Emerging diagnostic approaches for evaluation of fetal and pregnancy well-being in the mare
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Abstract
Placental insufficiency is regarded as the primary factor contributing to late-term abortion, pre-term delivery and perinatal death of foals. Often when problems associated with late-term pregnancy in the horse manifest the condition is well advanced and therapeutic intervention may not be effective in rescuing the pregnancy. If a compromised pregnancy could be identified early, the pregnancy might be sustained through appropriate medical intervention. The challenge is determining the most effective diagnostic approach that will correctly assess the status of the pregnancy and the uterine environment. It is highly unlikely that any one test will serve this purpose; rather a combination of diagnostic approaches may be necessary to accurately assess the well-being of the pregnancy and the fetus. As in human pregnancy, the most common cause of later term pregnancy loss and pre-term delivery is associated with some form of placental insult or deficiency which places the pregnancy at “high risk”. Thus, this review will focus on current diagnostic approaches (i.e., endocrine markers, transabdominal/transrectal ultrasound, Doppler ultrasound) utilized in equine medicine and also describe emerging techniques in human medicine (i.e, protein biomarkers in amniotic or cervico-vaginal fluids, bioluminescence imaging technology) that could be applied to the mare to identify “at risk” pregnancies.

Keywords: Emerging diagnostics, at risk pregnancies, equine

Introduction
It is well documented that pre-term deliveries in women are a leading cause of neonatal mortality and morbidity and increase the likelihood of other pathologies occurring in the surviving neonate. Offspring of preterm deliveries that do survive display delayed developmental progress and also have a greater chance of contracting disease and developing other chronic disorders in early life. Additionally, it is known that intra-amniotic infections are implicated as one of the causative factors of preterm birth. Similarly, pregnancy loss during late gestation and death of foals weakened by abnormal periparturient events constitute a large percentage of fetal and neonatal mortality in horses. Placental infection due to opportunistic pathogens (i.e., Streptococcus equi subspecies zooepidemicus or Escherichia coli) is the single most common cause of abortion, stillbirth and premature delivery in horses. Based on pathology records of horses, Giles and colleagues reported that 60% of fetal abortions, stillbirths and foals that died within 24 h of birth were associated with placental insufficiency, of which a third of the abortions and fetal deaths were associated with placental infections. Moreover, mares most commonly afflicted tend to be pluriparous. Due to the expense of breeding contracts and the long gestation of the mare, late-term fetal death represents a major financial loss and time investment for the breeder. Early identification of placental insufficiency would make it possible to sustain the pregnancy through medical intervention. For example, when placental function is compromised in mares suffering from fescue toxicosis, timely drug therapy improves pregnancy outcome. Likewise, if diagnosed early, placentitis can be treated with systemic antibiotics, progestins (progesterone, 17α- hydroxyprogesterone, 5α-pregnanes), non-steroidal anti-inflammatory drugs or corticosteroids in an attempt to hasten fetal organ maturation. However, despite this knowledge, a full understanding of the underlying pathophysiology of preterm birth is still lacking, and diagnostic tools, which can accurately and reliably identify “at risk” pregnancies, do not readily exist.

This review will focus on current diagnostic approaches (i.e., endocrine markers, transabdominal/transrectal ultrasound, Doppler ultrasound) utilized in equine medicine and also describe emerging techniques in human medicine (i.e, protein biomarkers in amniotic or cervico-vaginal fluids, bioluminescence imaging technology) that could be applied to the mare to identify “at risk” pregnancies.

Endocrine markers
Late gestation abortions and pre-term deliveries in the mare are commonly associated with disruption of normal endocrine function due to placental insult from infectious pathogens, and/or pathological changes to the fetoplacental unit or due to serious maternal illness (i.e., colic) or disease. LeBlanc has classified three groups of hormones involved in pregnancy based on their physiological roles: for example those involved in uterine quiescence (i.e., progestins), those that stimulate uterine contractions (i.e., prostaglandins, oxytocin) and those
involved in fetal maturation (i.e., glucocorticoids). One may add relaxin, a small polypeptide hormone that is found during pregnancy in many species including the equine. To this end, serum-based hormone assays have been used in the past to determine pregnancy and fetal viability in the horse and are likely to continue to be used to monitor pregnancy well-being in the equine.

Progestins

In most mammalian species, progesterone (P4) is essential for maintenance of pregnancy and may be produced by the ovary as in the sow and/or the placenta as in the mare. This C-19 steroid controls the duration of pregnancy by maintaining myometrial quiescence. The horse is unique among domestic species in that at about Day 35 of gestation a discrete thickening of the trophoblast develops on the outer surface of the chorion to form what is described as the chorionic girdle and later form endocrine-secreting structures known as the endometrial cups. From approximately Day 40 through to Day 70 of gestation these structures secrete equine chorionic gonadotropin (eCG) that has luteinizing hormone-like activity which ovulates successive waves of follicles stimulated to develop on the ovary under the steady release of pituitary derived follicle stimulating hormone (FSH) during the first half of pregnancy. These secondary corpora lutea secrete progesterone and persist until such time that the choioallantoic placenta is well enough established to take over completely the supply of sufficient progesterone to maintain the pregnancy until term (see review). However, during the second half of pregnancy P4 is rapidly metabolized into several related progestagens (pregnananes and pregnenes) as previously reported, but little is known about the biological activity of these P4 metabolites. Thus, from Day 40 to Day 100 of gestation, progesterone could be used as a marker of pregnancy well-being. However, by Day 85 of gestation, the fetoplacental unit begins to synthesize large quantities of steroid hormones utilizing fetal C-19 precursors secreted by the enlarged fetal gonads for the production of estrogens and maternal C-21 precursors for the synthesis of progesterone and large quantities of 5α-reduced progestagens. Near term, additional pregnenolone is secreted by the fetal adrenal glands so that the mare exhibits the unusual phenomenon of foaling while maternal serum progestagen concentrations are increasing and estrogen concentrations are decreasing. Thus, this mare is unusual among domestic species in that progesterone is only prominent during the early stages of gestation while plasma concentrations become negligible from mid-gestation to just prior to parturition when plasma concentrations begin to rise markedly. Consequently, a few studies have investigated the merits of monitoring progestagens as markers of pregnancy well-being in the mare.

Earlier studies have reported that progestagen concentrations are elevated in maternal plasma of mares as normal parturition approaches and in mares experiencing placental abnormalities leading to fetal losses or premature deliveries in late gestation. In contrast, those mares that abort their pregnancies during the first four months of gestation tend to have low plasma progesterone concentrations. Unfortunately, the absolute concentration of progesterone needed to maintain pregnancy at this stage of gestation has not been defined but typically circulating plasma concentrations greater than 2.0 ng/ml are thought to be necessary to support early pregnancy. While progesterone may be useful as an endocrine marker of pregnancy well-being during the early stages of gestation, it may have less potential during the second half of gestation due to lack of availability of commercial assays for the progestin metabolites found in systemic blood. Although luteal production of progesterone may persist beyond Day 120 of gestation, by Day 70 of gestation there is a measurable production of progesterone by the placenta. By Day 200 of gestation the placenta is the only source of progesterone which is rapidly metabolized to form 5α-pregnan metabolites. There are approximately nine progestagens other than progesterone that are quantitatively more important and may reach plasma concentrations ranging from 5 to 50 ng/ml. Two of these, 20α-hydroxy-5α pregnan-20-one (20αP) and 5α-pregnan-3β,20α-diol (3β,20α-diol) may reach circulating concentrations as high as 500 ng/ml which are not detectable by most conventional progesterone assays. Moreover, there are quantitative differences in the total progestagen concentrations between different breeds of horses but individual progestagens isolated from maternal plasma show remarkable consistency between breeds.

Measurement of circulating progestins has been advocated as a means of diagnosing placental dysfunction and pending pre-term delivery in the mare. In a 2005 study, Ousey and colleagues measured plasma concentrations of a cohort of progestagens during late gestation in mares with normal and compromised pregnancies. Total progestagen plasma concentration was measured using a commercially available ELISA assay system (Immulite Progesterone, Euro/DPC Ltd., Llanneris, Wales, UK) while nine specific metabolites were quantified using gas chromatography-mass spectrometry (GC-MS). The outcome of these studies was that mares with placentitis had increased concentrations of pregnenolone (P5), a primary precursor to progesterone, and/or increased progesterone and several metabolites including 5α-pregnan-30,20-dione, 20αP and 3β,20α-diol. The author speculated that this
increase was due to increased fetal production of P5 and/or P4 and increased metabolism in the utero-placental tissues in response to chronic stress. On the other hand, mares experiencing placental pathologies other than from infection had a mixed response and no clear link was demonstrated between maternal plasma concentrations of P4, or any of the other progestagens and the maintenance of pregnancy. Others have suggested that in addition to measurement of progestin concentrations, transrectal ultrasonography may also be used in the diagnosis of impending pregnancy loss. A study was undertaken to evaluate the accuracy in identifying mares with feto-placental compromise by experimentally induced placentitis using transrectal ultrasonography of caudal uterus in conjunction with the assessment of plasma progestin profiles. The outcome of such a dual diagnostic approach revealed that 20 of 22 mares were correctly identified with respect to their pregnancy outcome. The merits of ultrasonography as a diagnostic tool will be a subject of discussion later in this review.

Estrogens

Estrogens are not essential for the maintenance of pregnancy in the mare during late gestation but pregnancy is prolonged if there is disruption of normal fetal function leading to abnormal estrogen production which may result in poor uterine contractility and blood flow.33 The mare differs from other domestic species in that the feto-placental unit produces copious amounts of estrogens during the second half of pregnancy including estrone, estradiol 17β and the equine-unique ring B unsaturated estrogens, equilin and equilenin.7,29 The primary source for the estrogen precursors (i.e., dehydriopandrostosterone) in the horse is the fetal gonads33,34 and the aromatization of these androgen precursors occurs in the placenta.25 While the role of estrogens in pregnancy maintenance and parturition in the mare is not well understood, a few investigations have indicated that estrogen may promote synthesis of prostaglandins, an increase in oxytocin receptors and myometrial gap junctions thereby facilitating a switch in the amplitude and frequency of uterine contractions as parturition approaches.10 Other studies have demonstrated that uterine activity is elevated during the final week pre-partum and this activity is more pronounced during the nocturnal hours correlating with nocturnal increase in plasma estradiol 17β concentrations.35,37 Whether estrogens could be employed as useful diagnostic indicators of pregnancy well-being in the mare has not been adequately explored. However, some practitioners from clinical experience have reported that total serum estrogen concentrations <1000 ng/ml from approximately Day 150 to Day 300 of gestation is indicative of fetal stress while concentrations <500 ng/ml is generally indicative of impending abortion and the pregnancy is probably unresponsive to treatment.38 On the other hand, studies in late-term mares stressed by medical or surgical problems or induced abortions concluded that maternal and serum concentrations of estrogens (i.e., estrone sulfate) were not a sensitive indicator of fetal compromise or death and were observed only to decline after severe fetal stress or abortion was imminent or had a occurred.29,39

Relaxin

The placenta is the primary source of the small polypeptide hormone relaxin,40 more specifically the placental trophoblast cells.41,43 Relaxin is an important hormone of pregnancy, not only for facilitating uterine and mammary gland development, but also in the maturation of the cervix to facilitate normal delivery.16 Relaxin has several important functions during pregnancy and at time of parturition. Relaxin is essential for the live delivery of pups in the rat44,45 and pigs in the sow.46,47 Furthermore, relaxin treatment has been reported to improve calving in beef48,49 and dairy heifers by advancing the dilatation of the cervix and increasing pelvic area, thereby decreasing the incidence of dystocia.50 Relaxin can be measured in systemic blood of a number of mammalian species in high concentrations51,56 and thus has the potential to be a valuable clinical tool for both diagnosing placental and fetal well-being during late gestation. There is evidence that the aborting dog exhibits depressed serum relaxin prior to pregnancy loss.52,57 In addition, women with symptoms of impending miscarriage in early pregnancy had lower concentrations of relaxin compared to women with normal pregnancies.58,59 A similar pattern was observed in dogs exhibiting early embryonic loss.60 This led to the suggestion that relaxin might be a useful epidemiological tool in predicting pregnancy outcome in women.59 Little is known concerning the role of relaxin in equids during pregnancy and parturition. There is some evidence that relaxin secretory pattern is compromised during at risk pregnancies and complicated deliveries. Stewart and co-workers reported that plasma relaxin concentrations were low in Standardbred mares with abnormal termination of pregnancy and suggested that relaxin might be a useful indicator of placental function.61 Others have observed improved relaxin profiles and pregnancy outcomes in pony mares with at-risk pregnancies following treatment for fescue toxicosis.62
experimentally infected to induce placentitis. While relaxin profiles were found to be compromised in some of the clinical cases observed, the reliability of using circulating relaxin as a predictor for drug efficacy following the treatment of a threatened pregnancy due to uterine infections could not be determined satisfactorily due to high variability in relaxin values. Furthermore, there is some variation among breeds with respect to relaxin profiles during pregnancy, however, the functional significance of these differences in terms of actions of relaxin is unknown. In dogs, systemic relaxin near term varies considerably between individuals but lower than normal concentrations were associated with embryonic loss and spontaneous abortion. These data in dogs appear to support observations that individual mares with compromised placentas show variable systemic relaxin and this variability is sustained regardless of therapeutic intervention. Accordingly, relaxin may be less reliable in predicting pregnancy outcome following therapeutic intervention but useful in assessing loss of placental function.

Assessment of fetoplacental well-being by ultrasonography

Ultrasonography allows for the detailed and, to all intents and purposes, safe analyses of the function of the utero-placental unit and fetus during pregnancy. Imaging of the placenta and fetus began more than forty years ago with the initial introduction of the ultrasound with regular B-mode (2-dimensional grey-scale) which has evolved in the intervening years with the development and use of color Doppler, 3- and 4-dimensional imaging and the more recent introduction of contrast ultrasonography in human obstetrics and gynecology. Ultrasonography is now routinely used in human clinics, not only for assessment of placental function and vascular development, but also for identifying disorders of pregnancy including fetal growth restriction, pre-eclampsia, hydrops, oligohydramnios, and pathogenic infectious processes. The same level of sophisticated technology is not as readily available to most veterinary practitioners due to cost of equipment, but ultrasonography is used in most practices for assessment of ovarian function, pregnancy determination, placental and fetal well-being in both domestic livestock and companion animal species. Much of the breakthrough in the application of ultrasonography as it relates to pregnancy in the mare was undertaken by Ginther and colleagues in the early 1980s. Moreover, anomalies of the utero-placental unit using abdominal and transrectal ultrasonography have been important factors in the evaluation and assessment of fetal well-being and pregnancy outcome in normal and at-risk pregnancies in cattle (see review), horses and sheep. Transrectal ultrasonography has been especially useful in identifying those mares with placental disorders (utero-placental thickening, placental separation, placentitis) or mares with pending abortion due to disease or non-pregnancy-related illness. From a practitioner’s perspective, transrectal ultrasonography permits: 1) the measurement of the combined thickness of the uterus and placent (CTUP) at the cervix, 2) evaluation of placental edema or premature separation, 3) evaluation of allantoic and amniotic fluids, and 4) observation of the amnion and the measurement of its thickness. These parameters will be explored in more detail below.

Evaluation of changes in fetal fluid volume or echogenicity has proved to be of some diagnostic value. Ultrasonographic measurement of decreased fluid volume (oligohydramnios) is an abnormal finding in the mare and is usually associated with poor pregnancy outcome. Moreover, changes in the echogenicity of equine fetal fluids have been associated with various fetal diseases in foals including placentitis, mare reproductive loss syndrome (see reviews) septicaemia and peripartum asphyxia syndrome. However, echogenicity in fetal fluids also increases as term approaches in normal pregnancies and thus may not be as useful a predictor of pregnancy well-being. Amniotic fluid can be sampled to predict fetal lung maturity by evaluating the lecithin to sphingomyelin ratio (L/S) or performing amniotic lamellar body counts. Although amniocentesis is regarded as a safe procedure to perform in pregnant women it is a much more difficult procedure to perform in late gestation domestic animals with a reported incidence of abortion of 8% in cattle and as high as 25% in the mare. Transvaginal ultrasound-guided aspiration is a technique currently used with reasonable success by some practitioners to reduce unilateral twin vesicles in the mare. However, in our hands, both transabdominal and transcervical ultrasound-guided amniocentesis in the pony mare proved to be highly problematic leading to spontaneous abortion (D. Christiansen, personal communication). On a more positive note, ultrasonography can also be used to provide some predictable outcomes of pregnancy by measuring several biophysical parameters including fetal heart rate, fetal breathing movements, fetal body movements and measurements of specific organ parameters such as the stomach, heart, kidneys, fetal gonads, and fetal aortic and trachea diameter.

In a comprehensive study to develop criteria for assessing fetoplacental well-being in the mare, Bucca and colleagues employed both transrectal and transabdominal ultrasonography to monitor several biophysical parameters including evaluation of fetal heart rate, respiration, activity and assessment of vital organs, echogenicity and depth of fetal fluids and uteroplacental membrane thickness from mid-gestation to term. These authors have provided a detailed profile of normal biophysical parameters of the fetus at monthly intervals from gestation month
six (150-180 days) through to gestation month 12 (330-360 days) that could be evaluated as useful prognostic parameters. An interesting observation was the steady decline in both the mean heart rate at rest and during activity. Other consistent markers were the steady increase in fetal aortic and orbital diameters. However, transrectal measurements of CTUP, which were obtained at the ventral aspect of the cervical pole, showed considerable variation but the mean ventral CTUP showed a consistent increase from month to month. Renaudin and colleagues have reported a marked increase in CTUP in cases if ascending placentitis that were later confirmed by histopathology. In a more recent study, Morris and coworkers measured endocrine markers (progestin profiles) in conjunction with transrectal ultrasonography, particularly the CTUP in mares with experimentally induced placentitis. These investigators found a consistent relationship between placentitis and abnormal progestin profiles associated with an increase in CTUP above 1 cm. By performing the two tests, they reported that pregnancy outcomes were correctly predicted in twenty of the twenty two mares in the study. Ultrasonography has now become standard practice for evaluation of reproductive function, pregnancy confirmation and feto-placental well-being in most equine practices and is often supported by endocrine analysis when evaluating pregnancy well-being.

Doppler ultrasonography

While Doppler ultrasonography has been around for some time as a means of assessing vascular blood flow to vital organs it has become in more recent times an important clinical tool for assessing placental performance in healthy and high-risk human pregnancies and useful in predicting later complications and outcome of pregnancies that otherwise appear uncomplicated. This technology has been particularly useful in predicting pre-eclampsia and intrauterine growth restriction. Doppler ultrasonography has the advantage as a clinical tool in that it is capable of assessing fetal (umbilical artery), maternal (uterine arteries) and placental circulations (intraplacental circulation). Doppler ultrasonography has been used to characterize uterine blood flow throughout gestation in a number of species including the cow, dog and cat. A recent study characterized the functional hemodynamics of the utero-placental arterial vessels in the rabbit during pregnancy as well as in the umbilical cord, aorta and caudal vena cava of fetuses. In this study, the authors observed throughout gestation a significant (P <0.05) increase in the systolic peak velocity and end diastolic velocity in maternal and fetal vessels, whereas pulsatility index and resistance index decreased (P <0.05), except in utero-placental vessels. This led the authors to suggest that the pregnant rabbit could be used as an experimental animal model to assess by Doppler ultrasonography, functional hemodynamic changes in placental and fetal vessels under both normal and pathophysiological conditions.

In the equine, there has been limited use of this technology for diagnostic purposes of high-risk pregnancies since the initial report on the detection of fetal circulation in the mare and cow using Doppler ultrasonography. However, its application and potential usefulness in the mare has been evaluated. In contrast, much research has been undertaken in this field with regard to ovarian function, implantation and early fetal development. A limiting factor in the past was the size of the equipment and the cost, but recent technological advances have led to the production of battery powered, hand-held color Doppler machines. However, the expense of these devices may still undermine the economical use of Doppler technology for most large animal practitioners. To the best of our knowledge, there is no comprehensive report on the use of Doppler ultrasonography as a means of assessing fetal, placental and uterine circulation during late gestation in the mare, and whether such an approach would yield useful predictive information for determining pregnancy outcome is unknown. Thus, color Doppler ultrasonography is an area that may need further investigation as to its suitability as a diagnostic approach for assessing and identifying high-risk pregnancies during late gestation in the mare.

Qualitative and quantitative evaluation by ultrasound of fetal fluid, utero-placental unit and combined thickness of the uteroplacental unit and biophysical parameters such as fetal heart and respiration rates can be useful prognostic markers of feto-placental well-being. The most common clinical conditions requiring the need for assessment of feto-placental health in the mare are history of problematic pregnancies, placentitis, trauma, systemic illness or major surgery. However, it has been suggested that such measurements as a routine means of fetal monitoring is of limited benefit in the equine due to the inability to accelerate precocious fetal maturation and safely induce parturition. In addition to the use of ultrasonography as a means of monitoring pregnancy well-being, major advances have also been made in utilizing other non-invasive approaches to identify novel biomarkers associated with compromised pregnancies.
Proteomic analysis of amniotic and cervico-vaginal secretions

Recent advances in proteomic technology have sparked interest in identifying protein biomarkers of preterm pregnancies in humans. The premise behind these studies is that the proteomic profile of unhealthy tissues is drastically different than that of normal tissues. From these studies, valuable knowledge and research techniques can be gained and possibly applied to preterm pregnancies in the equine. To this end, proteomic technology has the potential to allow for the discovery of preterm pregnancy biomarkers that would permit early detection of “at risk” pregnancies so that adequate medical intervention can be provided to the pregnant mare and fetus.

Sampling

In the search for preterm pregnancy biomarkers, one of the primary considerations is to utilize a safe and relatively non-invasive sampling technique that is capable of revealing differences between normal and compromised pregnancies without inflicting harm upon the pregnancy. Much of the literature surrounding the detection of preterm pregnancy biomarkers in humans utilizes either amniotic fluid or cervico-vaginal fluid (CVF) for proteomic analysis. However, amniocentesis is a fairly invasive procedure and can increase the risk of miscarriage, so if the goal is to utilize a minimally invasive sampling technique, then CVF would be the most ideal candidate to use in the mare. Cervico-vaginal fluid can be obtained non-invasively simply by swabbing the inside of the vaginal wall. The swab is then placed into a polystyrene tube containing a buffer solution, and the contents of the tube are centrifuged, allowing the supernatant to be collected for storage at -80 °C or immediate analysis. Accordingly, this approach would provide a non-invasive means for acquiring a diagnostic sample, avoiding the risks associated with performing amniocentesis while still providing a sample for proteomic analysis.

Proteomic analysis—a basic description

Once an adequate sample is collected, it must be subjected to proteomic analysis which allows for the isolation, detection and quantification of small amounts of proteins within a complex mixture of other proteins, thus allowing protein expression patterns to be discovered when comparing normal and compromised pregnancies. The diagnostic sample is analyzed by one of the many proteomic techniques that exist. Generally, this is a two step process, whereby the samples are first subjected to a separation process, such as liquid chromatography or two-dimensional gel electrophoresis (2DGE), followed by analysis by one of the many mass spectrometry techniques (MS), such as matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), electrospray ionization (ESI), or surface-enhanced laser desorption/ionization (SELDI) MS which identify the chemical properties that are unique to every protein. The information from the MS analysis is compared to data stored in enormous bioinformatic databases, and the proteins from the sample are identified. This is obviously an over-simplified description of the elaborate steps in the process, yet it provides a concise depiction of this technique. Ultimately, the goal is to characterize disorders, such as preterm birth, based upon the type and amount of protein present in a given sample in the hope of creating a panel of biomarkers that would be indicative of mares at risk of delivering early. The disadvantage is that veterinary clinics would have to submit samples to an appropriate diagnostic laboratory for processing and currently it is cost-prohibitive for use in general practice.

Current progress in human medicine

Often, human medical knowledge stems from work performed in animals; however, in the case of identifying proteomic biomarkers for pregnancy complications in the equine, such as preterm birth and intra-amniotic infection (IAI), much can be learned from the recent work involving clinical research in humans. One of the more promising studies was conducted by Buhimschi and colleagues in which amniotic fluid samples from 104 patients were evaluated. Utilizing SELDI MS, these authors identified a unique panel of four proteins, comprised of neutrophil defensins-1 and -2 and calgranulins A and C, that was associated with women eventually diagnosed with IAI. Further work included assigning the patients a score value (ranging from 0 to 4), which gave the patients of neutrophil defensins-1 and -2 and calgranulins A and C, that was associated with women eventually diagnosed with IAI but absent in non-infected patients,97 amniotic fluid revealed that calgranulin B and a proteolytic fragment of insulin like growth factor binding protein-1 (IGFBP-1) were found to be present in the amniotic fluid of patients with IAI but absent in non-infected patients,97 revealing two additional potential protein biomarkers of IAI in humans. There are also data to support the use of
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these proteins is also crucial. Among the many proteins that have been identified as potential biomarkers of preterm

Physiological role of differentially expressed proteins
Identifying proteins that are up-regulated in compromised pregnancies is an important step towards fully
describing the pathophysiology associated with preterm birth; however, understanding the physiological function of
these proteins is also crucial. Among the many proteins that have been identified as potential biomarkers of preterm
pregnancies, many of which are not mentioned in this paper, there appear to be eight which are consistently
associated with the occurrence of preterm birth and IAI in humans: annexins II and V, proteolytic fragments of
IGFBP-1, neutrophil defensins 1 and 2, and calgranulins A, B, and C.97,98 It is plausible that these eight proteins may one day comprise a panel of biomarkers to aid in the diagnosis of preterm pregnancies; therefore, discussion of their function in relation to preterm pregnancies and IAI cannot be disregarded.

Annexins are a group of proteins that have a wide variety of physiological functions within cells, including
inflammatory and defense responses.98 By binding to calcium and phospholipids, annexins regulate prostaglandin
formation, a process that involves the mobilization of arachidonic acid from cell membrane phospholipids through
the action of phospholipase A2, which requires calcium for proper function.109,110 Thus, through regulation of
annexin protein expression, prostaglandin production can also be controlled throughout pregnancy and at parturition.

Throughout gestation, the expression of insulin-like growth factor (IGF) is necessary for many events
including implantation and fetal growth.111,112 In addition to IGF, IGFBP-1, localized in the decidua,113 is also vital
for a successful pregnancy through its regulation of IGF activity.112 The presence of IGFBP-1 in amniotic fluid
would not be unexpected; however, cases of IAI in which a proteolytic fragment of IGFBP-1 was found to be over-
expressed in amniotic fluid97 and CVF2 should cause concern. The presence of an IGFBP-1 proteolytic fragment in
the amniotic fluid and CVF has been suggested to be indicative of a response to infection that involves a protease-
related mechanism.98 Furthermore, the presence of an IGFBP-1 proteolytic fragment in the CVF is of great concern
because it signifies a failure of tissue junctions located at the choriodecidual interface, allowing chorionic-decidual
products to leak into the vaginal cavity.113 Therefore, the presence of proteolytic fragments of IGFBP-1 in the
amniotic fluid97 and CVF2 of patients with IAI can be explained as a response to invading pathogens that causes the
cleavage of IGFBP-1, and additionally, the presence of an IGFBP-1 proteolytic fragment in the CVF would indicate
a failure of membrane junctions, possibly preceding preterm birth.
Neutrophil defensins 1 and 2 are small cationic peptides that are stored in the primary granules (azurophil) of neutrophils and are known to provide immune protection from bacterial, fungal, and viral infections. Information surrounding neutrophil defensins suggests that these antimicrobial proteins exert their antimicrobial activity through a non-oxidative process, whereby they disrupt the structural integrity of the microbial cell membrane, ultimately causing cell death. Additionally, neutrophil defensins provide a link between innate and adaptive immunity through their recruitment of adaptive immune cells such as monocytes and T-cells, leading to a prolonged immune response. Thus, it is not surprising to find these two proteins (neutrophil defensins 1 and 2) up-regulated in the amniotic fluid of patients with IAI. The presence of these proteins suggests an immune response by the patient’s body that will provide both an immediate action, through the antimicrobial properties of defensins, and a long term immune response through the recruitment of adaptive immune cells such as monocytes and T-cells.

In addition to defensin proteins, calgranulins A, B, and C are also expressed in neutrophils and are classified as members of the S100 protein family. Calgranulin C is considered to be a minor calgranulin in neutrophils but still provides bactericidal properties against gram-negative bacteria. Together, calgranulins A and B form a zinc and calcium binding heterodimer protein referred to as calprotectin, which exerts a biostatic effect on a wide variety of microorganisms by competing against microorganisms for calcium and zinc, which are vital for cellular activity. Furthermore, in addition to its antimicrobial properties, calgranulin B is also implicated in the normal process of parturition given that its expression level increases in the cervix and myometrium at the onset of term labor. Additionally, calgranulin A and B play a role in prostaglandin production by promoting arachidonic acid transport in the cervix and myometrium during normal parturition. Understanding the physiological function of calgranulins A, B, and C provides insight into why they are up-regulated in cases of IAI and preterm birth. For cases of IAI, the up-regulation of the calgranulins provides an immediate immune response to invading pathogens by competing for minerals that are vital for the pathogen’s growth and survival. Additionally, in cases of preterm birth, premature up-regulation of calgranulins A and B possibly leads to an untimely increase in prostaglandins, ultimately inducing labor before term.

From the studies described, it is apparent that protein biomarkers of preterm birth and IAI are present in the amniotic fluid and cervico-vaginal fluid of humans. This affords many opportunities for equine researchers and clinicians to apply proteomic technology to the study of equine preterm birth in the hope of identifying similar protein biomarkers for preterm birth. Ultimately, the goal would be to utilize the information from such studies for the development of simple and rapid screening tests that would afford early detection of mares at risk of delivering before term so that adequate medical intervention can be provided to the mare and developing fetus.

**Biophotonic imaging technology and pathogenesis in reproduction**

Biophotonics involves the union of photonics and biology, and deals with the interactions between light, biological matter and its application to biomedical science. Nature has harnessed the photon (light) in many ways as a basic principle of life; whether through photosynthetic pathways in plants, as methods of communication among insects (e.g., firefly) or a multitude of other examples from across the natural world. The use of photonics in experimental models of human conditions has become a key diagnostic and research tool for understanding physiological systems in normal and diseased states that were not previously attainable with other detection systems. The last 10 to 15 years has seen unprecedented adaptations of this technology for investigating a variety of physiologically relevant systems in situ, including single cell, whole plants, Drosophila and rodents. Real time imaging technology using luciferase reporter genes to monitor physiological events in vivo has become an important and meaningful approach to studying, in real time and over time, biological responses including efficacy of drugs for cancer therapy, pathogenesis of bacterial pathogens or the regulation and expression of specific genes during specific physiological events.

Recently, we have employed a transgenic mouse model in which a promoter region of the vascular endothelial growth factor receptor 2 (VEGFR2) gene, is cloned up stream of the luciferase gene and when activated, in the presence of exogenous substrate (i.e., luciferin), can be non-invasively monitored using a highly sensitive imaging system to study regulation of vascular development under different physiological conditions. The VEGFR2 gene is transcriptionally regulated during angiogenesis under the influence of VEGF, an important angiogenic peptide. Currently, we are employing this mouse model to investigate the effects of intra-uterine growth restriction as a consequence of dietary deficiencies in pregnant females by monitoring the expression pattern of the VEGFR2-lux gene in fetal-placental tissues (Greene and Ryan, 2010, unpublished observations). Recent applications of biophotonic paradigms in large animal models including swine and sheep, and the development of methods to facilitate deep tissue photon capture (e.g., optical clearing techniques) across tissues.
(skin and intestine) suggests in vivo as opposed to ex vivo or post-mortem imaging may be feasible in the near future using minimally invasive detection procedures. Moreover, we have demonstrated the benefits of using genetically modified bacteria with the lux operon and bioluminescence imaging technology for evaluating pathogen progression in the gastro-intestinal tract of swine, \(^{132}\) in ex vivo studies of fetal lambs delivered to infected ewes \(^{133}\) and the ex vivo bovine reproductive tract. \(^{136}\) Through further development of the paradigm outlined in the above reported studies, applications might be extended to provide a more resolved model for understanding the progression of events (e.g., bacterial-induced endocrine changes, bacterial invasiveness of the fetal environment) that leads to preterm delivery and pregnancy failure in mares, and may have applications for evaluation of therapeutic interventions with the goal of reducing antenatal mortality. \(^{137}\) In addition, the postpartum clearance of bacteria following abortion and therapeutic intervention is another potentially valuable application for this technology. \(^{137}\)

**Conclusion**

This review explored the merits and disadvantages of present day diagnostics used as indicators of pregnancy well-being and introduced the potential application of new and/or emerging techniques currently employed in human medicine (i.e., proteomic marker analyses of amniotic and cervico-vaginal fluids). In addition, the potential merits of utilizing bioluminescence imaging technology to better understand the pathogenic process that lead to compromised pregnancies warrants continued investigation. The application of this novel imaging technology with lux-modified organisms may facilitate not only the development of more targeted therapeutic interventions, but also better diagnostic approaches to assess fetal and placental well-being. In addition, the ability to utilize cervico-vaginal swabs for evaluating pregnancy well-being in the mare has exciting possibilities in moving the diagnostic process forward. Ultimately, identifying “at risk” pregnancies in a timely manner may require a combinatorial approach using a number of emerging and current diagnostic tools as reliable predictors of pregnancy outcome.

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