New diagnostic biomarkers to evaluate late term pregnancy in mares
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Introduction
In populations of Thoroughbred mares in central Kentucky and in Great Britain, pregnancy loss rates in mares from approximately Day 40 of gestation to term vary between 6.3 to 12.9%.1-3 As such, these losses represent a substantial economic loss to breeders, and considerable effort in clinical approach and in research have been directed toward the identification of risk factors, diagnosis and treatment of the many and varied causes of these losses. In particular, a majority of abortions and stillbirths in the mare are associated with placental insufficiency or placentitis, and biomarkers for placental function have been of considerable interest.4,5 This review will examine current understanding of biomarkers for evaluation of equine pregnancy during the late fetal period (last trimester of pregnancy).

Steroid hormones
The equine fetoplacental unit produces pregnanes, androgens and estrogens during pregnancy which can be detected in maternal circulation. The application of techniques such as mass spectrometry has provided a better understanding of the specific steroids present in circulation compared to immunoassay techniques which may lack specificity in identifying individual steroids because of antibody cross-reactivity to related molecules. In a recent study, we characterized changes in maternal concentrations of steroids in pregnant mares throughout gestation.6 Although mass spectrometry has been used previously to examine changes in steroid concentrations during pregnancy, this is among the first study to examine these changes across the whole of gestation in the mare.

Estrogens
Production of estrogens by the equine conceptus has been identified as early as Days 8 to 107,8 although changes in circulating concentrations of estrogen in maternal blood are not detectable until about Day 40 of pregnancy when secretion of estradiol from the corpus luteum results in an elevated serum estradiol in pregnant mares.9 Production of estrogens by the fetoplacental unit beginning at around Day 80 result in an elevated estrogen concentration in maternal blood which peaks at around Day 210 of gestation10 and parallels the hypertrophy of the equine fetal gonads. Estrone and its sulfon conjugate are the predominant estrogen formed by the equine fetoplacental unit, and synthesis of estrogens requires steroidogenic activity by both the fetal gonads and placenta. The equine fetal gonads synthesize dehydroepiandrosterone (DHEA) while placental conversion of DHEA to androstenedione and subsequently estrone and estradiol complete the synthesis of estrogens by the placenta.11,12 In addition to the classic phenolic estrogens, the pregnant mare also has high concentrations of ring B unsaturated estrogens (equilin and equilenin) which are produced via secretion of an androgen (7-dehydro-DHEA) by the fetal gonads with subsequent aromatization by the placenta to the ring B unsaturated estrogens.13 Unfortunately, little is known about the biological significance of these estrogens nor their role as possible biomarkers for pregnancy well-being in the mare.

A number of studies have attempted to assess fetal well-being based upon determination of various estrogens in the blood of pregnant mares. Measurement of estrone sulfate concentrations have been used for pregnancy detection in mares14-17 with elevations in peripheral estrone sulfate concentrations as early as Day 37 of gestation. Because the initial elevation in maternal estrogens is likely associated with luteal production estrogens under the influence of increasing equine chorionic gonadotropin (eCG),9 fetoplacental estrogen synthesis does not appear to be increased until approximately Day 80. Induction of fetal death by administration of prostaglandin F2α or surgical removal of the fetus in pregnant mares at Days 44-89 resulted in a decline in maternal estrone sulfate concentrations that was coincident with fetal death. Determination of estrogens has been suggested as a method to assess fetal well-being during later gestation in the mare. Experimental induction of placentitis in mares at nine
months of gestation by intracervical inoculation of Streptococcus equi subsp zooepidemicus resulted in decline in maternal estradiol-17 that was detectable within four days after inoculation (three to six days prior to abortion) compared to gestationally age matched control mares. In contrast, estrone sulfate concentrations were decreased only at the day of abortion in mares with experimentally induced placentitis. The differences in the timing of changes between free estradiol-17 and estrone sulfate are likely due to the relatively longer half-life of the conjugated estrone in blood compared to estradiol-17. In a large field study conducted on Thoroughbred mares in Japan, serum estradiol concentrations after Day 201 of gestation were lower in mares that aborted or produced dead foals compared to mares that delivered live foals. Similarly, clinical observations reported by Douglas, found lower “total estrogen” concentrations between Days 150-280 in pregnant mares that aborted with placentitis than in mares delivering live foals. Together, these studies indicate that determinations of serum estradiol concentrations in late gestation may be useful for assessment of fetoplacental well-being in equine pregnancy. A few caveats apply in making this statement. Conjugated estrone sulfate concentrations appear to change more slowly and may not be useful for assessment of fetal well-being in the mare.

Interpretation of estradiol concentrations in the late pregnant mare requires normal reference values for the gestational age being evaluated. These reference values will be particular for the assay being used since these immunoassays may vary in their cross-reactivity and specificity for estrogens in equine sera. Interpretation of estradiol concentrations in late pregnancy (> 300 days) may be more difficult because estradiol concentrations are falling in normal mares during this period.

Androgens

As noted above, fetal gonadal production of androgens increases along with gonadal hypertrophy with a peak at approximately 175 days GA. In particular, concentrations of dehydroepiandrostosterone (DHEA), and its sulfoconjugate (DHEA-S), increase in maternal circulation peaking at approximately 175 days of pregnancy. We hypothesized that concentrations of DHEA-S appearing in maternal blood might be increased in mares in which placental dysfunction secondary to placentitis occurred. To test this hypothesis, we examined changes in maternal DHEA-S concentrations in mares with experimentally induced placentitis and control mares. Unfortunately, no significant differences were found in mares with or without placentitis indicating that maternal androgens do not appear to be as useful biomarker for placental dysfunction in mares.

Pregnanes

The mare has a complex steroidogenic scheme for the production of pregnanes during pregnancy which involves both luteal as well as fetoplacental contributions to progestational support of pregnancy in the mare. Initially, the primary corpus luteum formed at ovulation is responsible for progestogen production; however, progestrone concentrations are low to undetectable during the second half of gestation in mares. The secretion of eCG initiates the formation of accessory corpora lutea which result in an increase in maternal progesterone concentrations beginning around Day 40. Although progesterone is the major progestogen secreted during this time, concentrations of 5 -dihydroprogesterone (5 -DHP) are also elevated after ovulation, and DHP is a bioactive progestogen which is capable of supporting pregnancy in the mare in the absence of progesterone. As the endometrial cups and accessory CLs regress, there is a shift from luteal to fetoplacental production of progestogens around Day 110 of gestation. Placental production of pregnanes is characterized by increasing 5 -DHP for the remainder of pregnancy, and it is likely that 5 -DHP is the major progestagenic steroid during this portion of gestation. In addition, a number of other pregnanes are also produced by the equine placenta, some in very high concentrations. The biologic activity, if any, of these remaining pregnanes in equine pregnancy are unknown although some are present in very high concentrations during late pregnancy.

A number of investigators have examined the use of pregnanes to assess fetoplacental well-being in the mare. Because all of the studies except one, relied upon immunoassays for detection of pregnanes, it is difficult to make direct comparisons across these studies. Immunoassays vary widely in their cross-reactivity to different pregnanes which may be present in the mare in late gestation.

Clinical Theriogenology • Volume 8  Number 3 • September 2016 272
Therefore the reported values obtained may vary widely from laboratory to laboratory. In one study where determination of pregnane concentrations was based upon GC-MS, mares with clinical placentitis had elevated peripheral concentrations of several metabolites including pregnenolone (P5) and/or progesterone as well as metabolites of 5α-DHP: P5ββ, ββ-diol, βα-diol, 20α-5P compared to normal pregnant mares.24 When measured by immunoassay, total pregnanes were elevated in all seven mares affected with placentitis during late gestation.24 It appears that metabolism of progesterone to 5α-DHP is increased in mares with placentitis and that several other metabolites of 5α-DHP are increased in maternal circulation. These increases appear more likely to occur with chronic placental disease, and it has been hypothesized that chronic fetal stress leads to an increased P5 production by the fetal adrenal glands which subsequently drives an increased pregnane production by the placenta.24,28 Conversely, more acute disease was associated with a reduced fetal P5 production and a decrease in pregnane concentrations in the mare.24,28 Based upon immunoassay data, several studies have demonstrated an increase in pregnane concentrations in mares with placentitis or other abnormalities in pregnancy during late gestation. In a large field study conducted on Thoroughbred mares in Japan, serum progesterone concentrations after Day 201 of gestation were higher in mares that aborted or produced dead foals compared to mares that delivered live foals.19 Douglas also reported that mares with placentitis had higher serum progestogen concentrations between Days 150-280 than did mares with normal pregnancies.20 In summary, it appears that placentitis, particularly as it becomes more chronic, is often associated with elevations in serum progestogen concentrations in the mare. More acute placentitis may be associated with a decline in serum pregnane concentrations although this may occur very shortly before abortion. Future studies using more specific techniques such as mass spectrometry may shed more light on which pregnanes are more related to placental pathology. Clinical application of progestogen determinations in pregnant mares during late gestation currently is based upon evaluation of serial (three or more) samples taken at 48-72 hour intervals looking for a greater than 50% increase or decrease in progestogen concentrations compared to the initial sample.28 Again, these changes are more difficult to interpret after 300 days GA because progestogen concentrations are normally increasing beyond this time in normal pregnancy. Interestingly, mares exposed to endophyte-infected tall fescue (fescue-toxicosis) fail to demonstrate a rise in serum progestogen concentrations during the last month of gestation.29

Relaxin

Relaxin in the mare is a polypeptide hormone produced specifically by the placental trophoblast with increases in circulating concentrations noted from around Day 80 of gestation to term.30 Relaxin concentrations increase during labor,30,31 and relaxin has been evaluated as a possible biomarker for placental function in mares.32 Although the function of relaxin during pregnancy and labor in mares is not well studied, there is some information suggesting that relaxin secretion is altered in abnormal pregnancy in the mare. In a series of clinical cases, relaxin concentrations were reduced in mares with abnormalities such as hydrops, placentitis, and premature placental separation compared to normal pregnant mares.32 However, relaxin concentrations were also highly variable between mares with breed differences in relaxin concentrations noted in one study.33 At present, equine-specific immunoassays for relaxin are not available clinically although this biomarker warrants further research to assess its utility in evaluating fetal well-being and placental function in the mare.

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a major protein present in fetal circulation as well as in allantoic and amniotic fluids. An early description of an immunoassay for equine fetal protein described an increase in measured concentrations of equine fetal protein (EQFP) in serum of mares with clinical placentitis, embryonic loss and twin pregnancy.34 Unfortunately, characterization of EQFP was not provided, and it is unclear if this protein was equine AFP. Further studies using this ELISA for EQFP were not reported in the literature and so no additional information regarding this assay is available. Alpha-fetoprotein is a major protein constituent of equine allantoic and amniotic fluids,35 and AFP was identified in high concentrations in equine fetal serum as well as in equine fetal fluids based upon a heterologous assay for
human AFP (Immulite® 1000 platform; Siemens Healthcare Diagnostics Tarrytown, NY). Alpha-fetoprotein was detected based upon the heterologous ELISA in plasma from pregnant mares, but not in plasma from geldings or nonpregnant mares. In mares with experimentally induced ascending placentitis, plasma AFP concentrations were increased by 7 days after inoculation compared to control mares. We hypothesize that increased concentrations of AFP in maternal plasma of mares with placentitis may be related to altered vascular permeability, but this mechanism requires confirmation. In women, elevated AFP concentrations in maternal circulation have been associated with increased risk for preterm birth; however, meta-analysis of 24 studies incorporating more than 200,000 pregnancies concluded that elevated concentrations of AFP in maternal circulation alone were not a predictive marker for preterm birth unless AFP was combined with other markers including hCG and estriol. In mares, AFP may have utility to predict abnormal pregnancy outcome; however, larger studies are required and incorporation of other markers such as estradiol or progestogens may be required to realize this potential.

**Acute phase proteins**

Acute phase proteins (APP) are synthesized primarily by the liver as part of the acute response of the innate immune system to stimuli such as trauma, infection, neoplasia or inflammation. In horses, acute phase proteins include serum amyloid A (SAA), haptoglobin, and fibrinogen. Fibrinogen is probably the most commonly used APP in horses; however, a number of recent studies have examined SAA and haptoglobin as indicators of acute inflammation in the horse. In particular, two studies reported to date have examined changes in APP in mares with experimentally induced placentitis. In the first study, maternal concentrations of SAA increased (>7mg/L) within two to seven days after experimentally induced placentitis, and peak concentrations of SAA varied from 274 to 4386 mg/L. In a group of nine mares that were experimentally inoculated with *Streptococcus equi subsp zooepidemicus*, treatment (trimethoprim-sulfa, altrenogest, pentoxifylline) was initiated at onset of clinical signs of placentitis. In these treated mares, SAA did not increase in six of nine mares, and all of these six mares delivered a live foal. In the remaining three mares, SAA was persistently elevated in one mare which aborted whereas SAA was transiently elevated in the remaining two mares which delivered live foals. These data indicated that changes in SAA in the mare are rapid after experimentally induced placentitis with *Streptococcus* and that changes in SAA concentrations may be predictive of outcome after initiation of treatment. In a second study, changes in the APP, SAA, haptoglobin and fibrinogen were examined in mares with experimentally induced placentitis secondary to inoculation of *Streptococcus equi subsp zooepidemicus*. In this study, SAA concentrations increased significantly within two days after inoculation whereas haptoglobin concentrations increased significantly at Day 3 after inoculation. Interestingly, neither fibrinogen nor total white blood cell counts differed between control and inoculated mares in this study.

Although APP appear to be a very sensitive biomarkers for models of experimental placentitis, it is important to note that they are also very nonspecific. Other inflammatory stimuli can elicit a pronounced rise in SAA, and we have seen marked rises in SAA in mares subsequent to routine immunization, for example. In clinical cases of placentitis, SAA appears to be a much less consistent biomarker for placental inflammation. In a large prospective field study in which mares (n = 700) were sampled weekly during late gestation, SAA was not consistently elevated in mares with placentitis diagnosed by histopathologic evaluation of the placenta (unpublished data). It is unclear whether this is due to differences in the causative organism, duration or chronicity of the disease or possibly some other factor.

**microRNAs**

MicroRNAs (miRNAs) are small (18–24 nucleotides) non-coding RNAs that function to regulate translation and degradation of specific messenger RNAs (mRNA). MicroRNAs make up approximately 1% of the genome of multiple species, are highly conserved across species and are believed to regulate at least of 30% of genes. Approximately 10 x more stable than messenger RNAs, miRNAs have an average half-life of approximately five days and are abundant in circulation where they are found bound
to carrier proteins or within small membrane bound vesicles termed exosomes. MicroRNAs appear to have a wide range of biological functions and have attracted considerable attention as potential diagnostic markers as well as therapeutic targets for a wide range of diseases. MicroRNA represents a source of cell-free nucleic acid derived from the placenta which is readily available in maternal circulation.

Although much work remains to decipher the roles of placental-derived microRNAs, it appears likely that these small, regulatory RNAs function in fetal-maternal communication in areas such as immunoprotection of the fetal allograft. Other proposed roles include implantation and placentation, angiogenesis, proliferation and decidualization, apposition/adhesion of the embryo to the endometrium and embryonic migration and invasion (reviewed in ). There are a number of pregnancy-associated miRNAs in women, and it appears that fetal miRNAs in the maternal circulation increase exponentially during the first trimester. In the human, many of the miRNAs found in maternal circulation are derived from a single cluster located on chromosome 19 which appear to play a role in protecting the placenta against viral infections. Additionally, miRNA levels change significantly with abnormal pregnancies such as ectopic pregnancy, idiopathic, recurrent pregnancy loss, low fetal birth weight, high fetal birth weight, pre-term labor and pre-eclampsia in women. Our laboratory is currently investigating the use of miRNA in the horse as potential biomarkers for placental function.

Conclusions

Determination of reliable biomarkers for fetoplacental well-being in the mare has many potential applications but also present a number of challenges. Steroids, particularly progestogens and estradiol appear to be good biomarkers for diseases such as placentitis; however, normal values need to be established across the gestational ages since these steroids have variable serum concentrations across different stages of pregnancy. Likewise, normal values need to be established for the particular immunoassay being used for measurement of steroids, particularly progestogens, due to varying cross reactivities of different assays for progestogens. Alpha-fetoprotein may also be a possible biomarker for placentitis in the mare since elevated concentrations of AFP were observed in mares with experimental placentitis. Although acute-phase proteins such as SAA demonstrate a rapid and large increase after experimentally induced placentitis, experience to date suggests that SAA is not consistently elevated in clinical cases. It is likely that ultimately a multiparameter biomarker panel will be required to assess fetoplacental well-being in mares. At present, such a panel might constitute estradiol, progestogens and alpha fetoprotein.

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References