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CLINICAL CASE SESSION

Proteomic analysis of sperm with impaired acrosomal exocytosis from a subfertile stallion

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Stallion subfertility due to impaired acrosomal exocytosis has been reported in Thoroughbred stallions that carry the genotype A/A-A/A for 2 SNPs in exon 5 of the FKBP6 gene (ECA13). These animals are subfertile despite having otherwise normal sperm quality and good breeding management. Mass spectrometry-based technologies are a powerful tool to investigate the potential causes of unexplained subfertility in the stallion and might further improve our understanding of the molecular processes that take place during fertilization. Herein, we describe a preliminary experiment conducted to compare the sperm proteome from a fertile stallion (percycle pregnancy rate = 60%) and a subfertile stallion that carries the susceptibility genotype for IAE (percycle pregnancy rate = 30%). Fresh semen from each stallion was processed to induce spontaneous acrosomal exocytosis (AE) using a lactate-only-containing modified Whitten's medium (Lac-MW). At 0, 4, and 6 hours of incubation, sperm aliquots were analyzed for sperm viability (VIAB) and the rate of AE in viable sperm (AE/VIAB) via flow cytometry (FITC-PSA and fixable live/dead red stain). Also, at each period, the sperm proteomes from each stallion were analyzed via data-independent acquisition mass spectrometry. Student's t-test was used to assess differences between experimental groups. During incubation in Lac-MW, VIAB was similar ($p > 0.05$) between both stallions and was not affected by incubation time. AE/VIAB increased ($p < 0.05$) over time (0 hour: 3%, 4 hours: 32%, and 6 hours: 56%) for the fertile stallion, but not ($p > 0.05$) for the subfertile stallion (0 hour: 3%, 4 hours: 5%, and 6 hours: 5%). Mass spectrometry analysis detected a total of 2,252 proteins in sperm (false discovery rate < 1.0%). Of these, 144 proteins exhibited differences in relative quantity between the fertile and subfertile stallion (log2 fold change; $p < 0.01$). Data analysis using the PANTHER protein class annotation system revealed that most of the proteins with lower abundance (▼) in the subfertile stallion belonged to the calcium-binding protein ($p = 3.36 \times 10^{-5}$), and the metabolite interconversion enzyme ($p = 1.11 \times 10^{-7}$) groups, whereas proteins with higher abundance (▲) included those of the chaperonin ($p = 8.25 \times 10^{-4}$), protease ($p = 5.21 \times 10^{-4}$) and histone ($p = 3.22 \times 10^{-11}$) groups. Among these, proteins of interest that were identified and are known to be related to sperm capacitation/acrosomal exocytosis and stallion fertility included: ADAM7 (▼), Annexin-A2, -A4, and -A5 (▼), Calpain-5 (▼), Kallikrein-1E2(▼), and CRISP-2 (▼). Current experiments are focused on determining the potential relation between the FKBP6 gene genotype and the changes observed on the sperm proteome of more IAE-affected stallions.

Keywords: Stallion subfertility, acrosomal exocytosis, FKBP6, proteome

Thoraco-omphalopagus conjoined twins in a Standardbred mare

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An eight-year-old Standardbred mare presented to The Ohio State University College of Veterinary Medicine in dystocia of 30 minutes duration. No breeding records were provided at presentation. Pregnancy had been confirmed in this mare at 14 days postovulation with 1 embryonic vesicle visible. On vaginal palpation, the fetus was in posterior presentation, dorsosacral position with hindlimbs extended and there were no signs of fetal viability. General anesthesia was induced to allow for better manipulation of the fetus for extraction. During controlled vaginal delivery, 2 hind limbs were identified and a fetotomy was performed at the level of the tibiotarsal joint. Following removal of 2 hind limbs, another hind limb was identified on palpation and removed via fetotomy at the level of mid-femur. Following removal of 3 hind limbs, 2 distinct pelvises were identified on palpation. Retropulsion of 1 pelvis was attempted, however no progress could be made to separate the suspected twins. Caesarean section was recommended, but humane euthanasia was elected due to financial constraints. Postmortem examination revealed a thoraco-omphalopagus conjoined twin following a ventral midline approach to the uterus. Prior to necropsy of the conjoined twin, a computed tomography scan was performed. The calvarium of the conjoined twin was fused at the level of the facial crest caudal to the orbit. Two independent vertebral columns with separate spinal cords were present connecting at a single sternum. One thoracic limb was present on

either side of the joined sternum. There was another fused thoracic limb that formed a bipedal hoof below the metacarpophalangeal joint with the metacarpal bones fused. There were 2 prepuces and 2 anuses present with meconium present in both anuses. Two tails were also present. There was an umbilical hernia present with small intestines protruding through the hernia. Respiratory and digestive tracts were fused at the larynx with a single trachea and esophagus. There was a single pair of lungs along with a single heart with 2 descending aortas. The single esophagus entered into a single stomach. The jejunum was divided into 2 ~ 90 cm oral to the ileocecocolic junctions. Caudal to that point, there were 2 ilea, ceca, and large intestines present with fecal balls in both small colons. Thoraco-omphalopagus conjoined twins have been reported in other species, including humans. To our knowledge, this is the first reported case of thoraco-omphalopagus conjoined twins in the horse.

Keywords: Mare, dystocia, twins, thoraco-omphalopagus

Anaphylactic reaction following intrauterine administration of misoprostol in a mare

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Deep horn intrauterine application of misoprostol, a synthetic prostaglandin E1 (PGE1), has been demonstrated to improve fertility in mares suspected of oviductal dysfunction.[1] Adverse reactions in horses included mild abdominal discomfort and soft feces after oral misoprostol and in women anaphylactic reactions were observed after oral and vaginal treatment. An 18-year-old Friesian mare weighing ~ 650 kg was presented for unexplained infertility of 3 years duration. Misoprostol (600 µg, Greenstone LLC, Peapack, NJ) dissolved in sterile water was deposited at the tip of each uterine horn.[1] Ten minutes after treatment the mare collapsed in the stall. Her mucous membranes appeared dark red with a prolonged capillary refill time of 4 seconds and she demonstrated tachycardia of 100 beats per minute, cool extremities, tachypnea of 60 breaths per minute, and was minimally responsive. Dexamethasone (50 mg) and flunixin meglumine (750 mg) were given intravenously in addition to 6 mg epinephrine. A 14-gauge intravenous catheter was placed and 5 liters of lactated Ringer's saline (LRS) was given. While the mare was laterally recumbent, a cuffed intrauterine catheter was inserted and the uterus lavaged with 9 liters of LRS in 3 liter aliquots. Fifteen minutes after the onset of treatment, the mare's heart rate and respiratory rate improved and she was able to achieve sternal recumbency and stand with encouragement. However, 15 minutes later she again became tachycardic and tachypneic and collapsed a second time. An additional 6 mg of epinephrine was given intravenously in conjunction with continuous bolus fluids and the uterus was again lavaged with 9 liters of LRS. Ten minutes after the second epinephrine the mare improved and regained ability to stand. Her vital signs gradually normalized, she passed normal manure and began to graze. Over the course of the 1.5-hour treatment window the mare received intravenously 50 mg dexamethasone, 750 mg flunixin meglumine, 12 liters of LRS, 2 doses (6 mg each) epinephrine, and twice 9 liters of uterine lavage with LRS. Following recovery, no further ill effects of the incident were noted. The mare was bred with fresh cooled semen 2 weeks later but failed to become pregnant. She had no previously reported medication allergies or history of drug reaction. This is the first reported adverse event of its kind associated with intrauterine PGE_1

Keywords: Misoprostol, intrauterine, adverse reaction, anaphylaxis

Reference

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Next generation sequencing in deciding to discontinue antibiotic treatment in a stallion

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A 10-year-old Morgan stallion presented for test cooling of semen with a history of mares not conceiving from cooled shipped semen. Five weeks prior, the stallion was collected for semen shipment. The semen was greyish in color and had 60% progressively motile sperm (PMS), mild teratozoospermia, 58% morphologically normal sperm, and 4.48 x 10^9 total number of sperm. Penis was washed thoroughly before collection to minimize debris in the semen sample from the penis as a possible cause of semen discoloration. Semen was collected using a Missouri artificial vagina, and the semen sample was again greyish in color and had 3.4 x 10^9 total number of sperm, 60% PMS, and 66% morphologically normal sperm. Cytological examination of the semen sample with Diff-Quik stain had no leukocytes or germ cells. Large numbers of branching rods were observed on cytology and an aerobic culture was performed. A fastidious and slow-growing *Actinomyces* species was identified by culture. Antibiotic susceptibility was not possible due to the bacteria's slow-growing nature. *Actinomyces* has been reported to infect the testes and accessory sex glands in humans. Empirical antibiotic selection was based on published reports and oral doxycycline (10 mg/kg) was prescribed for 8 weeks. Stallion was presented again 43 days later. Semen appeared normal with improved number of total sperm (7.45 x 10^9), 60% PMS, and 90% normal morphology. A culture was performed, and the sample was submitted for next generation sequencing (NGS) of 16S rRNA for bacteria and ITS for fungi. The culture was negative, and NGS had 59% *Klebsiella oxytoca*, 18% *Petrimonas* sp., 7% *Streptococcus uberis*, 3% *Corynebacterium kroppenstedii*, 2% *Luteococcus* sp., and 2% *Proteiniphilum* sp. for bacteria and 91%

of ITS reads were *Mucor circinelloides* and 8% *Pleospora herbarum*. Traditional culture method is of limited use to identify when antibiotic treatments can be discontinued due to antibiotics' inhibitory effects on culture results. The NGS results had several mixed bacteria and fungi that are more resistant to antibiotics. *Actinomyces* species was not identified, which we interpreted as a positive sign. The improved spermiogram, negative culture, and lack of *Actinomyces* detected by NGS were indications that antibiotic treatment could be discontinued. Ideally, NGS should have been performed prior to the start of antibiotic treatment to be used as a baseline, and further studies are indicated to determine thresholds of potentially pathogenic bacteria on fertility. Availability of a commercial clinical NGS laboratory service for the veterinary industry is a potentially groundbreaking advancement. One potential use of this technology may be to identify when long-term antibiotic treatments can be safely discontinued.

Keywords: Stallion, *Actinomyces* species, next generation sequencing

Thromboembolic disorder in a postcesarean section bitch

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Pregnancy and the postpartum period substantially increase the risk for venous thromboembolism (VTE) in women, a disease that leads to pulmonary embolism and deep venous thrombosis. The most important risk factor contributing to a thrombotic event is the hypercoagulable state that occurs during a normal pregnancy, with the highest risk occurring the first 6 weeks postpartum. Risk factors among women are age (> 35-years-old), cesarean delivery, hypertension, heart disease, obesity, and the presence of infection postpartum. To the authors' knowledge, this condition has not been reported in dogs. A 3-year-old female intact primiparous Labrador Retriever presented as an emergency due to fever (104.3 °F), lethargy, and anorexia. History included dystocia due to secondary uterine inertia with fetal distress (stillborn fetus) that was resolved by emergency cesarean section 4 days prior to presentation. On initial evaluation, the patient was quiet and responsive, obese with a body condition score of 8/9, and had a moderate amount of lochia with no foul-odor. Bloodwork revealed a normocytic, normochromic, regenerative anemia (hematocrit of 27%, absolute reticulocytes 99.6 x 10^3/µl), leukocytosis with a neutrophilia characterized by a left shift and monocytosis (WBC 43.6 x 10^3/µl, reference interval [RI] 5.7 - 14.2; segmented neutrophils 35.8 x 10^3/µl, RI 2.7 - 9.4; band neutrophils 0.4 x 10^3/µl, RI 0.0 - 0.1; monocytes 2.6 x 10^3/µl, RI 0.1 - 1.3), hyperproteinemia (8.0 g/dl, RI 5.9 - 7.8) and hypoalbuminemia (1.8 g/dl, RI 3.2 - 4.1). Considering the clinical condition, medical intervention and hospitalization for further diagnostic testing were pursued. Medical management consisted of intravenous plasma-lyte A fluids (60 ml/kg/day), ampicillin/sulbactam (Unasyn, 30 mg/kg every 8 hours), and maropitant citrate (Cerenia®, 1 mg/kg every 24 hours). A disseminated intravascular coagulation panel indicated significantly increased D-dimers (4965 ng/ml, RI 0 - 575), decreased antithrombin III activity (50%, RI 65 - 145), and an increased aPTT (19.6 seconds, RI 8.5 -15.5). Abdominal ultrasonography revealed a diffusely, severely mottled spleen with innumerable hypoechoic regions of acute, multifocal infarction, suggesting the presence of splenic infarcts and no evidence of peritonitis. That evening, the patient developed labored breathing with a respiratory rate of 36 breaths per minute and pulse oxygenation of 93% (RI > 95%). An arterial blood gas revealed an alveolar-arterial oxygen gradient of 30 mmHg (RI 10 - 25). These findings were concerning for pulmonary thromboembolism. The patient improved after 24 hours of supplemental oxygen, and the supplementation was discontinued. Subsequent echocardiogram revealed no evidence of pulmonary hypertension. The patient was discharged 48 hours after hospitalization with oral amoxicillin/clavulanate (13.75 mg/kg every 12 hours for 14 days). Considering the clinical manifestation and increased risk reported with cesarean delivery and peripartum obesity in women, it was presumed that the pregnancy-related hypercoagulable state and postpartum period led to the development of a VTE in the postcesarean section bitch.

Keywords: Mare, pregnancy, placenta, cervix, ultrasonography

Funding: Cesarean section, hypercoagulable, pulmonary embolism, venous thromboembolism

Polled intersex syndrome in a Finnish Landrace lamb

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An apparently healthy, 4-month-old female Finnish Landrace lamb was presented for the presence of female and male external genitalia. The lamb was polled and a twin to a normal female. On initial examination the lamb had a short anogenital distance of 3.5 cm and a normal appearing vulva with a mildly enlarged clitoris. Left gonad was descended and palpable in the inguinal area, whereas the other gonad was suspected to be retained intra-abdominally. Left inguinal ring was severely dilated and the descended gonad could be moved freely intra- and extra-abdominally. Abdominal ultrasonography revealed a retained intra-abdominal right gonad that appeared testicular in origin with a hyperechoic mediastinum testis and a less echogenic parenchyma as seen in a normal testis. A fluid filled uterus was also identified, but ovaries were unable to be identified. Lamb underwent a midline exploratory laparotomy with a bilateral castration/hysterectomy. Uterus was identified and the uterine horns were followed to the location of the ovaries where testicular appearing structures with a pampiniform plexus

and epididymis were identified. The testicular tissue was attached to the broad ligament as the uterus and ovaries would be. Both testes were ligated and removed, the uterus was removed via a routine hysterectomy. Incision was closed in a routine fashion, the lamb recovered from anesthesia with no complications. Lamb was euthanized at a later time. Uterus and gonads were submitted for histopathology. Histopathology revealed that gonads contained hypoplastic testicular tissue. Seminiferous tubules were diffusely hypoplastic with a complete lack of spermatogenesis. Epididymis was hypoplastic with compressed ducts. There was marked congestion in the uterus with the lumen diffusely filled with erythrocytes consistent with intraluminal hemorrhage and the endometrial lamina propria was expanded by edema. No ovarian tissue was identified in the sections of the testis that were evaluated. However, due to the difficulty associated with identifying ovarian tissue, the presence of ovotesticular tissue could not be ruled out. Blood was submitted to the Texas A&M College of Veterinary Medicine, Molecular Cytogenetics Laboratory for karyotyping. The results revealed a genetically female sheep with a normal sheep karyotype (54 XX). On polymerase chain reaction, the lamb was negative for the presence of the Y-linked SRY gene and positive for the X-linked androgen receptor gene. No chromosomal abnormalities were observed. Intersex conditions in goats and in sheep are believed to be linked with the polled gene. Affected animals are genetically female (XX), SRY negative and believed to be homozygous for the polled gene. In this case the lamb is an intersex, consistent with polled intersex syndrome sex reversal but, may be a true hermaphrodite.

Keywords: Sheep, polled, intersex, sex reversal, chromosome, hermaphrodite

Reduction of equine monozygotic twins using craniocervical dislocation via colpotomy

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Twin management is a very important facet of equine reproduction management. Compared to dizygotic twins, monozygotic twins are a relatively rare occurrence in mares. Management of monozygotic twins is considerably more complex when it comes to preserving the safety and welfare of the mare and developing pregnancy. Of the options of twin reduction after day 50 of pregnancy, craniocervical dislocation (CCD) has been reported to be a superior choice.[1] We have modified the surgical approach for a CCD from the reported flank laparotomy to colpotomy. In our hands, successful reduction of dizogotic twins by CCD via a colpotomy approach has been 71% (n = 29). To-date we have managed 2 cases of monozygotic derived twins with a 50% success rate. Our hypothesis for the current case was that CCD via colpotomy is a successful technique for monozygotic twin management given its successful application on our first case of monozygotic twin reduction. An embryo recipient mare that received 1 embryo was presented at 65 days of pregnancy for the reduction of 1 fetus after diagnosed to have monozygotic twins via ultrasonography by the referring veterinarian. The referring veterinarian was actively monitoring the unilateral twin pregnancy for natural reduction. Once the pregnancy reached 60 days with no reduction, the case was referred to us for CCD. After confirmation of monozygotic twins (i.e. 2 separate amnions were observed within 1 allantoic sac), a CCD was performed on 1 fetus via a colpotomy approach. Unfortunately, both fetal heartbeats were lost on detection by the referring veterinarian and presumably aborted between 4 and 6 weeks after CCD procedure. Other cases of monozygotic twins have been reported in recipient mares that received a single embryo.[2-4] Several management techniques were employed in each of these cases with varying outcomes. CCD via colpotomy is a novel approach and was chosen in this case due to the age of the fetuses and a desire to reduce the most craniad that we presumed would have less chorionic attachment and lower development capacity as the pregnancy progressed.[1] The importance of this case is in demonstrating that CCD via colpotomy can be a useful method for management of monozygotic twins; however, prognosis for successful development of the remaining fetus is guarded due to the orientation of the twin fetuses enclosed within a single allantochorion.

Keywords: Twins, monozygotic twins, twin management, embryo recipient, embryo transfer

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Chronic seminal vesiculitis and blocked ampullae in a stallion

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Seminal vesiculitis is a rare condition in the stallion; however, it can result in blocked ampullae. A 12-year-old Gypsy Vanner stallion with a previous successful breeding history was presented for persistent polyspermia, manifesting as an abnormal grey

ejaculate. Semen collected at presentation had pus with no sperm. Testes felt normal but were mildly enlarged with firm epididymides. Ultrasonography findings were normal. Transrectal palpation of the ampullae and seminal vesicles (SV) elicited a painful response. Transrectal ultrasonography revealed fluid-filled ampullae and SV with thickened walls (1.5 cm). Stallion demonstrated normal libido during 3 collections and did not exhibit pain. Cultures were obtained from the unwashed urethral fossa, the distal urethra before and after ejaculation, and from the gel fraction. *Streptococcus equi* subspecies *zooepidemicus* was isolated, confirming the presumptive diagnosis of seminal vesiculitis. Microscopic examination of fresh ejaculate and Diff-Quik stained smears revealed azoospermia, high number of neutrophils, and bacteria in both fractions of the ejaculate in all 3 collections. Transrectal massage of the ampullae and oxytocin treatment immediately prior to the third collection did not alter the ejaculate. The diagnosis of blocked ampullae was confirmed by azoospermia and low concentration of alkaline phosphatase (30 IU/L) in the filtered fraction. Semen was collected 3 times a day and the stallion was treated orally twice a day with trimethoprim/sulfamethoxazole (TMS; 30 mg/kg) for 3 weeks at the owner's facility. Stallion was reexamined at the hospital. There were no changes to ampulla and SV. The first collection had > 70 ml heterogenous gel fraction and concentrated sperm-rich fraction (917 x 10^6 sperm/ml, 14.5 x 10^9 total) with marked number of detached heads and dead sperm (< 1% motility). Sequential collections had decreasing volume of gel, increasing number of sperm (up to 83.2 x 10^9), and an increasing trend in the motility rate in the ejaculate. Due to lack of complete response to TMS, local treatment of the SV was initiated using video endoscopy (5.9 mm x 110 cm). The local treatment consisted of vigorous lavage (500 ml lactate Ringer's saline per gland) followed by local infusion of ampicillin (2 gram in 18 ml) initially for 2 days followed by a compounded penicillin gel (5 MIU in 20 ml) every other day for a total of 3 treatments. Purulent material within the seminal vesicles markedly decreased throughout treatment. Stallion was discharged with instructions to have twice daily collections and continued TMS treatment at the owner's facility before readmission for follow-up. This case highlighted the importance of managing seminal vesiculitis aggressively, and the possible sequela of blocked ampullae due to local inflammation and accumulation of pus and sperm.

Keywords: Stallion, seminal vesiculitis, blocked ampullae