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The proteome of fetal fluids in mares with experimentally-induced placentitis

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ABSTRACT

Introduction: Placental inflammation (placentitis) is one of the leading causes of late-term abortion in mares. Although prognosis is good assuming early diagnosis and treatment, diagnostics are limited.
Methods: To better characterize the disease and identify potential biomarkers, we analyzed the proteome of fetal fluids (amniotic and allantoic) in both control mares (n = 5) and mares with experimentally-induced placentitis (n = 5) using LTQ-Orbitrap mass-spectrometry. Placentitis was induced via trans-cervical inoculation of *Streptococcus equi* ssp. *zooepidemicus*.
Results: In total, 130 proteins were identified in either amniotic fluid, allantoic fluid, or both, with amniotic proteins being more prevalent and better conserved across samples. A total of 18 proteins were up-regulated in amniotic fluid during placentitis, including haptoglobin, plasminogen isoform X2 and plasminogen-like isoform X1 which were found exclusively in samples from mares with placentitis. Five allantoic proteins were up-regulated, of which four were also found to be up-regulated in amniotic fluid, including alpha-1-antiproteinase and transferrin family members. A total of 19 proteins were down-regulated in amniotic fluid, with none decreasing significantly in allantoic fluid.
Discussion: We have performed the first proteomic analysis of amniotic and allantoic fluid during placental infection in any domestic livestock species. We identified a number of proteins with significantly altered expression, primarily those related to immune function. These findings provide information on the physiology of placentitis as well as identify potential biomarkers for future diagnostic work.

1. Introduction

Placental inflammation (placentitis) is one of the leading causes of late-term abortion in mares, affecting between 1 and 5% of pregnancies [1–3], with approximately 25% of placentas from aborted, stillborn or preterm foals having evidence of placentitis [4]. Typically originating from bacteria which traverse the cervix to colonize the chorioallantois and endometrium, placentitis may lead to myometrial contractility, separation of the chorioallantois from the endometrium and preterm birth or fetal demise if not promptly diagnosed and treated. Assuming neonatal survival, secondary health complications often occur due to preterm birth and exposure to inflammatory conditions [2].

There are remarkable similarities between placentitis in mares and chorioamnionitis in women [1], with the horse being an excellent disease model for chorioamnionitis. Gestational endocrinology is surprisingly similar between species [5], and there is a similar immune response to bacterial infiltration, specifically an increase in pro-inflammatory cytokines such interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-1β (IL-1β) [6–8]. Additionally, the mare is the only species other than humans with spontaneous placental infections, with the full course of disease closely mirroring chorioamnionitis including a similarly poor prognosis [9].

Despite the necessity of prompt diagnosis and treatment, diagnostic techniques for equine placentitis remain limited. The most common techniques include measurement of the combined thickness of the placenta and uterus (CTUP) via trans-rectal ultrasound [10], assays for steroid hormones [11,13] or acute phase proteins such as serum amyloid A (SAA; [14,15]). Unfortunately, each of these methods has its drawbacks; both CTUP and steroid

Abbreviations: CTUP, combined thickness of uterus and placenta; LC-MS/MS, liquid chromatography tandem mass spectrometry; Px, placentitis; PSM, peptide spectral match; SAA, serum amyloid A.

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hormones change rapidly during late gestation making it difficult to interpret results, and acute phase protein increases are inflammation-mediated, with levels increasing following inflammatory events, even those as innocuous as vaccinations [16].

Although fetal fluid sampling can be used for the confirmation of placental inflammation in women [17], with several mass-spectrometry based proteomic studies having been performed [18–21], much less is known about the composition of fetal fluids during normal or compromised pregnancies in the mare. Some of the first work in this area was done on the biochemical characteristics of amniotic and allantoic fluids in late gestation [22] and parturition [23,24], with more recent work evaluating changes beginning in early gestation [25]. Previous proteomic work has focused on the normal mare, with 34 proteins identified in amniotic fluid in a mass-spectrometry based study [26]. An overview of the lipidomics of equine amniotic fluid has recently been published as well [27].

In placentitis, most work has been done looking for specific proteins in circulation for potential use as biomarkers. Several positive acute phase proteins have been found to be increasing in circulation, including alpha-fetoprotein [15], serum amyloid A [14,28,29] and haptoglobin [14]. Alpha-fetoprotein is also found in late-gestation fetal fluids [15], suggesting that fetal fluid proteins may hold potential as biomarkers, either in circulation or by assessing the composition of fetal fluids themselves.

As in humans, fetal fluid sampling can be performed by ultrasound guided needle aspiration [30]. This technique is currently being used for other diagnostic tests, including genomic tests, chromosome evaluation in humans, as well as fetal lung maturation in both humans and horses [31,32]. This technique could likely be adjusted to serve as a diagnostic procedure for placentitis, assuming suitable biomarkers are identified.

The identification and evaluation of the proteome of amniotic and allantoic fluids in normal and compromised pregnancies will provide valuable information about the comparative physiology of amniotic and allantoic fluid, as well as information about the effects of bacterial placental infections on the fetal fluid proteome. Therefore, we have utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to evaluate the proteomic composition of the fetal fluids in mares with normal gestation and placentitis.

2. Materials and methods

All animal procedures were completed in accordance with the Institutional Animal Care and Use Committee of the University of Kentucky, following the guidelines of approved protocol #2014–1341. All chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise stated. Horses (*Equus caballus*) used in this study were pony mares ranging from 250 to 400 kg housed on pasture with free-choice grass hay available at all times.

Mares were bred via pasture breeding, with pregnancy in eight mares confirmed by transrectal ultrasonography between 18 and 35 days of gestation. Gestational age in these mares was determined based upon size and morphology of the embryo [33]. In two control mares (Con_4 and Con_5), breeding dates were unavailable and were estimated based upon fetal development at the time of euthanasia [34]. Beginning at day 280 of gestation (average gestation length = 335 ± 15 days), mares were randomly assigned to either placentitis (Px; n = 5), or control (Con; n = 5).

All Px mares were experimentally inoculated with an intracervical inoculation ranging from 5×10^6 to 25×10^6 colony forming units of *Streptococcus equi* subspecies *zooepidemicus* deposited approximately midway along the length of the cervical canal [14]. *Streptococcus equi* ssp. *zooepidemicus* represents one of the major causative agents of placentitis in mares, with this specific strain isolated from a clinical ascending placentitis case. One mare (Placentitis_4) did not develop placentitis following the initial inoculation and was subsequently re-inoculated with 25×10^6 colony forming units, at which point placentitis developed as expected. Control mares were not inoculated but were otherwise treated identically. Demographic data on the pregnancy and fetuses are presented in Table 1.

Beginning on the day of inoculation (day 0), all mares were evaluated by daily trans-rectal ultrasound to assess CTUP as well as the degree of placental separation [10]. Additionally, mares were assessed daily for vulvar discharge, premature mammary development and signs of cervical softening. When sufficient signs of placentitis were observed (CTUP increase of 3 + mm and >50% of original thickness, plus measurable placental separation), mares were euthanized with sodium pentobarbital. Control mares were euthanized in the same manner at an equivalent day following initialization of daily assessments. Fetal fluids were collected upon necropsy, taking care that the allantoic and amniotic fluid compartments did not cross-contaminate, with fluids frozen at −20 °C until use. Gross placental lesions with separation of the endometrium and chorioallantois were measured post-mortem to verify disease progression.

Additionally, histological samples were evaluated to confirm inflammatory changes in chorioallantois and endometrium. In brief, intact sections of chorioallantois and endometrium were fixed in formalin for >24 h prior to embedding in paraffin and were cut using a rotary microtome and affixed to slides. Histology was evaluated following hematoxylin and eosin staining using an automated Sakura Prisma stainer (Torrance, CA, USA).

2.1. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Prior to mass spectrometry, fetal fluids proteins were precipitated using acetone. For 25 μL of protein solution, three 50 μL additions of cold acetone were made while vortexing the sample. Proteins were allowed to precipitate during an overnight incubation of −20 °C. Precipitate was then collected by centrifugation at 12,000 × *g* for 20 min, followed by supernatant removal, with the pellet allowed to air dry for 20 min.

To digest the pellet, 3.3 μg of trypsin (Sigma T 6567, St. Louis, MO, USA) was added in 40 mM ammonium bicarbonate, followed by a 4 h incubation at 37 °C on a shaking plate. The sample was then reduced in 10 mM dithiothreitol for 30 min at 56 °C. This was followed by alkylation by 50 mM iodoacetamide in the dark at room temperature for 30 min. A second overnight (18 h) digestion at 37 °C occurred following the addition of 4 μg of trypsin. Upon completion of digestion, 95% HCOOH (1 μL) was added and sample volume was reduced by vacuum centrifugation to 20 μL for LC-MS/MS.

The ultimate nano-LC-MS/MS analysis was performed by injecting peptides into an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent, Dublin, CA, USA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed phase cHiPLC column (75 μm × 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid while B was acetonitrile with 0.1% (v/v) formic acid. A 50-min gradient condition was applied: initial 3% mobile phase B was increased linearly to 50% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of one segment with eight scan events. The 1st scan event was an Orbitrap MS scan (100–1600 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 7 most intense