35th Annual Symposium in Reproductive Science and Women's Health
Oral Presentation Abstracts

Abstract Title:	**Expression of Sex Hormone-Binding Globulin in the Horse: Preliminary qPCR Analysis**
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Abstract: Sex hormone-binding globulin (SHBG) is a glycoprotein that binds sex steroids. Androgens have the highest affinity for SHBG, and SHBG acts as their main carrier, regulating androgen bioavailability. There are at least two known isoforms of this protein, SHBG which is found in circulation and androgen-binding protein (ABP) which is found in the reproductive tract. Expression of SHBG seems to be species specific and varies according to age, gender, and pregnancy status. SHBG is expressed in liver in most species; however, there is no previous literature for SHBG in the horse. Our objective was to quantify the expression of mRNA for SHBG in the horse using qPCR in liver (stallion, mare, pregnant mare, fetal), kidney (stallion, mare, fetal), testis, and epididymis. All tissue was homogenized, and RNA was extracted using Trizol. Extracted RNA was DNAse treated using the DNA-free kit by Applied Biosystems and reverse transcribed to cDNA using MultiScribe RT by Invitrogen. Primers were designed using Primer-BLAST based on in silico Equus caballus mRNA Refseq (Gene ID:100073011). GAPDH and B2M were used as house-keeping genes. mRNA expression was detected in testis [(ΔCt =5.06±1.3) mean±STD] and stallion kidney (ΔCt=9.86). mRNA expression was negligible in all other tissues. To our knowledge, this is the first report that identifies SHBG in the horse; however, there was no expression of SHBG in liver at any of the stages evaluated, which seems to contradict expression patterns found in other mammals. Further experimentation is required to purify SHBG from circulation and more completely characterize its expression in the horse.

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35th Annual Symposium in Reproductive Science and Women's Health
Oral Presentation Abstracts

Abstract Title:	**Effects Of Urea Infusion On Uterine Environment On Mares During Diestrous**
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Abstract: High levels of dietary crude protein can elevate plasma urea nitrogen concentrations (PUN) in association with a decrease in intrauterine pH. Studies demonstrate that elevations in PUN can reduce fertility in cattle. To our knowledge, there are no publications in horses on the influence of elevated PUN on fertility. Our hypothesis is that intravenous infusion of urea in mares will acutely elevate PUN concentrations, decrease uterine pH and alter gene expression in endometrial tissue. Mares on day 7 after ovulation (n=10 per group) will be intravenously infused for 24 hours with saline or urea (0.01 g of urea/h per kg of body weight at a rate of 0.5 mL/min). Blood samples will be collected every 2 hours in order to measure serum progesterone and PUN, with ELISA and colorimetric spectrophotometric assay, respectively. Intrauterine pH will be measured using an adapted epoxy pH probe immediately after the infusion period. Additionally, an endometrial sample will be collected by transcervical endometrial biopsy for immunohistochemistry, histology and to examine changes in gene expression with RNA sequencing. Data will be analyzed using the mixed model procedure of SAS, analyzing the effect of treatment and the interaction of treatment and time. Data will be presented as mean and standard error. This study will elucidate the relationship between acute increase of PUN with lower uterine pH, lower progesterone levels and changes in the endometrial transcriptome. Therefore, this research will bring novel information that can be used in determining the ideal crude protein requirements for broodmares.

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Abstract Title:	**Regulatory Mechanisms of LH-induced Increases in Progesterone/PGR, EGF-like Factors, and Prostaglandins in Human Periovulatory Granulosa Cells**
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Abstract: The LH-surge stimulates production of autocrine/paracrine mediators including progesterone (P4), prostaglandins (PGs), and EGF-like factors to trigger ovulation. In the human ovary, however, little is known about mechanisms by which the LH surge induces these mediators and how these mediators coordinate ovulatory changes in periovulatory follicles. The present study explored these questions using in vivo and in vitro models. Pre- and periovulatory follicles were obtained before the LH surge and at early and late ovulatory phases after hCG administration in normally cycling women. Immunohistochemistry analysis showed that PGR is localized exclusively to granulosa cells of periovulatory follicles with intensive signals in late ovulatory follicles. In vitro human primary granulosa cells were isolated from IVF patients, acclimated in culture for 6 days, and treated with hCG. Real-time PCR analysis showed that hCG increased the levels of PGR, EGF-like factors, AREG and EREG, and PG synthases, PTGS2, PTGES, and AKR1C1, and transporters, ABCC4 and SLCO2A1. ELISA confirmed the increase in media concentrations of P4, PGE2, and PGF2a by hCG. To determine the function of hCG-induced P4/PGR and EGF-like factors, the cells were treated with RU486 (PGR antagonist) or AG1478 (EGFR tyrosine kinase inhibitor) ± hCG. Both RU486 and AG1478 suppressed the hCG-induced increases in mRNAs for EGF-like factors and PG synthases and transporters, and also abolished hCG-induced accumulation of PGE2 and PGF2a. This study documented that hCG increases 3 key mediators, P4/PGR, EGF-like factors, and PGs. Together, these data demonstrate for the first time complex cross-regulation among 3 key ovulatory mediators in human periovulatory follicles.

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35th Annual Symposium in Reproductive Science and Women's Health
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Abstract Title:	**Exogenous lactoferrin suppresses the expression tumor necrosis factor (TNF) in mares susceptible to persistent breeding-induced endometritis**
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Abstract: In the horse, breeding induces a transient innate immune response, known as mating-induced endometritis. There is a subset of mares that are unable to resolve this inflammation, and they are considered susceptible to persistent mating-induced endometritis (PMIE). These susceptible mares have been shown to have an abnormal immune response to breeding. At 6 hours post-breeding, the expression of the anti-inflammatory cytokines IL-1RN, IL-10, and IL-6 are suppressed in these mares, and a residual PMN infiltration is noted as long as 72 hours after insemination. In vitro, select seminal plasma proteins, specifically cysteine-rich secretory protein-3 (CRISP-3) and lactoferrin have been shown to affect the interaction between PMNs and spermatozoa, either by suppressing (CRISP-3) or promoting (lactoferrin) the binding between PMNs and spermatozoa based on their viability. The objective of this study was to determine if the addition of these select proteins had an effect on the mRNA expression on endometrial cytokines in susceptible mares after breeding. Six mares classified as susceptible to PMIE were bred during four consecutive estrous cycles with treatments in randomized order of: 1mg/mL CRISP-3, 150 ug/mL lactoferrin, seminal plasma, or lactated ringer's solution (LRS) to a total volume of 10mL combined with 1x109 spermatozoa pooled from two stallions. Six hours after treatment, an endometrial biopsy was obtained for qPCR analysis of selected genes associated with inflammation. Seminal plasma significantly increased the expression of the pro-inflammatory cytokines IL-1β and IL-8, while significantly suppressing the expression of TNF. Lactoferrin also suppressed the expression of TNF in comparison to the negative control of LRS and CRISP-3. In conclusion, seminal plasma had an overall effect on the cytokines studied at 6 hours after insemination. In addition, the seminal plasma protein lactoferrin also suppressed the expression of TNF, and may be considered as a modulator of the unregulated pro-inflammatory response seen in susceptible mares.

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35th Annual Symposium in Reproductive Science and Women's Health
Oral Presentation Abstracts

Abstract Title:	**Effects of Feeding a Yeast-Based Supplement Containing Selenized Yeast, Vitamin E and a DHA-Rich Microalgae on Sperm Motion Characteristics**
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Abstract: The use of frozen stallion semen has become quite popular. However, there are some stallions that have sperm that are quite susceptible to cold shock. Thus, there is a need for techniques that will alter sperm so that they can withstand the stress of freezing and thus improve pregnancy rates. Studies have shown that a diet high in omega-3 (n-3) fatty acids can improve the motility of cooled and frozen/thawed sperm. Many of the omega 3 fatty acid products for stallions have low levels of docosahexaenoic acid (DHA) and are based on fish oil, which may have reduced palatability. The objectives of this study were to determine if a DHA-rich microalgae meal would enhance the motility of fresh, cooled, and frozen-thawed stallion sperm. Twelve stallions, 3 to 12 y old were used. Semen was collected every other day for two weeks (July) and sperm motion parameters (total and progressive motility) were determined by computer assisted motility sperm analysis (CASA) on the last three ejaculates. These ejaculates were cooled to 5C (Equitainer, Hamilton Thorne) and held for 48 hr. Stallions were then paired based on CASA values for fresh and cooled semen, age of stallion, sperm output and body condition. Stallions were fed one of two dietary treatments for 60 d: A basal diet, Control, 0.4% BW as concentrate and 1.8% BW as grass hay, and DHA, basal diet plus 160 g of a yeast-based supplement containing selenized yeast, vitamin E and a DHA-rich microalgae (Schizochytrium limacinum CCAP 4087/2; Alltech Inc., Nicholasville, KY) to provide 2mg Se, 1000IU vitamin E and 15g DHA. Consumption of the supplement was accepted within a few days of feeding. Beginning on day 46, stallions were collected every other day until day 60. Sperm motion parameters were assessed with CASA. For cryopreservation, sperm were loaded into 0.5mL straws and cooled using a programmable freezer set to an appropriate cooling curve. Using flow cytometric analysis, sperm viability, mitochondrial membrane potential, lipid peroxidation, acrosomal status, and membrane fluidity were measured on frozen-thawed sperm. Sperm viability was measured using both a SYBR-14/PI stain as well as a Yo-Pro+/PI- stain. Sperm mitochondrial membrane potential was evaluated using JC-1, and lipid peroxidation measured using BODIPY 581/591 C11. Acrosomal status was analyzed using FITC-PNA/PI and membrane fluidity evaluated using Merocyanine 540/Yo-Pro. Data was analyzed using a mixed model and significance was set to $p \leq 0.05$. Although a significant improvement in total and progressive motility was seen in both the fresh and the cooled spermatozoa, there was no significant difference noted at any of the parameters measured in the frozen-thawed sperm.

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Abstract Title:	**Secretogranin II is a Novel Periovulatory Gene that is Induced by Human Chorionic Gonadotropin in Human and Rodent Granulosa Cells**
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Abstract: Secretogranin II (SCG2) is involved in hormone packaging and secretion, leukocyte migration, and angiogenesis. These processes are vital for ovulation; however, the involvement of SCG2 in ovulation is unstudied. Our hypothesis is that the LH surge increases SCG2 in human and rodent granulosa cells via classic LH receptor signaling pathways. For the human studies, granulosa-lutein cells (GLCs) from IVF patients were acclimated in culture to regain LH/hCG responsiveness and then treated with hCG (1 IU) for 0-24h. hCG significantly increased the mRNA levels of SCG2 at 6 and 12h when compared to the controls, and the mRNA levels of SCG2 return to control levels following 24h of hCG treatment (n=10; $p \leq 0.05$). To determine the signaling pathways that mediate SCG2 induction, the GLCs were cultured with vehicle control (DMSO), AG1478 (5μM; EGF receptor antagonist), hCG, or hCG+AG1478 for 0-12h. The mRNA levels of SCG2 were comparable between the controls and AG1478 groups. As expected, hCG significantly increased the mRNA levels of SCG2 at 6 and 12h, but the mRNA levels of SCG2 in the hCG+AG1478 group were significantly reduced when compared to the hCG group at 6h (n=5-8; $p \leq 0.05$). For the rodent studies, the immature PMSG/hCG mouse model was utilized. In granulosa cells, hCG significantly increased the mRNA levels of Scg2 at 4, 8, and 12h when compared to the controls (n=5-6; $p \leq 0.05$). Collectively, SCG2 is transiently induced by hCG in human and rodent granulosa cells, and EGF receptor signaling mediates the initial hCG-induced increase in human periovulatory SCG2 mRNA expression.

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35th Annual Symposium in Reproductive Science and Women's Health
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Abstract Title:	**Differential expression of microRNAs throughout gestation in the mare**
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Abstract: MicroRNAs (miRNAs) are small, non-coding RNAs which play an important role in regulating gene expression. Acting primarily to inhibit translation of mRNAs, miRNAs are differentially regulated between tissue types and may be released into circulation, making them attractive targets as biomarkers. Changes in the spectrum of circulating miRNAs have been well documented during pregnancy with differential expression noted in numerous gestational abnormalities. In humans, a cluster of miRNAs (C14MC) has been shown to be associated with pregnancy and appears to be conserved across eutherian mammals. In this project, we sought to characterize the normal profile of miRNAs throughout gestation in both chorion and serum of pregnant mares. To achieve this, we collected matched serum and chorion samples from pregnant mares at 4 mo (n = 3), 10 mo (n = 3) and post-partum (n = 3). Additionally, we collected serum samples from non-pregnant mares during diestrus (n = 6). Total RNA was extracted and then prepared for next-generation sequencing. There were an average of 8.26 x 106 mapped reads per chorion sample and an average of 3.88 x 105 mapped reads per serum sample, in total identifying 1,198 mapped miRNAs. Samples were normalized by global expression levels, then compared via one-way ANOVA. Correlation was evaluated using Spearman's rank coefficient, with significance set at $P < 0.01$. A total of 60 chorionic miRNAs were found to be affected by gestational age, whereas in serum, 45 miRNAs were differentially regulated. Three of these were affected in both chorion and serum. The level of miRNA was correlated ($\rho = 0.68$; $P < 0.001$) between the matched serum and chorion samples. Additionally, we found evidence of a differentially regulated miRNA cluster on chromosome 24, orthologous to human chromosome 14 (C14MC). The miRNAs present in this region are differentially regulated throughout gestation, clustered an average of 5.6 kb apart, and the miRNAs present in the cluster are almost identical to human C14MC. In addition, we noted four distinct clusters consisting of 15 miRNAs on the X-chromosome which were affected throughout gestation. In conclusion, miRNAs are differentially regulated during gestation in the mare, both in the chorion and in circulation, with expression patterns correlated between chorion and serum. We found distinct evidence of the C14MC miRNA cluster in the horse on chromosome 24, as well as additional miRNA clusters on the X-chromosome. The concentrations of these miRNAs were significantly altered during gestation and provide an intriguing target for biomarkers to assess placental health in the mare.

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35th Annual Symposium in Reproductive Science and Women's Health
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Abstract Title:	**A Prospective Case-Control Study of Biomarkers for Feto-Placental Well-Being in the Mare**
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Abstract: Late term abortion in the mare, due to feto-placental compromise, continues to be an area of research where better diagnostic markers are desired. Previous work has indicated that estradiol-17β (E2), progesterone (P4), alpha feto-protein (AFP), and serum amyloid A (SAA) may be useful indicators of abnormal pregnancy in the mare. The objective of this study was to assess changes in these biomarkers in a single serum sample from normal and abnormal pregnancy outcomes. Mares (n=700) had blood samples taken weekly beginning December 2013 through parturition or abortion. Mares (n=15) which aborted or presented placental abnormalities had placentas submitted for pathological evaluation. Placentas from control mares (n=30), selected based upon gestational age, were also submitted. E2, P4, AFP, and SAA concentrations were determined by immunoassay for the sample preceding abortion or parturition for both case mares and matched controls. Data were analyzed using nominal logistic regression. Diagnosis included: umbilical cord lesions (n=2), equine herpes virus 1 (n=1), idiopathic abortion (n=3), nocardioform placentitis (n=2), bacterial placentitis (n=5), and premature placental separation (n=2). Median gestational age of case mares was 329 days (range: 246-355 days). For the single sample from case mares, AFP was positively ($p<0.01$), E2 was negatively ($p<0.05$), and SAA trended ($p<0.1$) to be positively associated with abnormal pregnancy outcomes. This study suggests that decreases in E2 and increases in AFP, when comparing mares in question to uncompromised pregnancies, may be useful in predicting abnormal pregnancy outcomes in late gestation from a single serum sample.

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Abstract Title:	**Core Binding Factor Beta Knockdown Alters Ovarian Gene Expression and Function in the Mouse**
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Abstract: Core Binding Factor (CBF) is a heterodimeric transcription factor complex composed of a DNA-binding subunit, one of three RUNX factors, and a non-DNA binding subunit, CBFβ. CBFβ is critical for DNA binding and stability of the CBF transcription factor complex. In the ovary, the LH surge increases the expression of Runx1 and Runx2 in periovulatory follicles, implicating a role for CBFs in the periovulatory process. The present study investigated the functional significance of CBFs (RUNX1/CBFβ and RUNX2/CBFβ) in the ovary by examining the ovarian phenotype of granulosa cell-specific CBFβ knockdown mice; CBFβ f/f * Cyp19 cre. The mutant female mice exhibited significant reductions in fertility, with smaller litter sizes, decreased progesterone during gestation, and fewer cumulus oocyte complexes collected following induced superovulation. RNA sequencing and transcriptome assembly revealed altered expression of over 200 mRNA transcripts in granulosa cells of Cbfb knockdown mice following hCG stimulation in vitro. Among the effected transcripts are known regulators of ovulation and luteinization including Sfrp4, Sgk1, Lhcgr, Prlr, Wnt4, and Edn2, as well as many genes not yet characterized in the ovary. Cbfβ knockdown mice also exhibited decreased expression of key genes within corpora lutea and morphological changes in ovarian structure, including the presence of large antral follicles well into the luteal phase. Overall, these data suggest a role for CBFs as significant regulators of gene expression, ovulatory processes, and terminal differentiation of granulosa-lutein cells in the ovary.

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