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Alterations of Circulating Biomarkers During Late Term Pregnancy Complications in the Horse Part II: Steroid Hormones and Alpha-Fetoprotein

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ABSTRACT

Preterm labor and/or abortion causes considerable economic impact on the equine industry. Unfortunately, few experimental models exist for the induction of various pregnancy-related complications, and therefore extrapolations are made from the experimental model for ascending placentits, although inferences may be minimal. Certain steroid hormones (progestogens, estrogens) and fetal proteins (alpha-fetoprotein; AFP) might improve the diagnostics for abnormal pregnancy, but the utility of these markers in the field is unknown. To assess this, thoroughbred mares ($n = 702$) were bled weekly beginning in December 2013 until parturition/abortion. Following parturition, fetal membranes were assessed histopathologically and classified as either ascending placentitis ($n = 6$), focal mucoid placentitis ($n = 6$), idiopathic abortion ($n = 6$) or no disease ($n = 20$). Weekly serum samples were analyzed for concentrations of progesterone, estradiol-17β, and AFP. Samples were analyzed retrospectively from the week of parturition/abortion in addition to the preceding four weeks. For both ascending and focal mucoid placentitis, a significant increase in progesterone and AFP was noted, alongside a significant decrease in estradiol-17β and the ratio of estradiol-17β to progesterone in comparison to controls. In contrast, idiopathic abortions experienced a decrease in progesterone concentrations alongside an increase in AFP, and this was only noted in the week preceding parturition/abortion. In conclusion, spontaneous placental infection in the horse altered both endocrine and feto-secretory markers in maternal circulation, while minimal changes were noted preceding noninfectious idiopathic abortion. Additionally, this is the first study to report an alteration in steroid hormones and AFP during the disease process of focal mucoid placentitis, the etiology of which includes Nocardioform placentitis.

1. Introduction

In central Kentucky, placental infection is the leading cause of abortion in the horse [1,2]. The detection of microbes in the fetus and placenta is broadly referred to as placentitis, and can present in various pathologies, including ascending, hematogenous, or viral [3]. The experimental induction of ascending placentitis has advanced our understandings of the pathophysiology of this disease, in addition to providing prospective circulatory biomarkers for disease detection [4–15]. Unfortunately, no experimental model exists for the induction of focal mucoid placentitis, the etiology of which includes Nocardioform placentitis [16] or problems caused by noninfectious abnormalities, including umbilical torsion or premature placental separation. Therefore, the majority of diagnostic biomarkers have been extrapolated from the experimental induction of

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Ethical statement: All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky under the guidelines of the approved protocol #2013-1190.

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ascending placentitis toward other pregnancy-related complications, although inferences may be limited.

Clinically, ascending placentitis is diagnosed based on an increase in combined thickness of the uterus and placenta (CTUP) at the caudal pole of the cervix as noted by transrectal ultrasonography, in addition to clinical symptoms; including vaginal discharge, and premature mammary gland development and lactation [17–22]. Similar clinical modalities are utilized to diagnose focal mucoid placentitis, although the location of the lesion requires transabdominal ultrasonography, and vaginal discharge is typically not observed [23]. Unfortunately, physiological alterations such as lactation are believed to occur late in the disease process, allowing for limited therapeutic intervention. Because of this, circulatory biomarkers for early detection of the disease has been a topic of interest for researchers and clinicians alike, with focus on fetoplacental-specific markers at the forefront.

The steroid landscape of mid- to- late gestation in the horse is in constant fluctuation. Estrogens, progestogens, and androgens are produced in high concentrations by maternal and fetal tissues, and are metabolized within the placenta before being secreted into maternal circulation [24–26]. A shift in the production of progestogens occurs at roughly 100 days of gestation, as the ovarian-produced progesterone declines while 5α-reduced metabolites of this hormone increase at this time due to the enzymatic abilities of the fetoplacental unit and remain elevated until experiencing a precipitous decline in the final 3 to 7 days of gestation [27,28]. In contrast, immense estrogen production is noted in mid-gestation, reaching a summit at approximately 200 days, and then decline from this point until parturition [27]. In the horse, the experimental induction of ascending placentitis has been found to alter this endocrine response, and this includes an increase in progestogens [10], alongside decreasing estradiol-17β [6] preceding abortion. Additional markers of this disease have been investigated and include an elevation of fetal-derived alpha fetoprotein (AFP). The fetal analogue to albumin, this protein has been found to increase following the experimental induction of ascending placentitis, both in fetal fluids and maternal circulation [7].

While the experimental model for ascending placentitis offers considerable inferences into the pathophysiology of the disease, it is believed to be a more acute disease process in comparison to that noted in the field. Additionally, the etiology of focal mucoid placentitis and noninfectious pregnancy-related complications are poorly understood, and extrapolations made from the experimental induction of ascending infection may be inaccurate. Therefore, an assessment of the efficacy of the aforementioned diagnostic markers in predicting disease under field conditions requires evaluation of naturally-occurring disease in the clinical setting. The objectives of this study were to a) assess changes in steroid hormones and AFP in a naturally occurring disease process, and b) compare these alterations following specific pregnancy-related complications.

2. Materials and methods

2.1. Animal enrollment

2.1.1. Blood samples

All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky under the guidelines of the approved protocol #2013-1190. Horses (*Equus caballus*) used in this study were Thoroughbred mares ($n = 702$; 4-22 years of age) housed on 15 private farms located in central Kentucky, USA and samples were obtained with owner permission. Mares were bred via live cover during the natural Northern Hemisphere breeding season to various stallions, and then enrolled in the study based on farm residency. Beginning in December 2013, blood was obtained weekly via jugular venipuncture utilizing a vacutainer tube (10mL; Monoject; VWR, USA) for serum extraction, and sampling continued until either abortion or parturition occurred. Samples were transported back to the lab at ambient temperature and then centrifuged at 1,800 x g for 15 min. Serum was aliquoted and stored at -20°C until time of analysis.

2.1.2. Postpartum evaluation of placenta

Upon abortion and/or abnormal foaling outcome, fetal membranes from the affected mare were submitted to the University of Kentucky Veterinary Diagnostic Lab (UKVDL) for histopathology and disease diagnosis alongside the fetus if abortion occurred. Additionally, fetal membranes from control mares were enrolled based on farm residence, age, and parturition date, and evaluated by the same laboratory to confirm the absence of disease. Chorioallantois was assessed as previously described by Hong et. al (1992), in addition to fetal tissues if abortion occurred [1]. In brief, fetal membranes were weighed, and gross lesions recorded. Samples were obtained from lesion areas, in addition to the chorioallantois (body, gravid horn, and nongravid horn) and amnion. If abortion occurred, tissue samples were obtained from fetal liver, lung, heart, brain, and spleen, in addition to umbilicus. All tissue samples were stained with hematoxylin and eosin, and special stains (including Brown and Brenn Gram stain, Brown and Hopps Gram stain, Gomori's methanamine silver stain, and Warthin-Starry stain), were applied when needed. Samples of chorioallantois were cultured for bacteria and fungi as previously described [29]. Direct fluorescent antibody tests were conducted on tissue for *Leptospira spp*., equine arteritis virus, and equine herpesvirus, while blood and fetal fluids were collected and titrated for antibodies against *Leptospira spp.* by the microagglutination tests. Bacterial culture isolates were assessed via polymerase chain reaction (PCR) as described by Erol et al. (2015) [23]. In brief, DNA was isolated from surface swabs taken from the chorioallantois and bacterial culture isolates utilizing the MagNAPure Compact System (Roche Applied Science, Indianapolis, IN, USA) following manufacturer's instructions. PCR reactions were used to detect common nocardioform actinomycetes *Amycolatopsis spp.* and *Crossiella equi*. Clinical data was collected on the case and control mares regarding pregnancy history, breeding date, and dates of treatments or vaccinations the mare received.

2.2. Study design

The study was performed as a prospective enrollment of random mares before the retrospective analysis on the selected mares at the week prior to parturition/abortion (0–7 days prior to parturition/abortion) in addition to weekly, at -1 (8–14 days prior to parturition/abortion), -2 (15–21 days prior to parturition/abortion), -3 (22–28 days prior to parturition/abortion), and -4 weeks (29–35 days prior to parturition/abortion). As all ascending and focal mucoid placentitis mares delivered a viable neonate at term, this is shown as weeks prior to parturition, with control mares shown as weeks prior to parturition. As the idiopathic abortion group underwent abortion prior to full term gestational length, samples for this group were compared to control mares at a similar gestational length that were not immediately prepartum. Mares with no disease were selected based on specific criteria including last breeding date, age, and residence farm, and confirmed as having no disease upon postpartum diagnosis. Additional groups were chosen based upon postpartum diagnosis with ascending placentitis, focal mucoid placentitis, or idiopathic abortion before concentrations of progesterone, estradiol-17β, the ratio of E2:P4, and AFP were detected at these sampling points. For the group of idiopathic abortion, mares with no disease were matched at comparable gesta-

tional lengths to the sampling prior to abortion in the diseased mare category.

2.2.1. Progesterone

Total progesterone was measured as previously described by Wynn et al. (2018) [28]. In brief, a commercial immunoassay (Progesterone; Siemens Healthcare Diagnostics, Tarrytown NY, USA) was run on a chemiluminescence platform (Immulite 1000). Intra- and inner-assay CVs were 7.4% and 8%, respectively. The detection range for this assay is from 0.2 to 40 ng/mL, with an analytical sensitivity of 0.5ng/mL. Minimal cross-reactivity for this assay has been noted with other metabolites of progesterone [28].

2.2.2. Estradiol-17β

Estradiol-17β was measured as previously described by Canisso et al. (2017) [6]. In brief, a commercial immunoassay (Estradiol; Siemens Healthcare Diagnostics, Tarrytown NY, USA) was run on a chemiluminescence platform (Immulite 1000). Intra- and inner-assay CVs were 1.2% and 3.15% respectively. The detection range for this assay varies from 20 to 2,000 pg/mL with an analytical sensitivity of 15 pg/mL. The cross reactivity for this assay is non-significant for most adrenal and steroid hormones, although a cross reactivity with estrone (2.09%) is noted.

2.2.3. Alpha fetoprotein

Alpha Fetoprotein (AFP) was measured as previously described by Canisso et al. (2015) [7]. In brief, a heterologous commercial immunoassay (AFP; Siemens Healthcare Diagnostics, Tarrytown NY, USA) was run on a chemiluminescence platform (Immulite 1000). The AFP assay has a range of 0.2 to 200 IU/mL. Samples above the upper detection limited were diluted automatically by entering the dilution ratio in the platform with the diluent that accompanies the commercial kit.). Intra- and inner-assay CVs were 1.8% and 2.9% respectively According to the manufacturer, conversion of IU/mL to ng/mL of human AFP can be accomplished with a correction factor of 1.21.

2.3. Statistics

Statistical analyses were performed using SAS 9.4 using varying models depending on the data. The week prior to parturition/abortion, in addition to weekly sampling for 4 weeks prior to this, were analyzed for concentrations of each marker. All data were assessed for normality utilizing a Bartlett's test and a Modified Levene's test for equal variances. Weekly data were analyzed using a repeated measures Analysis of Variance (ANOVA). Group means for clinical data were analyzed utilizing an independent group *t*-test to make comparisons. Significance was set to $P < .05$ and trends at $P < .1$. Data is presented as the mean $\pm$ the standard error of the mean.

3. Results

3.1. Clinical data

Full analysis of the clinical data can be observed in part one of this two-part manuscript. Based on evaluation of the postpartum chorioallantois and neonatal outcome, mares were categorized into case and control groups. Mares having infection/inflammation at the cervical star area were classified as ascending placentitis ($n = 6$). Mares identified with focal mucoid lesions within the body or horns of the placenta were classified as focal mucoid placentitis ($n = 6$). Mares having noninfectious etiology and poor neonatal outcome (either abortion or term stillborn) were classified as idiopathic abortion ($n = 6$). Moreover, mares with no placental disease noted on postpartum histopathology were enrolled as controls ($n = 20$). No difference was noted in mare age when comparing focal mucoid placentitis, ascending placentitis, or noninfectious abortion (10.16 $\pm$ 1.74 y, 13 $\pm$ 1.5 y, 11.2 $\pm$ 1.4 y; respectively) to control (10.3 $\pm$ 0.8; $P = .40$). All control mares carried to term (347.9 $\pm$ 2.7d) and produced a viable foal. All ascending placentitis (6/6) and focal mucoid placentitis (6/6) mares carried to term and gestational length was not different from that of controls (338.6 $\pm$ 5.9d and 344 $\pm$ 4.9d respectively). In contrast, mares diagnosed with idiopathic abortion were significantly decreased in gestational length in comparison to controls in addition to the two placentitis groups (291 $\pm$ 16.1d; $P < .05$) and had poor fetal outcome, with none of the neonates surviving (0/6). A subset of mares in each group were treated with various therapeutics, including antimicrobials, anti-inflammatory drugs and synthetic progestins.

3.2. Clinical Variables

Gestational age had a significant effect on progesterone ($P < .01$), estradiol-17β ($P = .04$) and the ratio between estradiol-17β/progesterone ($P < .01$) but did not have a significant effect on AFP ($P = .71$). Treatment did not have an effect on progesterone ($P = .34$), estradiol-17B ($P = .47$) or AFP ($P = .34$).

3.3. Ascending placentitis

A significant increase in concentrations of progesterone ($P < .01$) and AFP ($P < .01$) was noted across time in mares with ascending placentitis (Fig. 1). This coincided with a significant decrease in the concentrations of estradiol-17β in addition to the ratio between estradiol-17β and progesterone ($P < .01$). When assessing individual weeks, progesterone concentrations were found to be significantly elevated at -3, -2, and -1 weeks in addition to the week of parturition in the ascending placentitis group in comparison to controls. Similarly, AFP was found to be elevated at -2, -1, and within the week of parturition in the diseased group when compared to controls. Concentrations of estradiol-17β were found to be decreased in the ascending placentitis group in comparison to controls and this was noted at both -2 and -1 weeks, in addition to the week of parturition. When the ratio of estradiol-17β to progesterone was assessed, the decrease in this ratio was noted in the diseased group at -4, -2, -1, and the week prior to parturition, while a trend was noted at -3 in comparison to controls.

3.4. Focal mucoid placentitis

A significant increase in concentration of progesterone ($P < .01$) and AFP ($P < .01$) was noted across time in mares with focal mucoid placentitis (Fig. 2). Additionally, a significant decrease in the concentrations of estradiol-17β ($P < .01$) in addition to the ratio between estradiol-17β and progesterone ($P < .01$) was noted across time in comparison to controls. Concentrations of progesterone were found to be elevated at -3, -2, and -1 weeks in addition to the week of parturition, and a similar profile was noted for AFP. Concentrations of AFP were elevated at -3, and -1 weeks in addition to the week of parturition, with a trend toward an increase at -2 weeks noted in the diseased mare group in comparison to controls. The decrease in concentration of estradiol-17β in the focal mucoid placentitis group was found to be significant at -3, -2, and -1 weeks, with a trend toward a decrease noted during the week of parturition. A decrease in the ratio of estradiol-17β to progesterone was noted in the focal mucoid placentitis group at -4, -3, -2, and -1 weeks prior to parturition, but this was not found to be significant in the week of parturition.

ASCENDING PLACENTITIS

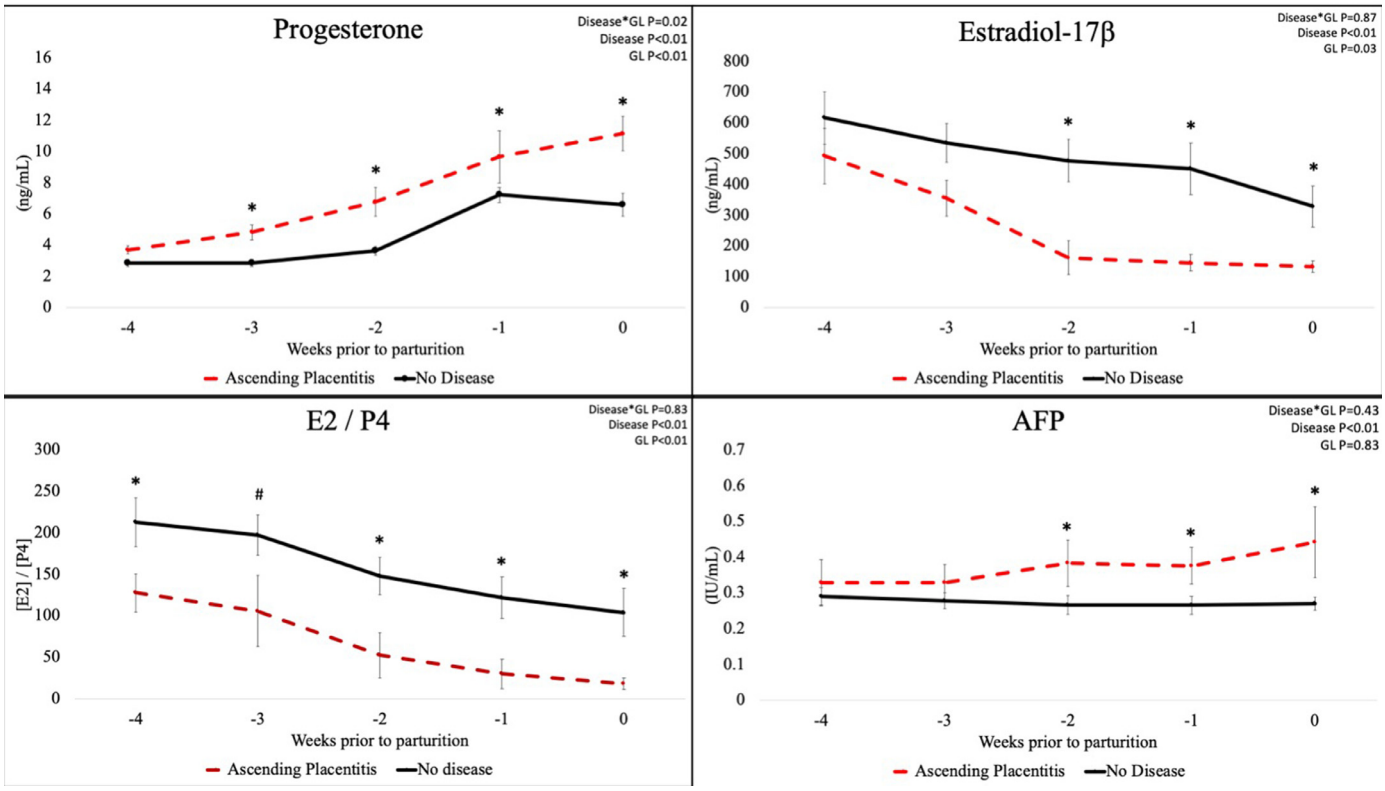

Fig. 1. Circulating concentrations of steroid hormones and AFP during ascending placentitis. Serum concentrations of progesterone, estradiol-17β, the ratio of estradiol-17β to progesterone, and AFP in mares with ascending placentitis ($n = 6$) compared to gestationally age-matched control mares ($n = 20$). A significant increase in progesterone and AFP was noted in ascending placentitis in the weeks preceding parturition. Additionally, a significant decrease in both estradiol-17β and the ratio of estradiol-17β to progesterone was noted in the diseased group when compared to controls. Asterisk (*) above data points indicates differences ($P < .05$), while pound sign (#) above the data points indicates differences ($P < .10$) within week between the diseased and control groups. Data is shown as the mean ± the standard error of the mean (SEM).

3.5. Idiopathic abortion

There was a significant decrease in progesterone noted in the idiopathic abortion group when compared to controls ($P < .01$), in addition to a trend toward an increase in AFP concentrations ($P = .08$; Fig. 3). In contrast, no alterations were noted when assessing concentrations of either estradiol-17β or the ratio of estradiol-17β to progesterone with regard to the idiopathic abortion group. The decrease in progesterone was noted both one week prior to parturition as well as within the week of parturition, while no significant increase was noted at -2, -3, or -4 weeks. The increase in AFP was noted at a trend during the week of parturition, with no significant alterations noted at -1, -2, -3, or -4 weeks prior to parturition when compared to controls.

4. Discussion

Alterations in the endocrine profile of mares experiencing ascending placentitis have previously been reported [6,10,30], but to our knowledge, this is the first study to report a similar alteration in these steroid hormones during the disease process of focal mucoid placentitis, the etiology of which includes Nocardioform placentitis. This included an increase in progesterone, a decrease in estradiol-17β, a decrease in the ratio of estradiol-17β to progesterone, in addition to an increase in the fetal secretory protein AFP. Additionally, minimal alterations were noted in endocrine or fetal secretory markers assessed when evaluating idiopathic abortion, although a decrease in progesterone alongside an increase in AFP could be noted in the week immediately prior to parturition, indicating the difficulty in detecting these noninfectious pregnancy-related complications.

The endocrinology of equine gestation is dynamic, with changes noted in estrogens, progestogens, and androgens. The steroid landscape of early pregnancy is dictated by the maternal gonads, with progesterone being the primary source of gestational support. This shifts at roughly 100d of gestation, upon which the fetal gonads experience marked growth in addition to the feto-placental unit acquiring the enzymatic activity to both metabolize and secrete steroids [25,31–33]. This leads to a surge in the production of metabolites of steroid hormones, which are then excreted into the fetal fluids, in addition to fetal and maternal circulation [26,31,34,35]. Metabolites of progesterone, deemed progestagens, increase in the final month of equine gestation, before experiencing a precipitous decline 3 to 7 days before parturition, and this profile was noted within the control group in the present study [28]. Additionally, the increase in fetal gonad size leads to a dramatic increase in estrogens, and specifically estrone sulfate, within maternal circulation in mid gestation, reaching its peak around 200 days of gestation, before declining toward parturition [24,25]. This was also noted within the current study, as the normal mare experienced a decline in circulating estradiol-17β during the final month of gestation.

In contrast to the healthy pregnancy, complications that occur during pregnancy have been associated with alterations in the en-

FOCAL MUCOID PLACENTITIS

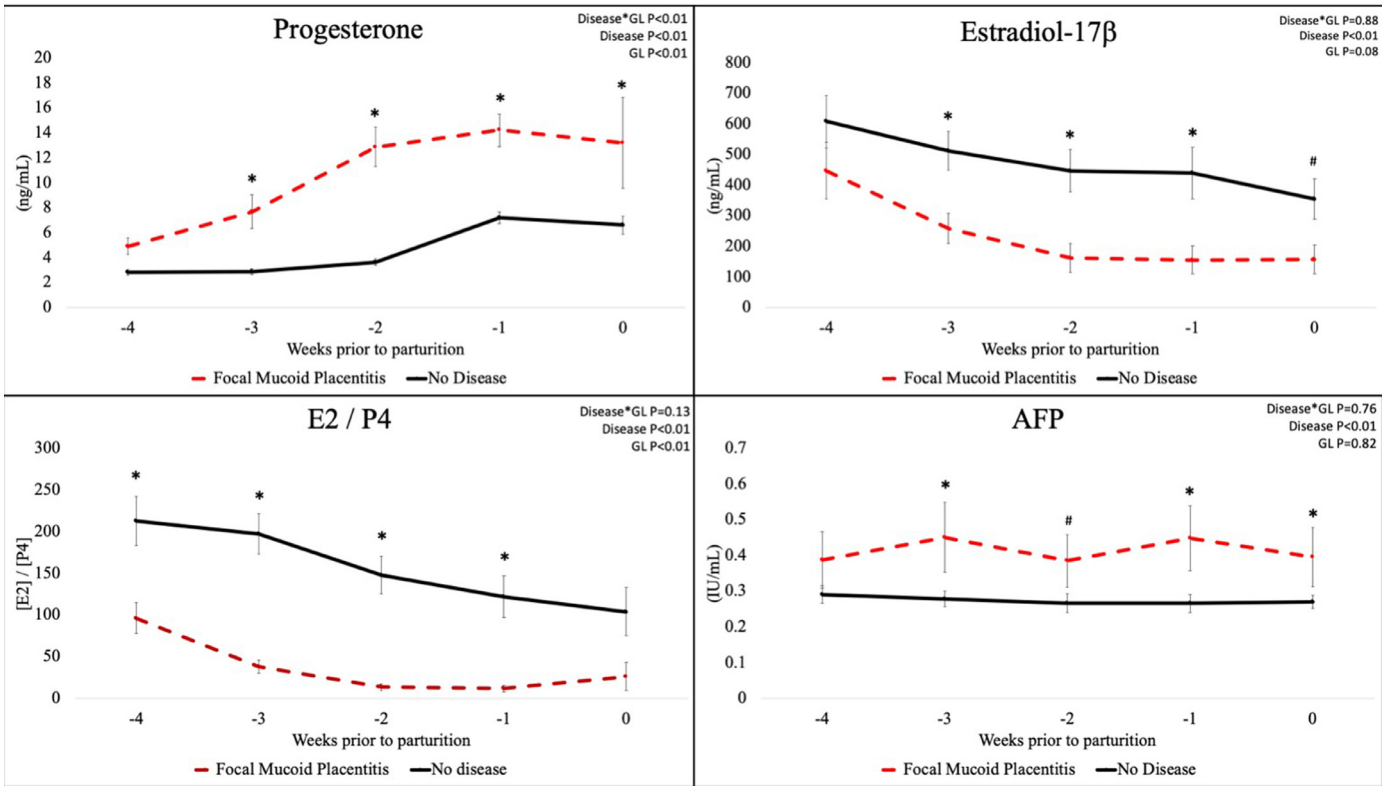

Fig. 2. Circulating concentrations of steroid hormones and AFP during focal mucoid placentitis. Serum concentrations of progesterone, estradiol-17β, the ratio of estradiol-17β to progesterone, and AFP in mares with focal mucoid placentitis (n = 6) compared to gestationally age-matched control mares (n = 20). A significant increase in progesterone and AFP was noted in focal mucoid placentitis mares in the weeks preceding parturition. Additionally, a significant decrease in both estradiol-17β and the ratio of estradiol-17β to progesterone was noted in the diseased group. Asterisk (*) above data points indicates differences (P < .05), while pound sign (#) above the data points indicates differences (P < .10) within week between the diseased and control groups. Data is shown as the mean ± the standard error of the mean (SEM).

docrine profile. Following the experimental induction of ascending placentitis, progestogen profiles were found to alter, but this was dependent on the severity or chronicity of the disease [10]. Wynn et al. (2018) found various progestogens (DHP, allopregnanolone, 20α-5P, $\beta\alpha$-diol, and 3β-5P) to increase in a sub-acute, or more chronic cases of placentitis, and this was confirmed by Shikichi et al. (2017) [30]. In contrast, the same progestogens decreased in comparison with controls when an acute placentitis was induced. Within the confines of this study, all placentae associated with focal mucoid and/or ascending placentitis produced a viable neonate, and therefore the disease may be considered chronic in nature. It is therefore unsurprising that ascending placentitis led to an increase in circulatory progesterone, and this was also noted in mares with focal mucoid placentitis. In the group of idiopathic abortion, a decrease in progesterone was noted within the week prior to fetal expulsion and may be indicative of the rapid nature of these pregnancy-related complications, which included umbilical cord torsions, idiopathic abortions, and stillborn neonates. As these complications result in an abortion, the increase in progesterone concentrations may be indicative of peripartum progesterone signaling and not due to the inflammatory-derived progestin decrease noted in infectious disease [10], but this could not be determined within the confines of this study.

In contrast to progesterone, concentrations of estradiol-17β were decreased in the mares experiencing chronic placentitis, and this included both the ascending and focal mucoid groups, indicating that the chronic nature of the infection/inflammation may play a larger role in the endocrine response to disease than the pathogen involved (*Nocardioform actinomycetes* vs. *Streptococcus ssp.*) or the etiology of the disease (ascending through the cervix vs. undetermined migration to the ventral aspect of the uterus). No alterations in estradiol-17β were noted preceding idiopathic abortion. While the decrease in estrogens during placental infection is supported by previous reports utilizing the experimental induction of ascending placentitis [6,30], varying time points and inoculums have been utilized, and therefore extrapolations are difficult to make toward naturally occurring disease. Canisso et al. (2016) found a decrease in estrogens to occur for 7 days preceding abortion, although no changes were noted prior to this time [6]. In the present study, a decrease in estradiol-17β production was noted 3 weeks prior to parturition in the focal mucoid placentitis group, and 2 weeks prior to parturition in the ascending placentitis group, indicating that the chronic nature of diseases investigated in the field may heighten this decrease in estrogen production, and prolong the response in circulation. This alteration in hormone concentration is further supported by Shikichi et al. (2017), who found estradiol-17β to decrease in mares that experienced fetal loss when compared to healthy pregnancies, and this began as early as 240 days of gestation [30]. The lack of alterations in estradiol-17β preceding noninfectious abortion may be due to inappropriate sampling time, as changes following the experimental induction of acute disease occurred nearer parturition/abortion. In the current study, samples were taken weekly, and more frequent sampling may be necessary to assess any alterations in estradiol-

IDIOPATHIC ABORTION

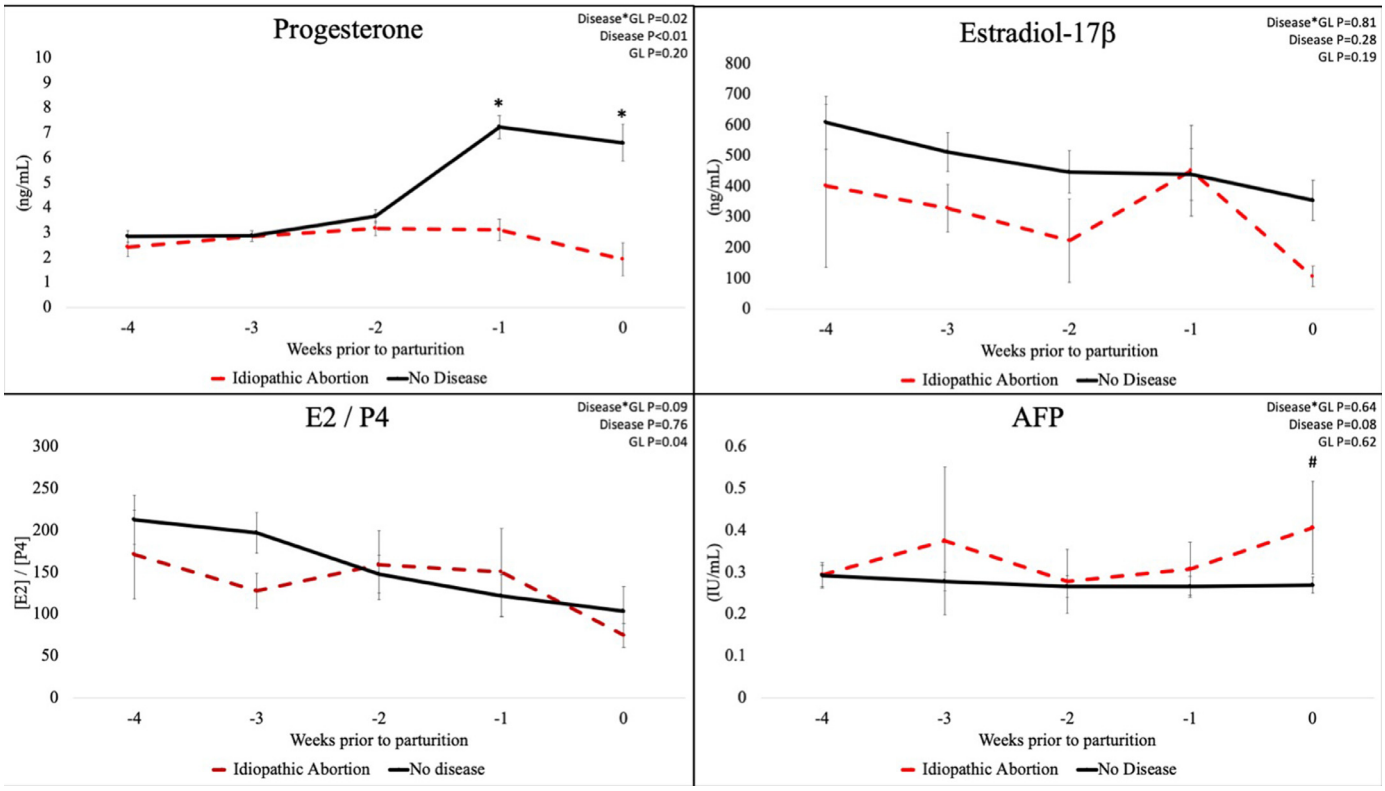

Fig. 3. Circulating concentrations of steroid hormones and AFP during idiopathic abortion Serum concentrations of progesterone, estradiol-17β, the ratio of estradiol-17β to progesterone, and AFP in mares with idiopathic abortion ($n = 6$) compared to gestationally age-matched control mares ($n = 20$). A significant increase in progesterone was noted both 1 week prior to abortion in addition to within the week of parturition. A trend toward an increase was noted in AFP concentrations within the week of parturition. No alterations were noted in either estradiol-17β or the ratio of estradiol-17β to progesterone preceding idiopathic abortion. Asterisk (*) above data points indicates differences ($P < .05$), while pound sign (#) above the data points indicates differences ($P < .10$) within week between the diseased and control groups. Data is shown as the mean ± the standard error of the mean (SEM).

17β preceding more acute pathologies. It is interesting to note that the ratio of estradiol-17β concentrations to the concentration of progesterone altered during the disease process of chronic placentitis, with an increase noted in both ascending and focal mucoid placentitis groups. Additionally, the combination of the two steroid hormones appeared to minimize the gestational age-related alterations that are noted in either progesterone or estradiol-17β within the final month of pregnancy. The ratio of E2:P4 experienced a linear decline preceding parturition, and yet mares undergoing either focal mucoid or ascending placentitis had decreased E2:P4 in comparison to controls. Therefore, the evaluation of the ratio between E2:P4 may be more sensitive for the detection of placentitis than either analyte alone, as the ratio minimizes the systemic variations of these endocrine markers noted in the normal pregnancy.

Alpha fetoprotein (AFP) is a secretory protein synthesized by the fetal liver and secreted through the placenta and fetal fluids to maternal circulation. The fetal analogue of albumin in adults, AFP is therefore found in high concentrations within fetal fluids of a variety of species, including the horse [7,36]. Circulating AFP has been found to increase following various pregnancy-related complications in the human, including placental inflammation [37], preterm premature rupture of membranes [38,39], in addition to preterm labor [40], and is hypothesized to increase due to it being an indicator of placental leakage [41]. Vincze et al. (2018) found AFP to be increased in mid gestation of the normal equine pregnancy (20–30 weeks) before experiencing a decline toward parturition, although considerable variation was noted between mares [42]. In the present study, little variation was noted among control mares, where concentrations of AFP remained stagnant for the duration of the sampling period. Following the induction of ascending placentitis, AFP has been found to increase retrospectively in an experimental setting, and this was also noted within the present study [7]. Both focal mucoid and ascending placentitis groups experienced an increase of AFP in the weeks prior to parturition, while mares with idiopathic abortion only experienced a trend toward this increase in the week immediately preceding abortion. This may be due to the more acute nature of the idiopathic abortion in comparison to the chronic nature of either placentitis group, as both groups resulted in a viable neonate. The basal concentrations of AFP during late gestation in the control mare group indicates that less frequent sampling may be necessary to utilize this marker of infection in the field in comparison to the endocrine markers which fluctuate in the normal mare, and this needs to be considered in future research.

The limitations of this study are described in depth in part one of this two-part publication. To summarize, by evaluating only the month preceding parturition, this study may limit both the time to intervention, as well as prohibiting the prospective prediction of disease. Additionally, this study evaluated a relatively small sample size in regard to pregnancy-related complications, in addition to investigating pregnancies that produced a viable neonate. The

pregnancy that supports a fetus to term has probable differences when compared to that of a pregnancy which aborts, and therefore comparisons are limited. Future research is required to assess these endpoints and should be done so with increased sample sizes in order to consider differences between term pregnancies associated with placental inflammation in comparison to abortions caused by placentitis.

In conclusion, naturally occurring chronic ascending placentitis was found to mimic the endocrine and AFP response noted during the experimental induction of a chronic ascending placentitis in the research setting, leading to an increase in progesterone, a decrease in estradiol-17β, and an increase in AFP. This the first study to report alterations in circulating markers during the disease of focal mucoid placentitis, indicating that the endocrine response of this disease to be comparable to that of a chronic ascending placentitis. These findings allow some inferences to be made into the elusive disease process of Nocardioform placentitits. No systemic alterations were noted in the evaluated markers preceding idiopathic abortion, indicating the unpredictability of monitoring their occurrence with circulating endocrine parameters. Future research is required to determine the sensitivity and specificity of these biomarkers and elucidate additional markers that may be added in order to detect early cases of placentitis in a prospective clinical setting.

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