Physiology and Endocrinology: Reproductive tissues, gametes, and embryo development

T316 Stem cell factor (SCF) activates AKT-p70RSK-S6 signaling in porcine trophoblast cells. Yurina Choi*, Wooyoung Jeong, Heejo Bang, Yujin Sung, and Jinyoung Kim, *Dankook University, Cheonan, Korea.*

During early pregnancy, a well-coordinated network between the conceptus and maternal uterus is especially crucial in pigs which involve a prolonged pre-attachment phase. This network is regulated by an astonishing amount of molecules such as growth factors. SCF (Stem Cell Factor) is a multipotent growth factor that elicits diverse biological actions on various types of tissues. In pig, SCF and their receptors are expressed in the uterine endometrium and conceptus during early pregnancy, but little is known about the biological role of SCF in the conceptus. Therefore, the aim of present study was to access SCF-induced intracellular signaling and cellular activities in porcine trophectoderm (pTr) cells. The effects of SCF were studied using pTr cells isolated from Day 12 pig conceptuses. Abundance of phosphorylated (p)-proteins relative to total proteins were determined by Western blot analyses of whole cell extracts 3 times and subjected to least squares using SAS software. In vitro cultured pTr cells were incubated with different concentrations of recombinant SCF (0–50 ng/mL). SCF dose dependently increased AKT phosphorylation, reaching 3.3-fold at 20 ng/mL. Within 30 min after 20 ng/mL of SCF treatment, levels of p-AKT, p-p70RSK and p-S6 proteins increased by 3.0-, 3.1- and 4.0-fold, respectively, and then returned to basal levels by 120 min. To ensure stimulatory effect of SCF on AKT signaling, cells were pre-incubated with AKT blocker (LY294002) 1 h before SCF treatment. Twenty μM of LY294002 decreased SCF-induced p-AKT, p-p70RSK and p-S6 proteins. Also immunofluorescence analyses found that p-S6 were localized abundantly within the cytoplasm of SCF-treated cells, but p-S6 was present at basal levels in the presence of LY294002. Furthermore, SCF increased pTr cell migration by 200%, but LY294002 significantly reduced this stimulatory effect to basal level. In conclusion, results of the present study suggest that SCF activates migration of trophectoderm through AKT signaling and supports the hypothesis that SCF is a critical regulatory factor of conceptus development during early pregnancy in pigs.

Key Words: SCF, early pregnancy, trophectoderm

T317 In vitro fertilization (IVF) from low or high antral follicle count pubertal beef heifers using semi-defined culture conditions. C. C. Chase, Jr.*[1], R. A. Cushman[1], A. K. McNeel[1], E. C. Wright-Johnson[1], O. L. Amundson[2], E. L. Larimore[2], B. N. Richardson[2], G. A. Perry[2], S. C. Tenley[3], J. R. Wood[3], A. S. Cupp[3], J. L. Vallet[1], D. D. Sypherd[1], and J. L. Miles[1], *[1]USDA, ARS, US Meat Animal Research Center, Clay Center, NE, [2]Dept. of Animal Science, South Dakota State Univ., Brookings, SD, [3]Dept. of Animal Science, University of Nebraska, Lincoln, Lincoln, NE.*

Our objective was to compare the in vitro maturation and fertilization of oocytes collected from low and high antral follicle count (AFC) heifers. Trans rectal ultrasonography was performed on 120 heifers to determine AFC and presence of a corpus luteum (i.e., pubertal). Those 10 heifers with the lowest AFC (avg. 14.2) and those 10 heifers with the highest AFC (avg. 29.9) all with evidence of estrous cyclicity (i.e., pubertal) were synchronized with 2 injections of $PGF_{2\alpha}$ and killed over 4 d; on d 5 to 6 of the estrous cycle. Nineteen heifers (n = 9 low and n = 10 high AFC) were at the appropriate stage of the estrous cycle. The IVF procedures and media were as described (P. J. Hansen's Laboratory, IVP Protocol). Cumulus-oocyte complexes (COCs) from follicles less than 8 mm in diameter were cultured in maturation medium (5% CO_2; 38.5°C) for 24 h. Matured COCs were fertilized using thawed frozen semen from a crossbred bull that was purified using Percoll separation procedures. Motile spermatozoa were added to COCs in fertilization medium at a final concentration of $1x10^6$ spermatozoa per mL. About 24 h later, presumptive zygotes were placed in micro drops of development medium under oil, and cultured (5% CO_2; 5% O_2; 38.5°C). On d 3 and 8 after fertilization, cleavage and blastocyst development, respectively, were assessed. Data were analyzed using the MIXED procedure of SAS and the model included the effects of collection d, group, and their interaction. Percentage data were analyzed using the GLIMMIX procedure with a binomial distribution and a logit link. Neither collection d nor the interaction differed ($P \geq 0.13$). High compared with low AFC heifers had greater numbers of COCs ($P < 0.01$; 27.0 ± 3.79 vs. 9.6 ± 3.97 per heifer), oocytes that cleaved ($P < 0.04$; 15.2 ± 2.63 vs. 6.1 ± 2.76 per heifer), and developed to blastocysts ($P < 0.007$; 4.08 ± 0.662 vs. 0.83 ± 0.695 per heifer). There was no difference ($P > 0.9$) in the percentage of COCs that cleaved (low = 52.3 ± 8.37%, high = 53.6 ± 7.98%) or in the percentage of COCs that developed to blastocysts ($P < 0.13$; low = 6.6 ± 2.79% vs. high = 13.7 ± 2.66). These results agree with our previous findings, high AFC heifers had greater numbers of COCs, oocytes that cleaved, and blastocysts compared with low AFC heifers; however, AFC does not appear to affect oocyte development and maturation through the blastocyst stage.

Key Words: heifer, IVF, antral follicle count

T318 Effect of L-carnitine in serum-supplemented IVM medium on mitochondrial membrane potential, ROS levels and subsequent embryo development of bovine oocytes. Beatriz C. S. Leao*, Nathália A. S. Rocha Frigoni, Priscila C. Dall'Acqua, and Gisele Z. Mingoti, *Laboratory of Physiology of Reproduction, School of Veterinary Medicine, Sao Paulo State University, Araçatuba, Sao Paulo, Brazil.*

This study aimed to evaluate the effects of supplementation with different concentrations of L-carnitine (L-car) during in vitro maturation (IVM) of bovine oocytes on their mitochondrial membrane potential (MMP), reactive oxygen species (ROS) levels, and subsequent embryonic development. Cumulus-oocyte complexes (COC) were matured for 22 h at 38.5°C, 5% CO_2 in air, on IVM medium (TCM-199 with bicarbonate, hormones and 10% FCS) supplemented with 0 (Control), 1, 5 or 10 m*M* of L-car. Matured and immatured oocytes (0 h) were stained with 500 n*M* of MitoTracker Red (Molecular Probes, Invitrogen), or with 5 m*M* of H_2DCFDA (Molecular Probes, Invitrogen). Stained oocytes (MMP: n = 191; and ROS: n = 250) were evaluated under an epifluorescence inverted microscope (excitation 579/495nm and emission 599/404nm, respectively) and the images analyzed by Q-Capture Pro image software to measure the arbitrary fluorescence units. The fluorescence intensity values were subtracted from mean values of "background" in the images. The 0h group was chosen as the calibrator, and each treatment value was divided by the mean of the 0h. In a second trial, COC (n = 1875) were IVM as above and then fertilized. The presumptive zygotes were cultured in SOF medium at 38.5°C, 5% CO_2 in air, for 7 d (Day 0 = IVF), when the blastocysts (Bl) rates were

evaluated. The averages were compared by ANOVA followed by the Tukey's test and data are presented as mean ± SEM. It was found a reduction ($P < 0.05$) on MMP after IVM with L-car 10mM (0.4 ± 0.0^{c}) in comparison to 1 m*M* (1.1 ± 0.1^{ab}) and 5 m*M* (1.0 ± 0.0^{ab}), as well as to 0h (1.0 ± 0.1^{a}); however, no treatment differed from the Control (0.8 ± 0.0^{abc}). In respect to ROS levels, we found an increase ($P < 0.05$) in oocytes matured with 10 m*M* (2.3 ± 0.1^{c}), in comparison to Control (1.8 ± 0.1^{b}), 1 m*M* (1.9 ± 0.1^{b}), 5 m*M* (2.0 ± 0.1^{b}) and 0 h (1.0 ± 0.0^{a}). Bl rates were similar ($P > 0.05$) in all treatments (25.2% ± 3.7 to 37.1% ± 2.7). In conclusion, the reduction on MMP after IVM of bovine oocytes with L-car 10 m*M* was followed by an increase of ROS level. However, there was no influence on their acquisition of capacity to Bl development. Financial support: FAPESP (#2012/10084–4 and #2013/07382–6)

Key Words: L-carnitine, mitochondrial membrane potential, ROS level

T319 Cell proliferation in ovarian follicles in nonpregnant ewes: Effects of plane of nutrition and arginine. Anna T. Grazul-Bilska*, Samantha L. Kaminski, Casie S. Bass, Kaitlyn K. Ebel, and Dale A. Redmer, *North Dakota State University, Fargo, ND.*

The aim of this study was to investigate the role of the NO system in ovarian function by determining if Arg supplementation affects cell proliferation in ovarian follicles in nutritionally compromised ewes. Ewes were stratified by weight and randomly assigned into either control (C; n = 35), overfed (O; 2xC; n = 37), or underfed (U; 0.6xC; n = 36) groups 8 weeks before Arg-treatment. Ewes from each nutritional group were randomly assigned to one of 2 i.v. treatments: saline (~10 mL) or Arg (L-Arg-HCl, 155 μmol Arg/kg of BW) which was initiated on d 0 of the estrous cycle and administered 3 times per day (0700, 1400, 2100 h) until ovary collection at the late-luteal stage of the first estrous cycle, or early or mid-luteal stages of the second estrous cycle. Ovaries were fixed in formalin solution followed by immunohistochemical localization of Ki67 (a marker of proliferating cells), and image analysis of granulosa (G) and thecal (T) layers. Data were analyzed by ANOVA using SAS. During nutritional treatment, C maintained BW, O gained 6 ± 1.2 kg, and U lost 14 ± 1.3 kg. Ki67 was detected in G and T layers, and other ovarian compartments. Cell proliferation in G and T of healthy follicles was affected by stage of the estrous cycle, but not plane of nutrition or Arg-treatment. Cell proliferation was 1.3–1.5-fold greater ($P < 0.001$) at early and mid than late luteal phase. For G and T of healthy follicles, interactions ($P < 0.05$) between plane of nutrition, Arg-treatment and/or stage were detected, demonstrating stimulatory effects of Arg-treatment on cell proliferation at the early luteal phase in O and U, and inhibitory Arg-effects at the mid-luteal phase in O ewes. Cell proliferation was greater ($P < 0.001$) in healthy antral than atretic follicles in all groups (14.4 ± 0.4 vs. 1.9 ± 0.1%). These data show that cell proliferation in G and T layers is affected by plane of nutrition, Arg-treatment and/or stage of the estrous cycle that likely affects follicular function. The mechanism of regulation of ovarian cell proliferation by diet and Arg remains to be elucidated. Supported by USDA-AFRI grant 2011–67016–30174, and Hatch Projects ND01748 and ND01754 to ATGB and DAR.

Key Words: plane of nutrition, arginine, ovary

T320 Comparison of mRNA expression of dominant follicle and follicular cyst in lactating dairy cows. Diego A. Velasco Acosta*[1,2], Tonja Egan[2], Cassandra Skenandore[2], Saige Sulzberger[2], Augusto Schneider[2], Fabio Lima[2], Marcio Corrêa[1], and Felipe Cardoso[2], *[1]Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brazil, [2]University of Illinois, Urbana, IL.*

Follicular cysts are an important disorder affecting 5 to 30% of dairy cows that leads to abnormal estrous cycle behavior and economic losses due to infertility. The aim of this study was to compare the mRNA expression in follicular cells of cows classified either as having a follicular cyst or cycling with dominant follicle (DF). Lactating dairy cows (n = 22) were examined weekly by ultrasound and 11 cows (Holstein, n = 7; Jersey n = 3; and Shorthorn n = 1) were classified as having a follicular cystic (CYS, follicle >25 mm with absence of corpus luteum) whereas 11 cows (Holstein, n = 11) were classified as cycling with a dominant follicle (CON, presence of corpus luteum and dominant follicle, n = 11). Cows were at 94.4 (30 – 382) DIM, milk yield 37.9 ± 12 kg /d, and parity 2.6 ± 1.3. Cows in CON had follicle diameter of 14.10 ± 3 mm whereas cows in CYS had follicle diameter of 35.73 ± 10.2 mm. Follicular fluid from each cow was aspirated and follicular cells were retrieved immediately by centrifugation, frozen in liquid nitrogen and stored at −80°C until RNA extraction. The mRNA expression for *LHCGR, STAR, 3β-HSD, P450scc, CYP19A1, IRS1, IGF, TLR4, TNF, IL1-β, IL8* and *IL6* was measured by real-time PCR. Statistical analysis was performed using the MIXED procedure of SAS. *LHCGR, 3β-HSD, CYP19A1, IRS1* mRNA expression was higher ($P < 0.05$) in CON (1.37 ± 0.4, 5.58 ± 1.2, 1.33 ± 0.2, 1.41 ± 0.3, respectively) than CYS (0.69 ± 0.3, 3.24 ± 0.9, 0.24 ± 0.01, 0.74 ± 0.2, respectively) with breed effect ($P < 0.07$). Expression of mRNA for *IGF, TLR4, TNF* and *IL8* was lower ($P < 0.05$) in CON (0.55 ± 0.07, 0.61 ± 0.2, 0.54 ± 0.3, 0.12 ± 0.01, respectively) than in CYS (4.62 ± 0.7, 3.40 ± 0.5, 1.58 ± 0.3, 0.52 ± 0.04, respectively), with no breed effect ($P > 0.20$). There was no difference ($P > 0.05$) for *STAR, P450scc, IL6* and *IL1-β* mRNA expression between CYS and CON. In conclusion, cows in CYS had lower expression of genes related to steroidogenesis and energy metabolism and greater expression of genes related to inflammation than CON. It seems that an inflammatory response may be involved in the follicular cyst etiology.

Key Words: follicle, cystic, inflammation.

T321 Colony-stimulating factor 2 affects development of the bovine preimplantation embryo differently for females than males. Luiz G. B. Siqueira*[1,2] and Peter J. Hansen[1], *[1]University of Florida, Department of Animal Sciences, Gainesville, FL, [2]Embrapa Gado de Leite, Juiz de Fora, MG, Brazil.*

Colony-stimulating factor 2 (CSF2) regulates early embryonic development by modifying the epigenome, reducing apoptosis, and altering ratio of cells in the trophectoderm (TE) and inner cell mass (ICM) of the blastocyst. Previously, CSF2 reduced trophoblast elongation in female embryos but increased elongation in males. Here it was tested whether sexual dimorphism in response to CSF2 can be observed as early as the blastocyst stage. Embryos were produced in vitro using X- or Y-sorted sexed semen (n = 1612 putative zygotes). On d 5 of culture, droplets were supplemented with 5 μL vehicle (control) or 10 ng/mL bovine CSF2. Blastocysts (n = 210) were collected at Day 7 and labeled with a nuclear dye (Hoescht 33342; total cells) and a TE cell marker (CDX2). Number of ICM cells was calculated by subtraction. Statistical analysis was performed using the Proc Mixed procedure of SAS; data represent least squares means ± SEM. Treatment of female embryos with CSF2 increased the proportion of zygotes ($P = 0.0213$) and cleaved embryos ($P = 0.0252$) to become a blastocyst but there were no effects in males ($P > 0.10$). The percent of zygotes becoming blastocysts on Day 7 was 14.7 ± 2.1 vs 21.5 ± 2.1% for control and CSF2 in females and 16.2 ± 2.0 vs 16.3 ± 2.0% in males. There was no effect of CSF2 treatment, sex, or the interaction on the total cell number or number of TE ($P > 0.10$). There was a tendency ($P = 0.0934$) for ICM number to be less in females (56.2 ± 3.1 vs 61.0 ± 2.9) and the TE:ICM ratio was greater (*P*

= 0.0217) for females (1.64 ± 0.91) compared with males (1.45 ± 0.09). Numerically (but not significantly), CSF2 tended to decrease ICM in females (53.9 ± 3.6 vs 58.6 ± 3.7) but not in males (60.4 ± 3.5 vs 61.5 ± 3.4). There was a tendency for a CSF2 by sex interaction ($P = 0.0955$) for TE:ICM ratio. In females CSF2 increased ratio (1.73 ± 0.11 vs 1.55 ± 0.11), but no effect was observed in males (1.41 ± 0.10 vs 1.50 ± 0.10). In conclusion, CSF2 exerts different responses on development of female and male preimplantation embryos. Consequences of actions of CSF2 on ICM and TE cell differentiation require further investigation. Support: NIH HD080855.

Key Words: embryo, colony-stimulating factor 2, sex

T322 Effect of fertility stressors on transcriptome of peripheral blood leukocytes (PBL) in dairy cows at the onset of conceptus implantation. Eduardo S. Ribeiro*[1,2], Rafael S. Bisinotto[1,2], Fabio S. Lima[1,2], Natalia P. Martinez[1,2], Leandro F. Greco[1,2], William W. Thatcher[1,2], and José E. Santos[1,2], *[1]Department of Animal Sciences, University of Florida, Gainesville, FL, [2]D.H. Barron Reproductive and Perinatal Biology Research Program, University of Florida, Gainesville, FL.*

Objectives were to investigate changes in transcriptome of PBL occurring at the onset of implantation and how they are affected by fertility stressors. Lactating cows (n = 481) had estrous cycle and ovulation synchronized to receive their first insemination (AI) postpartum. On d 19 after AI, PBL were isolated and mRNA extracted. A subsample of PBL mRNA from 36 cows was subjected to transcriptome analysis using the Affymetrix platform. Pregnancy was diagnosed on d 34 after AI. Two fertility stressors were evaluated, progesterone concentration during development of the ovulatory follicle and clinical uterine diseases (UTD). At the onset of synchronization, experimental design was established to have cows ovulating follicles that grow under low (LP) or high (HP) concentrations of progesterone. In addition, cows were evaluated daily on the first 10 d postpartum for diagnosis of UTD. Statistics was performed using Limma on R and FDR adjustment was used. LP during development of the ovulatory follicle reduced pregnancy per AI (P/AI; 34 vs 53%) and altered the transcriptome of PBL. In the HP group, pregnancy resulted in upregulation of classical interferon stimulated genes (e.g., *IFI6*, *ISG15*, *OAS1Y*); whereas in the LP group, pregnancy resulted in downregulation of a large number of inflammatory response genes (e.g., *HP*, *JUN*, *MYD88*). Particularly distinct transcriptome was observed in LP cows that failed to become pregnant, which indicated an inflammatory state. Cows that suffered from UTD also presented reduced P/AI (33 vs 50%) but only subtle differences in transcriptome, although potentially important. In pregnant cows previously diagnosed with UTD, expression of *OAS1X* was downregulated whereas *CD244* was upregulated compared with pregnant cows not diagnosed with UTD. Fertility stressors were associated with altered PBL transcript profiles at the onset of implantation. Differences observed might represent either a primary cause of subfertility or a consequence of impaired developmental potential of their conceptus and its ability to secrete signaling molecules to modulate the maternal immune system.

Key Words: cow, fertility, leukocyte

T323 Circulating concentrations of bovine pregnancy associated glycoproteins and late embryonic mortality in lactating dairy herds. Ky G. Pohler*[1], Marcos H. Pereira[2], Francisco R. Lopes[2], Jose L. M. Vasconcelos[2], Michael F. Smith[1], and Jon A. Green[1], *[1]Division of Animal Sciences, University of Missouri, Columbia, MO, [2]FMVZ–UNESP, Botucatu, SP, Brazil.*

In cattle, the incidence of late embryonic mortality (EM) has been reported to range from 3.2 to 42.7%. In some cases, the economic consequences of late EM are reported to be greater than that of early EM, because late EM can cause a significant delay in conception date. Although considerable effort has been directed toward understanding the causes of early EM, relatively little is known about the causes or mechanisms associated with late EM. The objectives of this experiment were as follows: (1) to determine the association between circulating concentrations of pregnancy associated glycoproteins (PAGs) and late EM in lactating dairy cattle following fixed-timed artificial insemination (TAI) on d 0 or fixed-timed embryo transfer (TET) on d 7, and (2) to identify a circulating concentration of PAGS on d 30 below which late EM would be likely to occur. Cows were diagnosed pregnant on d 30 of gestation based on presence of a fetal heartbeat and reconfirmed on d 60 of gestation. Late EM occurred when a cow had a viable embryo on d 30 of gestation but not on d 60 following TAI or TET. Only pregnant cows were included in the analysis (TAI-maintained, n = 413; TAI-EM n = 77; TET-maintained, n = 238; TET-EM, n = 47) which was subjected to Proc Glimix procedures of SAS. Cows that were pregnant at d 30 of gestation and maintained the pregnancy until d 60 had significantly greater ($P < 0.05$) circulating concentrations of PAGs at d 30 of gestation compared with cows that experienced late EM between d 30 and 60 of gestation in both TAI and TET. A receiver-operating characteristic curve was generated using MedCal Software to determine circulating concentration of PAGs on d 30 that should predict EM with ≥95 accuracy in both TAI and TET. Based on positive and negative predicative value analysis, a circulating concentration of PAGs below 1.4 ng/mL (TAI) and 1.85 ng/mL (TET) was 95% accurate in predicting EM (between d 30 - d 60) at d 30 of gestation. In summary, PAGs may provide a good marker for predicting EM between d 30 to 60 of gestation and may be able to accurately predict cows that will undergo late EM for the purpose of investigating mechanisms leading to late EM.

T324 Etiology of early pregnancy losses in Holstein dairy cows based on serum pregnancy-associated glycoprotein and progesterone concentrations. Maria J. Fuenzalida*, Paulo D. Carvalho, Milo C. Wiltbank, Pamela L. Ruegg, and Paul M. Fricke, *University of Wisconsin-Madison, Madison, WI.*

Our objective was to describe the mechanism and timing of pregnancy losses (PL) in cows after the first timed artificial insemination (TAI). A total of 136 cows that experienced PL were included in a matched case-control study. Cases were obtained from 3,164 cows from 4 dairy farms enrolled in a prospective cohort study. Cows with pregnancy-specific protein B (PSPB) ≥ 0.3 ng/mL on d 25 after TAI and were open based on transrectal ultrasonography 27 to 32 d (PG1) were defined as early PL (n = 49 cows). Cows that were pregnant at PG1 but open at subsequent evaluations were considered later PL (n = 87 cows). Cows that remained pregnant during the study period (from TAI to 90 d after TAI) were defined as Controls (n = 266 cows). Two Control cows were matched to each PL cow based on days in milk and parity. Progesterone (P4) and PSPB were measured weekly from 10 d before TAI until a cow was diagnosed open or remained pregnant and reached 90 d after TAI. Week of PL and cause of PL (embryonic death [ED] or corpus luteum regression [CLR]) was determined from weekly PSPB using a cutoff of the lowest PSPB concentrations of Controls (from 25 to 88 d after TAI) and weekly P4 concentrations. Data were analyzed by *t*-test, chi-squared and linear regression. For early PL, 30.6% (15/49), 16.3% (8/23) and 53.1% (26/49) were due to ED, CLR and undefined causes, respectively. For cows with later PL, 37.9% (33/87), 48.3% (42/87), and 13.8% (12/87) were due to ED, CLR and undefined causes ($P = 0.04$). Mean average days for occurrence of PL based on PSPB concentration

cutoff for ED was 48.6 (ranging from 28 to 88) and for CLR was 45.3 (ranging from 24 to 86) ($P = 0.29$). For early PL, P4 concentrations on d 25 were 5.8 ± 3.4 ng/mL and on d 32 were 6.6 ± 2.3 ng/mL ($P > 0.05$). PSPB concentrations on d 25 were 0.6 ± 0.6 ng/mL and on d 32 were 1.9 ± 1.5 ng/mL ($P < 0.05$). In conclusion, the occurrence of pregnancy loss due to ED occurred with more frequency in early PL near 32 d, whereas later PL were more likely due to CLR. Supported by AFRI Competitive Grant no. 2010–85122–20612.

Key Words: pregnancy loss, progesterone, pregnancy-associated glycoprotein

T325 Supplementation with insulin-like growth factor-1 during in vitro culture protects bovine embryos from deleterious actions of menadione. Nathália A. S. Rocha-Frigoni*, Beatriz C. S. Leão, Priscila C. Dall'Acqua, and Gisele Z. Mingoti, *Laboratory of Physiology of Reproduction, School of Veterinary Medicine, University of Sao Paulo State (UNESP), Araçatuba, Sao Paulo, Brazil.*

The objective of this study was to evaluate the protective effect of insulin-like growth factor (IGF-1) under oxidative stress condition induced by menadione (MD) on bovine embryos in vitro cultured (IVC). Cumulus-oocyte complexes were matured in TCM-199 with bicarbonate, hormones and 10% FCS for 22 h at 38.5°C in 5% CO_2 in air. After fertilization, the presumptive zygotes were IVC in SOF medium supplemented with 100 μM IGF-1, at 38.5°C in 5% CO_2 in air, for 7 d. On Day 6 the culture medium was supplemented with 5 μM MD. The cleavage rates and embryonic development were evaluated at Day 3 and Day 7, respectively (IVF = d 0). At d 7 blastocysts were stained to quantify the reactive oxygen species (ROS) levels with 5 μM H_2DCFDA (Molecular Probes, Invitrogen), the caspase activity (Image iT LIVE Red Caspase-3 and-7 Detection Kit, Molecular Probes) or the apoptotic index using TUNEL (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science). Stained embryos were evaluated under an epifluorescence inverted microscope (excitation 495/550–510–550nm and emission 404/595/590 nm, respectively for ROS, caspase and TUNEL). The images were analyzed by Q-Capture Pro image software for determining the fluorescent intensity. The results were compared by ANOVA followed by Student *t*-test ($P < 0.05$) and are presented as mean ± SEM. The cleavage rates did not differ ($P > 0.05$) among groups (77.1% ± 1.9 to 82.7.5% ± 2.2). The blastocyst rates were 38.1% ± 2.50^{ab} (Control), 38.9% ± 1.97^{a} (IGF), 21.39% ± 2.93^{c} (MD) and 25.7% ± 3.32^{bc} (IGF+MD). The intracellular levels of ROS were 111.1 ± 1.7^{b} (Control), 118.3 ± 2.1^{a} (IGF), 118.8 ± 2.1^{a} (MD) and 112.1 ± 3.6^{ab} (IGF+MD). The caspase activity did not differ ($P > 0.05$) among groups (28.2 ± 2.7 to 41.1 ± 3.2). Although, the rates of apoptosis were 12.8% ± 1.0^{b} (Control), 9.1% ± 0.75^{c} (IGF), 22.3% ± 2.3^{a} (MD) and 15.6% ± 1.6^{b} (IGF+MD). In conclusion, the supplementation with IGF-1 during IVC reversed the detrimental effects of MD on embryonic levels of ROS and apoptosis, as well as improved the embryo development.

Key Words: IGF-1, reactive oxygen species, apoptosis

T326 Menadione induces oxidative stress and reduces embryo development. Priscila C. Dall'Acqua*, Nathália A. S. Rocha-Frigoni, Beatriz C. S. Leão, and Gisele Z. Mingoti, *1Laboratory of Physiology of Reproduction, School of Veterinary Medicine, University of Sao Paulo State (UNESP), Araçatuba, São Paulo, Brazil.*

Oxidative stress during the in vitro production of embryos culminates with a rise in reactive oxygen species (ROS) levels and apoptosis. Menadione (MD) induces ROS generation and can be used as a tool to understand the actions of oxidative stress on embryo development. This study evaluated the effects of different MD concentrations during in vitro culture on bovine embryo development, intracellular levels of ROS and apoptosis rate. Cumulus-oocyte complexes were matured in vitro in TCM-199 with bicarbonate, hormones and 10% FCS during 22 h at 38.5°C in 5% CO_2 in air. After fertilization, zygotes were cultured in SOF medium at 38.5°C in 5% CO_2 in air, for 7 d (IVF = Day 0). On Day 6, SOF was supplemented with 0 (Control group), 2.5 μ*M* MD (MD 2.5) or 5.0 μ*M* MD (MD 5.0), The cleavage and blastocyst rates were evaluated at Days 3 and 7, respectively. Day-7 blastocysts were stained with H_2DCFDA (Molecular Probes, Invitrogen) or with TUNEL (in situ cell death detection kit, Roche Life Science) to evaluate the ROS levels and the apoptotic rates, respectively. Stained embryos were evaluated under epifluorescence (excitation 495/510–550 nm and emission 520/590 nm, respectively for H_2DCFDA and TUNEL) and the images were analyzed by Q-Capture Pro Image Software to determine the fluorescence intensity. Data were analyzed by ANOVA followed by Tukey's test ($P < 0.05$) and are presented as Mean ± SEM. The cleavage rates were similar ($P > 0.05$) among groups (78.2% ± 1.14 to 80.5% ± 0.88). The blastocyst rates were lower ($P < 0.05$) in MD 5.0 (25.9% ± 2.06^{b}) compared with Control (36.4% ± 1.51^{a}), and both were similar ($P > 0.05$) to MD 2.5 (32.5% ± 1.94^{ab}). The ROS levels were higher in MD 2.5 (0.75 ± 0.05^{b}) and MD 5.0 (1.11 ± 0.07^{b}) compared with Control (0.72 ± 0.04^{a}). The rates of apoptosis were higher ($P < 0.05$) in MD 5.0 (19.24% ± 0.69^{c}) compared with MD 2.5 (13.28% ± 0.71^{b}) and Control (10.0% ± 0.45^{a}). In conclusion, MD concentrations of 2.5 and 5.0 μ*M* were effective in inducing oxidative stress in bovine embryos produced in vitro and the detrimental effects were dose-dependent. The higher the oxidative stress, the more detrimental were the effects, causing reduction in the embryonic development, increasing on the intracellular ROS levels and apoptosis.

Key Words: menadione, oxidative stress, apoptosis

T327 Antioxidants improve membrane integrity and acrosome and sperm mitochondrial activity in ram sperm after cryopreservation. Elenice A. Moraes*[1], Wildelfrancys L. Souza[1], Jonathan M. S. Costa[1], and James K. Graham[2], *[1]Federal University of San Francisco Valley, Petrolina, PE, Brazil, [2]Colorado State University, Fort Collins, CO.*

There has been a renewed interest in the effects of oxidative damage to human sperm, as this damage to mitochondrial DNA and membrane architecture may explain the impaired fertility of the cryopreserved sperm. Different antioxidants have been tested to improve sperm quality but distinct and consistent beneficial effects are lacking. The objective was to determine if adding a combination different antioxidants improved ram sperm quality after cryopreservation. Thirty ejaculates, from 3 rams, were split and diluted into an egg-yolk-tris diluent containing different antioxidant treatments: control; 100 μ*M* melatonin (MEL) plus 0.05% ascorbic acid (MEL+AA); 100 μ*M* MEL plus 90 μL Trolox-C (MEL+TRO); 90 μL TRO plus 0.05% AA (TRO+AA); and 100 μ*M* MEL plus 0.05% AA plus 90 μL of TRO (MEL+AA+TRO); to final concentration 200 × 10^6 sperm/mL. The samples were cooled to 5°C/2 h, packaged into 0.5-mL straws, and frozen in static liquid nitrogen vapor for 15 min before being plunged into liquid nitrogen. Straws were thawed (37°C/30 s). The motility was determine using CASA, and to plasma membrane integrity the samples were stained with 2 L of Hoechst 33342 and 2 μL PI and incubated at 37°C/8min. Acrosomal integrity was determined, visually, after staining the sperm with 50 μL FITC-PNA at 37°C/20min. The sperm mitochondrial function was assessed using Rhodamine and PI (samples were incubated at 37°C/8

min with at 2 µL Rhodamine and 2 µL PI) and the samples assessed for the proportion of viable spermatozoa with high mitochondrial activity. Variables were determined by ANOVA using a Tukey test. Sperm treated with MEL+AA (29.1%) and MEL+AA+TRO (28.9%) maintained higher percentages of cells with intact plasma membranes after thawing ($P < 0.05$) than other treatments. The antioxidant combination (MEL+AA+TRO) resulted in the highest percentages of sperm with intact acrosomes (84.5%) and mitochondrial activity (96.4%) compared with other treatments ($P < 0.05$). All antioxidant treatments exhibited higher motility (61.4% versus 53%), acrosome integrity (78.1% versus 68.2%) and mitochondrial activity (92.7% versus 88.6%), than control ($P < 0.05$).Therefore, adding a combination of MEL+AA+TRO to ram sperm improved cell cryosurvival. Supported by FACEPE/CAPES.

Key Words: melatonin, Trolox-C

T328 Testosterone enhances basal, FSH- and IGF-I-stimulated aromatase gene expression in porcine granulosa cells in vitro. Leon J. Spicer*, John R. Evans, and Nicole B. Schreiber, *Oklahoma State University, Stillwater, OK.*

Ovarian follicular development is regulated by systemic as well as autocrine and paracrine factors such as estradiol (E2). E2 is produced by growing follicles via aromatase conversion of androgens into estrogens. Both FSH and IGF-I induce aromatase activity in granulosa cells (GC) of several species. To further evaluate the effects of FSH, IGF-I and testosterone on abundance of aromatase (*CYP19A1*) mRNA, GC from small porcine follicles (1 to 5 mm) were cultured for 2 d in medium containing 5% fetal bovine serum and 5% porcine serum followed by 48 h in serum-free medium containing control (no addition), FSH (30 ng/mL), or FSH plus IGF-I (30 ng/mL) in the absence or presence of 500 ng/mL of testosterone in a 2 × 3 factorial design. Cellular RNA was isolated and relative *CYP19A1* mRNA abundance was measured using real-time PCR with *18S* rRNA as a housekeeping gene. Data were analyzed via GLM procedure of SAS. Alone, FSH increased ($P < 0.05$) *CYP19A1* mRNA abundance 63-fold. Addition of IGF-I to FSH-treated cells increased *CYP19A1* mRNA abundance 6.5-fold above that seen with FSH alone. Testosterone alone increased ($P < 0.05$) *CYP19A1* mRNA abundance 30-fold and further amplified both FSH- and FSH plus IGF-I-induced *CYP19A1* mRNA abundance by 3- to 4-fold. In a second experiment, GC were cultured as in the first experiment except that medium (containing 500 ng/mL of testosterone as E2 precursor) was collected after 1 and 2 d of treatment for E2 measurement via RIA. FSH increased ($P < 0.05$) E2 production by 1.3- and 1.5-fold after 1 d and 2 d of treatment, respectively. IGF-I increased ($P < 0.05$) FSH-induced E2 production by 4.7- and 9.2-fold after 1 d (0.19 vs. 0.94 ± 0.06 ng/10^5 cells) and 2 d (0.21 vs. 1.94 ± 0.09 ng/10^5 cells) of treatment, respectively. In conclusion, testosterone-amplified basal and hormone-induced aromatase gene expression indicates that androgens produced by theca cells may act as a paracrine factor to increase aromatase activity in GC in addition to serving as E2 precursors for GC. This intra-ovarian regulatory mechanism may be vital for optimal follicular growth in pigs.

Key Words: aromatase, granulosa cells, pig

T329 Ovarian follicular dynamics in early- and late-maturing Brahman cows. Rui A. d'Orey Branco*[1,2], Thomaz H. Welsh[2], Don Neuendorff[1], and Ron D. Randel[1], *[1]Texas A&M AgriLife Research, Overton, TX, [2]Department of Animal Science, Texas A&M University, College Station, TX.*

Age at first calving is greater in *Bos indicus* than in *Bos taurus* cattle. Two different populations of Brahman cows exist based on age at first calving. Specifically, cows that are capable of calving by 26 mo of age are defined as early maturing (EM). Conversely, those cows that must be over 33 mo of age to calve are considered late maturing (LM). The objective of this trial was to compare follicular dynamics of EM and LM cows. This study's herd (located near Overton, Texas) was comprised of 120 pure bred Brahman cows. The EM group (n = 7) calved before 26 mo of age and the LM group (n = 7) calved at 33 mo of age or later. Cow BCS was between 4.5 and 7.5 and all of the cows calved at least 60 d before the trial began. Follicular evaluation was performed by transrectal ultrasound (US; Medison Co. SA-600; 7.5 mHz probe) and began after a naturally occurring estrus detected by a heat check bull. Cows were examined daily by US until signs of a new estrus were detected. During this second estrus the cow was examined by US every 4 h. Data were analyzed using PROC GLM of SAS. The EM and LM groups did not differ ($P > 0.1$) with respect to the diameter of the pre-ovulatory follicle or the time elapsed from the onset of estrus to ovulation. There were 3 follicular waves in 85.7% (6/7) of the LM and 14.3% (1/7) in EM cows (chi-squared; $P < 0.05$). The size of the largest subordinate follicle on the day before estrus was greater ($P < 0.01$) in EM (10.42 ± 2.38 mm) than in LM (5.23 ± 2.31 mm) cows. The number of subordinate follicles (>4mm) one day before estrus in EM cows (4.28 ± 2.55) was greater than in the LM cows (1 ± 0.76; $P < 0.01$). The total of follicular diameters one day before estrus was greater ($P < 0.01$) in EM (32.43 ± 5.63 mm) than in LM (7.43 ± 5.63 mm) cows. These results suggest that early and late maturing cows have differing aspects of follicular dynamics such as the number of follicular waves, the number of subordinate follicles, and the diameter of the second largest follicle. Further studies to determine whether this phenotypic difference is present in cows of other breeds are warranted.

Key Words: follicular dynamics, early maturing, late maturing