Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

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

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The aim of this study was to investigate the efficacy of tissue fixatives Bouin, Methacarn and Formaldehyd 10% in bovine ovaries. Pairs of ovaries from Nelore heifers (n = 5) were obtained from a slaughterhouse 30 km away from the Laboratory Animal of Reproduction - UEL, Londrina, Paraná. Immediately after collection, the ovaries were washed with a modified PBS solution (Cultilab®, Campinas-SP, Brazil), and sectioned with a 6 mm dermatological punch, resulting in 9 fragments approximately 3x3x1 mm removed from the cortical region of each ovary. Then, the ovarian fragments were immersed in one of the following fixatives: Bouin (B) or Formaldehyd 10% (F) for 6, 12 or 24 hours, or Methacarn (M) for 1, 2 or 4 hours, comprising 9 experimental groups. The determination of the fixation period of 6, 12 and 24 hours for Bouin and Formaldehyd 10%; 1, 2 and 4 hours for Methacarn was defined according to the manufacturer's instructions. Each fragment was placed individually in flasks containing 20 times the volume of the fixing solution. After this period, the fragments were kept in 70% alcohol until processing. For each fixative and time, five replicates were made. For histological processing, the fragments were dehydrated in increasing concentrations of alcohol, diaphanized in xylol and included in paraffin. Then, 5 µm serial sections were made in a rotating microtome (Leica®, Wetzlar-Germany), followed by the slide assembly and staining with periodic acid from Schiff (PAS) and Hematoxylin. A total of 135 slides with 810 histological sections were evaluated. The preantral follicles were classified as intact or degenerate. Degeneration was considered by the presence of at least one of the following aspects: retraction of the cytoplasm, pycnotic nucleus, cytoplasmic vacuoles, displacement of the granulosa cells and rupture of the basement membrane. For statistical analysis, PROC GLM was used followed by the Tukey test ($p < 0.05$). The Bouin fixative provided the best ($p<0.0001$) conditions of morphological integrity 84.5%^{a} (355/420) in comparison to the others, Methacarn 65.7%^{b} (276/420) and 15.5%^{c} (70/450). For integrity of primordial and primary follicles, the best results were also achieved with Bouin in relation to the other fixativesrespectively 53.3%^{a} (222/420); Methacarn 25.61%^{b} (106/420) and Formaldehyd 7.77%^{c} (35/450) for primordial; Bouin 26.75%^{a} (114/420); Methacarn 36.69%^{a} (153/420) and Formaldehyd 5.33%^{b} (24/450) for primary ($p < 0.0001$). However, in relation to the integrity of the secondary follicles, there was no difference ($p > 0.1$) among the fixatives. Regarding the fixation times for all tested fixatives, there was also no significant difference. In conclusion, Bouin proved to be the most effective fixative in the morphological preservation of preantral follicles in bovine ovarian tissue. In addition, the times tested in this study showed similar results, regardless of the fixative employed.

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Folliculogenesis, oogenesis, and superovulation

Evaluation of foetal bovine ovarian cortex fragments submitted to xenotransplantation*

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Ovarian tissue xenotransplantation may be a method to promote the development of ovarian follicles frozen of women undergoing chemotherapeutic. In cattle, there are reports of follicular development to the antral phase when xenotransplanted in mice, obtaining embryos of five to eight cells. Ovarian foetal tissue xenotransplantation, in case of death of pregnant cow in late gestation, may be useful since it has suffered less environmental interference, has a greater reserve of primordial follicles and could be used as a tool to decrease the generation interval. The aim was to evaluate oxidative stress and levels of cell degeneration and apoptosis in foetal bovine ovarian tissue fragments submitted to xenotransplantation. Ten pairs of foetal bovine ovaries collected in slaughterhouse were fragmented (3x3x1 mm) and divided in 3 groups: fresh control (FC) and xenotransplanted in 20 Balb C line female mice, recovered after 7 (X7) or 14 days (X14). The fragments from all groups were prepared for confocal analysis. Fragments were washed (PBS with 5% fetal bovine serum), in dark room, in solution with 1µl/ml H2DCFDA (DCF) (30`), 10µM/ml YO PRO 01 (YOPRO) (30`) and 100µg/ml propidium iodide (PI) (15`) for reactive oxygen species (ROS) emission analysis, amount of cells in apoptosis and degenerate cells, respectively. Fluorescence intensity at different depths was performed with LSM 710 ZEISS® microscope according to excitation ranges of each probe: DCF 495/519 nm; YOPRO 491/509 nm; IP 543/616 nm. Images were analyzed in the Zen 2008 program. Statistical analyses were performed using Sigma Plot 11.0 with F test in 5% significance. Means ± standard error followed by different letters attest to significant difference. Fragments submitted to xenotransplant, regardless of being X7 or X14, presented lower levels of cellular degeneration (1.25 ± 0.10b and 1.02 ± 0.06b)and ROS (1.78 ± 0.13b and 3.25 ± 0.49b), compared to FC (4.5 ± 0.26a and5.86 ± 0.36a, respectively). They had also lower levels of apoptosis, but, those recovered after 7 days had the lowest rate (X7 = 1.17 ± 0.03c, X14 = 2.28 ± 0.21b, FC = 3.29 ± 0.10a). In conclusion, foetal bovine ovarian fragments xenotransplantated had satisfactory tissue viability, given lower levels of degeneration, ROS and apoptosis than in fresh tissue. This studywasfinanced in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – FinanceCode 001; the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG, and Conselho Nacional de Pesquisa (CnPQ).

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Do sheep breed effect influence superovulatory responses and embryos yields?

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The aim was evaluated the breed effect on superovulatory responses and embryo yields in Brazilian native ewes submitted to a mid-term progesterone-based protocol and gonadotrophic treatment with 133 mg of pFSH. The study was conducted in the northeast of Brazil in ewes of three breeds: Morada Nova (MN; n=10); Santa Inês (SI; n=10) and Somalis (SO; n=10). On random day of estrous cycle (Day 0) all ewes received an intravaginal device of progesterone (0,3g of P4, CIDR®, Zoetis, Brazil) for nine days. On Day 7 started the pFSH (133mg, Folltropin V®, Vetoquinol, Brazil) treatment in six decreasing doses (25-25-15-15-10-10%) administered i.m. at 12h intervals. On Day 9 were injected two equal doses of d-cloprostenol (37.5 µg, Prolise®, AgenerUnião, Brazil) at 12h interval. All ewes showed estrus and were mated by fertile rams. To prevent corpora lutea (CL) regression, ewes received three administration i.m. of flunixin- meglumine (24.9mg; Banamine®, MSD, São Paulo, Brazil) on Days 12, 13 and 15. On Day 16 was performed non-surgical embryo recovery (NSER) after cervical dilation using d-cloprostenol and estradiol benzoate at 16h and ocytocin at 20 min before. Transrectal 7.5 MHz B-mode ultrasound (Z5 Vet®, Mindray, China) were performed at 36h after P4 device removal (Day 11) and at 12h before of the NSER (Day 15) to see the ovarian population. Data was analyzed using ANOVA followed by Tukey test ($p<0.05$). The number of medium follicle (4.0-5.9mm) on Day 11 was lesser ($P=0.002$) in MN (3.90±0.77) than SI (10.00±1.26) and SO (8.80±1.44). However, the other variables on Day 11: number of total antral follicles (MN=18.70±2.23, SI=31.30±5.49 and SO=25.10 ± 2.88), small follicles (≤ 3.9mm) (MN=13.90±1.87, SI=20.20±5.18 and SO=16.20±1.70) and large follicles (≥ 6.00mm) (MN=0.90±0.58, SI=1.10±0.48 and SO=0.10±0.10) did not differ among breeds. The number of CL on Day 15 was greater ($P<0.0001$) in SI (15.30±1.40) compared to MN (7.83±2.23) and SO (10.11±0.84). And, the variables on Day 15: numbers of recovered structures (MN=7.60±2.25, SI=13.33±5.33 and SO=6.13±1.27) and viable embryos (MN=6.60±2.71, SI=10.78±5.14 and SO=5.25±1.44) did not showed difference. In conclusion, the number of medium follicles on Day 11 and the number of Cls showed to influence of the breed effect; however, the viable embryos not altered among breeds. Financial support: Embrapa (02.13.06.026.00.02; 02.13.06.026.00.04); FAPEMIG (PPM 00201-17).

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Effect of quercetin addition on the in situ culture system of Bosindicus ovarian fragments on agarose gel support

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This study evaluated the effect of adding quercetin to the *insitu* culture medium on agarose gel support from *Bosindicus* ovarian fragments. Six pairs of ovaries (n = 12) of Nelore heifers obtained from a slaughterhouse located 30 km from the Animal Reproduction Laboratory - UEL, Londrina, PR were used. In the slaughterhouse, each pair of ovaries was washed in saline solution, divided into 9 fragments of approximately 3x3x1 mm and 1 fragment of each animal was fixed in Bouin for 24 hours, and the rest (n = 8) were transported to the laboratory at a temperature of 4ºC in minimal essential medium (MEM) supplemented with 100 mg/mL of penicillin and 100 mg/mL of streptomycin.The fragments from the same ovary were distributed one for each concentration of 0 (MEM), 10 (Q10), 25 (Q25) or 50 µg/mL (Q50) of quercetin added to the MEM supplemented with ITS, pyruvate, glutamine, hypoxanthine, BSA, penicillin and streptomycin for 5 or 10 days of culture. The medium was changed two days apart. The fragments were processed by classical histology at the end of each culture. For follicular analysis, the slides were stained with periodic acid from Schiff (PAS) and Hematoxylin.The preantral follicles were classified as primordial or in development (primary and secondary), and according to integrity (intact and degenerate), considering 30 follicles per fragment.For statistical analysis, the Chi-square test was used (P<0.05).After 5 days of culture, the highest percentage of intact preantral follicles (P=0.04) was found with the addition of 25 µg/mL of quercetin 67.3%^{a} (105/156), in comparison a uncultivated control 52.9%^{b} (92/174), MEM 52.0%^{b} (79/152) and Q50 53.8%^{b} (64/119), but similar to the Q10 57.0%^{ab} (69/121).The best results with 10 days of culture were Q25 57.1%^{a} (76/133) and MEM 60.8%^{a} (93/153) compared to Q10 39.1%^{c} (54/138) and Q50 43.4%^{bc} (75/169), P=0.001.Both in the 5 or 10 days of culture there was a higher proportion of developing follicles (P<0.05) in relation to the primordial, regardless of the evaluated treatment.The highest proportion of developing follicles 94.3%^{a} (P<0.0001) was found at Q25, when comparing uncultured control 73.9%^{b}, MEM 73.4%^{b}, Q10 81.2%^{b} and Q50 71.9%^{b} at 5 days of culture.However, with 10 days of cultivation, all treatments were similar in relation to this proportion (P>0.1). It was concluded that supplementation of 25 µg/mL of quercetin was the best concentration to maintain the integrity and development of the preantral follicles culture for 5 days. Keywords: *in vitro* culture, antioxidant, cattle, folliculogenesis.

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Correlations between biometrics parameteres and vascularization of corpora lutea and serum progesterone concentration in superovulated sheep

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In vivo embryo production performs an important role in small ruminants, however the efficiency of this biotechnology has high variability. In this context, the occurrence of luteal dysfunction is exacerbated in superovulated females (Rodriguez et al., 2015) and it is an unwanted response wich may be related to low embryo production. Thus, it is possible to highlight the need to expand knowledge related to the corpora lutea functionality, as well as determinative tools for ultrasound diagnosis. The aim of this study was to test correlations between biometric variables, luteal vascularization and serum progesterone concentrations. Twenty-seven Santa Inês ewes were submitted to estrus synchronization protocol based on the use of progesterone device (0.3 g of P4, Eazi-breed CIDR®, Zoetis, Brazil) for eight days and two equal doses of d-cloprostenol (37.5 µg, Prolise®, AgenerUnião, Brazil) i.m. on Days 0 and 8. Superovulatory treatment started on Day 6, when the females were randomly divided into three groups according to the total exogenous FSH dose consisted of 100, 133 or 200 mg (Folltropin V®, Vetoquinol, Brazil) in eight decreasing doses every 12 hours (Day 6 to Day 9). On Day 6, the females received 200 IU eCG i.m. (Novormon®, Zoetis, Brazil). The females were subjected to natural breeding. Ovarian ultrasound evaluations (B-mode and Color Doppler) were performed daily during the early luteal phase, between Days 11 (beginning of luteogenesis) and 15 (corresponding to videolaparoscopy and surgical embryo recovery). From these evaluations, the diameter, area and volume of the corpora lutea were determined, as well as the percentage and volume of luteal vascularization. Blood samples were collected at all times of ultrasonographic evaluation for subsequent measurement of serum progesterone concentrations by the radioimmunoassay technique. Biometric variables, luteal vascularization and serum progesterone concentrations were correlated by Pearson's correlation test ($p < 0.05$). The animals treated with 100, 133 and 200 mg of FSH showed no difference regarding to the total amount of corpora lutea (classified as normal or regressed) in the ovaries ($p = 0.0822$, 0.7092 and 0.2252, respectively). Positive correlations ($p < 0.0001$) were observed between serum progesterone concentrations and luteal diameter ($r = 0.24$), luteal area ($r = 0.24$), luteal volume ($r = 0.24$) and vascularization volume ($r = 0.18$) in all treatments. However, there was no correlation between serum progesterone concentrations and the percentage of vascularization. In conclusion, luteal vascularization is an indicative of corpus luteum functionality in superovulated ewes.

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Is the ovarian vascularization correlated with superovulatory response and *in vivo* embryo production in native brazilian sheep?

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The use of color Doppler ultrasonography in multiple ovulation and embryo transfer (MOET) protocols for sheep allows the real-time monitoring of ovarian blood flow and potentially can be a tool to predict embryo production in sheep. Therefore, the present study tested the correlation of ovarian vascularization at 36 h after the end of the superovulation treatment and at 12h (Day 15) before the non-surgical embryo recovery (NSER) (Day 16) with estrus response (ER%), duration of estrus (DE), number of corpus luteum (CL), number of anovulatory follicles (AF), number of structures recovered (SR), number of viable embryos (VE; Grade 1-3). A total of 40 ewes (Morada Nova and Santa Inês ewes, *n* = 20/breed) received a new (G-new, *n* = 20) or reused (G-reused, *n* = 20) intravaginal progesterone device (Eazi-breed CIDR®, Zoetis, New Zealand) for 9 days. At 60 h before CIDR® removal, the pFSH (133mg, FolltropinV®, Vetoquinol, Brazil) treatment was initiated in six decreasing doses (25-25-15-15-10-10%) i.m. administrated 12 h apart. On day of CIDR® removal two equal doses of d-cloprostenol (37.5µg, Prolise®, AgenerUnião, Brazil) were applied 12 h apart. On Days 12 to 15 three equal doses of flunixinmeglumine (6.75 mg; Banamine®, MSD, Brazil) were i.m. injected. All ewes showed estrus and and were mated with fertile rams. Color Doppler and B-mode transrectal ultrasounds (Z5Vet®, Mindray, China) were performed at 36 h after CIDR® removal and 12 h before NSER.Females that had more than four corpus luteum bodies were administered d-cloprostenol (37.5 µg) and estradiol benzoate i.m. (1 mg, Estrogin®; Biofarm, SP, Brazil) at 8 pm the previous day and oxytocin i.v. (50 IU Oxytocin forte®; UCB, SP, Brazil) 20 minutes before NSER. Pearson's correlation was performed between ovarian vascularization and variables of estrus, superovulatory response and embryo yields ($P<0.05$). The ovarian vascularization at 36 h did not correlate ($P>0.05$) with any variable, whereas the ovarian vascularization at 12h was positively correlated ($P<0.05$) with CL ($r = 0.41$), ER ($r = 0.43$) and VE ($r = 0.44$). In conclusion, the ovarian vascularization in the early luteal phase has positive correlation with superovulatory response and embryo yields, whereas at the preovulatory period it does not correlate with any evaluated variable. Financial support: Embrapa (02.13.06.026.00.02/ 02.13.06.026.00.04), FAPEMIG (PPM 00201-17), Capes (88882.344029/2019-01).

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Cervical relaxation protocol in sheep decreases Oct-4 and Nanog gene expression temporarily

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Hormonal protocols aiming at dilation of the sheep cervix were developed to allow access to the uterus. Despite the proven efficacy of these protocols, little is known about these drugs used on embryonic quality. Thus, this study evaluated the influence of the hormonal protocol of cervical dilation (based on estradiol benzoate, d-cloprostenol and oxytocin) on the expression profile of genes related to incidence of apoptosis (Bax and Bcl-2), stress (Hsp90 and PRDX1) and pluripotency maintenance (Oct-4 and NANOG). For this, two experiments were conducted. In experiment one, 40 Santa Ines ewes were submitted to the superovulation protocol followed by natural mating (Pinto et al., Theriogenology, 113:146-52, 2018). The animals were divided into two groups: i) treated group (n = 20) – consisting of estradiol benzoate (100 µg, i.v.) and d-cloprostenol (0.12 mg; i.m.) 12 h before embryo collection associated with oxytocin (100 IU, i.v.) 15 min before collection procedure; ii) control (n = 20) – the animals received saline solution to replace the hormones used at the same intervals and quantities. All embryos were collected by laparotomy and classified according to the IETS. Three pools of five blastocysts (grade I or II) per group were used for total RNA extraction with RNeasy Micro Kit (Quiagen, Valencia, USA) and SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, USA) for reverse transcription. Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software. For normalization of results, GAPDH, UXT and H2AZF reference genes were used. In experiment two the same design was used and the embryos retrieved from each group (treated = 22; control = 21) were cultured *in vitro* for 24 h in synthetic oviduct fluid (SOF) medium with 10% fetal bovine serum, in humidified atmosphere of 5% CO_2 in air at 38.5 ºC. After the cultivation period, the embryos were frozen for gene expression analysis. The expression of BAX, Bcl-2, PRDX1 and HSP90 transcripts was similar ($P > 0.05$) in both groups. However, a smaller ($P < 0.05$) number of NANOG and OCT4 transcripts was found in embryos of the treated group. Nevertheless, when these embryos were cultured *in vitro* for 24 h, this difference did not persist ($P > 0.05$). In conclusion, the cervical relaxation protocol based on the administration of estradiol benzoate, d-cloprostenol and oxytocin may temporarily alter the expression of NANOG and OCT4 genes in surgically recovered sheep embryos.

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Folliculogenesis, oogenesis, and superovulation

Effect of the GnRH antagonist on the sheep follicular population

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The present study evaluated the ovarian follicular population dynamics followed treatment with different doses of the GnRH antagonist in sheep. A total of 18 ewes were submitted to short-term estrus synchronization protocol (OLIVEIRA et al., Proceedings of the CBRA, 2009). On the seventh day after ovulation of synchronized estrus, females were randomly divided into one of three groups (n=8/group) according to the dose of the GnRH antagonist (Firmagon®; LaboratóriosFerring Ltda) subcutaneously administered: Control (placebo treatment); Lower dose: 215µg/kg; and Higher dose: 235µg/kg of body weight. B-mode ultrasound exams of the ovaries were conducted daily from one day before treatment with GnRH antagonist until the females showed estrous behavior. Ultrasound (MyLab Vet®, Esaote) coupled to a transrectal linear transducer with a frequency of 6 and 8 MHz was used. Data were compared by ANOVA using Tukey's as post hoc test. There was no interaction (P>0.05) between treatment or days after treatment on ovarian follicle population. The number of small follicles (2 - 3.49 mm) was greater (P=0.0002) in the Lower dose (5.4±0.4) compared to the Control (4.1±0.3) and Higher dose (3.5±0.2). The number of large follicles (≥4.5 mm) was lower (P=0.01) in the Higher dose (0.2±0.0) compared to the Control (0.5±0.1) and Lower dose (0.4±0.1). The number of medium follicles (3.5-4.49 mm) and the average diameter of the follicles in the three categories of diameter did not differ (P>0.05) between groups. The number of medium follicles differed (P=0.01) between Days 8 and 15 after synchronized estrus. The number of large follicles on Day 6 differed (P=0.0002) of Days 8, 9, 10, 11, 12, 16 and 17. The average diameter of medium follicles differed (P=0.01) between Days 8 and 10. The number of small follicles and the average diameter of small and large follicles did not differ (P>0.05) among days. In conclusion, administration of the 235µg/kg of GnRH antagonist suppresses the development of large antral follicles, while the administration of 215µg/kg increases the small follicles population in sheep. Financial support: CNPq (Process nº 422597 / 2016-3). Keywords: dominant follicle, follicular wave synchronization, ultrasound, ewe.

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ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

Luteal diameter and P4 concentration in mares synchronized with intravaginal progesterone implant (1.9g) and eCG

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Studies demostrated that a larger ovulatory follicle tends to produce a larger corpus luteum (CL) and greater progesterone (P4) production, favoring successful pregnancy. The objective of this work was to evaluate the luteal diameter and the amount of P4 produced in mares submitted to an estrus synchronization protocol based on P4 intravaginal implant, associated or not with eCG. In the experiment, 15 mares, crossbred, were evaluated in the transition season (September/2018) and in the breeding season (January/2019). The animals were between 4 and 8 years old and they had a body condition score between 2.5 and 3.5 (1 to 5). The experiment was carried out in the city of Esmeraldas, MG (CEUAUFMG 57/2018). The same hormonal protocol was performed in the transition season (ET) and later repeated in the same mares in the reproductive period (ER). Day 0 of the protocol consisted of the application of 4 mg of estradiol benzoate (4 mL, IM; Gonadiol®, Zoetis), 5 mg of Dinoprost (1mL, IM; Lutalyse®, Zoetis) and insertion of the intravaginal P4 implant (1.9 mg; CIDR®,Zoetis). The P4 implant remained for 10 days. On the tenth day, the implant was removed and 5 mg (1mL) of Dinoprost was administered. Additionally, the animals were randomly assigned to receive 400 IU of equine chorionic gonadotropin (eCG; 2ml, im; Novormon®, Zoetis; Ecg Group; n = 8) or 2 mL of saline (Control group;n=7). After removal of the implant, the mares were evaluated twice a day, to perform ovarian ultrasound monitoring every 12 hours, until the time when it was possible to see a 35 mm pre-ovulatory follicle in the ovarian ultrasound exam, where in this moment, human chorionic Gonadotropin (hCG; 1,750 IU, 0.7 mL, IV; Vetecor®, Ceva) was applied. At 9 days after ovulation was detected, measurements of the luteal diameter were performed by ultrasound and taken from blood samples for P4 measurement by chemiluminescence. The parameters (means ± standard deviation) of the two reproductive periods were analyzed by the variance test and the F test, using the software R 3.6.1, considering 5% of significance. No interaction was observed between treatment and season for luteal diameter (P=0.6212) or for P4 measurement (P=0.7784). There was no treatment effect (control=29.10±1.28 mm;eCG=29.90±1.13 mm;P=0.7250) or season (ER:28.40±1.09mm; ET: 30.60±1.31 mm; P=0.2084) for the 9-day CL diameter. There was also no treatment effect (Control:6.31±0.67ng/mL; eCG 6.09±0.61ng/mL; P=0.8077) or season (ER:7.06±0.59ng/ml; ET:5.33±0.69ng/mL; P=0.0894) for serum P4 measurement. Considering the luteal evaluation performed at 9 days after ovulation, it was concluded that the association of eCG with the protocol containing P4 intravaginal implant (1.9g) did not influence the CL size or the serum P4 concentration. However, regardless of the use of eCG protocol used allowed the performance luteal satisfactory in mares that were in the transition station, showing to be similar to the mares of the breeding season.

Animal Reproduction

ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

Ovulatory parameters of mares synchronized with intravaginal implant of progesterone (1,9 g) and ecg during the transition season and the breeding season

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Although eCG is used in superovulation protocols in horses, few studies have investigated the use of this associated with progesterone (P4) in the ovulatory response of follicles >35 mm in mares. This factor may become interesting especially when used in the seasonal transition period. In the experiment, 15 mares, crossbred, were evaluated in the transition season (ET; September/2018) and in the breeding season (ER; January/2019), so that 30 estrous cycles were evaluated per animal. The animals were between 4 and 8 years old and they had a body condition score between 2.5 and 3.5 (1 to 5). The experiment was carried out in the city of Esmeraldas, MG (Protocols CEUA UFMG 57/2018). The same hormonal protocol was used in ET and subsequently repeated in the same mares in the ER . Day 0 (D0) of the protocol consisted of the application of 4 mg of estradiol benzoate (4 mL, IM; Gonadiol®, Zoetis), 5 mg of Dinoprost (1mL, IM; Lutalyse®, Zoetis) and insertion of the intravaginal P4 implant (1.9 mg; CIDR®, Zoetis). The P4 implant remained for 10 days. On the tenth day (D10), the implant was removed and 5 mg (1mL) of Dinoprost was administered. Additionally, the animals were randomly assigned to receive 400 IU of equine chorionic gonadotropin (eCG; 2ml, im; Novormon®, Zoetis; Ecg Group; n = 8) or 2 mL of saline (Control group; n = 7). After the removal of the implant, the mares were evaluated twice a day, to perform the ovarian ultrasound monitoring every 12 hours, until the moment when it was possible to visualize a pre-ovulatory follicle ≥35 mm, when human chorionic Gonadotropin was applied (hCG; 1,750 IU, 0.7 mL, IV; Vetecor®, Ceva). The data (means ± standard deviation) were analyzed by the variance test, using the software R 3.6.1, considering 5% of significance. The ovulation was observed in 83% (25/30) of the evaluated animals, being detected at a higher (P = 0.042) rate of ovulation in ER (100%; 15/15) compared to ET (67% ; 10/15). There was no difference (P= 0.697) between treatments [control = 79% (11/14); eCG = 87.5% (14/16)] nor difference (P= 0.0386) among the 4 experimental groups [ER / control= 100% (7/7); ER/eCG= 100% (8/8); ET/control= 57% (4/7); ET/eCG= 75% (6/8)]. The average ovulation occurred 26,2 ± 10,5 h. It was detected the difference in time between induction with hCG and ovulation, where animals ER had a lower (P= 0.031) range for the occurrence of ovulation (33.7 ± 10.3 h) compared to ET animals (39.0 ± 10.4 h) ,when there were not differences (P= 0.455) between the control group (34.0 ± 7.8 h) and eCG (38.7 ± 13.2 h) and no interaction (P = 0.291) between them (ER/Control= 31.7 ± 8.3 h, ER/eCG = 36.0 ± 12.6 h; ET/control= 37.2± 6.6 h; ET/eCG= 40.3± 12.8 h). In Conclusion, the mares of the breeding season had better ovulatory parameters compared with the animals in transition station, irrespective of eCG association when it was removal the intravaginal implant progesterone.

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ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

Effects of lipopolyssacharide on follicular reserve in cattle

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Mammals, including cattle, are born with a finite follicular reserve and their depletion is associated with the end of the fertility. The quality and useful life of this follicular reserve is greatly influenced by inflammatory processes. The objective of the study was to verify the follicular reserve of beef cows challenged with the application of lipopolysaccharide (LPS). Ten healthy heifers, managed in a confined system were randomly divided into two groups: LPS group (n=5), which received two intravenous applications 0.5µg/kg body weight of LPS diluted in 2 mL of saline (0.9% NaCl) with an interval of 24h and control group (n=5), which received two applications of 2mL of saline (0.9% NaCl) in the same range. The first application of LPS was performed on day 1, and on day 5, the animals were slaughtered, and a fragment of the ovary was collected for histological and follicular evaluation and a fragment for real-time PCR of AMH expression. Body temperature data were measured up to 24 hours after the first application. The results were analyzed using the t test in the GraphPad Prism 7 program. Significance was considered at $P< 0.05$. The animals in the LPS group showed an increase in temperature above physiological values, thus demonstrating that the treatment was effective in causing an acute inflammatory response. However, the frequency of healthy follicles was similar between the control and LPS groups, being 66.49% and 66.35%, respectively, there was no difference in the follicular population total, as well as in the different follicular categories. Despite these results, based on the work carried out by Bromfield and Sheldon (Biology of Reproduction, 88: 1-9, 2013), we believed that exposure to LPS was capable of triggering a higher rate of follicular abnormality and also of follicle passage primordial to primordial in transition, both effects being caused directly by LPS and by inflammatory mediators.It is known that the binding of the molecular patterns associated with pathogens or LPS to Toll Like Receptors stimulates the production of inflammatory cytokines in the circulatory stream and locally in the ovary, which seem to act by increasing the rate of atresia and follicular activation. However, when investigating an ovarian reserve indicator gene (AMH), the challenge with LPS did not modulate the gene expression of animals challenged with LPS. It is known that AMH is an important factor of follicular development, recent studies on follicular number and AMH in cattle have shown a particularly high variation in the ovarian reserve, both affected by inheritable conditions and also maternal underutrition. Thus, it is inferred that in our study, because it is not one of these conditions, but rather a specific challenge, it was not possible to observe changes in the gene expression. The exposure of cattle to two doses of LPS at 24 hour intervals does not affect the follicular reserve, supported by the number of follicles and AMH expression.

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ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

In vivo embryo production of cows submitted to a novel superovulation protocol with constant or decreasing doses of FSH and inseminated with ULTRAFertility sex-sorted sperm

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The study evaluated embryo production of non-lactating Holstein cows submitted to a superovulation (SOV) program with different regimens of FSH and inseminated with sex-sorted sperm with distinct fertility. Cows were assigned to 1 of 4 groups in a 2x2 factorial design. All cows were synchronized with a modified Double-Ovsynch, called "Super Double-Ovsynch" (50µg lecirelin [GnRH] on D-16, 0.5mg cloprostenol [PGF] on D-9, 25µg GnRH on D-6, 50µg GnRH on D0, FSH from D1.5 to D6, PGF on D5, 5.5 and 6, and 50µg GnRH on D7). In groups D-FSH (n=25), cows received 300mg FSH (Folltropin, Vetoquinol) divided in 10 decreasing doses every 12h, whereas groups C-FSH (n=25) received 10 constant doses. AI was done 12 and 24h after GnRH with frozen/thawed Sexed ULTRA^{TM} (Sexing Technologies) semen ($4x10^6$ sperm/dose) from sires classified as ULTRAfertility (UF; 25 SOVs and 3 sires) or not UF (NUF; 25 SOVs and 3 sires), resulting in 4 groups: D-FSH/UF, D-FSH/NUF, C-FSH/UF and C-FSH/NUF. There were 4 SOVs in a crossover arrangement. Embryos were recovered by uterine flushing on D15. Statistical analyzes were performed by PROC GLIMMIX of SAS 9.4 ($P \leq 0.05$). Considering all cows, CL number on D15 (11.5±0.8), recovery rate (53.6±4.6%) and recovered structures (6.8±0.7) were similar among groups. Ten cows (20%) did not respond to the protocols or failed to yield at least 2 structures and were removed from analyses of embryo quality. There were no interactions between FSH regimen and sire fertility. All variables shown below were similar when comparing C-FSH (n=21) and D-FSH (n=19), respectively, such as CL on D15 (11.7±1.1vs. 14.5±1.11), recovery rate (63.5 vs. 67.7%), structures (7.1±0.8vs. 9.8±1.0), viable (IETS) embryos (3.4±0.6 vs. 4.4±0.8), and percentage of viable embryos (49.2 vs. 49.2%). Moreover, percentage of unfertilized oocytes (34.2 vs.34.6%) and degenerated embryos (16.6 vs. 16.2%) was also similar between C- and D-FSH. Regarding effect of sex-sorted sperm, CL on D15 (12.7±1.0 vs. 13.3±1.1), recovery rate (69.3 vs. 62.0%), recovered structures (9.0±1.0 vs. 7.9±0.9), and percentage of degenerated embryos (18.2 vs. 14.8%) were similar between UF (n=19) and NUF (n=21), respectively. The UF sires produced greater numbers and percentages of viable embryos (5.7±0.6 [68.9%] vs. 2.2±0.5 [31.4%]), and much lower numbers and percentages of unfertilized oocytes (1.2±0.4 [13.0%] vs. 4.4±0.9 [53.8%]). In conclusion, the Super Double-Ovsynch either using constant or decreasing doses of FSH induced similar responses, with relatively high efficiency based on the antral follicle count at the onset of SOV and with only 20% of cows yielding ≤1 structure. Therefore, superstimulating with constant doses of FSH facilitates management without compromising embryo yield and the use of UF sex-sorted sperm resulted in noteworthy production of high-qualityembryos in non-lactating Holstein cows. Acknowledgements: FAPESP Grant #2018/037987, CAPES, CNPq and Sexing Technologies do Brasil.

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Differences between Bos taurus and Bosindicus oogenesis and folliculogenesis are highlighted by chromatin condensation in immature oocytes: a preliminary study

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Despite the development and commercial application of the production of bovine embryos *in vitro* (IVP), the efficiency of this biotechnology is still limited to an average of 30 to 40% of embryos produced. This is probably due to the heterogeneity of ovarian antral follicles that are punctured for oocyte collection, which reflects in differences in developmental competence (Landry &Sirard, Biol. Reprod., 99:877-887, 2018). Determining the degree of chromatin configuration according to the follicular diameter could allow the selection of oocytes with greater competence. As most of the studies related to this event were carried out in *Bos taurus*, there is a need to compare the results with *Bosindicus*. Thus, the aim of this study was to evaluate the chromatin configuration of bovine oocytes derived from different follicle sizes of *Bos taurus* and *Bosindicus* cows. Cumulus oocyte complexes (COC) were obtained by puncture of different follicle diameters after dissection of abattoir-derived ovaries from Nellore (n=79) and Angus (n=78) cows. Selected COCs were divided into three groups according to the follicular diameter: small (S; 0 to 2 mm), medium (M; >2 to 6 mm) and large (L; >6 to 9 mm). The control group (C) was obtained by puncture of 3 to 8 mm follicles. Immature COCs were mounted in Vectashield with DAPI® solution and evaluated under an epifluorescence microscope to determinate the chromatin configuration at germinal vesicle (GV) stage oocytes, classified as GV0, GV1, GV2 and GV3 as condensation progresses (Lodde et al. Mol. Reprod. Dev., 74:740–749, 2007). Data are presented as percentage (%) and were analyzed by qui-squared test ($P<0.05$). There was a significant difference ($P<0.05$) between the two breeds on M follicles, with less GV1 oocytes on Angus (17.9%), compared to Nellore (47.8%). In addition, no oocytes in GV0 stage were found in M follicles and there were no differences ($P>0.05$) in rates of GV2 (25% vs. 21.7%, respectively to Angus and Nellore) and GV3 (57.1% vs. 30.4%, respectively). No differences were found in GV stages in small follicles (GV0: 25% vs. 5.6%; GV1: 50% vs. 33.3%; GV2: 6.3% vs. 22.2%; and GV3: 18,8% vs. 38,9%; respectively to Angus and Nellore), large follicles (GV0: 0%; GV1: 30.8% vs. 58.8%; GV2: 23.1% vs. 17.6%; and GV3: 46.2% vs. 23.5%; respectively), and even in the control group (GV0: 4.8% vs. 0%; GV1: 47.6% vs. 23.8%; GV2: 28.6% vs. 28.6%; and GV3: 19% vs. 47.6%; respectively). In view of these preliminary results, we can suggest that there are differences regarding the chromatin configuration of oocytes included in follicles with different diameters in size, according to the breed. The continuity of studies on this topic should elucidate these differences and assess their potential impact on IVP, serving as a subsidy for the adaptation of *in vitro* culture systems to improve oocyte maturation, with the potential to increase embryo production.

Animal Reproduction

Folliculogenesis, oogenesis, and superovulation

Evaluation of hydrogel as a potential vehicle for intraovarian treatments

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Most drugs and hormones used in veterinary reproductive practice are injected SC, IM, or IV, and reach the target tissues by the systemic route. Among the drawbacks of this route are the high concentrations required to achieve therapeutic effects, losses due to metabolization and excretion, and the possibility of residues in milk and meat. Direct intraovarian administration may be an alternative to deliver drugs directly on the target tissue. The present study aimed to evaluate the innocuity of a hydrogel for intraovarian use. The biopolymer used was developed by the laboratory of Nanotechnology (LabNano), Embrapa, and presented viscosity and rheologic properties suitable for injection. The innocuity of the hydrogel was tested *in vivo* using Nelore cows. In a first trial, cows (N=9) received intradermic injections of 0.1mL hydrogel, saline, or were only punctured, always using 27G needles. Skin thickness at the site of injection was measured immediately before (0h), and 48 and 96h after the procedure. In a second trial, cows (N=10) received an intravaginal implant of progesterone (1g), an injection of 2mg estradiol benzoate and 0.5mg cloprostenol, and four days later were randomly distributed into two groups: intraovarian injection of 0.5mL DPBS (control) or 0.5mL hydrogel, performed using and adapted OPU system and 20G needles. The cows were evaluated for clinical endpoints (heart rate, respiratory rate, rectal temperature, ruminal movements, mucosal redness score) and had blood samples collected for evaluation of hemogram, leucogram, and blood biochemistry (alkaline phosphatase, creatinine, creatinine kinase, gamaglutamyl transferase, oxalacetic transaminase, and urea) at 0, 24, 48, and 168h after injection. Ovaries were scanned at 0, 24, 48, 72, 96 and 168h, using B mode and color Doppler ultrasound. Data were analyzed using the SAS MIXED model with a repeated measures statement to account for the effects of treatment, time, and interactions. In the first trial, there was a transitory increase ($P<0.0015$) in skin thickness at 48h, but no difference ($P>0.05$) was observed among groups. In the second trial, all parameters remained within the physiological range for the breed throughout the evaluation period. Cows injected with hydrogel had a lower ($P<0.0221$) respiratory rate at 24h when compared to the control group. However, this difference is likely to be associated with the decrease ($P<0.0001$) observed in both groups in rectal temperature at the same moment. There was no difference ($P>0.05$) among groups in any other clinical or hematological endpoint. There was also no difference ($P>0.05$) in antral follicle count or vascularization score among groups, and no sign of edema or inflammation was observed in the ovaries after 24h of injection. In summary, the hydrogel used is safe as a vehicle for intraovarian injection of drugs and hormones.Acknowledgements: Santé Laboratório Veterinário, Embrapa Project 30.19.90.006.00.00, CAPES

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ABSTRACTS: 34TH ANNUAL MEETING OF THE BRAZILIAN EMBRYO TECHNOLOGY SOCIETY (SBTE)

Folliculogenesis, oogenesis, and superovulation

Aloe vera increases mRNA expression of antioxidant genes after vitrification of bovine ovarian tissue

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Vitrification of ovarian tissue is a conducive approach to the conservation of the genetic material from animals of high genetic value and for those threatened with extinction. However,it is known that the vitrification procedure generates reactive oxygen species (ROS) through different mechanisms that can cause damage to the structure of the cytoskeleton, lipids membrane, proteins and DNA. These effects, however, can be limited by the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidases that regulate the concentration of ROS in the cell. Therefore, antioxidant substances that can be added to the vitrification solution to increase the activity of these enzymes have been extensively studied.On this way, the extract of *Aloe vera* (AV) is an excellent alternative, since it hasmore than 75 different potentially active compounds in its solid content, showing several pharmacological properties like antioxidantactivity.Thus, the aim of this study was to evaluate the effects of AV extract (10 or 50%) as a supplement of vitrification solution of bovine ovarian tissue in the expression of mRNA for *SOD, CAT, PRDX6* and *GPX1* after vitrification. For this purpose, cortical fragments from bovine ovaries (3x3x1mm) were transferred individually to a vitrification solution, consisting of α-MEM, 10% fetal bovine serum, 0.25M of sucrose, 3M dimethylsulfoxide (vitrification control) or supplemented with 10% and 50% of the extract of *Aloe vera* for 5min. Then they were vitrified on a solid surface and stored in cryobiological cylinders for two weeks. After this period, the fragments were thawed and subjected to analysis of gene expression by qPCR in which transcripts were evaluated for *SOD, CAT, PRDX6* and GPX1. The ΔΔCt method was used to normalize the relative gene expression. Concerning to statistical analysis, data were submitted to the Kruskal-Wallis test followed by Dunn's comparison. The differences were statistically significant when $P<0.05$. The presence of AV(10 or 50%) in the vitrification solution did not influence the levels of mRNA for CAT. However, supplementation with AV, in both concentrations, significantly increased mRNA levels for *PRDX6* and *GPX1*. In addition, the presence of 10% of AV provided a significant increase in the levels of mRNA for *SOD* in comparison with the fresh control. In conclusion, the addition of AV extract in the vitrification solution promotes a significant increase in the mRNA expression for *SOD*, *PRDX6* and *GPX1* in bovine ovarian tissue submitted to vitrification, showing the antioxidant activity of the AV extract. Key words: Antioxidant activity.Cryopreservation.Oxidative stress. Pre-antral follicles.

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Folliculogenesis, oogenesis, and superovulation

Effect of Aloe vera on follicular viability and maintenance of the extracellular matrix in bovine ovarian tissue after vitrification

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Vitrification of ovarian tissue is a technique successfully used to preservethe fertility. However, some factors can compromise the quality of the tissue and vitrified follicles. Among them, an overproduction of reactive oxygen species (ROS), which can deregulate the expression of metalloproteinase and damage the collagen fibers of the extracellular matrix. Given the importance of maintaining the integrity of the extracellular matrix, an improvement of the vitrification solution with the addition of antioxidant substances has been promising. In this sense, the aim of this study was to investigate the effect of *Aloe vera* extract on follicular viability and maintenance of the extracellular matrix from bovine ovarian tissue after vitrification.Therefore, cortical fragments from bovine ovaries were distributed in the following treatments: fresh ovarian fragments (control), vitrified without *Aloe vera* and vitrified with *Aloe vera* at a concentration of 10 and 50%.The ovarian fragments (3x3xmm) were transferred individually to a vitrification solution, consisting of α-MEM, 10% fetal bovine serum, 0.25M of sucrose, 3M dimethylsulfoxide (vitrification control) or supplemented with 10% and 50% of the extract of *Aloe vera* for 5min. Then they were vitrified on a solid surface and stored in cryobiological cylinders for two weeks. After this period, the fragments were thawed and subjected to histological analysis to evaluate the morphology followed by staining with Picrosirius Red (Abcam Kit)to measure the levels of collagen fibers.The average percentage of normal follicles were evaluated by the tukey test, followed by the Kruskal-Wallis test. Collagen fibers were analyzed by the Kruskal-Wallis test, followed by Dunn's test. The differences were statistically significant when $p < 0.05$. In histological analysis, the percentage of morphologically normal follicles in treatments with *Aloe vera*10% (88%) and *Aloe vera*50% (84%) was higher than the treatment without the addition of *Aloe vera* (61%). In addition, the presence of *Aloe vera* (10% and 50%) in the vitrification solution maintained at the levels of the tissue matrix (63% and 62%), respectively, similar to that found in the control group (67%). According to these results, it can be concluded that the presence of *Aloe vera* extract in the vitrification solution maintained the ovarian tissue integrity, with preservation of normal follicles, in addition to maintaining adequate levels of collagen fibers in the extracellular matrix.Key-word: Cryobiology. Follicular viability. Cow. Ovary.

Animal Reproduction

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Folliculogenesis, oogenesis, and superovulation

Expression of genes for proliferation in granulosa cells of heifers that are carriers and non-carriers of the high fecundity Trio allele

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Follicles from cattle that are carriers of the high fecundity Trio allele grow at a much slower rate than non-carriers possibly due to reduced proliferation of granulosa cells (GC).The aim of this study was to evaluate the expression of genes for proliferation in GC at two different stages of follicle development (before and after deviation) from Trio carriers and non-carriers. A total of 35 Angus-cross heifers (19 carriers and 16 non-carriers) had synchronization of a new follicular wave using follicular aspiration (FA; all follicles >3mm in diameter). The CL were regressed using two treatments with 500ug of cloprostenol sodium and an intravaginal P4 implant was kept until the end of the protocol. Carriers and Non-carriers were randomized to have GC collected by follicular aspiration on Day 2 (before follicular deviation) or Day 4 (after deviation) following a previous follicular aspiration to produce 4 groups: Carriers D2 (C-2 n = 9), Non-carriers D2 (NC-2 n = 8), Carriers D4 (C-4 n = 10) and Non-carriers D4 (NC-4 n = 8). Follicular fluid (FF) and GC from individual follicles were recovered as follows: C-2=23, NC-2=25, C-4=11 and NC-4=8.The mRNA was extracted from GC of each follicle using RNeasy Plus Micro Kit (Qiagen #74034), reverse transcribed to cDNA (Applied Biosystems #4368814), and quantified using quantitative RT-qPCR. The mRNA expression of LH receptor (LHCGR; marker of dominance), SMAD6 (overexpressed mRNA in Trio GC), Cyclin B1(CCNB1) and Cyclin A2 (CCNA2) (markers of cell proliferation) was evaluated and intrafollicular E2 concentrations were determined. Statistical analyses were performed with SAS using ProcMixed and linear regression by ProcReg. As expected, carriers of Trio had greater (P<0.001) SMAD6 than non-carriers (17.7±1.78 vs. 2.6±0.76) for both days and follicular volume was greater on Day 4 than 2 and greater for non-carriers than carriers. In addition, follicles on D-4 had greater (P=0.001) LHCGR than D-2 (200 vs 1.85) for both genotypes and greater FF-E2 on D-4 than D-2. For both of the genes for proliferation, CCNA2 and CCNB1, there was a day effect (P<0.0001) with proliferative genes having lower expression on Day 4 than 2 and a genotype effect (CCNB1, P=0.003; CCNA2, P=0.02) with proliferative gene expression greater in non-carriers than carriers. There was also an interaction of Day by genotype for CCNA2 (P=0.025) but not CCNB1 (P=0.17) due to a greater difference between genotypes on Day 4 than on Day 2 for CCNA2. There was a negative correlation between SMAD6 for both CCNB1 (R2=0.23; P=0.0002) and CCNA2 (R2=0.21; P=0.0004), consistent with Trio carriers (high SMAD6) having lower proliferation gene expression. In conclusion, the expression of two genes for proliferation are consistent with the idea that GC have lower proliferation after follicle deviation. More importantly, these results support the idea that reduced proliferation of GC may underlie the reduced follicle growth rate in carriers of the Trio allele.