2 and GV3. It can be concluded that SF have the lowest capacity to reach metaphase II, and that the degree of chromatin condensation in GV, when evaluated by lacmoid stain, is not a good parameter to estimate oocytes competence in cattle.



A173 Folliculogenesis, oogenesis, and superovulation

Fibroblast growth factor 2 regulates apoptosis and viscosity of the extracellular matrix in bovine cumulus-oocyte complexes undergoing *in vitro* maturation

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In vitro maturation (IVM) is one of the main obstacles to improve IVF outcomes. There are evidence that cumulus expansion and apoptosis are influenced by in vitro conditions. Fibroblast growth factor 2 (FGF2) expression is upregulated by the LH ovulatory peak in bovine cumulus cells (CC). In bovine granulosa cells, FGF2 activates ERK1-2, a pathway known to regulate cumulus expansion and cell survival. Extracellular matrix (ECM) chemical and physical properties affect adhesive and invasive capacity of the COC and thus fertilization rates. In this study, we assessed whether activation of the ovulatory cascade with FSH increases FGF2 mRNA abundance in CC, and tested the effects of FGF2 on apoptosis, degree of expansion and viscosity of the ECM in bovine COCs undergoing IVM. Groups of 20 COCs (grades 1 and 2) from 3-8 mm follicles of abattoir ovaries were cultured in TCM 199 (0.4% BSA, 1µl/mL FSH, 22µg/ml sodium pyruvate, 75µg/mL ampicillin) supplemented with graded doses of FSH (0, 0.1, 1, 10 and 100 ng/mL; IVM for 12h; n=4/dose) or FGF2 (0, 1, 10 and 100 ng/mL; IVM for 22h; n=4/dose) at 38,5°C in humid atmosphere. FGF2 mRNA was assessed by real time RT-PCR using SybrGreen (LifeTech) and CYC-A as the reference gene, after RNA extraction from cumulus cells with RNEasy (Qiagen). Expansion degree was visually assessed and classified in grades 1 to 3 (1 poor expansion and 3 complete expansion). ECM viscosity was estimated by the number of pipetting movements necessary to completely denude the oocyte. To assess apoptosis, CC were tested for annexin V and caspase 3/7 staining in a flow cytometer (kits APC Annexin V, BD Pharmingen; CellEvent™ caspase 3/7, Thermo Scientific). Effects of treatments were tested by ANOVA and means were compared by the Fisher protected test. Treatment with FSH increased FGF2 mRNA levels in a dose dependent manner (mRNA relative values: 0.92±0.12a, 0.82±0.09a, 1.44±0.64a, 4.44±0.52b, 19.92±5.74c). FGF2 did not alter expansion degree, but increased ECM viscosity at all concentrations tested and there are empirical evidence indicating that cumulus ECM is far more viscous when maturation occurs in vivo in comparison to in vitro. FGF2 increased non-apoptotic cells (annexin V and caspase 3/7 negative; 80.48±2.43%a, 82.11±2.36%ab, 85.14±1.09%ab, 86.08±0.83%b) and reduced caspase 3/7 positive cells (17.64±2.06%a, 15.60±2.66%ab, 12.52±1.39%b, 12.25±1.02%b), suggesting an inhibitory action on the intrinsic pathway of apoptosis. In conclusion, the present data suggest that activation of the ovulatory cascade increases transcription of FGF2 in bovine cumulus cells, which appears important for the control of ECM viscosity and apoptosis in the bovine COC. These data have potential practical implications for IVM and suggest novel actions for FGF2 as a mediator of the ovulatory cascade favoring cumulus cells survival and fertilization.



A174 Folliculogenesis, oogenesis, and superovulation

Lactation influence on antral follicles count (AFC) in Holstein cows reared in the semiarid

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This study was conducted at the Experimental Station of São Bento do Una (ESSBU/IPA) Pernambuco, Brazil (Latitude 08 31' 35'' S and Longitude 036 27' 34.8'' W), with the objective of evaluating the influence of lactation on the diameter and count of ovarian antral follicles (AFC) in Holstein cows submitted to hormonal protocol. Eighty-seven lactating cows aged 36 to 96 months, body condition score 2-3 and between 50 and 120 days in milk (DIM) or non-lactating were used. The cows were kept in a semi-intensive system, receiving a diet composed of cactus pear (*Opuntia ficus-indica* Mill), sorghum silage (*Sorghum bicolor* (L) Moench) and protein concentrate with 28% crude protein along with mineral supplement and water ad libitum. All cows after clinical and gynecological evaluation were distributed in six experimental groups (G1 to G6). In groups G1, G2 and G3 cows were not submitted to hormonal protocol, being G1 (n = 15) composed of non-lactating cows, G2 (n = 15) by lactating cows with average production (15-20 kg / milk / day) and G3 (n = 15) lactating cows with high production (21-35 kg / milk / day). The G4, G5 and G6 groups were submitted to hormonal protocol, with G4 (n = 14) being non-lactating cows, G5 (n = 14) being cows with average production, and G6 cows with high production. The hormonal protocol consisted of an intravaginal device containing 1g of P4 and 2mg of Estradiol Benzoate in D0, 0.530mg of Cloprostenol along with 200 IU of FSH in D4. All females were submitted to ultrasound examination for AFC and measurement of follicular diameter in D6. An ultrasound equipped with a 7.5 MHz microconvex transducer coupled to a transvaginal guidewire was used. The antral follicles of both ovaries were measured obtaining the diameter of the largest and the smallest follicle in each ovary. The data were submitted to ANOVA and to the Tukey test considering the level of 5%. There was no difference ($P > 0.05$) in the diameter of the large follicles, being observed variations from 9.6 ± 0.75 to 11.9 ± 0.87 mm. As for the smaller diameter follicles varied from 3.3 ± 0.99 to 4.7 ± 1.02 mm ($P < 0.05$). In the AFC cows from G4 had an average of 25.8 ± 1.72 follicles, being superior ($P < 0.05$) to the other groups. However, G1 cows had 18.7 ± 1.54 follicles and were also superior ($P < 0.05$) to G2 cows (8.7 ± 1.04), G3 (12.4 ± 1.16), G5 (11.3 ± 1.12) and G6 (10.3 ± 0.93). Therefore, it was concluded that lactation exerted influence on AFC in Holstein cows, even with the use of hormonal protocol.



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Lipids of bovine oocytes recovered from different follicle sizes

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Oocyte development is a complex process involving sequential molecular, biochemical and ultrastructural changes. The aim of the present study was, therefore, to access the amount of cytoplasmic lipid droplets and lipid profile fluctuation in oocytes derived from different follicle sizes and to associate with the oocyte mRNA levels of key lipid metabolism-related genes, and follicular fluid metabolic molecules. Cow ovaries collected from a slaughterhouse were used for the recovery of oocytes and follicular fluid from follicles with ≤ 2 mm, 3-5 mm, 6-8 mm and > 8 mm diameter. The diameters of follicles were carefully determined with caliper device followed by volume monitoring. Only oocytes with homogeneous cytoplasm and with more than three layers of cumulus cells were selected and denuded after trypsin and acidic tyrode's solution treatment. Oocyte lipid content and profiles were accessed by Sudan Black B staining (n=20 to 30 oocytes per group) and MALDI-MS (35 to 65 oocytes per follicle size, n=7 to 13 per group), respectively. Relative abundance of oocytes mRNA transcripts for ACSL3, ELOVL5, ELOVL6, SREBP and LXR α genes were determined by quantitative realtime PCR (n=4, total of 60 oocytes per follicle size). The patterns of follicular fluid molecules were investigated through commercial biochemical assays. The data were analyzed by ANOVA using the PROC GLIMMIX of SAS and MetaboAnalyst 3.0. Oocytes recovered from follicles > 8 mm had higher cytoplasmic lipid content when compared with the oocytes from other follicle sizes (6.0 ± 0.4 ; $P < 0.05$). The other groups showed no difference in lipid content (≤ 2 mm: 4.8 ± 0.4 ; 3-5 mm: 4.1 ± 0.3 e 6-8 mm: 4.2 ± 0.4 ; $P > 0.05$). The mRNA abundance of ELOVL6 was reduced ($P = 0.02$) in the oocytes recovered from 6-8 mm (5.3x) and > 8 mm (5.6x) follicles compared with the ≤ 2 mm group. The mRNA levels of ACSL3, ELOVL5, SREBP and LXR α were similar ($P > 0.05$) among groups. Follicular fluid of large follicles (6-8 mm and/or > 8 mm) presented higher ($P < 0.05$) levels of glucose, cholesterol, reactive oxygen species, glutathione and superoxide dismutase activity compared with small follicles (≤ 2 mm and/or 3-5 mm). Triglyceride concentration has reduced ($P < 0.05$) in large follicles (6-8 mm and > 8 mm) compared with small follicles (≤ 2 mm and 3-5 mm). Additionally, oocytes recovered from different follicle sizes have presented fluctuations of membrane lipids such as phosphatidylcholines and sphingomyelins. Therefore, the findings of this study reveal the following: i) oocytes recovered from follicles greater than 8 mm have higher lipid content; ii) the oocyte phospholipid membrane profiles varies among different follicle sizes; iii) the mRNA level of ELOVL6 in oocytes recovered from large follicles suggests lower elongation reaction rate of fatty acids containing 12 to 16 carbons; iv) follicular fluid triglyceride, cholesterol, and glucose concentrations may be influencing oocyte lipid content and profiles. Acknowledgements: CNPq and FAPESP.



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Increased expression of Mitofusin 2 in relation to Mitofusin 1 in oocytes impairs folliculogenesis in mice

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Mitochondria are highly dynamic organelles that change their morphology by undergoing fusion and fission processes. We previously addressed the role of mitochondrial fusion in oocytes by conditional knockout of Mfn1 (M1), Mfn2 (M2) and Mfn1+Mfn2 (DM) based on Zp3-driven expression of Cre recombinase. As a result, M1 and DM females were infertile, whereas M2 females were subfertile. Compared to the M1 group where infertility was caused by defective folliculogenesis, DM females were infertile due to ovulation of oocytes that arrested meiotic progression. These results suggested that ablation of Mfn2 in DM oocytes partially rescued the effect of Mfn1 deficiency. To further investigate this, using 5-week-old mice injected with eCG, we evaluated oocyte ultrastructure, ATP and FADH₂ level as well as gene expression in oocytes and ovaries. The number of follicular cells was determined using ovaries from 8-week-old females injected with BrdU. Data were evaluated by ANOVA followed by Duncan test (P<0.05), with at least biological triplicates. DM ovaries expressed normal levels of follicular markers that were downregulated (*Inhba*, *Inhbb*, *Fst* and *Fshr*) or upregulated (*Kitl* and *Lhcgr*) in M1 ovaries. In addition, these genes were not altered in M2 ovaries, except for *Fst* and *Fshr* that were increased. A similar pattern was found in oocytes since *Bmp15* and *Fgf8b* were downregulated in the M1 group, but remained unchanged in the WT, M2 and DM. These findings are in accordance with our previous results, suggesting the lack of Mfn2 enabled folliculogenesis in the DM group. In keeping with this, the number of follicular cells did not differ between the WT and DM groups, but it was decreased in the M1 and increased in the M2. The number of replicating cells was also decreased in M1 follicles, but unchanged among WT, M2 and DM. Ultrastructural analyses revealed that DM and M2 oocytes contained fewer mitochondria which were swollen in comparison to WT and M1 oocytes. Moreover, ultrastructure characteristics of mitochondria (i.e. increased number of inner membrane vesicles) were more often altered in the M1 and DM groups. Mitochondrial dysfunction in the DM group became more apparent after ovulation, as indicated by enhanced mitochondrial aggregation, increased amounts of FADH₂ and decreased levels of ATP and ATP/ADP. These defects possibly led to defective meiotic progression of DM oocytes. Therefore, ablation of Mfn2 in DM oocytes could have enabled follicular development through inducing replication of follicular cells. As a result, this might have compensated for the mitochondrial defect in DM oocytes until ovulation when the oocyte loses interaction with cumulus cells. This suggests an important role of Mfn1/Mfn2 ratio to oocyte development. Considering that Mfn1, but not Mfn2, were downregulated in oocytes from aged females, Mfn1/Mfn2 imbalance might underpin part of the effects of aging on oocyte competence.

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Mutant mitochondrial DNA is selectively eliminated in the mouse oocyte by mitophagy

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Several lethal and cureless diseases are caused by mutations in the mitochondrial DNA (mtDNA). However, since wild-type mtDNA molecules are preferentially transmitted to the next generation, understanding the underlying mechanism might enable manipulation of mitochondrial inheritance. Recently, we conditionally (driven by Zp3-Cre) knocked out mitofusins 1 (Mfn1) and 2 (Mfn2) in heteroplasmic oocytes (C57BL/6 (B6) and NZB/BINJ (NZB) mtDNA) to evaluate the effect of mitochondrial fusion on mtDNA inheritance. NZB mtDNA differs from B6 mtDNA in 91 nucleotides, which are known to affect mitochondrial function. We found that Mfn1-null oocytes did not differ (-10.3 ± 1.58) from wild-type (WT) oocytes (-10.1 ± 1.25) regarding the Δ NZB (difference between the percentage of NZB mtDNA in the oocyte or progeny and that present in the mother). However, the Δ NZB was mitigated in Mfn2-null (-3.0 ± 1.45) and Mfn1-null+Mfn2-null (DM-null) (-4.4 ± 1.21) oocytes. This indicates that during oocyte development B6 mtDNA is preferentially inherited by a mechanism relying on Mfn2 expression. Considering that Mfn2 seems to play a key role during mitophagy, this work aimed at associating the higher levels of NZB mtDNA in Mfn2-null oocytes with a possible defect on the autophagic flux. In this regard, we used heteroplasmic B6/NZB mice and estimated the effect on mitochondrial inheritance by quantitating the Δ NZB and the occurrence of autophagosomes in the oocyte. Data were analyzed by ANOVA followed by T test or comparison of means by Duncan. Differences with $P < 0.05$ were considered significant. First, we confirmed that the conditional knockout of Mfn2 in the oocyte resulted at weaning in pups with higher levels of NZB mtDNA (Δ NZB – WT = 8.1 ± 1.07 vs. Mfn2-null = 2.4 ± 1.77). In addition, both the level of NZB mtDNA in spleen (Δ NZB – WT = -22.4 ± 2.47 vs. Mfn2-null = -13.4 ± 3.51) and liver (Δ NZB – WT = 9.3 ± 3.06 vs. Mfn2-null = 19.9 ± 2.77) were increased in pups that were 100 days old. These tissues, respectively, eliminate and accumulate NZB mtDNA with aging, giving evidence that the lack of Mfn2 in the oocyte impacted on mitochondrial inheritance. Next, we investigated whether the underlying mechanism by which NZB mtDNA is eliminated was dependent on autophagy. To this end, heteroplasmic oocytes were *in vitro* matured in the presence (CQ) or absence (CT) of 100 μ M of chloroquine, an autophagic inhibitor. The higher amounts of autophagosomes in CQ oocytes, as indicated by anti-Lc3b immunofluorescence (CQ = 52.0 ± 2.51 vs. CT = 42.1 ± 5.11), suggested the treatment interfered with the autophagic flux. As a result, the treatment with chloroquine reduced NZB mtDNA elimination by $\sim 70\%$, agreeing with our results from Mfn2-null oocytes. In conclusion, these results provide evidence that mitochondrial inheritance is regulated in the oocyte by mitophagic elimination of mutant mtDNA.



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Ovarian response to hormonal stimulation treatment in collared Peccary (*Pecari tajacu* Linnaeus, 1758)

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The *Pecari tajacu* species, commonly known as collared peccary, has aroused commercial interest. The study of the animal reproductive physiology is necessary in order to assist captive management and biodiversity conservation. This study aimed to compare two different hormonal treatments in collared peccary females. The animals (n=4) were provided by Wild Animals Multiplication Center (CEMAS) located at Federal Rural University of Semi-Arid (UFERSA) and were subjected to two ovarian stimulation protocols with PG600[®] (eCG and hCG, MSD Saúde Animal, São Paulo, Brasil). The hormone was administered by single intramuscular injection and the treatments consisted of 5mL as recommended for swine ovarian stimulation (SD, Swine Doses) or 1.2mL as determined by allometric calculation (AD, Allometric Doses). Five days after hormone administration, ovaries were recovered and visualized follicles were classified as (S) small (<0.3cm), (M) medium (0.3-0.5cm) or (L) large (>0.5cm). The number of corpora lutea (CL) in each ovary was registered. Biopsies of follicles were achieved with Castroviejo scissors, weighted and stored in liquid nitrogen for downstream applications. Data were presented as mean \pm s.e.m and compared by Student t test at $P < 0.05$. The follicle numbers for each group (SD and AD, respectively) were 6.00 ± 0.71 and 12.00 ± 2.83 for S, 8.00 ± 0.71 and 8.00 ± 0.00 for M and 10.00 ± 1.41 and 4.50 ± 1.77 for L follicles. Despite numerically different, the number of S, M and L follicles were statistically similar ($P > 0.05$) between SD and AD groups. The biopsy weights of SD animals were 5.44 ± 1.71 mg for S, 11.92 ± 2.67 mg for M and 17.53 ± 2.92 mg for L follicles. While for AD group, the follicular biopsies weighted 2.09 ± 0.28 mg for S, 2.41 ± 0.36 mg for M and 2.69 ± 0.59 mg for L follicles. Thus, the mass of follicular wall increased significantly ($P < 0.05$) from S to M and from S to L follicles in DS, but not in AD group. Consequently, DS treatment yielded a greater amount ($p < 0.05$) of follicular tissue sample from L follicles than AD (378 ± 53.49 vs 28.4 ± 3.81 mg, respectively). Finally, both treatment produced similar ($P > 0.05$) ovulation rates (accessed by CL number) in females. Thus DS produced 2.50 ± 0.35 CL/animal and AD produced 3.00 ± 0.71 CL/animal. Additionally, a female of AD treatment presented two structures macroscopically classified as luteinized follicles. In conclusion, both SD and AD treatments produced similar ovarian stimulations (denoted by the number of follicles) and ovulation rates in collared peccary females. On the other hand, AD treatment generated luteinized follicles, which might indicate that the dosage is not completely optimal to induce ovulation. Finally, the follicular wall mass seems to increase with follicular size after SD, but not after AD treatment. Additional investigations about oocyte quality or molecular profiles of ovarian follicles can elucidate reproductive physiology of collared peccary.



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Embryonary recovery rates after the induction of ovulation using human chorionic gonadotropin (HCG) in mares with different follicular diameters

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Human chorionic gonadotropin is used as an ovulation inducer (OI) in mares and does not affect the embryo recovery rate. This hormone is important in embryo transfer programs in order to obtain an effective synchronization degree of ovulations between the donor and the recipient to obtain improved gestation rates. (JACOB, et al., 2011. A Hora Veterinária, v. 180, p. 9-13). However, it is unknown whether there is an influence in the embryo recovery rate after the induction of ovulation in follicles (FL) with diameters < 35mm. Observing this fact, the aim of this study was to evaluate the embryo recovery rate after the induction of ovulation with hCG in mares with follicles with different diameters. Estrous cycles (n=101) from embryo donor mares (n=40; with ages ranging from 3 to 20 years) were used in this study, between the 2015/2017 season in the State of Rio de Janeiro, Brazil. The estrous cycles were divided into two groups where: GI: control group (35 cycles), with spontaneous ovulation; and GII: hCG group (64 cycles), with ovulation induced using 1000 UI, iv., of hCG (Chorulon® - MSD Saúde Animal, Brasil), subdivided by follicular class: Follicles with diameter $\geq 32\text{mm} < 35\text{mm}$; FL $\geq 35\text{mm} \leq 37\text{mm}$; FL $> 37\text{mm}$ to 41.5mm. Only mares that had single ovulations were included in the study. The follicular diameters were accompanied in intercalated days using transrectal ultrasonography until the observation of a follicle ≥ 28 mm of diameter, when the ultrasonography was realized daily until the ovulation. The mares were inseminated with fresh semen as close as possible of the ovulation for the GI. For the GII the insemination occurred 24h after the induction of the ovulation with fresh semen. The embryos were collected 8-10 days (D8-D10) after the ovulation, using the transcervical method. The exact Fischer test was used for the statistical comparison of all data. The positive (CP) and negative (CN) embryo recovery rate of the GI was: CP 48.57% (17/35) and CN 51.42%(18/35) to follicles which had a variation in diameter of 37.5 - 52mm. The obtained results from GII was: CP= 46.96% (31/66) and CN= 51.42% (35/66). The GII was divided by class related to the follicle diameter: FL $\geq 32 < 35\text{mm}$ = CP 40% (10/25) and CN 60% (15/25); FL $\geq 35 \leq 37$ = CP 54.16% (13/24) and CN 45.83% (11/24); FL $> 37\text{mm}$ = CP 47.05% (8/17) and CN; 52.94% (9/17). There was no statistical difference between the embryo recovery rate in follicles induced with hCG and the control group (P=0.6), and also with the different diameters in follicles induced with hCG (P=0.09). We conclude that: 1) the embryo recovery rates did not differ between mares that spontaneously ovulated or had the ovulation induced with 1000UI of hCG; 2) there is no difference between the embryo recovery rates after ovulation induction of follicles with different diameters.