79 MICRORNA EXPRESSION IN BOVINE CUMULUS CELLS

K. Uhde, L. T. A. van Tol, T. A. E. Stout, and B. A. J. Roelen

Department of Farm Animal Health, Utrecht University, Faculty of Veterinary Medicine, Utrecht, the Netherlands

A mammalian oocyte within an ovarian follicle is surrounded by cumulus cells, together this structure is known as the cumulus-oocyte complex (COC). Cumulus cells are important for the development of the oocyte, they support the maturation process of the oocyte within the ovary and aid in sperm recognition. Because it is known that a Dicer knockout leads to infertility, microRNAs (miRNA) are focused to have an important role in oocyte development. MiRNAs are small noncoding RNA sequences that act as transcriptional regulators. Little is known about the expression of miRNA in cumulus cells or how cumulus-derived miRNA may regulate or be used to indicate the developmental competence of the maturing oocyte. Our aim was to investigate miRNA expression in oocytes and to identify and establish how specific miRNA influence the acquisition of developmental competence by bovine oocytes. Normalization of qPCR data requires stable reference genes. To this end, we tested the expression of various miRNA with respect to their ability to be used as reference miRNA for bovine cumulus cells; these included miR-103, miR-93, miR-26, let-7a, miR-191, and the small noncoding nuclear RNA U6. Cumulus-oocyte complexes were recovered from the ovaries of slaughtered cows and matured *in vitro*. Small samples of cumulus cells were collected from these COC before and after maturation. From the cumulus cell groups recovered at different stages, small RNA were extracted and cDNA was synthesised, followed by qRT-PCR. To identify the optimal combination of reference genes, the geNorm algorithm was used. MiR-26a and let-7a were identified as the most stably expressed miRNAs, whereas U6 showed the most variable expression levels. Future investigations are planned to identify miRNA in cumulus cells that can be used as markers for oocyte developmental competence. Using a single oocyte-embryo culture system will enable us to retrospectively relate cumulus miRNA expression to the developmental capacity of the oocyte.

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80 DNA METHYLATION OF INSULIN-LIKE GROWTH FACTOR 2 (IGF2) GENE IN DAY 14 *IN VITRO*-PRODUCED BOVINE EMBRYOS OF DIFFERENT SIZES

J. O. Carvalho[A], *M. M. Franco*[B], *G. M. Machado*[B], *and M. A. N. Dode*[B]

[A]University of São Paulo, Piracicaba, SP, Brazil;
[B]Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil

In mammals, a correct DNA methylation reprogramming and the maintenance of genomic imprinting after fertilization are essential for embryo development and pregnancy. One important imprinted gene, related to embryo development and placentation, is the insulin-like growth factor 2 (IGF2) gene. Therefore, embryos with different sizes could show differences in the methylation pattern of IGF2 gene. The aim of this study was to evaluate the methylation pattern of the differentially methylated region (DMR) located within exon 10 of the IGF2 gene, of *in vitro*-produced Nellore bovine embryos that were different in size on day D14 of development. The embryos were produced from oocytes obtained by follicular aspiration of slaughter house ovaries. On D7 after *in vitro* fertilization only grade I blastocysts were selected and, in groups of 10 embryos, were transferred non-surgically to the uteri of previously synchronized recipients with similar conditions. Seven days after being transferred, embryos were collected (Day 14 of development) and measured using Motic Images Plus 2.0 program (Motic, Richmond, BC, Canada). Embryos >45 mm were considered large (L) and those <25 mm were considered small (S). After being measured, a portion of each trophoblast layer was biopsied and used to determine the methylation status of the IGF2 gene by bisulfite sequencing. The methylation pattern was evaluated on individual embryos considered as separate replicates. At least 5 to 8 clones were evaluated per embryo and the sequences were analysed with the BiQAnalyser software (Max-Planck-Institut für Informatik, Saarbrücken, Germany), using the GenBank sequence NM_174087.3 as reference. The methylation pattern of the different groups was compared using Kruskal-Wallis test ($P < 0.05$). No differences in DNA methylation were found between S ($26.7 \pm 8.3\%$, $n = 37$ clones, 5 embryos) and L ($34.8 \pm 2.9\%$, $n = 20$ clones, 4 embryos) embryos. It is already known that the region studied is hypermethylated in sperm and hypomethylated in oocytes and, in some somatic cell types, it is expected to be around 50% methylated, being an imprinted region. Although we found a lower percentage of methylation than that expected for an imprinted region, this pattern may be the physiological pattern for trophoblast cells. This is the first report describing the methylation pattern of this region of the IGF2 gene in Day 14 bovine embryos of different sizes. It can be concluded that the methylation pattern of the intragenic DMR on exon 10 of IGF2 gene of *in vitro*-produced embryos on Day 14 of development is not affected by embryo size.

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81 JAK-STAT SIGNALLING IS CRITICAL FOR INNER CELL MASS DEVELOPMENT IN BOVINE BLASTOCYSTS

F. Meng, B. Forrester-Gauntlett, H. Henderson, and B. Oback

AgResearch, Ruakura Research Centre, Hamilton, New Zealand

The inner cell mass (ICM) of mammalian blastocysts comprises 2 transient lineages, namely hypoblast and epiblast, which develop into extra-embryonic and embryonic tissues, respectively. In the mouse, epiblast cells autocrinally secrete fibroblast growth factor (FGF) to induce hypoblast differentiation, and pharmacological FGF/mitogen-activated protein kinase (MAPK) signal inhibition converts all ICM cells into epiblast. We conducted a chemical screen for additional signal enhancers of epiblast identity in bovine Day 8 blastocysts. From the morula stage onwards, *in vitro*-fertilised (IVF) embryos were cultured in the presence of 9 small molecule inhibitors, targeting 9 principal signal pathway components. Inhibitors included SB431542, LDN193189, BIBF1120, Forskolin, BI-D1870, A66/TGX 221/ZSTK474, and AZD1480, targeting TGFβ-RI, BMP-RI,

VEGFR/PDGFR/FGFR, adenylate cyclase, ribosomal S6 kinase (RSK), PI3K, and JAK2 signalling, respectively. Using (1) blastocyst quality (by morphological grading), (2) cell numbers (by differential stain), and (3) lineage-specific candidate gene expression (by quantitative PCR) as readouts, we sought to identify positive and negative regulators of ICM development and lineage determination. Based on our previous digital mRNA profiling data (McLean *et al*. 2014 Biol. Reprod., in press), we selected discriminatory epiblast-specific (*FGF4, NANOG*) and hypoblast-specific (*PDGFRα*, *SOX17*) markers for qPCR analysis. Each inhibitor was compared, alone or in combination, to an appropriately diluted dimethylsulfoxide (DMSO) vehicle control in at least 3 biological replicates. Statistical significance was determined using a generalised linear mixed model with binomial distribution and logit link for developmental data and REML for log cell counts and log gene expression data, applying fixed treatment effects and random run and sample within run effects. Blocking TGFβ1-, BMP- or VEGF-/PDGF-/FGF-signalling did not affect blastocyst development, ICM *v.* trophectoderm (TE) cell numbers, or gene expression. Repression of PI3K signals via AG66 and TGX, but not ZSTK alone, modestly decreased grade 1–2 blastocyst development ($P < 0.05$) but had no effect on cell numbers or gene expression. Stimulating adenylate cyclase activity increased *NANOG* levels (2.5-fold; $P < 0.05$), while RSK inhibition reduced *FGF4* and *PDGFRα* expression (4-fold and 2-fold, respectively; $P < 0.05$). Suppressing JAK-STAT signalling, on the other hand, consistently compromised grade 1–2 blastocyst development and ICM numbers relative to DMSO controls (18/235 = 7% *v.* 59/159 = 29%, $n = 5$ IVF runs; 12 *v.* 47 ICM cells, $N = 25$ and $N = 7$ embryos counted, respectively; $P < 0.0001$). Epiblast and hypoblast markers were up to 40-fold reduced (*FGF4, NANOG, SOX17*; $P < 0.0001$) or completely abolished (*PDGFRα*; $P < 0.0001$). This effect was specific to the ICM because TE numbers and TE-specific gene expression (*CDX2, KTR8*) were not significantly altered. In summary, we have established Day 8 blastocysts as a useful chemical screening platform and demonstrated that bovine ICM development critically depends on JAK-STAT signalling.

82 STRUCTURAL REMODELLING OF THE NUCLEAR ENVELOPE IN BOVINE PRE-IMPLANTATION EMBRYOS

J. Popken[A,B], *A. Graf*[C], *S. Krebs*[C], *H. Blum*[C], *T. Guengoer*[B], *V. Zakhartchenko*[B], *E. Wolf*[B], *and T. Cremer*[A]

[A]Division of Anthropology and Human Genetics, LMU Biocenter, Planegg-Martinsried, Bavaria, Germany;
[B]Chair for Molecular Animal Breeding and Biotechnology, Gene Center, LMU, Munich, Bavaria, Germany;
[C]Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU, Munich, Bavaria, Germany

In the present study, we investigated the changes of the nuclear envelope and its underlying lamina, as well as features of higher order chromatin organisation in bovine embryos generated by *in vitro* fertilization during pre-implantation development. We used super-resolution, 3-dimensional structured illumination microscopy combined with 2-colour immunostaining of the nucleoporin Nup153 and lamin B serving as markers for nuclear pore complexes (NPC) and the nuclear lamina, respectively. DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). We examined 20 nuclei for the zygote (10 male pronuclei and 10 female pronuclei; $n = 10$) and the blastocyst (10 trophectoderm and 10 inner cell mass nuclei; $n = 1$) stage, and 10 nuclei for each the 2-cell ($n = 5$), 4-cell ($n = 3$), 8-cell ($n = 2$), 19-cell ($n = 1$), and morula ($n = 1$) stages. We report 4 major findings: (1) At the onset of major genome activation (MGA) nuclei showed a peripheral location of chromosome territories (CT), separated by wide IC channels and surrounding a major lacuna depleted of chromatin. The NPC were exclusively present at sites where DAPI-stained DNA contacted the nuclear lamina, whereas extended lamina regions without such contacts lacked NPC. In post-MGA nuclei, the CT formed a higher order chromatin network distributed throughout the entire nuclear space and the major lacuna disappeared. In line with a switch to a ubiquitous lining of DNA at the lamina, NPC were also uniformly distributed throughout the entire nuclear envelope. These findings shed new light on the conditions that control the integration of NPC into the nuclear envelope. (2) The switch from maternal to embryonic production of mRNA was accompanied by an increased amount of nuclear lamina invaginations covered with NPC, which may serve the increased demands of mRNA export and protein import. (3) Other invaginations, as well as interior nuclear segments and vesicles without contact to the nuclear envelope, were exclusively positive for lamin B. Because an increase in these lamin B positive structures occurred in concert with a massive nuclear volume reduction, we suggest that they reflect a mechanism for fitting the nuclear envelope and its lamina to a shrinking nuclear size throughout bovine pre-implantation development. (4) Throughout the cytoplasm, randomly distributed extranuclear clusters of Nup153 without associated lamin B were frequently observed from the zygote stage up to MGA. These clusters may represent a deposit of maternal Nup153 and likely other nucleoporines not studied here. Corresponding RNA-Seq data revealed deposits of spliced, maternally provided *NUP153* mRNA and little unspliced RNA before MGA, which increased strongly at the initiation of embryonic *NUP153* expression at MGA. After MGA, these clusters were exclusively located at or near the nuclear border and were no longer present at the morula stage and later. In conclusion, our findings demonstrate the dynamic adaptation of the nuclear envelope to the special needs of bovine pre-implantation development and show the necessity of chromatin association for the integration of nuclear pores into the nuclear envelope.

83 STAGE-SPECIFIC PROTEOME SIGNATURES IN EARLY BOVINE EMBRYO DEVELOPMENT

D. R. Deutsch[A], *T. Fröhlich*[A], *K. A. Otte*[A], *A. Beck*[B], *F. A. Habermann*[C], *E. Wolf*[A,B], *and G. J. Arnold*[A]

[A]Gene Center, Laboratory for Functional Genome Analysis LAFUGA, Ludwig-Maximilians-Universität München, Munich, Bavaria, Germany;
[B]Chair for Molecular Animal Breeding and Biotechnology, Department of Veterinary Sciences and Gene Center, Ludwig-Maximilians-Universität München, Munich, Bavaria, Germany;
[C]Chair for Anatomy, Histology and Embryology, Department of Veterinary Sciences, Ludwig-Maximilians-Universität München, Munich, Bavaria, Germany

Development of early embryonic stages before activation of the embryonic genome depends on sufficiently stored products of the maternal genome and adequate activation, deactivation, and relocation of proteins. To establish protein function, several posttranslational events (e.g. proteolytic activation, phosphorylation, or secretion) are frequently essential and thereby prevent prediction of protein abundance from transcript abundance. Consequently, proteomic studies are indispensable to characterise the molecular processes governing early embryonic development and to establish

corresponding regulatory networks. Here, we present a quantitative proteome analysis of bovine zygotes and embryos at the 2-cell and 4-cell stage. Cumulus-oocyte complexes (COC) were prepared from bovine ovaries obtained from a local abattoir and selected for a compact layer of cumulus cells. *In vitro* maturation, fertilization, and embryo production were performed according to standard procedures. For quantitative *isobaric tags for relative and absolute quantitation (iTRAQ)*-liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, protein from batches of 50 MII oocytes (serving as a reference), zygotes, 2-cell and 4-cell stage embryos, respectively, was extracted. Quantitative proteome analysis of iTRAQ-labelled tryptic peptides was performed on an Orbitrap XL instrument (Thermo Fisher, Waltham, MA, USA) coupled to an Eksigent nano-liquid chromatography system (AB Sciex, Framingham, MA, USA). The tandem MS data were analysed by MASCOT and filtered for a false discovery rate (FDR) of $<1\%$. Quantification of iTRAQ signals was accomplished with the Q+ module of the Scaffold software (Proteome Software Inc., Portland, OR, USA). *t*-Tests, ANOVA and principal component analysis (PCA) analysis were performed using R (R Core Development Team, Vienna, Austria). From 4 biological replicates, 1072 proteins were identified and quantified. Eighty-seven differed significantly in abundance between the 4 stages ($\log_2$ fold change $\geq |0.6|$, $P \leq 0.05$). The proteomes of 2-cell and 4-cell embryos differed most from the reference MII oocyte, and a considerable fraction of proteins continuously increases in abundance during the stages analysed. Bioinformatic analysis of abundance altered proteins provided evidence that the proteins RPS14 and HNRNPK involved in the p53 pathway play a major role during early development, as well as proteins of the lipid metabolism, in particular APOA1. Furthermore, a group of proteins (e.g. SPTBN1, PPP1CC, RABGAP1, STMN1, and WEE2) is engaged in mitosis. In addition, we detected relevant differences between transcript and protein abundance levels; for example, for WEE2. In conclusion, this study identified and quantified numerous proteins important for early embryogenesis so far not described in the mammalian system, and contributed protein profiles for key players previously described. Our results highlight the importance of innovative proteomic tools and workflows to complement transcriptome data of early embryogenesis.

84 PAIRS OF BLASTOMERES FROM BOVINE DAY 5 MORULAE ARE ABLE TO CONTRIBUTE TO INNER CELL MASS AND TROPHECTODERM IN CHIMERIC EMBRYOS GENERATED BY AGGREGATION WITH TWO DAY 4 MORULAE

K. Simmet[A], *M. Reichenbach*[B], *S. Jung*[C], *R. Fries*[C], *T. Grupp*[B], *C. Gschöderer*[B], *J. Scherzer*[B], *H. D. Reichenbach*[D], *and E. Wolf*[A]

[A]Chair for Molecular Animal Breeding and Biotechnology, Ludwig-Maximilians-Universität München, Oberschleissheim, Germany;
[B]Bayern-Genetik GmbH, Grub, Germany;
[C]Chair for Animal Breeding, Technische Universität München, Freising, Germany;
[D]Bavarian State Research Center for Agriculture, Institute of Animal Breeding, Grub, Germany

The multiplication of high-value embryos by chimera formation using asynchronic aggregation is a promising alternative to embryonic cell nuclear transfer. Single blastomeres from a donor embryo are aggregated with 2 host embryos, thus several chimeras can be constructed per donor embryo. Due to the advanced developmental stage, the donor blastomeres are likely to contribute to the inner cell mass (ICM) and later give rise to the embryo proper, whereas the host embryos form extra-embryonic tissues. To test if pairs of blastomeres from Day 5 morulae are able to form the ICM when aggregated with 2 Day 4 host embryos, we produced transgenic donor embryos carrying a fluorescent reporter gene (enhanced green fluorescent protein, eGFP) by using semen from an eGFP transgenic bull (Reichenbach *et al*. 2010 Transgenic Res. **19**, 549–556) for *in vitro* fertilization and *in vitro* host embryos produced by a standard procedure. The zona pellucida of all embryos was removed by treatment with 1 mg mL^{-1} pronase. Donor embryos were assessed for eGFP expression by fluorescence microscopy and disaggregated by gentle pipetting after incubation in Mg^{2+}- and Ca^{2+}-free medium. Pairs of blastomeres were then placed between 2 host embryos and cultured individually in a well-of-the-well culture dish. On Day 6 after aggregation, fully developed blastocysts were assessed for eGFP fluorescence. In 3 replicates, $n = 30$ chimeras were produced by aggregation; 13 (43%) developed to blastocysts, of which 2 (15%) showed local eGFP expression in the ICM and 7 (54%) showed a generalized expression. From the results of this study we conclude that Day 5 morulae may be multiplied in an efficient manner by using the chimera formation technique, which makes this approach applicable to *ex vivo*-derived embryos. In future investigations we will study the effect of using donor blastomeres from either the inside or outside of the donor morula and test the use of tetraploid host embryos to increase the rate of blastocysts with the desired genotype in the ICM. Finally, we aim to introduce this multiplication approach to the production of genotyped embryos with a genomic estimated breeding value (gEBV) and intend to produce calves with identical gEBV.

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85 LOW-MOLECULAR-WEIGHT METABOLITES IN BOVINE *IN VITRO* PRODUCTION CULTURE MEDIA AS EMBRYO QUALITY MARKERS

M. Nõmm[A], *E. Mark*[A], *K. Kilk*[B], *S. Kõks*[A,B], *and Ü. Jaakma*[A,C]

[A]Estonian University of Lifesciences, Tartu, Estonia;
[B]University of Tartu, Tartu, Estonia;
[C]Competence Centre on Reproductive Medicine and Biology, Tartu, Estonia

The need for noninvasive embryo quality assessment techniques has increased as the *in vitro* production of cattle embryos has become more popular and necessary in the beef and milk production industries. In this study, we assessed the metabolomic profile of embryo culture media to determine whether it is possible to evaluate differences in low-molecular-weight metabolites in the culture media composition of morula stage embryos

compared with embryos that develop to the blastocyst stage. Single bovine embryos were cultured in 60-μL SOF+0.4% BSA droplets under mineral oil. Twenty microliters of culture media was removed at Day 2, 5, and 8 post-fertilization. Cultured droplets without a zygote served as the control samples. A total of 42 samples were analysed using liquid chromatography-mass spectrometry (Q-Trap 3200, Ab Sciex, Framingham, MA, USA), followed by principal component analysis. Our preliminary results indicated significant differences ($P < 0.00001$) in 10 low-molecular-weight compounds between the groups. Three of those compounds (588, 589, and 702 Da) were represented in higher concentrations only in embryos that advanced into the blastocyst stage. These first results could allow the identification of embryos with improved viability and give better understanding of the development of pre-implantation embryo.

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86 BIRTH OF HEALTHY CALVES AFTER INTRAFOLLICULAR OOCYTE TRANSFER

M. Hoelker[A], *A. Kassens*[B], *E. Held*[A], *C. Wrenzycki*[C], *U. Besenfelder*[D], *V. Havlicek*[D], *H. Sieme*[B], *D. Tesfaye*[A], *and K. Schellander*[A]

[A]Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, Bonn, Germany;
[B]Reproductive Medicine Unit, University of Veterinary Medicine Hannover, Hannover, Germany;
[C]Clinic for Veterinary Obstetrics, Gynecology and Andrology, JLU Giessen, Giessen, Germany;
[D]Reproduction Center Wieselburg, VetMed University, Vienna, Austria

The *in vitro* production (IVP) of bovine embryos is a well-established technique that has been available for nearly 20 years. However, there remain major differences between IVP-derived blastocysts and their *in vivo*-derived counterparts. Many studies have pointed out that most of these differences are due to the *in vitro* developmental environment. To circumvent these negative effects due to *in vitro* culture conditions, a new method – intrafollicular oocyte transfer (IFOT) – was established in the present study. Using modified ovum pick-up (OPU) equipment, *in vitro*-matured oocytes derived from slaughterhouse ovaries were injected into the dominant preovulatory follicle of synchronised heifers (follicular recipients) enabling subsequent ovulation, *in vivo* fertilization, and *in vivo* development. A total of 810 *in vitro*-matured oocytes were transferred into 14 heifers. Subsequently, 222 embryos (27.3%) were recovered after uterine flushing at Day 7. Based on the number of cleaved embryonic stages, 64.2% developed to the blastocyst stage, which did not differ from the IVP-derived embryos (58.2%). Interestingly, lipid content of IFOT-derived blastocysts did not differ from the fully *in vivo*-produced embryos, whereas IVP-derived blastocysts showed significantly higher lipid droplet accumulation compared with fully *in vivo*-derived and IFOT-derived blastocysts ($P < 0.05$). Accordingly, IFOT blastocysts showed significantly higher survival rates after cryopreservation than complete IVP-derived embryos (77% *v.* 10%), which might be attributed to a lower degree of lipid accumulation. In agreement, transfer of frozen-thawed IFOT blastocysts to synchronized recipients (uterine recipients) resulted in much higher pregnancy rates compared with transfer of IVP-derived blastocysts (42.1 *v.* 13.8%) but did not differ from frozen-thawed *ex vivo* blastocysts (52.4%). Of these presumed IFOT pregnancies, 7 went to term, and microsatellite analysis confirmed that 5 calves were indeed derived from IFOT, whereas 2 were caused by fertilization of the follicular recipient's own oocyte after AI. Taken together, IFOT-derived blastocysts closely resemble *in vivo*-derived blastocysts, confirming earlier suggestions that the ability to develop to the blastocyst stage is already determined in the matured oocyte, whereas the quality in terms of lipid content and survival rate after cryopreservation is affected by the environment thereafter. However, to the best of our knowledge, this is the first study reporting healthy calves after intrafollicular transfer of *in vitro*-matured oocytes.

87 OVARIAN RESERVE, EMBRYO PRODUCTION, AND THEIR CORRELATION WITH ANTI-MÜLLERIAN HORMONE (AMH) IN HOLSTEIN COWS

J. Verstegen and A. Rozner

MOFA Global LLC, Verona, Wisconsin, USA

Anti-Müllerian hormone (AMH) is a small peptide hormone that has been associated with ovarian follicular reserve in humans and in some animal species including bovine. Profiles of AMH, as well as the relationship between serum AMH to oocyte number and *in vivo* embryo production, were evaluated in Holstein cows. AMH levels were determined in 15 unstimulated cows at monthly intervals for 4 months and in 394 male and 399 female developing Holstein animals from birth to adulthood. Also, AMH was measured in 41 heifers at the time of ovum pick-up (OPU) and 125 heifers at the time of embryo flushing. Superovulation was induced before OPU or embryo flushing using a modified Ovsynch protocol with 4 days of decreasing FSH (Pluset H®, MOFA Global, Verona, WI, USA). Blood samples were collected using serum tubes and spun within 2 h. The samples were stored at −20°C until evaluated for AMH using the AMH-Bovine specific immunoassay® (MOFA Global). AMH levels in males and females peaked at 2 months of age and then decrease as they reached adulthood. The average AMH level of adult cows was stable for each of the 4 monthly measurements, with a high correlation between all values per animal ($r^2 = 0.9077$; $P < 0.01$), suggesting that AMH levels are consistent for at least 4 consecutive months. However, AMH levels were lowest during the summer months, suggesting a seasonal change in AMH secretion. Animals repeatedly ovarian stimulated showed decreasing AMH levels (509 ± 295, 299 ± 210, 211 ± 119) with subsequent stimulations. There was also a significant decrease in the number of embryos recovered (5.7 ± 4, 2.2 ± 1.9; $P = 0.02$); however, the number of oocytes was not altered by multiple stimulations (9.9 ± 9.8, 8.1 ± 6.2; $P = 0.57$). Because AMH and embryo numbers decreased after multiple stimulations, the first AMH value and results of the first OPU or embryo flush were used for the correlation of AMH to the number of oocytes or embryos. Animals were separated into 3 AMH categories: low (<100), normal (100–400), and high (>400 pg mL^{-1}). High AMH OPU animals had significantly higher numbers of oocytes than the normal or low AMH groups (13.8 ± 9.2, 9.2 ± 5.3, 5.6 ± 3.9; $P = 0.001$). High AMH flushed animals had significantly higher numbers of embryos than animals with low AMH (10.9 ± 8.0, 5.7 ± 5; $P = 0.002$). Statistical analyses were performed using Statview 5. Differences were

determined using Student's *t*-test; $P < 0.05$ was considered significant. In conclusion, AMH serum concentrations are consistent over multiple months; however, blood should not be taken for animal selection by AMH after ovarian stimulations have begun and should be interpreted with caution during the summer months. AMH is highly associated with superovulation response and oocyte and embryo production and should improve efficiency of multiple-ovulation embryo transfer.

88 CHANGING MATERNAL NUTRITION IN EARLY PREGNANCY MODIFIES FETAL OVARY DEVELOPMENT IN NELLORE COWS

H. F. Costa[A], *M. C. V. Miguel*[A], *A. M. Pedroso*[B], *S. P. Gobbo*[A], *F. L. Lopes*[A], *J. R. Peiro*[A], *and G. P. Nogueira*[A]

[A]UNESP-FMVA-DAPSA, Aracatuba-SP Brazil;
[B]EMBRAPA, Sao Carlos

Environmental influences such as nutritional restriction during early gestation in cattle may impair fetal development and compromise functions in adulthood. During the first trimester of gestation fetal gonads are formed. We hypothesised that either restriction or excess of nutrients ingested by cows during the first third of pregnancy interferes with fetal body weight (BW) and ovary development. Twenty-one uniparous Nelore cows (BW = 488 ± 24 kg, body condition score, BCS = 3.1 ± 0.1) were subjected to timed AI with sexed semen (female) of a single bull and individually allocated to different diets. The diet of the control group (C) met the maintenance requirements, and the groups of high (A) and low (B) were either 180% or 60% of maintenance respectively. Live weight and BCS were assessed weekly to adjust the diet according to the individual weight of each animal. At 60 days of gestation, 9 fetuses (3/group) were removed by colpotomy (accessed through vagina), weighed, and their ovaries were dissected and weighed. One fetal ovary (of each pair) was analysed by RNA-seq (mRNA). The effects of treatments on both ovarian and fetal weight were compared by ANOVA (proc GLM, SAS 9.3, SAS Institute Inc., Cary, NC, USA). During the 60 days of treatment, cows from Group A gained 66 kg (from 474 ± 6 kg to 541 ± 10 kg) and 1.25 points in BCS (from 3.0 to 4.25), whereas the cows in group B lost 61 kg (from 458 ± 3 kg to 397 ± 6 kg) and 1 point in BCS (from 3.2 to 2.2). Fetal ovary weight (sum of the two) was lower in group B (0.007 ± 0.001 g; $P < 0.04$) than in groups A (0.02 ± 0.004 g) and C (0.013 ± 0.007 g), which did not differ ($P > 0.08$) between each other. Fetuses in group B weighed less (12.8 ± 1.14 g; $P < 0.006$) than in groups A (20.56 ± 2.2 g) and C (20.03 ± 0.8 g). Maternal nutritional status during the first 60 days of gestation changed the transcriptome of fetal ovaries. There were differences in the pattern of gene expression between the control, high, and low intake groups. A total of 79 genes out of 20 657 showed differential expression between treatments (false discovery rate 0.05), some of which were related to embryonic and ovarian development. Thus, we conclude that changing maternal nutrition during the first 60 days of gestation will change the transcriptomic profile of fetal ovaries. Poor maternal nutrition jeopardizes ovarian size and weight and fetal weight, suggesting impairment on the production of ovarian follicles. This is a critical period in fetal ovarian development, as oocytes grow and differentiate, and need to escape from degenerative processes to remain in the ovaries. Thus, the developmental impairment at the beginning of meiosis could reduce the number of oocytes in the fetal ovary. Histological examination of fetal ovaries is underway to evaluate the number of oocytes.

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89 EFFECT OF NONESTERIFIED FATTY ACIDS ON *IN VIVO* OR VITRO EMBRYO PRODUCTION IN KOREAN NATIVE CATTLE (HANWOO)

J.-J. Park[A], *H.-J. Yoo*[A], *K.-W. Kim*[A], *E.-J. Chu*[A], *H.-W. Cho*[B], *K.-S. Cheong*[B], *S.-H. Lee*[C], *and S.-H. Yeon*[C]

[A]Animal Reproduction & Biotechnology Center, Myung-Poom Hanwoo Consulting, Hoengseong, South Korea;
[B]South Branch of Gangwondo Veterinary Service Lab, Wonju, South Korea;
[C]Hanwoo Experiment Station, National Institute of Animal Science, R. D. A, Pyeongchang, South Korea

Recent reports suggest that high concentrations of nonesterified fatty acids (NEFA) negatively affect oestrous cycle, fertility, *in vitro* oocyte maturation, embryo quality, and viability. This study was performed to determine the relationship between plasma concentration of NEFA and embryo quality in Hanwoo cattle. In experiment 1, embryo recovery rate from superovulated donor cows 7 days after AI was evaluated. Donors, at random stages of the oestrous cycle, received a CIDR (Day 0). On Day 7, 200 mg of FSH was administered followed by 40, 30, 20, and 10 mg of FSH in declining doses twice daily by intramuscular injection for 4 days. On the third day of FSH administration, 25 mg of prostaglandin F_{2a} was given and the CIDR was withdrawn. After FSH injections were complete, donors were artificially inseminated twice on Day 11 and 12 at 12-h intervals. At first AI, 250 μg of gonadotropin-releasing hormone (GnRH) was administered. During embryo collection, plasma samples were obtained from jugular veins to measure NEFA concentrations by chemistry analyzer. Next, the effect of NEFA on embryo development *in vitro* was examined (experiment 2). After *in vitro* maturation and fertilization of abattoir oocytes using standard procedures, zygotes were cultured in mSOFaa supplemented with 5% (vol/vol) oestrous cow serum (ECS) collected from 8 random cows representing 8 different NEFA concentrations (72.6, 126, 175.5, 244.6, 311, 393, 527.3, and 979 g dL^{-1}) and compared with mSOFaa without serum (no-serum control). Statistical analysis was performed by ANOVA (SAS 9.1, SAS Institute Inc., Cary, NC, USA) and Duncan's multiple range tests where appropriate. Recovery of total and transferable morulae and blastocysts was related to NEFA levels of donor cows. The donor group with the lowest plasma NEFA levels yielded the most embryos, of which most were high quality ($n = 3$, 173 ± 11 g dL^{-1} NEFA; 14 ± 3 total recovered embryos of which 85 ± 7% were transferable). Higher NEFA plasma levels reduced both the absolute number of embryos recovered and the fraction of transferable embryos ($n = 8$, 301 ± 20 g dL^{-1} NEFA with more than the 10 recovered of which 56 ± 5% were transferable; $n = 6$, 301.5 ± 37 g dL^{-1} NEFA with 8 ± 2 total embryos recovered of which 19 ± 8% were transferable; $n = 4$, 288.5 ± 58 g dL^{-1} NEFA with <7 embryos recovered of which 45 ± 4% were transferable). In experiment 2, cleavage and blastocyst rates were not significantly different ($P > 0.05$) between the groups. However, embryos exposed to the 2 lowest concentrations of NEFA showed a higher hatching rate compared with control and embryos exposed to the 3 highest NEFA levels (126 and 175.5 g dL^{-1} NEFA: 75.33, 77.67%

hatching respectively *v.* control, 979, 527.3, 393 g dL^{-1} NEFA: 48.33, 32.78, 45.33, 44.52% hatching; $P < 0.05$). However, embryo developmental rate was highly variable. Our data suggest that high plasma NEFA concentrations can have negative effects on *in vivo* and *in vitro* embryo production, whereas low levels may be beneficial.

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90 DESTABILIZATION OF COHESIN REC8 CAUSES ANEUPLOIDY AFTER THE SECOND MEIOSIS IN MURINE POST-OVULATORY AGED OOCYTES

G. Shimoi[A], *K. Kudoh*[B], *Y. Kameyama*[A], *and R. Hashizume*[A]

[A]Faculty of Bioindustry, Tokyo University of Agriculture, Abashiri, Hokkaido, Japan;
[B]Hachinohe Industrial Research Institute, Aomori Prefectural Industrial Technology Research Center, Hachinohe, Aomori, Japan

We have previously reported that the early embryos derived from post-ovulatory aged oocytes frequently exhibit aneuploidy, resulting from abnormalities in the cleavage apparatus of MII oocytes. Other studies have also described a potential mechanism that results in aneuploidy, which is attributed to the failure of a cell cycle checkpoint. The spindle assembly checkpoint (SAC), which acts during metaphase, is a monitoring system that equally distributes sister chromatids by correctly attaching spindle fibres to the appropriate centromere. Cohesin, a functional protein complex of the SAC, includes the REC8 subunit, and acts as an adhesion factor for sister chromatids. Segregation of sister chromatids occurs following the degradation of the cohesin complex by the separase enzyme. The segregation process can be mediated by meiosis-specific REC8, which contains a recognition site for separase. In this study, we examined the expression of meiosis-specific REC8 protein in murine post-ovulatory aged oocytes, and verified the association with aneuploidy induced during second meiosis. Superovulated oocytes from the ICR mouse strain were aged by culture for 3 to 24 h *in vitro*. To eliminate the male genome factor, chromosomal analysis was performed using oocytes activated by $SrCl_2$, without fertilization. The expression level of REC8 in oocytes, before and after activation, was analysed by Western blot, using a rabbit anti-REC8 antibody (primary) and horseradish peroxidase-conjugated anti-rabbit antibody (secondary). In the 6- and 12-h aged groups, 23.8% and 40.3% of oocytes, respectively, exhibited aneuploidy after the second meiosis. The rate of aneuploidy in the 12-h aged group was significantly higher than that in the fresh oocyte group (10.3%; $P < 0.05$). It could be speculated from our previous data that this fact contributed to the occurrence of aneuploidy in early embryos derived from the aged oocytes. In 3-, 6-, 12-, and 24-h aged groups, the results of semiquantitative analysis of REC8 levels in MII oocytes (non-activated) were 1.32 ± 0.38, 1.30 ± 0.58, 1.15 ± 0.21, and 0.98 ± 0.14, respectively. REC8 levels in the 24-h aged group were significantly lower than in the fresh group (1.86 ± 0.56, $P < 0.05$). The expression levels of REC8 in activated oocytes at 3 and 6 h were 0.53 ± 0.01 and 0.55 ± 0.04, respectively. REC8 levels in the 12-h (1.00 ± 0.03) and 24-h (0.95 ± 0.04) groups were significantly higher than in the fresh group (0.49 ± 0.09; $P < 0.05$). The significant reduction of REC8 levels at anaphase, after oocyte activation, was not observed in oocytes aged 12 h or more. In MII oocytes, REC8 levels tend to decrease gradually with post-ovulatory age. Destabilisation of the cohesin REC8 subunit may contribute to the nondisjunction of sister chromatids during second meiosis in post-ovulatory aged oocytes, ultimately resulting in aneuploidy.

91 SILDENAFIL CITRATE MODIFIES FETOPLACENTAL DEVELOPMENT IN A RABBIT MODEL OF INTRAUTERINE GROWTH RESTRICTION

J. López-Tello[A], *M. Arias-Alvarez*[B], *A. González-Bulnes*[C], *S. Astiz*[C], *R. M. García-García*[D], *M. Rodríguez*[A], *P. L. Lorenzo*[D], *and P. Rebollar*[A]

[A]Dpto. Producción Animal, ETSIA, UPM, Madrid, Spain;
[B]Dpto. Producción Anima, Facultad Veterinaria, UCM, Madrid, Spain;
[C]Dpto. Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, Madrid, Spain;
[D]Dpto. Fisiología Animal, Facultad Veterinaria, UCM, Madrid, Spain

The failure of fetuses to achieve their full growth potential is known as intrauterine growth restriction (IUGR). Sildenafil citrate (SC) is a phosphodiesterase 5 (PDE-5) inhibitor, which enhances nitric oxide (NO)-dependent vasodilatation, and it may have a potential therapeutic role in the treatment of IUGR. The aim of this study was to evaluate the effect of SC on placental and fetal development in a diet-induced rabbit model of IUGR. A total of 24 rabbits does weighing 4.3 ± 0.49 kg on average were used. At Day 9 of pregnancy, females were randomly allocated into 3 experimental groups: one group was fed ad libitum during pregnancy (Group C; $n = 8$); the rest of the does had 50% restricted daily intake and were treated or not with 20 mg of SC daily from Day 22 of pregnancy until parturition (Groups SC and R, respectively, $n = 8$ for both). At Day 28 of pregnancy, half of the pregnant does from each group were euthanised to study fetoplacental development, while the remaining does were allowed to deliver. At Day 28, weight, length, and thickness of fetal and maternal placentas, and fetal weight and size [crown-rump length (CRL), and transversal thoracic diameter (TD)] were assessed. A fetus was considered IUGR when it weighted less than the 10th percentile for its normal gestational weight. Statistical analysis was performed using the PROC GLM procedure. Nutritional restriction induced a higher rate of fetuses IUGR than control group (31.0% *v.* 15.1%; $P < 0.05$). The percentage of fetuses with IUGR was 23% in SC group (no significant differences with groups C and R). However, SC increased the thickness of maternal and fetal placentas compared to group R (0.4 ± 0.02 *v.* 0.2 ± 0.02 cm; 0.6 ± 0.02 *v.* 0.3 ± 0.02 cm; $P < 0.05$ respectively), being similar to group C (0.4 ± 0.02 and 0.5 ± 0.03 cm). Maternal placental weight in group C showed higher values (1.5 ± 0.08 g; $P < 0.05$) than both restricted groups (1.2 ± 0.07 g). CRL in group SC was larger than in group R (10.5 ± 0.12 *v.* 10.0 ± 0.12 cm; $P < 0.05$) and similar to that in group C (10.5 ± 0.15 cm). The neonates in group SC showed higher values for CRL (10.9 ± 0.15 cm) than those from groups R and C (10.5 ± 0.11, 10.2 ± 0.20 cm; $P = 0.05$). Regarding TD, fetuses in group SC showed higher values than group R (2.3 ± 0.04 *v.* 2.1 ± 0.03 cm; $P < 0.05$) and equaled

that of group C (2.3 ± 0.03 cm). In conclusion, maternal malnutrition prejudices fetoplacental development, causing IUGR. Treatment with SC in the last third of gestation counteracts fetal growth retardation by favouring placental development and function and, thus, fetal growth. These results confirm that administration of SC may have a potential benefit in pregnancies complicated by placental insufficiency and IUGR.

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92 NUCLEAR INVAGINATIONS ADAPT TO RABBIT EARLY EMBRYONIC DEVELOPMENT

J. Popken[A,B], *M. Dahlhoff*[B], *T. Guengoer*[B], *V. J. Schmid*[C], *A. Strauss*[D], *T. Cremer*[A], *V. Zakhartchenko*[B], *and E. Wolf*[B]

[A]Division of Anthropology and Human Genetics, LMU Biocenter, Planegg-Martinsried, Bavaria, Germany;
[B]Chair for Molecular Animal Breeding and Biotechnology, Gene Center, LMU, Munich, Bavaria, Germany;
[C]Institute of Statistics, LMU, Munich, Bavaria, Germany;
[D]Division of Genetics, LMU Biocenter, Planegg-Martinsried, Bavaria, Germany

Nuclear invaginations carrying nuclear pores may facilitate increased mRNA export and protein import to areas inside the nucleus remote from the nuclear border. In this study on rabbit embryos, we investigated whether large early embryonic nuclei and the increased import/export demands around major embryonic genome activation (MGA) at the 8-cell stage affected the quantity of nuclear invaginations carrying nuclear pores. To achieve this objective, we used super-resolution 3-dimensional structured illumination microscopy on 10 pronuclei or nuclei per stage of 23 *in vivo*-fertilized and *in vitro*-cultured embryos stained with antibodies for the nucleoporin NUP153 and lamin B and stained with 4′,6-diamidino-2-phenylindole (DAPI) for chromatin. Statistical comparisons between stages were performed using the Wilcoxon rank-sum test. At the zygote stage, the female pronucleus displayed on average 16.5 and the male pronucleus featured on average 15.25 wide and narrow nuclear envelope invaginations, carrying large or tiny amounts of cytoplasm. Subsequent stages showed predominantly wide invaginations targeting nucleoli. The contact areas between nucleoli and invaginations were free of nuclear pores. In contrast, narrow invaginations, which are the almost exclusive type of invaginations during cattle and mouse pre-implantation development, were not in contact with nucleoli. At the 2-cell stage, the number of invaginations increased to an average of 27.3 invaginations per nucleus ($P < 0.05$) and increased again to peak at the 4-cell stage with 51.2 invaginations per nucleus ($P < 0.01$). At the 8-cell stage (MGA), the amount of nuclear invaginations was reduced to 25.4 invaginations per nucleus ($P < 0.01$). The reduced number of nuclear invaginations at the 8-cell stage could be associated with a significant decrease in average nuclear volume from 2593 μm^3 at the 4-cell stage to 960 μm^3 at the 8-cell stage ($P < 0.001$) and a subsequent reduced average distance from areas inside the nucleus to the nuclear border. Nuclear invagination numbers continued their decline at the 21-cell stage with 5.2 invaginations per nucleus ($P < 0.001$), whereas nuclear volumes decreased to 618 μm^3 ($P < 0.001$). The morula stage, with 6.9 invaginations per nucleus ($P = 0.9$), and the blastocyst stage, with 4.5 invaginations per nucleus ($P = 0.5$), showed no more significant changes. Large NUP153 cytoplasmic clusters present before MGA may represent a maternally provided NUP153 deposit. MGA may mark the switch from the use of a NUP153 deposit to on-demand production. Additionally, over- and under-representation analyses on mitotic blastomeres revealed that NUP153 association with chromatin is initiated during metaphase before the initiation of the regeneration of the lamina ($P < 0.001$; chi-squared goodness-of-fit test). In conclusion, rabbit embryonic development is accompanied by stage-dependent changes of the amount of nuclear invaginations carrying nuclear pores. Although cattle and mouse embryos almost exclusively feature narrow invaginations restricted to the nuclear periphery and not in contact with nucleoli, rabbit embryos feature additional wide invaginations that can reach across the nucleus and target nucleoli.

93 THE INVOLVEMENT OF E-CADHERIN IN THERMOPROTECTIVE FUNCTION OF INSULIN-LIKE GROWTH FACTOR-1 IN 4-CELL HAMSTER EMBRYOS

A. C. Trejo[A], *I. B. Abad*[A], *V. M. V. Meza*[A], *A. M. Villa*[B], *J. Z. Abad*[C], *M. C. Navarro-Maldonado*[C], *and D. G. Ambriz*[A]

[A]Univeridad del Papaloapan, Loma Bonita, Oaxaca, México;
[B]Benemerita Universidad Autónoma de Puebla, Tecamachalco, Puebla, México;
[C]Universidad Autónoma Metropolitana, Iztapalapa, Distrito Federal, México

Studies have demonstrated that the early pre-implantation embryo is very sensitive to effects of heat stress *in vitro*. Heat stress reduces the total cell number in blastocysts and increases apoptosis in blastomeres. Insulin-like growth factor-1 (IGF-1) has been widely studied as a thermoprotective agent for its anti-apoptotic actions. Addition of IGF-1 to the culture medium decreases the effects of heat stress on blastocysts but has no effects on 2-cell embryos. Molecular mechanisms by which IGF-1 decreases apoptosis involve activation of the PI3K/Akt pathway. It is also known that adherens junctions contribute to PI3K/AKT activation mediated by the transmembrane glycoprotein E-cadherin, which is involved in Ca^{2+}-dependent cell-cell adhesion. Within 2- to 8-cell embryos, E-cadherin is mainly inactive and has cytoplasmic localization. 6-Dimethylaminopurine (6-DMAP) induces premature cell flattening and E-cadherin redistribution to adhesion sites in 4-cell embryos. The aim of this study was to induce E-cadherin redistribution in 4-cell hamster embryos and evaluate the thermoprotective function of IGF-1 in these embryos. Four-cell embryos were incubated in the presence of 6-DMAP to induce E-cadherin redistribution to adhesion sites and cultured for 24 h under conditions of heat stress and compared with controls without 6-DMAP. Culture medium was supplemented with IGF-1. At the end of culture, developmental stage and rate of apoptosis were determined and analysed by ANOVA using the General Linear Model (GLM) of SAS (SAS Institute Inc., Cary, NC, USA) procedure with statistical significance at $P < 0.05$. E-Cadherin redistribution induced by 6-DMAP increased development to the 6-cell stage after 24 h (63.57% *v.* 38.81%, respectively; $P < 0.05$) and reduced apoptosis (25% *v.* 33%, respectively; $P < 0.05$) under heat-stress conditions. In conclusion, we hypothesise a role for E-cadherin-mediated cell flattening in promoting IGF-1-mediated thermoprotection in pre-compact 4-cell hamster embryos. Further studies are required to confirm this link.

94 CHRONOLOGICAL TRANSITION OF GONOCYTES TO SPERMATOGONIAL STEM CELLS DURING PREPUBERTAL AND PUBERTAL PERIODS IN DOMESTIC CATS

N. Tiptanavattana[A], *A. Radtanakatikanon*[B], *S. Buranapraditkun*[C], *P. Hyttel*[D], *H. M. Holmes*[D], *P. Setthawong*[A], *M. Techakumphu*[A], *and T. Tharasanit*[A]

[A]Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; [B]Pathology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand; [C]Allergy and Clinical Immunology Unit, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; [D]Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences (SUND) University of Copenhagen, Frederiksberg C, Denmark

The pubertal age of domestic cat (*Felis catus*) as defined as a complete spermatogenesis has been reported to occur around 8 months of age. During the initial phase of testicular development, the transition of gonocytes to spermatogonial stem cells (SSC) takes place within the seminiferous cords. This stage-specific transition has been demonstrated to facilitate SSC isolation and enrichment. Because information for this aspect in domestic cats is limited, this study aimed to identify the phase transition of gonocytes to SSC during newborn to puberty. Cat testes were collected and classified by age into 3 groups: group 1: 0–4 months ($n = 5$), group 2: 4–6 months ($n = 5$), and group 3: 6–12 months ($n = 5$). Testes were studied for conventional histology, transmission electron microscopy (TEM), and FACS analysis on GFRα-1 expression, a SSC marker. For histology, tissues were fixed, sectioned, and stained with H&E. Serial changes of germ cell development within the testes were observed using light microscopy. In addition, ultrathin sections (60 nm thickness) of testes were cut and examined with TEM for ultrastructure analysis. Immunolabelling and flow cytometry of GFRα-1 were used to identify the SSC population after testicular cell dissociation. The percentages of spermatogonia per tubule were analysed by one-way ANOVA, and data are presented as mean ± s.e. The development of testicular germ cells from gonocyte to spermatozoon was gradually demonstrated in histological sections, depending on age of the cats. For group 1, the gonocytes were clearly presented in the seminiferous cord. These gonocytes were in proliferative phase, as they frequently contained homogeneous euchromatin and less organelles. In group 2, the gonocytes transformed to spermatogonia as indicated by their small size (range 8.11–13.55 μm) with oval to flattened shape, chromatin condensation, and darkened cytoplasm. These cells migrated and settled onto the basement membrane of seminiferous cord. At this stage, mitochondria and small clumps of heterochromatin increased when compared with group 1. Some spermatogonia occasionally developed through the meiosis by 6 months of age (group 2), whereas complete spermatogenesis was first identified in 9-month testes (group 3). The percentage of spermatogonium/tubule in group 2 (15.84 ± 0.67) was significantly higher ($P < 0.001$) than group 1 and 3 (1.99 ± 0.22 and 6.88 ± 0.53, respectively). Because the SSC-like cells (based on their histological morphology) were predominantly found in group 2, the testes ($n = 5$, 4–6 months of age) were additionally digested to confirm GFRα-1 expression. Of total testicular cells, a high proportion of GFRα-1 positive cells ($12.32 \pm 6.31\%$) were identified by FACS. In conclusion, this study provides information regarding the age-dependent development of testicular germ cells in domestic cats. The findings provide the transition period of gonocytes to SSC that occurs around 4 to 6 months of age. This study can be applied for the enrichment of feline SSC upon testicular digestion.

95 DOSAGE COMPENSATION OF X CHROMOSOME INACTIVATION CENTER (XIC)-LINKED GENES IS ALREADY ACHIEVED IN PORCINE BLASTOCYST

J. Y. Hwang[A], *J.-N. Oh*[A], *D.-K. Lee*[A], *C.-H. Park*[B], *and C.-K. Lee*[A]

[A]Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, Korea; [B]Institute of Green Bio Science and Technology, Seoul National University, Kangwon-do, Korea

X-chromosome inactivation (XCI) is an epigenetically essential process for balancing dosage of X-linked genes between male and female eutherian. Importance of this complex and species-specific event has been highlighted recently in developmental and stem cell biology. However, the process has been confirmed only in restricted species, even though the species-specific studies are needed for comprehensive understanding of XCI in specific species. XCI is regulated by the various genes, many of which are coded on the X chromosome inactivation centre (XIC). Among the XIC-linked genes, especially non-coding RNA (ncRNA) like *XIST*, which is master gene for XCI, are known to regulate XIC. But the centre is not identified in various species. In this study, we identified XIC in pig and analysed the dosage differences of XIC-linked gene in porcine embryos. At first, the centre was searched in pig. The genomic length of the porcine XIC was similar to human XIC and the order and coding strand of the counterparts in pig XIC were same as the human XIC-linked genes. However, sequence comparison between human XIC-linked gene and its porcine counterpart showed that ncRNA around *XIST* were less conserved rather than protein-coding genes. This would be caused by rapid evolution of genomic region harboring ncRNA. The expression of XIC-linked genes was compared between male and female porcine embryonic fibroblast (PEF) to confirm that dosage compensation is completed in PEF. Most of the genes were not expressed sex-specifically, but two genes, *XIST* and an uncharacterized gene, *LOC102165544*, were expressed female preferentially in PEF. Interestingly, *LOC102165544*, which had low sequence homology with human *JPX*, was expressed about 2-fold higher in female PEF. This means that *XIST* and *LOC102165544* are XCI-escaping genes. Among the XIC-linked genes, *CHIC1*, *XIST*, *LOC102165544*, and *RLIM* were stably expressed in embryonic stage, and *XIST* and *LOC102165544* were up-regulated after morula formation. As *XIST* accumulation is a requisite for XCI initiation, expression levels of the 4 genes between male and female blastocysts were compared. Interestingly, expression levels of *CHIC1* and *RLIM* were not different in male and female blastocysts. This means their dosage would be already compensated in porcine blastocyst. Additionally, to confirm loci of the 2 genes *CHIC1* and *RLIM* harbor one of the inactive alleles in female blastocyst, the DNA methylation pattern was examined. One of the *CHIC1* alleles was inactive but *RLIM* CpG site was hypo-methylated in female blastocyst. This would indicate that one of the *RLIM* alleles is transcriptionally inactivated by chromatin modification rather than by DNA methylation of the allele. Regulatory regions of *XIST* and *LOC102165544* were demethylated in blastocyst and this showed XCI was not finished in porcine blastocyst. Conclusively, our results demonstrate the XCI already occurs in porcine blastocyst at least one gene but it is not completed.

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96 EFFECT OF *MOF* GENE ON PREIMPLANTATION DEVELOPMENT OF PIG PARTHENOGENETIC EMBRYOS

R. Wu, D. Song, Z. Cao, Y. Li, F. Fang, and Y. Zhang

College of Animal Science and Technology, Anhui Agricultural University, Hefei, Anhui Province, China

The *Mof* gene (males absent on the first) is crucial to X-chromosome dosage compensation in the fly. It acts specifically to catalyse acetylation of histone H4 lysine 16 (H4K16ac) as one histone acetyltransferase of the MYST family, playing essential roles during mammalian development. However, little is known about *Mof* gene in pigs. The present study was designed to explore effects of *Mof* on pre-implantation development of pig parthenogenetic embryos obtained as reported by Cao *et al*. (2012 Zygote *20*, 229–236). Immunofluorescent staining was performed to examine protein expression level of porcine Mof (pMof) and H4K16ac, and fluorescent intensity was measured by Image J software (NIH, Bethesda, MD, USA). Data are presented as mean ± standard error, and statistical analyses of the fluorescent intensity value, embryo development rate, and quality were performed using ANOVA with SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA), and *P*-value <0.05 was considered significant. First, the coding sequence (CDS) of the pMof gene was cloned and the spatio-temporal expression patterns of the CDS were determined in pig oocytes, early embryos, and other tissues. A 1471-bp-long cDNA of pMof was obtained with 99.34% and 98.25% amino acid sequence homology and 92.88% and 88.96% nucleotide sequence homology to the human and mouse *MOF* homologues. We observed that pMof is expressed predominantly in oocytes and early embryos but at low levels in sperm and other organs. We found that pMof decreased from pronuclear to 8-cell stages and remained low until the blastocyst stage based on RT-qPCR results ($n = 10$ for each stage embryos). In contrast, pMof protein expression as examined by immunofluorescent staining ($n = 15$ for each stage embryos) remained high throughout the pre-implantation development period. After porcine embryonic genome activation, pMOF remained detectable in 4-cell ($n = 10$) and 8-cell ($n = 10$) embryos despite amanitin treatment for 24 h. Thus, the mRNA level of *MOF* was not decreased after transcription inhibition suggesting that *MOF* is a maternal gene. To assess functional significance, we examined the expression of H4K16ac, a target of pMof, and found that the level of H4K16ac was constantly low from pronuclear to morula stage, but increased dramatically in blastocysts. When we knocked down pMOF by cytoplasmic injection of siRNA into porcine MII oocytes, rate and cell number of blastocysts declined significantly [blastocyst rate: uninjected ($n = 228$) *v.* negative-siRNA ($n = 220$) *v.* MOF-siRNA ($n = 230$) $= 65.8 \pm 3.75\%$ *v.* $57.5 \pm 4.30\%$ *v.* $46.3 \pm 5.72\%$; cell numbers: uninjected *v.* negative-siRNA *v.* MOF-siRNA $= 84.73 \pm 5.25$ *v.* 77 ± 5.50 *v.* 55.08 ± 6.56]. A marker for DNA double-strand breaks and repair, γ-H2AX, increased in parallel to more apoptotic cells. Knockdown of MOF reduced H4K16ac. Overall, pMof is highly conserved among human, mouse, and pig; pMof is essential to pre-implantational development of pig parthenogenetic embryos involved in regulating H4K16ac.

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97 MAGNITUDE AND SPECIFICITY OF EFFECTS OF MATERNAL AND PATERNAL GENOMES ON THE FETO-PLACENTAL UNIT

R. Xiang[A,B], *C. A. S. Estrella*[A,B], *C. J. Fitzsimmons*[A,B], *Z. A. Kruk*[A,B], *D. A. Thomsen*[A,B], *D. L. Rutley*[A], *K. L. Kind*[A,B], *C. T. Roberts*[B,C], *and S. Hiendleder*[A,B]

[A]JS Davies Epigenetics and Genetics Group, School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy Campus, Roseworthy, South Australia, Australia;
[B]Robinson Research Institute, The University of Adelaide, Adelaide, South Australia, Australia;
[C]School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, South Australia, Australia

The placenta, a major determinant of fetal growth in eutherians, facilitates maternal-fetal cross talk and mediates programming of postnatal phenotype via genetic and epigenetic mechanisms. However, magnitude and specificity of effects of maternal and paternal genomes on placental and fetal phenotype and their relationships are unclear. Using an outbred bovine intra-species model with well-defined *Bos taurus taurus* and *Bos taurus indicus* maternal and paternal genetics, we generated purebred and reciprocal cross fetuses (Animal Ethics No. S-094-2005) to dissect and quantify effects of parental genomes, fetal sex, and nongenetic maternal effects (maternal weight and post-conception maternal weight gain) on 41 gross and histomorphological feto-placental parameters. Analysis of data from 73 fetuses recovered at midgestation (Day 153) with general linear models (Xiang *et al*. 2014 JBMR http://dx.doi.org/10.1002/jbmr.2263) using the GLM procedure of R version 22.14 (R Development Core Team, Vienna, Austria) revealed that maternal and paternal genome combined explained the highest proportion of variation (47.2–99.5%) in 30 investigated parameters with significant ($P < 0.05$–0.0001) models. Fetal sex accounted for up to 32.2% ($P < 0.05$–0.0001) and nongenetic maternal effects for up to 25.1% ($P < 0.05$–0.001) of variation in 11 and 14 parameters, respectively. Partitioning of parental (epi)genome variation showed that the maternal genome predominantly contributed to variation in gross (80.3–95.7%; $P < 0.05$–0.0001) and histomorphological (51.5–82.1%; $P < 0.05$–0.0001) placental parameters, fetal weight (54.1%; $P < 0.0001$), and fetal organ weights (43.7–73.1%; $P < 0.05$–0.0001), whereas the paternal genome predominantly contributed to fetal fluids weight (73.0%; $P < 0.001$), umbilical cord weight (73.9%; $P < 0.05$) and length (73.2%; $P < 0.01$), and placental (69.6%; $P < 0.05$) and umbilical cord (83.2%; $P < 0.0001$) efficiency. Our finding that the maternal genome determined placental phenotype (i.e. nutrient source) and the paternal genome determined umbilical cord and fetal fluid phenotype (i.e. nutrient flow) is in line with predicted expression patterns of genomic imprinting effects by both maternal-offspring coadaptation (Wolf and Hager 2006 PLoS Biol. **4**, e380) and conflict-of-interest (Moore and Haig 1991 Trends Genet **7**, 45–49) hypotheses in the feto-placental unit. Furthermore, there were 4 maternal genome determined relationships between placental weights and umbilical cord phenotype ($P < 0.05$–0.0001) and 28 paternal genome and/or fetal sex-determined relationships between fetus-, organ- and fetal fluid weights and umbilical cord phenotype ($P < 0.05$–0.0001). The finding of specific relationships between placenta and fetus merging in clusters differentiated by maternal and paternal genome effects suggests the existence of (epi) genetic-regulated morphological modules within the feto-placental unit.

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98 UTERINE INVOLUTION AND VASCULAR PERFUSION DURING EARLY POSTPARTUM IN MARES

R. P. Arruda[A], *K. M. Lemes*[A], *L. A. Silva*[B], *E. C. C. Celeghini*[A], *M. A. Alonso*[A], *G. Pugliesi*[A], *H. F. Carvalho*[A], *F. J. Affonso*[A], *D. F. Silva*[A], *and T. G. Leite*[A]

[A]Laboratory of Semen Biotechnology and Andrology, Department of Animal Reproduction, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil;
[B]Department of Veterinary Medicine, Faculty of Animal Sciences and Food Engineering, University of São Paulo, Pirassununga, São Paulo, Brazil

In horses, a rapid involution of the uterus occurs right after parturition, allowing the reestablishment of a favourable uterine environment for embryo development. However, limited evidence is found regarding the vascular events during puerperal period in mares. We aimed to evaluate the morphological (size of uterus and uterine fluid content) and haemodynamic (endometrial and mesometrial vascular perfusion) characteristics of the uterine involution process. Mares ($n = 10$) were evaluated by transrectal ultrasonography from the first day postpartum (d1) to the sixteenth day after first ovulation (D0 = ovulation). For ultrasound exams, a duplex B-mode and Doppler ultrasound instrument (M5 VET; Mindray Medical International Limited, China) equipped with a transrectal transducer was used. The previously pregnant (PH) and nonpregnant (NPH) horns were individually evaluated. Data were analysed for the main effects of horns (PH and NPH), day, and their interaction, using the PROC MIXED procedure of SAS software (9.3 version; SAS Institute Inc., Cary, NC, USA). Discrete variables were analysed by ANOVA. A reduction ($P < 0.05$) in the uterine diameter was observed during the first 7 days postpartum, but the rate of uterine involution (decrease in uterine size) decreased thereafter. The involution was complete around the d21 for the NPH and around d24 for the PH. Presence of uterine luminal fluid (mm) was increased between d1 (no fluid) and d2 (31.41 ± 2.88) postpartum, followed by a decrease between d4 (30.43 ± 4.52) and d7 (10.20 ± 1.76). No fluid was observed after d16 postpartum or after the third day postovulation (D3). For endometrial and mesometrial vascular perfusion, only a day effect ($P < 0.05$) was observed. An increase in the endometrial and mesometrial vascularization was detected, respectively, between d1 and d4, and between d1 and d2. Vascular perfusion did not differ after d4 for endometrial tissue, whereas it was reduced ($P < 0.05$) between d2 and d10 for mesometrium. For the vascular perfusion after ovulation, an increase ($P < 0.05$) from D0 to D5, followed by a decrease ($P < 0.05$) between D5 and D11 and an increase ($P < 0.05$) between D11 and D14 was observed in the endometrial and mesometrial tissues. The profile of the vascular perfusion in endometrium and mesometrium after first ovulation postpartum is similar to that observed during oestrous cycles and early pregnancy, indicating a return of the uterus to the pre-pregnant uterine characteristics in mares.

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99 GLUCOCORTICOID RECEPTORS ARE EXPRESSED IN OVARIES OF NEWBORN AND ADULT FEMALE HORSES

D. Scarlet[A], *I. Walter*[B], *and C. Aurich*[A]

[A]Centre for Artificial Insemination and Embryo Transfer, Vetmeduni Vienna, Austria;
[B]Institute of Anatomy, Histology and Embryology, Vetmeduni Vienna, Austria

In contrast to other domestic animal species, *in vitro* maturation (IVM) of oocytes in the horse is still not successful. Oocytes for IVM are obtained either from slaughterhouse ovaries or via ovum pick-up from living mares. Both situations may be associated with a stress-induced glucocorticoid release. So far, neither an involvement of glucocorticoids in follicle and oocyte maturation nor the presence of glucocorticoid receptors (GCR) in ovarian tissue has been investigated in the horse. We hypothesised that GCR are expressed in equine ovarian tissue independent of the animal's age and stage of the oestrous cycle. Ovaries ($n = 40$) were collected from killed newborn female foals ($n = 10$) and killed or slaughtered adult mares ($n = 10$). For assessment of GCR mRNA expression, ovarian samples were fixed in Tissue-Tek O.C.T. Compound (*Sakura* Finetek, Zoeterwoude, the Netherlands) and stored at −80°C. Various cell populations were isolated using laser capture microdissection on cryosections. After RNA extraction, samples were analysed by qualitative RT-PCR and real time-PCR. For analysis of GCR protein, tissue was fixed in Bouin's solution and histological slides immunostained using a monoclonal antibody for GCR (Ab2768, Abcam, Cambridge, UK), followed by visualisation with diaminobenzidine. One tertiary follicle per slide (40×; light microscopy) was analysed and percentages of cells staining positive for GCR calculated. Statistical analysis was done with the SPSS Statistics 21 software (SPSS Inc., Chicago, IL, USA). Expression of mRNA for GCR was detected in oocytes, cumulus cells, granulosa, and theca cells, independent of age and stage of the oestrous cycle. In both neonates and adults, nuclei of the oocytes and cumulus cells stained positive for GCR regardless of stage of folliculogenesis. Also, GCR were constantly expressed in granulosa cells from both preantral and antral follicles. Percentage of granulosa cells staining positive for GCR (adult: 73.6 ± 3.2, fillies: $72.4 \pm 1.9\%$) was higher ($P < 0.001$) than of theca cells (adult: 56.8 ± 3.9, fillies: $57.2 \pm 1.9\%$), but not affected by age. GCR were lacking in ovarian stroma of adults but not of neonates. In periovulatory follicles from adult mares, GCR were abundant in developing luteal cells. GCR were also detected in the nuclei of luteal cells in corpora haemorrhagica and corpora lutea. Follicular atresia was associated with a decrease of GCR independent of cell type and age. This study describes for the first time the expression of GCR in horse ovaries, which are present independent of age of the animal, stage of folliculogenesis, and oestrous cycle stage. Results suggest that glucocorticoids are involved in follicular and oocyte maturation, ovulation, and luteal function in the horse. Presence of GCR in the ovaries of newborn horses suggests a role of glucocorticoids in ovarian tissue maturation. Nevertheless, detrimental effects of excess glucocorticoid secretion due to stress on follicular development, oocyte maturation, and luteal function cannot be excluded in the mare.

Early Pregnancy

100 EMBRYO SURVIVAL AND CONCEPTUS ELONGATION FOLLOWING ASYNCHRONOUS EMBRYO TRANSFER IN CATTLE

F. Randi, B. Fernandez, M. McDonald, C. Johnson, N. Forde, and P. Lonergan

University College Dublin, Dublin, Ireland

Maternal progesterone (P4) regulates early conceptus growth and development in ruminants. Early embryo transfer studies in sheep and cattle demonstrated a need for close synchrony between the embryo and the uterine environment of the recipient. However, manipulating P4 may be one way of strategically regulating the temporal changes that normally occur in the uterine environment in order to allow flexibility in the timing of embryo transfer. For example, previous studies have demonstrated that P4 administration during the first few days of the oestrous cycle facilitates pregnancy establishment with older embryos. The aim of this study was to examine the effect of embryo-uterine synchrony on conceptus elongation in cattle. Oestrous cycles of crossbred beef heifers were synchronised using an 8-day P4-Releasing Intravaginal Device (PRID Delta®, CEVA, Mountain View, CA, USA) with administration of a prostaglandin $F_{2\alpha}$ analogue (Enzaprost®, CEVA; 5 mL equivalent to 25 mg of dinoprost) given on the day before PRID removal. Heifers were checked for signs of oestrus 4 times per day commencing 30 h after PRID withdrawal. Only those seen in standing oestrus ($n = 50$) were randomly assigned to 1 of 5 treatment groups to receive Day 7 *in vitro*-produced blastocysts ($n = 10$ per recipient) (1) on Day 5 post-oestrus; (2) on Day 5, with P4 supplementation via PRID from Day 3 to 5 + 750 IU of eCG at PRID insertion; (3) on Day 5, PRID Delta from Day 3 to 5 plus 3000 IU of hCG at PRID insertion; (4) on Day 7, or (5) on Day 9. At embryo age Day 14, all heifers were slaughtered and the uterus was flushed to recover and measure conceptuses. Data are summarised in Table 1. Fewer recipients yielded conceptuses ($P < 0.05$) and fewer conceptuses overall were recovered ($P < 0.05$) following transfer on Day 5 compared with Day 7 or Day 9. Supplementation with P4 resulted in short cycles (evidenced by corpus luteum regression and/or a recent ovulation at slaughter) in 33.3 to 54.5% of recipients receiving embryos on Day 5. Mean conceptus length was greater ($P < 0.05$) following transfer to an advanced uterus. In conclusion, transfer of embryos to a retarded (Day 5) uterine environment results in poor embryo survival. Supplementation with P4 shortened the interoestrous period in a significant number of heifers. Transfer to an advanced uterine environment promotes conceptus elongation, presumably driven by P4.

Table 1. Embryo survival and conceptus length data

Treatment	Day 5	Day 5 P4/eCG	Day 5 P4/hCG	Day 7	Day 9
No. of recipients	12	11	10	10	11
No. (%) of recipients yielding conceptuses	7 (58.3)	4 (36.4)	5 (50.0)	9 (90.0)	11 (100.0)
No. (%) of conceptuses recovered	24 (20.0)	12 (10.9)	20 (20.0)	51 (51.0)	54 (49.1)
No. (%) of short cycles	1 (8.3)	6 (54.5)	3 (33.3)	1 (10.0)	0 (0.0)
Mean (± s.e.) conceptus length (mm)	0.56 ± 0.06	1.7 ± 0.34	1.5 ± 0.26	3.2 ± 0.45	21.8 ± 2.38

101 SUPPLEMENTATION WITH SUNFLOWER SEED ALTERS THE ENDOMETRIAL LIPID COMPOSITION IN BEEF COWS

C. M. B. Membrive[A,B], *M. B. Cordeiro*[B], *D. Pazzanese*[C], *G. Pugliesi*[D], *M. F. Sá Filho*[D], *and M. Binelli*[D]

[A]São Paulo State University, Dracena, São Paulo, Brazil;
[B]São Paulo State University, Araçatuba, São Paulo, Brazil;
[C]University of São Paulo, Piracicaba, São Paulo, Brazil;
[D]University of São Paulo, Pirassununga, São Paulo, Brazil

Embryo death between 15 and 19 days of pregnancy is caused by the increase in the release of endometrial prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) involved in the luteolysis process in cattle. Compounds rich in linoleic acid, such as sunflower seeds, provide lipid changes in the endometrium, and may be involved in the ability of $PGF_{2\alpha}$ biosynthesis. Previous studies observed that the conception rate increased in Nelore cows supplemented with sunflower seed for 22 days from the timed AI (66.7% *v.* 46.3%; Peres *et al.* 2008, Acta Sci. Vet. **36**, 639) and in crossbred heifers submitted to timed embryo transfer (55.66% *v.* 36.94%; Membrive *et al.* 2013 Acta Sci. Vet. **36**, 603). We aimed to test the hypothesis that supplementation with sunflower seed promotes endometrial changes in lipid composition. Thus, we compared the composition of fatty acids in endometrial tissue in cows supplemented or not with sunflower seed. Nelore ($n = 30$) cows received an intravaginal device containing progesterone (1 g; DIB, Syntex Biochemistry & Pharmaceutical Industries SA, Buenos Aires, Argentina) associated with an im injection of oestradiol benzoate (2 mg; Benzoate HC, Hertape Calier Animal Health SA, Juatuba, MG, Brazil). The devices were removed after 8 days, when cows were treated im with cloprostenol sodium (2 mg; Sincrocio®, Ourofino Animal Health Ltd., Cravinhos, SP, Brazil), oestradiol cypionate (0.5 mg; ECP®, Zoetis Ltda., São Paulo, Brazil) and eCG (300 IU; Folligon®, Intervet Veterinary Ltda., Cotia, Brazil). Two days after removal of the device, females were assigned into 6 groups to receive 1.7 kg/animal/day of 40% soybean meal, 44% crude protein (CP) + 60% sunflower seed for 6 ($n = 4$), 14 ($n = 5$) and 22 days ($n = 6$), or 53% soybean meal, 44% CP + 47% corn for 6 ($n = 4$), 14 ($n = 5$) and 22 days ($n = 6$). Both diets were formulated with 72% total digestible nutrients and 24% CP. Females were slaughtered 24 h after the end of supplementation and endometrial tissue was isolated and stored at −196°C. The fatty acids in endometrial tissue were

assessed by gas chromatography. Data were analysed by SAS Proc GLIMMIX (SAS Institute Inc., Cary, NC, USA). The fatty acid profile (54 compounds) was analysed and 43 fatty acids were present in the endometrial tissue. The lacking fatty acids in endometrial tissue were C4:0, C11:0, C12:1, C: 13:0, C13:0 iso, C13:0 anteiso, C14:0 iso, C15:1, C18:1 *trans*-16, C18:2 *cis*-12, *trans*-10, and C21:0. The fatty acids that showed a higher percentage compared with the Control group were C18:1 *trans*-10-*trans*-11-*trans*-12 and C10:1. The fatty acids that showed low percentage compared with the Control group were C15:0 iso, C20:5, C20:3n-3, C23:0, C24:0, and C22:5. In conclusion, supplementation with sunflower seed promotes changes in the endometrial lipid profile that may reduce the pregnancy loss in beef cows.

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102 SUPPLEMENTATION WITH ESTRADIOL CYPIONATE AT THE ONSET OF A SYNCHRONIZED PROESTRUS ALTERS THE UTERINE GENE EXPRESSION OF SUCKLED ANESTROUS BEEF COWS

M. F. Sá Filho[A], *G. Pugliesi*[A], *A. M. Gonella-Diaza*[A], *M. Sponchiado*[A], *M. F. Mendanha*[A], *R. S. Ramos*[A], *S. C. S. Andrade*[B], *G. Gasperin*[B], *M. D. Goissis*[A], *F. S. Mesquita*[A,C], *L. L. Coutinho*[B], *P. S. Baruselli*[A], *and M. Binelli*[A]

[A]Departament of Animal Reproduction, FMVZ-USP, University of Sao Paulo, Sao Paulo, SP, Brazil;
[B]Laboratório de Biotecnologia, Depto. de Zootecnia. ESALQ-USP, Piracicaba, SP, Brazil;
[C]Universidade Federal do Pampa, Curso de Medicina Veterinária, Uruguaiana, RS, Brazil

The present study evaluated the effect of oestradiol cypionate (ECP) supplementation at the onset of a proestrus [i.e. at progesterone (P4) device removal of the synchronization of ovulation protocol] on the global endometrial gene expression of suckled anestrous beef cows. A total of 12 suckled cows presenting absence of corpus luteum detected by ultrasound received 2 mg of oestradiol benzoate IM and an intravaginal P4 device. Eight days later, P4 devices were removed, and the cows received 500 mg of sodium cloprostenol IM. Cows were blocked according to body condition score and diameter of largest follicle (LF) at P4 device removal and were randomly assigned to receive an IM treatment with 1 mg of ECP (ECP, $n = 6$) or not (CON, $n = 6$) at the P4 device removal. All cows received 10 μg of buserelin acetate 48 h after P4 device removal and were timed AI immediately after. Cows presenting a new corpus luteum formed 6 days after the gonadotropin-releasing hormone treatment had an endometrial fragment collected by transcervical biopsy. RNA-Seq analysis was performed and the transcriptome profile was obtained. The significance of differential gene expression was assessed with the package DESEqn 2 *v.*1.2.10 and the differential expression estimation was based on the GLM followed by the Wald test. The significance threshold was established as an FDR- Benjamini-Hochberg-adjusted *P*-value of <0.1. The integrated analysis was performed using DAVID database. In total, 135 transcripts were differentially expressed between ECP and CON groups, of which 73 genes were up-regulated by ECP supplementation and 62 genes were up-regulated in the CON cows. Two pathways were overrepresented by the ECP-induced transcripts: pathways in cancer ($n = 5$ genes) and small cell lung cancer ($n = 3$ genes). On the other hand, ECP-inhibited transcripts indicated the enrichment of 3 pathways: Parkinson's disease ($n = 3$ genes), oxidative phosphorylation ($n = 3$ genes), and Alzheimer's disease ($n = 3$ genes). More specifically, ECP-induced transcripts associated with pathways in cancer [gene symbol (fold change); respectively] were *LAMC3* (1.55), *PTCH1* (1.51), *PTCH2* (1.52), *PIK3R3* (1.22), and *PIAS1* (1.18), whereas ECP-inhibited transcripts associated with oxidative phosphorylation were *ATP5F1* (1.18), *ATP5J* (1.24), and *NDUFB3* (1.37). Therefore, ECP supplementation at onset of the synchronized proestrous slightly alters the uterine transcriptome. The enriched pathways affected by the ECP supplementation described in this work need to be studied more but these results show candidate pathways that can be associated with uterine environment and receptivity and with possible regulation by oestradiol supplementation given at the onset of the proestrous.

103 CIRCULATING MICRORNAS FOR EARLY DIAGNOSIS OF BOVINE PREGNANCY

J. Ioannidis[A], *C. Ashworth*[A], *R. Raue*[B], *and X. Donadeu*[A]

[A]The Roslin Institute, Edinburgh, Midlothian, United Kingdom;
[B]Zoetis Research and Development, Brussels, Belgium

Early diagnosis of pregnancy can shorten calving intervals, improve annual milk production and increase overall profits from modern dairy herds. At present, accurate diagnosis is only possible after the third week of pregnancy. Circulating microRNAs (miRNAs) have been proposed as diagnostic biomarkers for numerous human conditions such as cancer and diabetes. Moreover, distinct circulating miRNA profiles have been associated with different stages of human pregnancy. The objective of this study was to determine whether differential miRNA profiles occur in circulation during early pregnancy (Day 24 or earlier) in cattle that could be used for diagnostic purposes. Holstein-cross heifers were oestrous-synchronised and artificially inseminated (AI, $n = 11$) or sham-inseminated (control, $n = 8$) at first detected oestrus. Plasma samples were collected on Days 0, 8, 16 and 24 after insemination. Circulating miRNA levels were independently determined in pooled plasma samples ($n = 3$ pools for each of pregnant Day 24 and nonpregnant Days 0, 8, and 16) using Qiagen qPCR arrays (Qiagen, Valencia, CA, USA) and in individual samples ($n = 11$ samples for each pregnant Days 16 and 24, and 8 samples for each of nonpregnant Days 0, 8, and 16, respectively) using Illumina miRNA sequencing. The qPCR array data were analysed using the ΔΔCq method. The miRNA sequencing data were normalised using EdgeR. Differential expression between pregnant and nonpregnant groups was determined using 2-sample *t*-tests with false discovery rate (FDR) adjustment. Differences in miRNA expression were validated by RT-qPCR. Out of a total of 191 miRNAs analysed in pooled samples using qPCR arrays, 8 were differentially expressed (<3-fold, FDR <0.1) in Day 24 pregnant heifers relative to nonpregnant heifers (Days 0, 8, and 16 combined). No miRNAs were differentially expressed (FDR >0.1) between nonpregnant time-points. Changes in levels of 11 miRNAs were validated by RT-qPCR in individual plasma samples; although expression trends for these miRNAs were the same as in pooled samples, none of the changes in individual samples were significant after FDR adjustment ($P > 0.1$). Deep sequencing (96 million miRNA reads) identified 231 miRNAs in bovine plasma. There were no significant differences

(FDR >0.1) in the expression of any miRNAs between pregnant heifers (Days 16 or 24) and nonpregnant (Days 0, 8, and 16 individually or combined). In addition, no significant differences were identified among nonpregnant time-points. In summary, we successfully performed miRNA profiling of bovine plasma using both deep sequencing and qPCR; however, we did not detect differences in miRNA expression between early pregnant (Day 16 or 24) and nonpregnant heifers. Changes in circulating miRNA levels may involve low abundance miRNAs that cannot be accurately quantified using current technology. Alternatively, changes in circulating miRNA levels may only occur later during pregnancy in cattle.

104 EXPRESSION OF ACTIVIN A AS A LOCAL REGULATOR IN THE BOVINE OVIDUCT

Y. Yamamoto, *Y. Kobayashi*, *Y. Yoshimoto*, *and K. Okuda*

Okayama University, Okayama, Japan

Activin (ACV) is known as a local regulator of several reproductive functions including follicular development and implantation in mammals. ACVA is a glycopeptide belonging to the transforming growth factor β superfamily, and is a homodimer of inhibin ßA (INHBA) subunits. Follistatin (FST), an ACV-specific binding protein, inhibits ligand-receptor binding. ACV receptor (ACVR) is a hetero-tetramer consisting of 2 kinds of protein, ACVR1 or ACVR1B and ACVR2A or ACVR2B. The oviduct provides an optimal environment for sperm capacitation, fertilization, and early embryonic development. Previous reports have demonstrated that ACVRs were expressed in bovine oocytes and embryos, and that early embryonic development is facilitated by ACVA *in vitro*. ACVA produced by the bovine oviduct may affect gametes and embryos as well as oviductal cells as a local regulator in cow. Bovine oviductal samples were classified into 6 stages of the oestrous cycle (day of ovulation; Days 2–3 after ovulation; Days 5–6; Days 8–12; Days 15–17; Days 19–21). We examined (1) protein expression of ACVA and FST in oviductal fluid collected from the ampulla and isthmus, (2) mRNA expression of *INHBA* and *FST* in the ampullary and isthmic oviductal tissues during the oestrous cycle, (3) the effects of oestradiol-17β (E2: 0.1, 1, 10 nM) and progesterone (P4: 1, 10, 100 nM) on mRNA expressions of *INHBA* and *FST* in cultured oviductal epithelial cells isolated from the ampulla and isthmus, and (4) mRNA expression of *ACVRs* in tissues and in cultured epithelial and stromal cells. The main findings were as follows: (1) Both ACVA and FST were detected throughout the oestrous cycle in the oviductal fluid of the ampulla and isthmus. (2) *INHBA* expression was higher in the isthmus than in the ampulla. *FST* expression in the ampulla was lowest at peri-ovulation, *INHBA* expression in the isthmus was highest on the day of ovulation and *FST* in the isthmus during Days 2–6 was highest. Because an increase of ACVA production and a decrease of FST production raise ACVA bioactivity, ACVA seems to be most active at peri-ovulation in both the ampulla and isthmus. (3) In the cultured isthmic oviductal epithelial cells, 10 nM E2 significantly stimulated *INHBA* expression, but tended to suppress *FST* expression. Therefore, the bioactivity of ACVA seems to be controlled by E2 during the oestrous cycle in the isthmus. (4) The expression of *ACVR1B* and *ACVR2A* was clearly detected in the tissues as well as in cultured epithelial and stromal cells. The overall findings suggest that ACVA secreted into oviductal fluid plays an important role in oviductal functions, including fertilization in the ampulla and sperm motility and viability in the isthmus. It is also suggested that ACVA acts on both epithelial and stromal cells as a local regulator of cellular functions, such as cellular proliferation and secretion in the cow.

105 EFFECT OF LYSOPHOSPHATIDIC ACID ON PROSTAGLANDIN PRODUCTION IN THE BOVINE OVIDUCT

Y. Yoshimoto[A], *Y. Yamamoto*[A], *Y. Kobayashi*[A], *I. Woclawek-Potocka*[B], *E. Sinderewicz*[B], *and K. Okuda*[A]

[A]Laboratory of Reproductive Physiology, Graduate School of Environmental and Life Science, Okayama University, Okayama, Japan; [B]Department of Reproductive Immunology and Pathology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

The oviduct is an essential organ for successful pregnancy in mammals. The transport of gametes and early embryos is mainly induced by contraction and relaxation of smooth muscle. The contraction and relaxation of bovine oviductal smooth muscle are induced by prostaglandin (PG) $F_{2\alpha}$ and PGE2, respectively. Lysophosphatidic acid (LPA), a type of phospholipid, is involved in various physiological actions such as promoting inflammation and cellular proliferation in various organs. LPA acts through at least 6 G protein-coupled receptors. Both LPA and LPA receptors are expressed in endometrium and, moreover, LPA affects PG production by the endometrium in cow. Based on the above findings, we hypothesised that LPA is locally involved in PG production by oviductal cells to promote motility of oviductal smooth muscle in cow. Oviductal samples ipsilateral to a corpus luteum or a dominant follicle at peri-ovulation (0–6 and 19–21 days after ovulation) were collected in abattoir. Messenger RNA expression of LPA receptors (*LPAR1–6*) and LPA-producing enzymes (*ATX*, *PLA1α*, *PLA1β*) was examined in ampullary and isthmic tissues. Expression in cultured epithelial and stromal cells isolated from the bovine oviduct were also examined to determine the cells possessing LPA receptors and LPA-producing enzymes. In addition, the effect of LPA (0.1, 1, 10 μM) on the expression of cyclooxygenase (*COX*)*-1* and *COX-2* (PG-synthesising enzymes) and on PGE2 and $PGF_{2\alpha}$ production by cultured epithelial and stromal cells was investigated. The significant differences ($P < 0.05$) were determined by Student's *t*-test for 2 groups, and by one-way ANOVA followed by Tukey's multiple comparison test for more than 3 groups. *LPAR1–6*, *ATX*, *PLA1α*, and *PLA1β* were expressed in both ampullary and isthmic tissues as well as in both cultured epithelial and stromal cells. The expression of *LPAR1–3* was significantly lower in the isthmic tissues than in the ampullary tissues, whereas the expression of *LPAR4–6* was significantly higher in the isthmic tissues than in the ampullary tissues. The expression of *COX-2* was significantly stimulated by 10 μM LPA in cultured isthmic stromal cells. In addition, LPA significantly stimulated both PGE2 and $PGF_{2\alpha}$ production by cultured isthmic stromal cells. In the isthmus of the oviduct, LPA produced by epithelial and stromal cells may stimulate the expression of COX-2 in the stromal cells, followed by increased PG production. Because the mRNA expression of *LPAR4–6* is higher in the isthmus than in the ampulla, those effects of LPA might be mediated by activation of LPAR4–6. The overall findings suggest that LPA is one of the regulating factors for transport of gametes and early embryos by controlling the motility of smooth muscle in the bovine oviduct.

106 THE RECEPTIVE BEEF COW ENDOMETRIUM: POTENTIAL KEY FEATURES FAVOURING THE COMMUNICATION BETWEEN EMBRYONIC AND MATERNAL TISSUES

V. Van Hoeck[A], *M. R. França*[A], *G. Gonella*[A], *A. Langbeen*[B], *G. Pugliesi*[A], *and M. Binelli*[A]

[A]São Paulo University, São Paulo, SP, Brazil;
[B]University of Antwerp, Wilrijk, Antwerp, Belgium

A receptive state of the endometrium is crucial for proper communication with the pre-implantation embryo and, thereby, a perquisite for a successful pregnancy. Both reduction of transmembrane mucin and initiation of apoptotic events have been proposed as key features prompting embryo/endometrial cell interaction and facilitating maternal receptivity of the murine and human embryo. In beef cows, however, the signature for receptivity needs to be elucidated. Therefore, we aimed to characterise "receptive" versus "refractory" endometrial tissues with the focus on (1) transcription profiles related to mucin, apoptosis and proliferative pathways, and 2) phenotypic features; that is, epithelial transmembrane mucin and apoptotic cell rates (ACR). Using a bovine model, preovulatory follicle growth was manipulated to produce 2 groups: cows with larger preovulatory follicle, longer proestrus, and higher receptivity (LF-LCL) versus cows with smaller preovulatory follicle, shorter proestrus, and lower receptivity (SF-SCL). Seven days post-induced ovulation, endometrium was collected. Transcriptome profiles of endometrial tissue ($n = 3$/group) were determined using Illumina RNA sequencing analyses. In addition, paraffin embedded endometrial samples ($n = 6$/group) were stained with Alcian Blue for semiquantitative analyses of mucin staining intensity and treated with antibodies against activated Casp3 to determine ACR. RNA-seq data showed that cell surface *MUC1* gene expression was drastically reduced (fold-change −3.33) in LF-LCL tissue ($P < 0.05$). In contrast, the LF-LCL endometrial tissue displayed an up-regulated expression of genes involved in apoptosis pathways, such as *Casp9*, *CRADD*, *DAPL1* (fold-change 1.80, 2.10, and 3.60, respectively; $P < 0.05$). Moreover, the expression of genes related to proliferation, such as *MYC* and *NOV*, was significantly down-regulated in LF-LCL endometrial tissue (fold-change −2.2 and −6.3, respectively; $P < 0.01$). Histology of endometrial samples revealed that the signal for transmembrane, anti-adhesive, mucin at the LF-LCL epithelium was consistently low, whereas the SF-SCL tissue group displayed variable amounts of transmembrane mucin at the apical epithelial boarder. Immunohistochemistry showed that gland cells from LF-LCL endometrial tissue displayed a significantly higher ACR compared with gland cells of the SF-SCL tissue (29.6 ± 1.52% *v.* 11.9 ± 1.26%; $P < 0.05$). In the surface epithelium and the stromal tissue, Casp3 positive cells were rare and ACR was similar between groups. In conclusion, a down-regulated expression of the transmembrane mucin might indicate a receptive condition of the endometrium. Furthermore, the prominent apoptotic characteristics in the LF-LCL tissue suggest that the receptive endometrium must surpass the proliferative status in order to differentiate to more specialised functions, such as embryotrophy.

107 PRODUCTION OF EPIDERMAL GROWTH FACTOR BY BOVINE ENDOMETRIAL CELLS AND ITS EFFECTS ON EMBRYONIC INTERFERONT AND PROSTAGLANDIN

T. J. Acosta[A,B] *and K. Takatsu*[A]

[A]Okayama University, Okayama, Japan;
[B]Obihiro University, Obihiro, Japan

A dynamic interaction between bioactive products of the embryo (blastocyst) and the endometrium is crucial for the successful establishment of pregnancy. In ruminants, the principal signal for maternal recognition of pregnancy is interferon-τ (IFNT) secreted by the trophoectoderm between Days 8 and 20 post-fertilization. Epidermal growth factor (EGF) produced by the endometrium acting through EGF receptors (EGFR) present in the blastocyst seems to regulate embryonic production of IFNT. Epidermal growth factor and IFNT have been shown to play crucial roles in controlling luteolytic prostaglandin (PG) $F_{2\alpha}$ (PGF) and luteotropic PGE2 production by bovine endometrium. However, it is unknown how these bioactive molecules regulate uterine function during maternal recognition of pregnancy. To clarify the main source of EGF in bovine endometrium and the mechanisms regulating the interaction between the hatched blastocyst and maternal uterine environment, the production of EGF by cultured endometrial epithelial and stromal cells and the effects of EGF on embryonic IFNT and PG were investigated. In addition, the effects of EGF on PGE2 and PGF production by cultured epithelial or stromal cells were examined. Endometrial epithelial and stromal cells were enzymatically isolated on the day of ovulation, seeded at a density of 100 000 viable cells mL^{-1}, and cultured at 38°C in a humidified atmosphere of 5% CO_2 in air. After the cells reached 90% confluence, they were cultured in the presence or absence of EGF (0.1, 1.0, 10, and 100 ng mL^{-1}) for 24 h. Cells cultured in the absence of EGF and their cultured media were collected separately for protein analysis. Hatched bovine blastocysts (Days 8–10) were also cultured and exposed to EGF (1, 10, and 100 ng mL^{-1}) for 24 h. Protein concentrations of EGF and IFNT in the cultured media were determined by commercial enzyme immunoassay kit. Hormonal concentrations were analysed by ANOVA followed by Fisher's protected least-significant difference procedure (PLSD) as a multiple comparison test by StatView (Abacus Concepts Inc., Berkeley, CA, USA). The concentration of EGF in the culture media of epithelial cells cultured in the absence of EGF was significantly ($P < 0.05$) higher than in the cultured media of endometrial stromal cells. Epidermal growth factor (10 and 100 ng mL^{-1}) increased embryonic production of IFNT and luteotropic PGE2 production but not luteolytic PGF by hatched blastocyst. EGF (100 ng mL^{-1}) increased both PGE2 and PGF production ($P < 0.05$) by cultured endometrial epithelial and stromal cells. The overall results suggest that endometrial epithelial cells rather than stromal cells are the main source of EGF. Epidermal growth factor produced by epithelial cells stimulates the production of IFNT by bovine trophoblasts. The capacity of conceptus to increase IFNT and luteotropic PGE2 production rather than luteolytic PGF in response to EGF stimulation may be essential for the establishment of pregnancy in cattle.

108 CHARACTERIZATION OF BOVINE OVIDUCTAL EXOSOMES FROM *IN VIVO* AND *IN VITRO* ORIGIN

C. Almiñana[A], *E. Corbin*[A], *G. Tsikis*[A], *C. Soleilhavoup*[A], *L. Galio*[B], *O. Sandra*[B], *and P. Mermillod*[A]

[A]INRA, UMR7247 Physiologie de la Reproduction et Comportements, Nouzilly, France;
[B]INRA, UMR1198 Biologie du Développement et Reproduction, Jouy-en-Josas, France

Successful pregnancy requires an appropriate communication between the mother and the embryo(s). Recent studies indicate that exosomes, small (30–200 nm) membrane vesicles of endocytotic origin, could act as intercellular vehicles in this unique communication system. Exosomes have been identified *in vivo* in all body fluids including follicular, uterine, and oviductal fluids and can be secreted by most cell types *in vitro*. Bovine oviductal epithelial cells (BOEC) have been thoroughly used to study embryo-maternal communication and to improve embryo development *in vitro*. Hence, our objective was to provide a morphologic and proteomic characterisation of exosomes secreted by BOEC *in vivo* in the oviductal fluid and *in vitro* in the conditioned media. Oviducts from cows were flushed to recover *in vivo* exosomes and then BOEC were scraped in order to derive primary cultures. *In vitro* exosomes were collected from conditioned media of BOEC primary cultures after reaching confluence (10 days). Isolation of exosomes from *in vivo* and *in vitro* origin was performed by ultracentrifugation. The presence of exosomes was confirmed in oviductal flushings and conditioned media by electron microscopy. Further characterisation of exosomes was carried out based on morphology (transmission electron microscopy), size (dynamic light scattering, DLS), and protein composition (protein profile analysis by SDS-PAGE and Western immunoblotting). Preliminary results by DLS revealed different size distribution profiles in exosome samples (*in vivo*: mean size of 93.41 nm; *in vitro*: 433.5 nm). Because exosomes are considered as "micromaps" of the originating cells, protein patterns expressed by *in vivo* exosomes and *in vitro* exosomes were compared with scraped and cultured BOEC, respectively. Protein profile analysis by SDS-PAGE showed quantitative and qualitative differences among the exosome samples, their cells of origin, and the milieu (conditioned media or flushing). Exosome-specific protein bands were detected and will be further characterised. In addition, exosomes from *in vivo* and *in vitro* origin exhibited distinct proteomic profiles. Western blot analysis demonstrated that (1) both exosomal protein samples were positive for HSP70, a known exosomal protein, and negative for Grp78, an endoplasmic reticulum marker detected in BOEC; (2) *in vivo* exosomes expressed oviductal glycoprotein (OVGP), heat shock protein A8 (HSPA8), and myosin 9 (MYH9), 3 oviductal proteins with known roles in fertilization and early pregnancy. However, only HSPA8 and MYH9 were detected in *in vitro* exosomes. Our results provide the first extensive characterisation of oviductal exosomes from *in vivo* and *in vitro* origin, an essential step in furthering our understanding of the early embryo-maternal cross talk.

109 EXPRESSION OF GENES RELATED TO ENDOMETRIAL RECEPTIVITY IN EQUINE ENDOMETRIUM DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

M. Hitit[A], *A. Guzeloglu*[A], *C. Ozel*[A], *M. O. Atli*[B], *E. Kurar*[C], *and S. A. Kayis*[A]

[A]Selcuk University, Konya, Turkey;
[B]Dicle University, Diyarbakir, Turkey;
[C]NEU, Konya, Turkey

A set of genes that display differential expression levels in the reproductive tract could serve as beneficial markers of endometrial receptivity. SERPINA14 is present in the uterus during pregnancy and suppresses lymphocyte accumulation. Osteopontin is the ligand of integrin β3 and enables trophoblast communication during implantation. Leukemia inhibitory factor (LIF) is involved in inflammatory cell signalling and contributes to implantation by regulating immune cells. The objective was to assess the expression of SERPINA14, osteopontin, and LIF mRNAs in the equine endometrium during the oestrous cycle and early pregnancy. Biopsies were obtained from mares on day of ovulation (d 0, $n = 4$), late diestrus (LD, $n = 4$, high progesterone [P4]), and after luteolysis at the beginning of oestrus phase (AL, $n = 4$, <1 ng mL^{-1} P4) of the cycle. Biopsies were also taken on days 14 (P14, $n = 4$), 18 (P18, $n = 4$), and 22 (P22; $n = 4$) of pregnancy. Relative mRNA expression levels of genes were quantified using real-time quantitative RT-qPCR in duplicate. Data were analysed using one-way ANOVA, and l.s.d. test was applied. Both the oestrous cycle and early pregnancy increased SERPINA14 mRNA levels compared to d0. Expression of LIF mRNA was not significantly regulated except for a decline at AL. Expression of osteopontin mRNA was up-regulated during the oestrous cycle at LD while early pregnancy inhibited this up-regulation. The results suggest that the genes studied related to endometrial receptivity are strictly regulated accordingly to the stage of oestrous cycle, probably by circulating ovarian steroids, specifically progesterone, and pregnancy-associated factors are also involved in this regulation.

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110 BARLEY SUPPLEMENTATION AT MID-GESTATION IN BROODMARES DOES NOT AFFECT FETAL DEVELOPMENT AND IS ACCOMPANIED BY MINIMAL PLACENTAL ADAPTATIONS

M. Robles[A], *P. Peugnet*[A], *C. Dubois*[B], *L. Wimel*[B], *A. Tarrade*[A], *and P. Chavatte-Palmer*[A]

[A]INRA, UMR 1198 Biologie du Développement et Reproduction, Jouy en Josas, France;
[B]IFCE, Chamberet, France

Modifications of maternal environment could alter fetal growth and development through the placenta and thus health in adulthood. The developmental origins of health and disease suggest that maternal nutrition during pregnancy may affect offspring development and subsequent energy metabolism. To understand the effect of common feeding practices during gestation, 24 saddlebred mares were allocated to 1 of 2

groups: group B was supplemented twice a day with barley (B) and group F was fed only with fodder (F) between month 7 of gestation and foaling. B mares maintained an optimal body condition score through gestation, with an increase in glycaemia and insulinemia after each meal and insulin resistance in month 9 of gestation. F mares lost condition as assessed by body condition score in the last part of gestation, leading to a moderate undernutrition and a transitional increase in nonesterified fatty acid plasma concentrations. Diets had no effect on feto-placental biometry or on placental structure. In contrast, an increase in microcotyledonary vessel volume was observed in F placentas, indicating placental adaptation, possibly to increase fetomaternal exchanges. There was no overall difference in the expression of genes involved in vascularization, nutrient transfer, growth, and development between placentas from B and F mares. Nevertheless, as seen in other species, sex-specific effects of maternal nutrition were observed in placentas from female foals, with differences in the expression of endogline, kinase insert domain receptor, insulin-like growth factor 2 and insulin-like growth factor 1 receptor genes. This study demonstrates that breeding practices such as supplementation in concentrate at mid-gestation do not seem to affect fetal development. More work is ongoing to evaluate postnatal health.

111 EXPRESSION OF PHOSPHOLIPASE A2 ISOFORMS IN EQUINE ENDOMETRIUM DURING THE ESTROUS CYCLE AND EARLY PREGNANCY

C. Ozel[A], *A. Guzeloglu*[A], *M. Hitit*[A], *M. O. Atli*[B], *E. Kurar*[C], *and S. A. Kayis*[A]

[A]Selcuk University, Konya, Turkey;
[B]Dicle University, Diyarbakir, Turkey;
[C]NEU, Konya, Turkey

Phospholipase A2 (PLA2) is involved in the synthesis of prostaglandins (PG) as it releases arachidonic acid from the membrane phospholipids to be a precursor for cyclooxygenase enzymes. Therefore, it is critically important during luteolysis and at the time of maternal recognition of pregnancy. The embryo must attenuate endometrial $PGF_{2\alpha}$ production, whereas PGE2 is considered to be luteoprotective. Furthermore, implantation also requires the action of PGs. A balance should be maintained in PG synthesis in the endometrium. The objective of this study was to evaluate the expression of isoforms of PLA2 (cytosolic [cPLA2], secretory [PLA2], and calcium independent [iPLA2]) in equine endometrium during the oestrous cycle and early pregnancy. Biopsies were obtained from mares on the day of ovulation (d0, $n = 4$), late diestrus (LD, $n = 4$, high progesterone [P4]), and after luteolysis in the beginning of oestrus phase (AL, $n = 4$, <1 ng mL^{-1} P4) of the cycle. Biopsies were also taken on days 14 (P14, $n = 4$), 18 (P18, $n = 4$), and 22 (P22; $n = 4$) of pregnancy. Relative mRNA expression levels of genes were quantified using real-time quantitative RT-qPCR. Data were analysed using one-way ANOVA and l.s.d. test. Compared with the day of oestrus (d0), steady-state levels of cPLA2 and sPLA2 were down-regulated at LD, where P4 was high, and expression of both were up-regulated again at AL. In contrast, iPLA2 expression was higher at LD and then decreased again at AL. Pregnancy decreased expression of cPLA2 and sPLA2 mRNA at P14 and P18 compared with their respective cycle days. Late diestrus elevation in the expression of iPLA2 was suppressed by pregnancy at P14; however, it was up-regulated later in pregnancy at P22. The results suggest that expression of cPLA2 and sPLA2 is negatively correlated with circulating progesterone concentrations. Pregnancy further inhibits their expression in the equine endometrium. However, iPLA2 expression seems to be positively correlated by progesterone presence and its expression increased as equine pregnancy advanced.

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112 EFFECT OF ASYNCHRONOUS EMBRYO TRANSFER ON GLUCOSE TRANSPORTER EXPRESSION IN EQUINE TROPHECTODERM

T. A. Stout, *C. Gibson*, *and M. de Ruijter Villani*

Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

Equine pregnancy is characterised by an unusually long pre-implantation period (40 days) during which the conceptus is entirely dependent on uterine secretions for nutrient provision; although glucose is an important nutrient during development post-blastocyst formation, little is known about its transport into the early horse conceptus. Equine embryos are also known to tolerate an unusually large degree of uterine asynchrony following embryo transfer (ET). However, negative asynchrony (recipient behind the donor) of more than 5 days markedly retards conceptus growth and development, and thereby offers a unique tool for studying the effect of the uterine environment on early development. In a preliminary study, we detected abundant mRNA expression for the facilitative glucose transporters (SLC2As) 1–3, 5, 8 and 10 and sodium-glucose co-transporter (SLC5A)11 in Day 14 to 28 equine conceptus membranes. In the current study, we evaluated the effect of uterine asynchrony on trophectodermal glucose transporter expression. Day 8 horse embryos were transferred to recipient mares that ovulated on the same day (synchronous; $n = 10$) or 5 days after (asynchronous; $n = 10$) the donor mare. The conceptuses were collected 6 or 11 days after ET (Day 14 or 19 of embryo development: $n = 5$ per group). Trophectodermal mRNA expression for glucose transporters was evaluated by RT-qPCR, and the effects of asynchronous ET and stage of pregnancy were analysed by two-way ANOVA followed by independent-samples *t*-tests. Gene expression for SLC2A3 and 8 was stable over time and treatment. Expression of SLC2A1 and SLC5A11 decreased between Days 14 and 19 in synchronous pregnancies only ($P < 0.05$). SLC2A2 expression increased markedly on Day 19 in synchronous ($P < 0.01$) but not asynchronous pregnancies ($P < 0.05$). SLC2A5 expression was lower in the asynchronous group on Day 14, but increased beyond expression levels in synchronous pregnancies by Day 19 ($P < 0.05$). In summary, expression of SLC2A1 and 3, the major placental glucose transporters, was not affected by asynchronous ET. The marked up-regulation of SLC2A2 expression between Days 14 and 19 of synchronous but not asynchronous pregnancy suggests a stage-specific function, whereas the increase in SLC2A5 at Day 19 after asynchronous ET could be a compensatory response to growth retardation.

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113 PRELIMINARY CHARACTERIZATION OF OXYTOCINASE IN EQUINE SERUM

M. Diel de Amorim, K. Nielsen, and C. Card

Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

Oxytocinase/insulin regulated aminopeptidase (IRAP) or leucyl-cystinyl aminopeptidase (LNPEP) is an enzyme that is involved in the regulation of hormones such as oxytocin, vasopressin, and angiotensin in both humans and sheep. Historically, very low levels of this aminopeptidase were reported in monthly samples obtained from cycling and pregnant mares using an enzymatic colourimetric method. The regulation of oxytocin in horses is of interest because of its central role in uterine clearance, luteal maintenance, parturition, passage of fetal membranes, maternal foal bonding, and milk let-down. A preliminary study was performed with the objective of re-examining the level of serum oxytocinase in nonpregnant control ($n = 3$ mares sampled every other day; EOD of the oestrous cycle), and $n = 5$ mares sampled Day 12 to 15; oxytocin-treated ($n = 2$ mares sampled Day 12 to 15), and early pregnant mares ($n = 6$ mares sampled EOD), using more sensitive ELISA methodology. Mares were examined daily in oestrus until ovulation (Day 0) and from Days 10 to 21, using transrectal ultrasonography of the reproductive tract. Palpable changes in uterine and cervical tone, ultrasound measurement of dominant follicles, oedema scores (0 to 4 with 4 being maximal oedema), and changes in luteal echotexture and size were recorded. Pregnant mares were bred using AI (>200 million motile and normal sperm) from a proven stallion while in oestrus until ovulation, beginning when the dominant follicle was >35 mm. Oxytocin-treated mares were administered 60 IU IM oxytocin SID from Day 7 to 14. Blood was collected to obtain serum. Changes in serum oxytocinase levels were measured using a commercially available ELISA kit for Horse LNPEP according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA) and validated for use in our laboratory using serial dilutions of pooled serum with an intra-assay and inter-assay CV <15%. The lowest standard of the assay was 31.2 ng mL^{-1}. Preliminary results of this pilot study demonstrated in control cycles median serum oxytocinase levels below the lowest standard (2.0 to 30.1 ng mL^{-1}). Oxytocin-treated mares had median serum oxytocinase levels from 39 to 61 ng mL^{-1} on Days 12 to 15. Early pregnant mares had detectable levels from Day 8 to 21 (medians ranging from 40 to 89 ng mL^{-1}). We concluded that serum oxytocinase levels were below the lowest standard in diestrus, and were low but detectable in oxytocin-treated mares. The highest oxytocinase levels were measured during early pregnancy. Further studies of serum oxytocinase in a larger population of mares, along with studies of tissue mRNA levels of oxytocinase, are required to better understand the regulation of oxytocin in horses.

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114 ASYNCHRONOUS EMBRYO TRANSFER AFFECTS THE EXPRESSION OF IMPRINTED GENES IN EQUINE TROPHECTODERM

C. Gibson, M. de Ruijter Villani, and T. A. Stout

Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

Gene imprinting is a form of epigenetic modification that results in parent-of-origin specific monoallelic expression. Imprinted genes play important roles during fetal-placental growth with paternally imprinted genes generally promoting and maternally imprinted genes suppressing fetal growth. Imprinted genes are therefore believed to have important effects on trophoblast differentiation and placental development, and in adjusting fetal nutrition to maternal supply. The horse is an interesting model of early placental development because of its unusually long pre-implantation period (40 days), during which the conceptus is dependent on uterine secretions for nutrient provision. Moreover, horse embryos tolerate a wide range of uterine asynchrony following embryo transfer (ET), offering a unique tool to study maternal influences on conceptus development. This study examined the effect of asynchronous ET on the expression of imprinted genes in equine trophectoderm. Twenty Day 8 embryos were transferred to recipient mares that either ovulated on the same day (synchronous; $n = 10$) or 5 days after (asynchronous; $n = 10$) the donor mare. The conceptuses were recovered 6 or 11 days after ET (Day 14 or 19 of conceptus development; $n = 5$ per group). Bilaminar trophectoderm was isolated and mRNA expression for a range of genes known to be imprinted in equine trophectoderm (*H19*, *PHLDA2*, *IGF2R*, *IGF2*, *PEG3*, *PEG10*, *SNRPN*, *INSR*, and *INS*) was investigated by RT-qPCR. The effects of asynchronous ET and stage of pregnancy on gene expression were analysed by two-way ANOVA followed by independent-samples *t*-tests. *IGF2*, *PEG3*, *PEG10*, *INSR*, *H19*, and *PHLDA2* all showed a significant up-regulation in gene expression between Days 14 and 19 of pregnancy; however, expression was higher in synchronous than asynchronous pregnancies at Day 19 ($P < 0.05$). *IGF2R* expression increased significantly from Day 14 to 19 in the synchronous pregnancies ($P < 0.05$), but did not differ between treatments at Day 19. *SNRPN* expression increased from Day 14 to 19, and was unaffected by asynchrony. *INS* mRNA was not detectable in trophectoderm. In conclusion, asynchronous ET had a significant effect on gene expression at Day 19 of gestation that was not evident at Day 14. This may be either a contributor to the delayed development that is observed in asynchronous pregnancies or a result/response; in either case, it may affect subsequent development.

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115 CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT (CRISPR)/CAS9-DIRECTED INACTIVATION OF PORCINE INTERLEUKIN-1B AS A MODEL TO STUDY ENDOMETRIAL RECEPTIVITY AND CONCEPTUS ATTACHMENT

J. J. Whyte, M. E. Hennessy, R. D. Geisert, and R. S. Prather

University of Missouri, Columbia, MO, USA

Embryonic losses in livestock range from 20 to 40%, with two-thirds of these losses occurring in the peri-implantation period. An understanding of successful pregnancy establishment in pigs is important for translational research and commercial pig production. Failure of conceptus-maternal communication is a major contributor to this loss, yet the molecular control of this process remains unclear. Engineered nucleases such as the bacterial