New Insights About Detecting Pregnant Mares at Risk: What We Have Learned from a Model of Placentitis

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In the early 2000's, the late Dr. Michelle LeBlanc and her research team, used ambitious methodology to establish a working model of induced placentitis in the mare. In this model, a clinically-isolated inoculum of *Steptococcus equi* subspecies *zooepidemicus* was placed mid-way in the cervix, disrupting the critical cervical plug without fully breaching the cervix. Bacteria migrated, cranially, and inhabited the cervical pole of the chorioallantois.[1] A second, more aggressive model of infection was developed by Lyle and co-workers by placing the bacterial inoculum directly on the chorioallantois. [2] The rationale for this model was to develop a more consistent mode of chorioallantoic infection. Using either model, establishment of placental infection varies broadly between mares under experimental conditions, as do clinical signs. While some argue that a model does not realistically represent the clinical condition, the information gained has positively improved the outcome of many mares affected with placentitis and other conditions affecting the late term, pregnant mare.

The pathophysiology of equine placentitis

Initial studies by LeBlanc and workers provided formative information regarding placentitis. [1] Placentitis induced in sixteen pony mares between 280 and 295 days of gestation resulted in 14/16 infected mares delivering dead or non-viable foals. Two foals born prematurely (days 311 and 313 of gestation) experienced accelerated maturation and lived with minimal neonatal care. The duration and intensity of uterine contractions were higher in infected mares. Concentrations of prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$) in allantoic fluid from mares with placentitis were elevated. Concentrations of IL-6 and IL-8, cytokines involved in inflammation, were elevated in the placentas of infected mares. All infected mares had gross thickening and purulent material at the cervical star of the placenta. Bacteria were located on the surface of the placenta, in the amniotic fluid and on umbilical cord. Bacteria were isolated in fetal lungs from 7 fetuses. The authors proposed that fetal infection occurred by passage of bacteria through fetal membranes and into the amniotic fluid which the fetus inhaled or swallowed. Interestingly, not all fetuses became infected from inoculated mares. These important data showed that both infection and inflammation were involved placentitis-induced preterm delivery, similar to other species. Therefore, efforts for diagnosing placentitis have been directed at identifying infection and/or inflammation.

Physical examination findings

Diagnosis of placentitis relies heavily on clinical changes which often lag behind the disease process. The most common clinical signs of placentitis include premature udder development and purulent vulvar discharge. Vulvar discharge is often the first sign of infection but is frequently undetected if the mare's perineal region is not monitored daily.[3] Premature mammary gland development can occur in mares with twin pregnancies as well as mares with placentitis, likely as a secondary response to activation of the hypothalamic pituitary axis. Some mares have scant to no vulvar discharge present and/or minimal udder development but have fulminant placental infection. These mares may suffer a subclinical infection that results in death of the fetus. Alternatively, some mares have extensive placental separation due to infection but will deliver viable foals. Vigilant monitoring of the perineal and mammary areas, particularly in mares "at risk," is often the best line of defense for early detection of placentitis. Data from a clinical study [4] showed that early detection of disease with physical and ultrasonographic

monitoring allowed for early treatment of mares and an over 30% reduction in prematurely delivered foals. Adding the tools of daily examination of the mammary gland and perineal area provides a simple, effective screening tool for early detection of placentitis.

Mammary secretion electrolytes

Measurement of electrolyte values in mammary secretions is a simple, semi-quantitative tool for determining imminent parturition as well as fetal readiness for birth. [5, 6] Traditionally, inversion of sodium and potassium concentrations and a rise in calcium signal impending parturition. These tools are widely used in a both laboratory measurements as well as in stall-side tests for calcium (carbonate) concentrations. More recently, change in mammary secretion pH has been used to predict imminent parturition in both normal and abnormal equine pregnancies. [7, 8] [9] While specific pH values predictive of impending delivery varied between studies, pH lower than 7.0 was predictive of foaling within 24 hours. This simple tool is easily applied to monitoring both the normal and abnormal, pregnant mare.

Ultrasonographic tools for diagnosis of placentitis

Transrectal ultrasonography in late gestation is useful for evaluating placental integrity (at the cervical star), fetal activity and fetal fluid character. The cervical star region of the placenta is the most frequently affected area in mares with placentitis due to the ascending route of infection. The combined uterine and placental unit is measured. Values of the combined thickness of the uterus and placenta (CTUP) for mares with normal pregnancies have been established. [10] Mares with placental infection or inflammation will have an increase in CTUP measures or separation of the membranes with presence of purulent material. [11] A caveat to this procedure is the inherent operator bias that occurs with two-dimensional ultrasound measurements. Repeat measures performed by the same ultrasonographer allows for more accurate diagnosis of disease.

Transabdominal ultrasonography is an excellent tool for evaluating fetal well-being, fetal fluid character as well as some areas of the placenta.[12, 13] The technique has been well described and is broadly used. Fetal health can be assessed through transabdominal ultrasonographic measures of fetal heart rate, tone, activity and size. Transabdominal ultrasound evaluation of the placenta provides the most accurate means of diagnosing nocardioform placentitis. Similarly, a transabdominal approach is the best/only method to identify late term twins with the ultrasound.

More recently, Doppler ultrasonography has been investigated as a tool for characterizing normal [14] [15] and identifying abnormal late term pregnancies, including mares with placentitis.[15] Doppler ultrasonography allows assessment of fetal blood flow and perfusion, with a particular emphasis on the umbilical artery.

Hormonal Assays and Biomarkers

The limitations in sensitivity of clinical or ultrasonographic parameters to aid in rapid diagnosis of equine placentitis have led to investigation of laboratory based tests to improve diagnosis. Several hormonal assays, and more recently blood-based biomarkers, contribute information that can improve diagnosis of placentitis.

Progesterone assays:

Several progestagens are synthesized by the feto-placental unit to support pregnancy in the latter two thirds of gestation. These progestagens are metabolites of progesterone (P4) and pregnenolone (P5). Ousey and co-workers[16] used gas chromatography-mass spectrometry to measure serial concentrations of progestagens throughout pregnancy in in mares with both

normal and abnormal pregnancies. All progestagens, except progesterone (P4), rose gradually in mare serum as pregnancy advanced. Mares with placentitis had higher levels of P4, P5 and metabolites when compared to normal, pregnant mares. Mild increases in P4 were noted in mares with placental pathology other than placentitis. Mares experiencing stress other than placentitis (colic, laminitis) showed normal, or slightly lower, levels of P4 and P5 metabolites. Accurate measurement of progestagens requires sophisticated mass spectrometry equipment. However, many P4 immunoassays (radioimmunoassay and ELISA) cross react with progestagens produced by the feto-placental unit. Cross-reactivity of assays must be determined for each commercial test used to measure progesterone. To aid in detection of placental pathology and/or response to therapy, it is recommended to monitor serial serum progesterone concentrations 3 to 4 times at one to two day intervals. These values can be assessed for trends including a premature, gradual rise (prior to day 300 gestation) or a rapid drop (impending delivery) that may be useful for directing therapies.

Estrogens:
Estrogens are broadly produced in equine pregnancy by the feto-placental unit. The fetal gonads produce estrogen precursors, which are aromatized by the placenta to produce a variety of estrogens including estrone, estradiol 17α and β, equilin and equilenin. Estrogen production increases from approximately day 80 of gestation, peaks at 210 days of gestation and gradually decreases prior to delivery. High maternal serum estrogen concentrations (usually measured as estrone sulfate or total estrogens) indicate a functional feto-placental unit and are a strong indicator of fetal viability. The usefulness of this tool to detect and monitor pregnancy health has been less well defined than measurement of serum progestagens. Douglas [17] reported that a total estrogen concentration of greater than 1000 ng/mL was consistent with a normal pregnancy between 150 and 280 days gestation. However, an association was made in mares having total estrogen concentrations less than 500 ng/mL and pregnancy loss. The value of measuring estrogens as predictors of placentitis was recently investigated by workers at the University of Kentucky.[18] Estrone sulfate and 17**β** estradiol sulfate were measured in normal pregnant mares and mares with ascending placentitis. Estrone sulfate was not different between groups. 17**β** estradiol sulfate dropped rapidly after bacterial inoculation in mares suggesting that this hormone might provide diagnostic information from mares with placentitis. The authors postulated that hormonal changes in naturally occurring infections might be more subtle therefore accurate detection of 17**β** estradiol sulfate in mares with placentitis might be more difficult.

Time resolved fluoroimmunoassay technology was recently used to assess steroid (progesterone and estrogen) hormones in plasma from a large number of pregnant mares. Samples were obtained from mares starting at 201 days gestation to foaling.[19] Using this methodology, the authors measured progestin and estrogen concentrations with anti-progesterone and anti-17β-estradiol antibodies. Mares were retrospectively assigned to two groups based on the health and outcome of the foal (healthy foal group and foal loss group). Between 240 and 320 days gestation, mares with abnormal pregnancies had higher progestins and lower estrogens than mares with normal pregnancies.[19] Mares who were administered treatments for placentitis (ritodrine, synthetic progestins and trimethoprim sulfamethoxazole) that delivered live foals showed changes in plasma steroid hormones that were consistent with normal, foaling mares. Further, the authors extrapolated cut off values for progestins and estrogens at 20 day intervals from Day 200 gestation forward that distinguished normal from abnormal pregnancies. The authors postulated that a one-time measurement of progestins and estrogens might be possible for predicting an abnormal pregnancy, particularly from Day 241 to 320 gestation.[19] These data suggest an alternative method for monitoring steroid hormones as predictors of abnormal pregnancy.

Relaxin:
Relaxin is a polypeptide hormone produced by the placenta. In mares, placental relaxin production begins around day 80 of pregnancy and continues until expulsion of the placenta. Relaxin concentrations have been advocated as a means of monitoring pregnancy health in mares and other species. Ryan and co-workers[20] used a homologous radioimmunoassay to measure relaxin concentrations in mares with normal pregnancies and mares with placentitis. They showed a positive relationship between low maternal serum concentrations of relaxin and poor pregnancy outcome. The authors noted a high degree of variability in relaxin concentrations in pregnant mares which they felt negatively affected accurate assessment with relaxin. To date, commercial assay of this hormone is not available.

Biomarkers
Serum biomarkers that reflect the health of the feto-placental unit are an area of great interest in both human and equine pregnancies. The scope of biomarkers that have been identified in human pregnancy is broad yet similarities between species are limited. Recently, acute phase proteins, such as serum amyloid A and haptoglobin (indicators of inflammation) have been examined in normal and abnormal equine pregnancies. Using ELISA technology, Coutinho da Silva and co-workers[21] measured serum amyloid A in normal pregnant mares and mares with experimentally induced placentitis. Serum amyloid A concentrations were shown to increase dramatically in the last 36 hours of normal pregnancy. Mares with placentitis demonstrated a premature rise in serum amyloid A. When serum amyloid A concentrations were monitored in mares administered treatment for placentitis, concentrations were shown to decline in some mares, presumably as a response to treatment. In a similar study,[22] SAA, haptoglobin, fibrinogen and white blood cell counts (WBC) were monitored in mares with normal pregnancies and mare with experimentally-induced placentitis. Similar to the prior study, SAA and haptoglobin increased rapidly after experimental infection and stayed elevated until abortion. However, in contrast to the previous study, SAA (and haptoglobin) did not rise prior to foaling in normal, pregnant mares. Fibrinogen and WBC counts were not affected by infection. Data from these studies demonstrate the usefulness of SAA as a screening tool for mares with placentitis. Serial measurements that can reveal trends in SAA concentrations as a result of infection, or in response to treatment, likely will yield the most useful information. As with previously validated tests for placentitis, changes in SAA concentrations are best used as an adjunct to ultrasonography and clinical signs.

Alpha-fetoprotein, a protein member of the albuminoid superfamily, has recently been measured in fetal fluids and plasma from normal, pregnant mares and mares with experimentally-induced placentitis.[23] The protein is produced by the fetal liver and has been used as a predictor of abnormal pregnancy in humans. [24] It has also been suggested that alpha-fetoprotein is predictive of placental abnormalities/insufficiencies, in addition to fetal anomalies such as neural tube defects. Plasma concentrations of alpha-fetoprotein from mares with experimentally-induced placentitis were elevated when compared to normal foaling mares.[23] Increased concentrations were most evident in the immediate days preceding delivery which limits the usefulness of the test as a diagnostic tool to initiate treatment for placentitis.

There has been increased interest in broad study of proteins in biologic and disease processes, termed proteomics. Kentucky workers recently performed protein analysis on fetal fuids (allantoic and amniotic fluid) from a population of normal mares and mares with experimentally-induced placentitis.[25] One hundred and thirty proteins were identified in one or both fluids. Unique proteins were more consistently identified in amniotic fluid. Amniotic fluid from mares with placentitis contained 3 highly expressed proteins (haptoglobin, plasminogen isoform X2

and plasminogen-like isoform 1).[25] Haptoglobin was also identified in allantoic fluid but not as consistently.[25] Some proteins were downregulated in amniotic fluid from mares with placentitis including members of the keratin family, para-aminobenzoic acid biosynthesis pathway (folate synthesis) and extracellular matrix proteins. [25] The significance of these findings has not been fully elucidated but may lead to improved diagnosis of placentitis (upregulation of fetal fluid proteins) and/or a better understanding of the pathophysiology of the disease (up and down regulation of proteins).

Conclusions

Diagnosis of pathologic conditions of the late term, pregnant mare, including placentitis, remains challenging. It is unlikely that a single, “silver bullet”, test will accurately predict conditions that threaten pregnancies. Several exciting technologies are being explored that will aid in earlier, accurate diagnosis of conditions such as placentitis. Regular, thorough physical examinations combined with pregnancy history and advanced diagnostic tools will likely be the most effective means of identifying conditions that threaten pregnancy, allowing for prompt treatment.

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