Characterization of the cervical mucus plug in mares

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Abstract

The cervical mucus plug (CMP) is believed to play an integral role in the maintenance of pregnancy in the mare, primarily by inhibiting microbial entry. Unfortunately, very little is known about its composition or origin. To determine the proteomic composition of the CMP, we collected CMPs from mares ($n=4$) at 9 months of gestation, and proteins were subsequently analyzed by nano-LC-MS/MS. Results were searched against EquCab2.0, and proteomic pathways were predicted by Ingenuity Pathway Analysis. Histologic sections of the CMP were stained with H&E and PAS. To identify the origin of highly abundant proteins in the CMP, we performed qPCR on endometrial and cervical mucosal mRNA from mares in estrus, diestrus as well as mares at 4 and 10 m gestation on transcripts for lactotransferrin, uterine serpin 14, uteroglobin, uteroferrin, deleted in malignant brain tumors 1 and mucins 4, 5b and 6. Overall, we demonstrated that the CMP is composed of a complex milieu of proteins during late gestation, many of which play an important role in immune function. Proteins traditionally considered to be endometrial proteins were found to be produced by the cervical mucosa suggesting that the primary source of the CMP is the cervical mucosa itself. In summary, composition of the equine CMP is specifically regulated not only during pregnancy but also throughout the estrous cycle. The structural and compositional changes serve to provide both a structural barrier as well as a physiological barrier during pregnancy to prevent infection of the fetus and fetal membranes.

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Introduction

The mucus formed in the equine cervix during pregnancy (cervical mucus plug – CMP) is believed to play a critical role as a barrier to microbial entry into the pregnant uterus. The cervix of the mare lacks anatomical features such as interlocking cervical rings noted in other large domestic animals (el-Banna & Hafez 1972, Senger 1999). The CMP may provide an additional physical and physiological barrier protecting the uterus from infiltration of bacteria during pregnancy (Lee *et al.* 2011).

In women, the CMP has been shown to be important in both the adaptive and innate immune systems. Levels of IgG and IgA are increased significantly in the CMP during pregnancy in women (Hein *et al.* 2005), and the CMP exhibits an intrinsic antimicrobial activity as well. The CMP is able to inhibit a wide range of both gram-negative and gram-positive bacteria *in vitro*, including *Streptococcus* spp., *Escherichia coli* and *Pseudomonas* spp. (Hein *et al.* 2002). A number of other immunologically important proteins such as matrix metalloproteases, azurocidin, cathelicidin antimicrobial peptide and neutrophil defensin have been identified in the CMP of women (Parks *et al.* 2004, Becher *et al.* 2010, Lee *et al.* 2011), although overall knowledge of the proteomic composition of the CMP in species other than humans is sparse.

Three studies have been performed looking at cervical mucus using mass spectrometry in women. The first looked at the overall protein content (Andersch-Bjorkman *et al.* 2007, Panicker *et al.* 2009), as well as O-glycosylation of mucins throughout the menstrual cycle (Andersch-Bjorkman *et al.* 2007), whereas the other looked specifically at the protein composition of the cervical mucus plug expelled during parturition (Lee *et al.* 2011). Additionally, an extensive study evaluating the proteome of the cervical mucus, uterine fluid and oviductal fluid in estrus and diestrus ewes was recently published (Soleilhavoup *et al.* 2016).

Mucins are a primary component of cervical mucus, with relative concentrations of specific mucins varying between pregnant and non-pregnant individuals. In the human, a number of gel-forming and transmembrane mucins are present, including MUC1, MUC4, MUC5B, MUC5AC, MUC6 and MUC16 (Gipson *et al.* 1999, Andersch-Bjorkman *et al.* 2007). The physical changes in cervical mucus around ovulation are well documented, with early works showing variation in mucin concentrations between the follicular and luteal phases (Chantler *et al.* 1989, Gipson *et al.* 1999, Gipson 2001). Recent evidence suggests that changes in the glycosylation of mucins vary throughout the menstrual cycle as well (Andersch-Bjorkman *et al.* 2007).

The origin of the proteins within the CMP is unclear. They may be produced by the cervical mucosa or it has been suggested that the proteins originate from the endometrium, the amnion or amniotic fluids (Andersch-Bjorkman *et al.* 2007, Lee *et al.* 2011). Lee and coworkers (2011) identified a number of proteins of a canonically amniotic origin, whereas Andersch-Bjorkman and coworkers (2007) identified both endometrial cells and proteins within the CMP. To the best of our knowledge, no one has examined whether transcripts of these proteins are produced within the cervical mucosa, the endometrium or the placenta during pregnancy.

Uterine infection during pregnancy is devastating in both humans and in horses. Placentitis is the leading cause of late-term abortion in the mare, causing approximately one-third of such losses (Giles *et al.* 1993). In ascending placentitis, the bacterial infection appears to originate at the cervix and migrates cranially, causing inflammation, tissue necrosis, placental separation and ultimately resulting in fetal demise (LeBlanc 2010). In the human equivalent of placentitis, chorioamnionitis, the CMP is believed to be key in preventing these infections (Chimura *et al.* 1993). In women at high risk for chorioamnionitis and preterm birth, the cervical mucus exhibits physically different qualities, including spinnbarkeit, the ability to stretch abnormally to 20 mm and beyond (Critchfield *et al.* 2013). Additionally, the mucus was significantly more permeable when challenged with fluorescent microbeads. There is some evidence that a predisposition for placentitis can be introduced by fetal factors, as a recent study showed 7/8 cloned equine pregnancies displayed some indication of placentitis, including 5/5 aborted foals (Pozor *et al.* 2016).

Cervical mucus plays a potentially critical role as a physical and biochemical barrier to prevent opportunistic infections; however, there is little to no information available about its composition or origin during pregnancy in the horse or other large domestic animals. Therefore, we aimed to characterize the late gestation cervical mucus plug histologically and by characterizing the protein composition using liquid chromatography tandem mass spectrometry (LC-MS/MS). Additionally, we identified a subset of canonically endometrial proteins, which were highly expressed in the cervical mucus plug and examined the expression of their transcripts throughout the estrous cycle and during late gestation. Expression of these transcripts was evaluated in both cervical mucosa and endometrium with quantitative real-time PCR (qRT-PCR).

Materials and methods

Animals and tissue collection

Animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of Kentucky

(#2014-1341, #2011-0840, #2010-0767). All chemicals were purchased from Thermo Fisher Scientific unless otherwise stated. All horses (*Equus caballus*) used in this study were light-breed horse and pony mares ranging from 250 to 550 kg housed on pasture with free-choice grass hay available at all times.

Cervical mucus was collected from four pregnant mares (*Equus caballus*) between 260 and 280 days of gestation by manual intracervical manipulation and extraction. These samples were removed from an unripened cervix without disturbing the pregnancy, so only a partial plug was obtained. Samples were primarily derived from the vaginal portion of the cervix; however, it was not possible to determine the exact orientation of the extracted CMP. Mucus was immediately snap-frozen in liquid nitrogen and stored at -80°C until ready to use.

Cervical mucosa and endometrial tissue samples for RNA isolation were obtained postmortem from mares during estrus ($n=8$), diestrus ($n=8$), 4-month gestation ($n=6$ /cervical mucosa; $n=4$ /endometrium) and 10-month gestation ($n=8$). Thirty clinically healthy horse and pony mares of different light breeds ranging from 3 to 18 years of age were used in this study. Estrus was defined by the presence of a periovulatory follicle (≥ 35 mm), and endometrial edema was observed upon transrectal ultrasonography, whereas diestrus samples were taken between 10 and 14 days after ovulation. Diestrus status was confirmed by the presence of a corpus luteum and determination of serum progesterone concentrations. Ovulation was confirmed by daily transrectal palpation and ultrasonography.

After collection, uterine and cervical tissues were dissected into mucosa and muscularis. Separate aliquots were preserved in RNAlater, maintained at 4°C for 24 h per manufacturer's instructions and then stored at -80°C until RNA isolation.

Histological evaluation

Cervical mucus was fixed in formalin for >24 h prior to embedding in paraffin. Sections were cut using a rotary microtome and affixed to slides. Histology was evaluated after hematoxylin and eosin staining using an automated Sakura Prisma stainer (Torrance, CA, USA), following manufacturer's instructions. Periodic acid-Schiff (PAS) staining was performed to localize mucins and other heavily glycosylated proteins. This technique used the DAKO PAS automated kit AR172 and the DAKO Artisan machine, as per manufacturer's instructions.

Protein isolation and liquid chromatography tandem mass spectrometry (LC-MS/MS)

Protein was isolated from cervical mucus by pulverizing samples under liquid nitrogen using a stainless steel mortar and pestle. Powdered mucus was weighed and reconstituted with T-PER protein extraction buffer with $1\times$ HALT protease inhibitor at a concentration of 1.0 mL to 0.1 g of mucus. Samples were incubated on ice for 30 min to allow for complete resuspension of proteins with regular vortexing. After incubation, samples were centrifuged at $16,000g$ for 15 min at 4°C to remove debris and then frozen at -20°C until processed for mass spectrometry.

Prior to mass spectrometry, proteins underwent acetone precipitation. Working with 25 µL of protein solution, three additions of cold acetone, 50 µL each, were made while vortexing the sample. Proteins were allowed to precipitate during an overnight storage at -20°C. Precipitate was collected by centrifugation at 12,000g for 20 min, the supernatant was removed and the pellet was allowed to air dry for 20 min.

To digest the pellet, 3.3 µg of trypsin (Sigma T 6567) was added in 40 mM ammonium bicarbonate, followed by a 4-h incubation at 37°C with shaking. 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 with 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.

Peptides were injected for nano-LC-MS/MS analysis. LC-MS/MS analysis was performed using 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, whereas 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 ions with collision-induced dissociation (CID) method.

The LC-MS/MS data were submitted to a local Mascot server for MS/MS protein identification via Proteome Discoverer (version 1.3, Thermo Fisher Scientific) against the *Equus caballus* genome (EquCab2.0; www.ncbi.nlm.nih.gov/genome). Parameters used in the MS/MS ion search were trypsin digest with maximum of two miscleavages, cysteine carbamidomethylation, methionine oxidation, a maximum of 10 ppm MS error tolerance and a maximum of 0.8 Da MS/MS error tolerance. A decoy database was built and searched. Filter settings that determine false discovery rates (FDR) were

used to distribute the confidence indicators for the peptide matches. Peptide matches that pass the filter associated with the strict FDR (with target setting of 0.01) were assigned as high confidence. For MS/MS ion search, proteins with two or more high confidence peptides were considered unambiguous identifications without manual inspection. Proteins identified with one high confidence peptide were manually inspected and confirmed. The presence or absence of any given protein in any individual horse is noted in [Supplementary Fig. 1](#) (see section on [supplementary data](#) given at the end of this article).

Ingenuity Pathway Analysis software (Qiagen) was used to determine which pathways are putatively present within the cervical mucus based on the proteomic analysis from LC-MS/MS evaluation of the CMP.

RNA extraction and qPCR analysis

Total cellular RNA was extracted from endometrium and cervical mucosa using TRIzol Reagent according to the manufacturer's recommendation. The RNA was precipitated as previously described ([Ball et al. 2013](#)). Quantification of RNA was performed via spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific), and samples with a 260/280 ratio of 1.95 or greater, and a 260/230 ratio of 2.0 or greater were used for analysis. RNA samples (1 µg/reaction) were treated with rDNase I for 30 min at 37°C, followed by treatment with DNase Inactivation Reagent (room temperature for 2 min), RNA was then reverse transcribed using the TaqMan Reverse Transcription Reagents.

We identified six proteins for further analysis. These proteins were selected for being highly abundant in the CMP and having previously been reported to be expressed by the endometrium. Specifically, we examined uterine serpin 14 (*SERPINA14*) ([Padua & Hansen 2010](#)), uteroglobin (secretoglobin family 1A member 1; *SCGB1A1*) ([Cote et al. 2014](#)), uteroferrin (acid phosphatase 5; *ACP5*) ([Padua et al. 2012](#)) lactotransferrin (*LTF*) ([Kolm et al. 2006](#)) and deleted in malignant brain tumors 1 (*DMBT1*) ([Yuan et al. 2014](#)). We also looked at transcripts from mucin 4 (*MUC4*), mucin 5B (*MUC5B*) and mucin 6 (*MUC6*). Unfortunately, we were unable to design a satisfactorily specific primer for equine mucin 5AC. The mRNA expression of select transcripts was quantified by real-time quantitative PCR (qPCR) in endometrium and cervical mucosa. Primers ([Table 1](#)) were designed using Primer-BLAST from the National Center for Biotechnology Information ([Ye et al. 2012](#)). Real-time qPCR of duplicate samples was performed using the

Table 1 Sequences of primers used in the quantitative real-time PCR experiments.

Gene name	Accession number	Forward primer	Reverse primer
<i>ACP5</i>	NM_001246672.1	GTTCTACACAGCCCGGGAAA	GAATGTCTCTGGAACCGCT
<i>B2M</i>	NM_001082502.3	GTGTTCCGAAGGTTTCAGGTT	ATTTCAATCTCAGGCGGATG
<i>DMBT1</i>	XM_014732986.1	TTTCAAGAGACGCCAGCTT	CACCTGGGCATCCTGAATGT
<i>EEF1A1</i>	NM_001081781.1	CAACATCGTCGTCATTGGGC	CAGCAGCCTCCTTCTCGAAT
<i>GUSB</i>	XM_014729943.1	GGGATTGCGACTGTGGCTGTCA	CCAGTCAAAGCCTTCCCTCGGA
<i>LTF</i>	NM_001163974.1	GGCAGCAAAATGCGCCAAATTCCA	TCTGCCTTGTTTCCCGCGATGG
<i>MUC4</i>	XM_014732832.1	TGAACGCCACCCTCAATCAG	AGCTTGGTCTTCCCGATGTG
<i>MUC5B</i>	XM_014729573.1	ATGACGAGGACGGCAACTAC	TGCACTGGATGCCATTAGGG
<i>MUC6</i>	XM_014729569.1	TGCCGTACAAGACTCGCAAT	TGTACACTGGAACACAGGC
<i>SCGB1A1</i>	NM_001081858.2	CACACCTGCCAGTTTCGA	GCTTTCATGTCTGCATCA
<i>SERPINA14</i>	NM_001242444.1	ACGCAGGACGATTGCACTTT	AGTGCACCAATCTCGTGTC

ViiA-7 Real-Time PCR System (Thermo Fisher Scientific). Reactions contained a mixture of cDNA (5 ng), primers (25 ng each) and a SYBR Green Master Mix. Cycle parameters of polymerase chain reaction were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and then a dissociation step of 95°C for 15 s. Melting curves for each sample were assessed to evaluate the specificity of the reaction. PCR efficiencies were calculated using LinRegPCR (version 2013.0) (Ruijter *et al.* 2009). All reactions were pipetted using the epMotion Automated Pipetting Systems (Eppendorf; Hauppauge, NY, USA).

The ΔC_T for each gene of interest was calculated by subtracting the C_T of the housekeeping gene from the C_T of the gene of interest. Using Normfinder software, the most stable normalization value in cervical mucosa was obtained by averaging the housekeeping genes β -glucuronidase (*GUSB*) and eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*), whereas β -2-microglobulin (*B2M*) was the most stable reference transcript for endometrium (Andersen *et al.* 2004). Gene expression data are presented as relative quantification values. Changes in relative abundance of specific transcripts were examined by calculating the fold change using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001).

Immunohistochemistry

Protein localization of mucin 4, uteroglobin and lactoferrin was evaluated by immunohistochemistry (IHC). Cervical mucosa and endometrial tissue samples were obtained postmortem from mares during estrus ($n=2$), diestrus ($n=2$) and late pregnancy ($n=3$). Tissues were fixed in 10% formalin for 24 h, and then stored in 100% methanol at 4°C until they were dehydrated and embedded in paraffin. Slides were sectioned at 5 μ m and stained with mouse anti-mucin 4 monoclonal antibody (1:100 dilution, sc-33654, Santa Cruz Biotechnology), goat anti-lactoferrin polyclonal antibody (1:00, sc-14434, Santa Cruz Biotechnology) or rabbit anti-uteroglobin (1:500 dilution, generous gift from Prof. Jörg Klug, University of Giessen) (Ellenberger *et al.* 2008). Slides were processed with the Leica BOND-MAX system (Leica Microsystems). Briefly, automated dewaxing and rehydration steps were followed by heat-induced (100°C for 20 min) antigen retrieval using pH 8.8 EDTA-based ready-to-use solution (Leica Biosystems). The slides were subsequently incubated with 3% hydrogen peroxide (5 min), optimally diluted primary antibody (15 min), a postprimary blocking reagent (to prevent nonspecific polymer binding) (8 min), horseradish peroxidase-labeled polymer (8 min) and diaminobenzidine substrate (10 min). All reagents were components of the Bond Polymer Refine detection system (Leica Biosystems). Primary antibodies were diluted to optimal concentration using Bond Primary Antibody Diluent (Leica Biosystems). Washing steps between each reagent were performed using Bond Wash Solution 10 $\times$ Concentrate (Leica Biosystems) diluted to a 1 $\times$ working solution with distilled water. Negative controls were prepared in the absence of primary antibody (data not shown). Slides were observed at 200 $\times$ magnification.

Statistics

The ΔC_T values for estrus, diestrus and pregnancy of endometrium and cervical mucosa for *SERPINA14*, *SCGB1A1*, *ACP5*, *LTF*, *DMBT1*, *MUC4*, *MUC5b* and *MUC6* were compared by one-way ANOVA. When there were differences between the three groups, ΔC_T values were compared by Tukey's *post hoc* analysis. Values for $\Delta\Delta C_T$ were converted to fold change in expression using $2^{-\Delta\Delta C_T}$. A P value of 0.05 or less was considered statistically significant. Statistical analyses were carried out using JMP 11.0 (SAS Institute, Cary, NC, USA).

Significance levels for pathways identified by Global Canonical Pathways in Ingenuity Pathway Analysis were calculated using the right-tailed Fisher exact test. Significance was set at $P<0.01$.

Results

Gross and histological evaluation

Equine cervical mucus is present in variable quantities between 260 and 280 days of gestation. Although the consistency of the CMP varied from mare to mare, overall, it tended to be a very viscoelastic material with a reddish-orange to pink color (Fig. 1).

Figure 1 Image of cervical mucus recovered from a mare at 9 months of gestation. Cervical mucus was removed by digital manipulation of cervix, with the quantity of mucus varying by mare.

Figure 2 Micrograph of equine cervical mucus recovered at 9 months of gestation. Cervical mucus was stained with either (A) hematoxylin and eosin or (B) periodic acid-Schiff stain. The cellular region is noted with 'c', the a-cellular region with 'ac'. Scale is indicated by the bar at the lower right corner of each image.

Histological evaluation revealed two very distinct cellular and a-cellular regions within the cervical mucus plug (Fig. 2A). The cellular region contained numerous cell nuclei that tended to be karyorrhexic or pyknotic. PAS staining for glycosylation reveals positive staining through both the a-cellular and cellular regions (Fig. 2B).

Protein isolation and liquid chromatography tandem mass spectrometry (LC-MS/MS)

Overall, 218 proteins from the *Equus caballus* genome (EquCab2.0) were identified in equine cervical mucus during late gestation using LC-MS/MS (Supplementary Table 1). Of these, 63 proteins had one or more isoforms that were indistinguishable, with an average of 2.5 isoforms for every affected protein. Discounting the non-distinguishable isoforms, a total of 179 distinct proteins were identified across all four samples. These proteins were primarily proteins with innate and acquired immune functions. The most abundant protein across all mares was lactotransferrin (LTF), followed closely

by immunoglobulins (Ig). Of the immunoglobulins, IgA was the most abundant, followed by IgG and then IgM. *SERPINA 14* was the most abundant uterine serpin. Numerous proteins related to the innate immune system were present including complement (C3, C6, Factor B and I), pantheinase, secretoglobulin, serotransferrin and chitotriosidase (Supplementary Table 1). There were multiple highly abundant proteins, which have previously been shown to be produced by the endometrium, including *SERPINA14*, *LTF*, *SCGB1A1*, *ACP5* and *DMBT1*. The most abundant mucin in each of the four mares was mucin-5B (*MUC5B*), followed by mucin-5AC (*MUC5AC*), mucin-4 (*MUC4*) and mucin-6 (*MUC6*) respectively.

Pathway analysis

We performed protein pathway analysis with Ingenuity Pathway Analysis to better assess putative function of the cervical mucus proteins identified in this study. In total, 38 pathways were denoted as present ($P < 0.01$; Table 2). These pathways were largely immunological in basis, with a total of 20/38 pathways with a reported immune function. The pathways with the lowest P values included liver X receptor/retinoic acid receptor (LXR/RXR) activation, clathrin-mediated endocytosis signaling, acute phase response signaling, bile acid receptor/retinoic acid receptor (FXR/RXR) activation, complement system and caveolar-mediated endocytosis signaling. Pathways with shared proteins are indicated in Supplementary Fig. 1.

qPCR analysis

Real-time quantitative PCR was performed on both cervical mucosa and the corresponding endometrium during diestrus, estrus and late pregnancy. In cervical mucosa, transcripts for *SERPINA14*, *ACP5* and *LTF* were expressed at significantly higher levels during gestation ($P < 0.05$) than during either estrus or diestrus, although *LTF* expression was only significantly higher at 10-month gestation, not at 4 months (Fig. 3A, B and D). Expression of *SCGB1A1* was significantly higher at 10m gestation than diestrus; however, there was no significant difference between estrus samples and pregnant samples (Fig. 3C). Additionally, *DMBT1* was significantly higher at 10m gestation than either estrus or 4m gestation (Fig. 3E). None of these transcripts exhibited differences between estrus and diestrus in the cervical mucosa.

Within the endometrial samples, three transcripts (*SERPINA14*, *ACP5* and *LTF*) were expressed at the highest level in pregnancy ($P < 0.05$; Fig. 4A, B and D). The expression of *ACP5* and *LTF* was significantly lower at 4m than 10m gestation, but still increased over non-pregnant samples. *SERPINA14* and *LTF* were differentially expressed between estrus and diestrus as well as pregnant and non-pregnant samples.

Table 2 Canonical pathways in cervical mucus. Pathways identified as present in cervical mucus proteome using Ingenuity Pathway Analysis ($P < 0.001$).

Pathway name	–log (P -value)	Molecules present
LXR/RXR activation*	9.92	LYZ, S100A8, TF, C3, CLU, ORM2, APOH, RBP4, PLTP, A1BG, ALB
Clathrin-mediated endocytosis signaling*	9.07	LYZ, S100A8, TF, CLU, ORM2, ACTC1, ACTA2, ACTA1, RBP4, ACTG2, UBC, ALB
Acute phase response signaling*	8.36	CFB, HP, TF, C3, CP, ORM2, APOH, C4BPA, RBP4, PLG, ALB
FXR/RXR activation*	7.28	TF, C3, CLU, ORM2, APOH, RBP4, PLTP, A1BG, ALB
Complement system*	5.64	CFB, C3, CFI, C4BPA, C6
Caveolar-mediated endocytosis signaling*	5.44	ACTC1, B2M, ACTA2, ACTA1, ACTG2, ALB
Pyruvate fermentation to lactate*	5.42	LDHC, LDHA, LDHB
Production of nitric oxide and reactive oxygen species in macrophages*	4.98	LYZ, S100A8, CLU, ORM2, RAP1B, RBP4, RAP1A, ALB
Leukocyte extravasation signaling*	4.68	EZR, ACTC1, RAP1B, ACTA2, ACTA1, THY1, RAP1A, ACTG2
Atherosclerosis signaling	4.06	LYZ, S100A8, CLU, ORM2, RBP4, ALB
Mechanisms of viral exit from host cells*	4.03	ACTC1, ACTA2, ACTA1, ACTG2
IL-12 signaling and production in macrophages*	3.89	LYZ, S100A8, CLU, ORM2, RBP4, ALB
MSP-RON signaling pathway*	3.83	ACTC1, ACTA2, ACTA1, ACTG2
Virus entry via endocytic pathways*	3.76	ACTC1, B2M, ACTA2, ACTA1, ACTG2
Primary immunodeficiency signaling*	3.76	IGLL1/IGLL5, IGHG3, IGHG4
Integrin signaling*	3.67	TSPAN1, ACTC1, RAP1B, ACTA2, ACTA1, RAP1A, ACTG2
Fcγ receptor-mediated phagocytosis in macrophages and monocytes*	3.67	EZR, ACTC1, ACTA2, ACTA1, ACTG2
Epithelial adherens junction signaling	3.67	ACTC1, RAP1B, ACTA2, ACTA1, RAP1A, ACTG2
RhoGDI signaling	3.28	CDH13, EZR, ACTC1, ACTA2, ACTA1, ACTG2
B cell receptor signaling*	3.26	CALM1, RAP1B, IGHG3, RAP1A, IGHG4
Calcium signaling	3.21	CALM1, ACTC1, RAP1B, ACTA2, ACTA1, RAP1A
Remodeling of epithelial adherens junctions	3.18	ACTC1, ACTA2, ACTA1, ACTG2
Agrin interactions at neuromuscular junction	3.15	ACTC1, ACTA2, ACTA1, ACTG2
RhoA signaling	3.13	EZR, ACTC1, ACTA2, ACTA1, ACTG2
Allograft rejection signaling*	2.83	B2M, IGHG3, IGHG4, GZMB
FAK signaling	2.78	ACTC1, ACTA2, ACTA1, ACTG2
Crosstalk between dendritic cells and natural killer cells*	2.74	ACTC1, ACTA2, ACTA1, ACTG2
Regulation of actin-based motility by Rho	2.71	ACTC1, ACTA2, ACTA1, ACTG2
Death receptor signaling*	2.69	ACTC1, ACTA2, ACTA1, ACTG2
VEGF signaling	2.69	ACTC1, ACTA2, ACTA1, ACTG2
Signaling by Rho family GTPases	2.6	CDH13, EZR, ACTC1, ACTA2, ACTA1, ACTG2
Autoimmune thyroid disease signaling	2.59	IGHG3, IGHG4, GZMB
Phospholipase C signaling	2.58	CALM1, RAP1B, IGHG3, RAP1A, IGHG4
Paxillin signaling	2.54	ACTC1, ACTA2, ACTA1, ACTG2
Corticotropin releasing hormone signaling	2.39	CALM1, KRT1, RAP1B, RAP1A
NRF2-mediated oxidative stress response	2.39	SOD3, ACTC1, ACTA2, ACTA1, ACTG2
Agranulocyte adhesion and diapedesis*	2.3	EZR, ACTC1, ACTA2, ACTA1, ACTG2
Actin cytoskeleton signaling	2.06	EZR, ACTC1, ACTA2, ACTA1, ACTG2

*Indicates a reported immune function.

A1BG, alpha-1-B glycoprotein; ACTA1, alpha-1 actin, skeletal muscle; ACTA2, alpha-2 actin, skeletal muscle; ACTC1, alpha-1 actin, cardiac muscle; ACTC2, alpha-2 actin, cardiac muscle; ACTG2, gamma-2 actin, smooth muscle; ALB, albumin; APOH, beta-2-glycoprotein; B2M, beta-2-microglobulin; C3, complement component 3; C4BPA, complement component 4 binding protein alpha; C6, complement component 6; CALM1, calmodulin 1; CDH13, cadherin 13; CFB, complement factor B; CFI, complement factor I; CLU, clusterin; CP, ceruloplasmin; EZR, ezrin; GZMB, granzyme B; HP, haptoglobin; IGHG3, immunoglobulin heavy constant gamma 3; IGHG4, immunoglobulin constant gamma 4; IGLL1/IGLL5, immunoglobulin lambda-like polypeptide 1; LDHA, lactate dehydrogenase A; LDHB, lactate dehydrogenase B; LDHC, lactate dehydrogenase C; LYZ, lysozyme; ORM2, alpha-1-glycoprotein 2-like; PLG, plasminogen; PLTP, phospholipid transfer protein; RAP1A, RAP1A, member of RAS oncogene family; RAP1B, RAP1B, member of RAS oncogene family; RBP4, retinol-binding protein 4; S100A8, S100 calcium binding protein A8; SOD3, superoxide dismutase 3; TF, transferrin; THY1, Thy-1 cell surface antigen; TSPAN1, tetraspanin 1; UBC, ubiquitin C.

SCGB1A1 showed higher expression in estrus and diestrus when compared to pregnancy (Fig. 4C), whereas *DMBT1* exhibited the highest expression in estrus and at 10m gestation, with estrus expression 750-fold higher than at 4m gestation (Fig. 4E). Overall, changes in expression levels were much more dramatic in endometrial samples than in cervical mucosa, with an average fold change of 16,600 seen in endometrial samples, compared to an average fold change of 1600 in cervical mucosa.

Interestingly, mucin production at 4m gestation was significantly lower than any other time point in both endometrial and cervical mucosa samples. Beyond this, there seemed to be no correlation between mucin expression in the endometrium and cervical mucosa. For *MUC4*, expression levels were highest in endometrial estrus samples, but lowest in the cervical mucosa estrus and 4m gestation samples ($P < 0.05$; Fig. 5A and B). Similarly, expression of *MUC4* was the lowest in pregnant endometrial samples, but highest in 10m gestation

Figure 3 Cervical mucosal transcript analysis during estrus, diestrus and pregnancy. Quantitative PCR was used to determine transcript levels in cervical mucosa during estrus, diestrus, 4 m and 10 m gestation. Transcripts evaluated include (A) uterine serpin 14 (SERPINA14); (B) uteroferrin (ACP5); (C) uteroglobin (SCGB1A1); (D) lactotransferrin (LTF); and (E) deleted in malignant brain tumors 1 (DMBT1). Fold change was calculated as $2^{-\Delta\Delta CT}$. Significance was set as $P < 0.05$. Transcripts with significant differences are denoted by varying superscripts. Error bars represent the 95% confidence interval.

cervical mucosa. Expression of *MUC5B* significantly varied through estrus, diestrus and pregnancy in both the endometrium and cervical mucosa, with the highest expression at 10 m gestation in both endometrial and cervical mucosa samples. However, during estrus, *MUC5B* was increased within the endometrium but decreased within the cervical mucosa (Fig. 5C and D). Within the endometrium, *MUC6* changed significantly between both stages of pregnancy, estrus and diestrus, with the greatest expression seen during estrus and the least expression during 4 m gestation ($P < 0.05$; Fig. 5E). The only significant difference in *MUC6* expression in the cervical mucosa was seen between the 4 m gestation and 10 m gestation samples (Fig. 5F).

Immunohistochemistry

Within the uterus, lactoferrin was detected at the highest level in the 10-month gestation samples and was localized to the endometrial glandular cells, as well as the lumen of the endometrial glands. Both SCGBA1A1 and *MUC4* were similarly localized within the glandular cells and glands, and all three proteins were present to some degree within the glandular epithelium in non-pregnant mares (Fig. 6). Within the cervix, all three proteins were specifically localized to the glandular epithelium (Fig. 7).

Discussion

This study reports, for the first time, the proteomic composition of the equine cervical mucus plug during late gestation. Given the protein composition, equine cervical mucus appears to play a major role in the maintenance of pregnancy and prevention of disease during late gestation, and it is composed of proteins derived from both the innate and acquired immune system. Histologically, the cervical mucus plug is composed of two distinct regions; a cellular region and an a-cellular region, as is seen in other species (Hansen *et al.* 2014). In the cellular region, the high concentration of pyknotic nuclei is suggestive of polymorphonuclear cells such as neutrophils, basophils and eosinophils. In pregnant women, the cellular portion of the CMP is associated with the vaginal portion of the CMP, whereas the a-cellular portion is directed toward the uterus (Hein *et al.* 2005). Unfortunately, the method of collection in this study precludes orientation. Both regions stain positively with PAS, indicating that there is a high level of polysaccharides throughout (Arike & Hansson 2016).

The proteins present within the cervical mucus plug could be loosely categorized into two categories; proteins with immune functions or pregnancy-associated proteins, with several proteins, including lactotransferrin,

Figure 4 Endometrial transcript analysis during estrus, diestrus and pregnancy. Quantitative PCR was used to determine transcript levels in endometrium during estrus, diestrus, 4 and 10 m gestation. Transcripts evaluated include (A) uterine serpin 14 (SERPINA14); (B) uteroferrin (ACP5); (C) uteroglobulin (SCGB1A1); (D) lactotransferrin (LTF) and (E) deleted in malignant brain tumors 1 (DMBT1). Inset graphs reduce the y-axis to allow visualization of all significant values, if required. Fold change was calculated as $2^{-\Delta\Delta CT}$. Significance was set as $P < 0.05$. Transcripts with significant differences are denoted by varying superscripts.

having a well-documented role in both (reviewed in Teng 2002). Of the top ten pathways identified by Ingenuity Pathway Analysis, nine pathways have a known role in supporting immune function. Clathrin-mediated endocytosis signaling, caveolar-mediated endocytosis signaling, the acute phase response system, the complement system, production of nitric oxide and reactive oxygen species in macrophages and leukocyte extravasation signaling all aid white blood cells (WBCs) in identifying and destroying foreign bodies. White blood cells produce energy by the conversion of pyruvate to lactate (Paul *et al.* 1987), and LXR/RXR and FXR/RXR have been shown to be present in peripheral mononuclear cells and help inhibit apoptosis induced by bacterial stressors, thereby fortifying innate immunity (Valledor *et al.* 2004, Schote *et al.* 2007). Although the 10th pathway, atherosclerosis signaling, does not appear to play a role in the immune system, each of the proteins identified in this pathway were also present in at least one of the other top ten pathways, demonstrating that atherosclerosis signaling is not likely present, as well as highlighting the importance of viewing pathway analysis results cautiously.

Overall, the protein composition of the human CMP and the equine CMP appeared to differ significantly. Only 21.1% of the equine CMP proteins were also found within the human CMP as reported by Lee and coworkers (Lee *et al.* 2011), whereas the 38.6% of the

proteins from the equine CMP were also found in cervical mucus from the non-pregnant ewe (Soleilhavoup *et al.* 2016). A subset of proteins reported in all three species include actin, alpha-1B-glycoprotein, alpha-2-macroglobulin, annexin 1 and 2, ceruloplasmin, clusterin, complement proteins C6 and B, IgGf-binding protein, IgG, lysozyme C, plasminogen, protein S100 A8, retinol-binding protein 4 and serotransferrin. Many of the most abundant proteins in the equine CMP were not identified in the human CMP; for example, LTF was absent, although it has been reported previously in the cyclic woman (Hein *et al.* 2005, Shaw *et al.* 2007, Lee *et al.* 2011). Some of the proteins had only minor changes between species, for example, serpin B11 was present in the horse, whereas serpin B5 and B10 were present in the ewe (Soleilhavoup *et al.* 2016) and serpin B3 was present in the human (Lee *et al.* 2011).

We found IgA to be the most abundant immunoglobulin within the CMP, followed by IgG, and then IgM. This is in contrast with the findings in the human, which showed IgG to be the most abundant immunoglobulin in the CMP throughout pregnancy (Kutteh & Franklin 2001, Hein *et al.* 2005). IgG is typically present in high concentrations in circulation; it is possible that the CMP in humans is composed of a higher proportion of serum proteins due to the human's hemochorial placentation. Conversely, IgA is known to be most abundant in mucosal surfaces. Interestingly,

Figure 5 Mucin transcript analysis during estrus, diestrus and pregnancy. Quantitative PCR was used to quantitate mucin transcript levels in (A, C, E) endometrium and (B, D, F) cervical mucosa during estrus, diestrus and 4 m and 10 m gestation. Mucins evaluated include (A, B) mucin 4 (MUC4); (C, D) mucin 5B (MUC5B); and (E, F) mucin 6 (MUC6). Fold change was calculated as $2^{-\Delta\Delta CT}$. Inset graphs reduce y-axis to allow visualization of all significant values, if required. Significance was set as $P < 0.05$. Transcripts with significant differences are denoted by varying superscripts.

IgG passes through the placenta to the fetus in the human (Nagendran *et al.* 2015), but not in the horse (Jeffcott 1974); this combined with the presence of amniotic proteins in the human cervical mucus plug (Lee *et al.* 2011) suggests that this may be a source of IgG in the human CMP. Alternatively, the differential expression of these immunoglobulins may be related to the differing profiles of pregnancy hormones, as immunoglobulin levels are known to be steroid hormone dependent (Kutteh & Franklin 2001, Lu *et al.* 2003).

To elucidate the origin of the CMP, we examined six proteins highly expressed in the CMP which have previously been shown to be produced by the endometrium. Transcripts for each of these proteins were found within the cervical mucosa as well as the endometrium and were consistently expressed at the highest level at 10-month gestation in the cervical mucosa. Although transcripts for each of these proteins were found within the endometrium, expression during pregnancy was not consistently higher than non-pregnant endometrium. In fact, expression of *SCGB1A1* was lowest within the pregnant endometrium. Furthermore, only four proteins present in the allantoic fluid in the horse were found in the CMP, including serum albumin, serotransferrin, alpha-2-macroglobulin and alpha-1B-glycoprotein (our unpublished data). It appears unlikely that allantoic fluid is contributing significantly

to the composition of the CMP, particularly as the most abundant protein in allantoic fluid, fibronectin, was not identified in the CMP. Although we cannot rule out the contributions of proteins derived from endometrium, chorioallantois or allantoic fluid, it appears that the cervical mucosa itself is primarily responsible for the formation of the CMP.

Interestingly, several proteins showed drastically different patterns of regulation between the cervical mucosa and endometrium, suggesting these proteins have varying roles based on location and that regulation for these proteins occurs locally. Uteroglobin (*SCGB1A1*) was upregulated 9-fold in cervical mucosa during pregnancy, but downregulated 160-fold in the pregnant endometrium. Rabbit endometrium exhibits a similar decrease in *SCGB1A1*, with levels dropping as early as 72 h after ovulation (Saeed *et al.* 2015). This decrease appears to be important for successful implantation (Vicente *et al.* 2013). In humans, endometrial *SCGB1A1* is present throughout the menstrual cycle and peaks during the early luteal phase (Muller-Schottle *et al.* 1999); however, it does not appear to be present in cervical mucus, either during the peri-ovulatory period (Daniel *et al.* 1987) or during pregnancy (Lee *et al.* 2011). To the best of our knowledge, *SCGB1A1* has not previously been identified in cervical mucosa in any species.

Figure 6 Immunolocalization of LTF, SCGB1A1 and MUC4 in cervical mucosa. Immunohistochemical localization of LTF, SCG and MUC4 in estrus (A, B, C), diestrus (D, E, F) and late pregnant (G, H, I) cervical mucosa. Magnification of 200x.

DMBT1 exhibited the highest expression during estrus in the endometrium, but expression in the cervical mucosa was lowest during the same period, consistent with findings in the ewe (Soleilhavoup *et al.* 2016). The high expression of *DMBT1* in the estrous endometrium is perhaps not surprising, as it appears to play a role in fertilization in both the pig and the horse (Ambruosi *et al.* 2013). The upregulation of *DMBT1* by the cervical mucosa during diestrus and late pregnancy may be explained by its role in host defense. Belonging to a family of scavenger receptor cysteine-rich genes (Mollenhauer *et al.* 1997), it is upregulated in response to inflammatory stimuli in lung tissue (Mollenhauer *et al.* 2002). Additionally, *DMBT1* has been shown to modulate bacterial recognition and inhibit invasion of a wide variety of bacteria (Bikker *et al.* 2002, Rosenstiel *et al.* 2007).

Lactotransferrin, an estrogen-responsive glycoprotein, was shown to be preferentially expressed during estrus in the non-pregnant endometrium (Fig. 4D) where it has been proposed to inhibit bacterial growth by sequestering free iron (Metz-Boutigue *et al.* 1984, Newbold *et al.* 1992, Baker *et al.* 1998, Kolm *et al.* 2006). The increased expression during estrus holds true in the mouse, rat, bitch, woman and rhesus monkey (Newbold *et al.* 1992, Teng *et al.* 2002a,b, Kida *et al.* 2006, Kolm *et al.* 2006). In sheep, there appears to be no increase in LTF within uterine fluids during estrus, and LTF actually decreases in ovine cervical mucus at this time (Soleilhavoup *et al.* 2016). There is a significant body of evidence that LTF protects against uterine and placental infections and may prevent preterm labor (Mania-Pramanik *et al.* 1999, Otsuki *et al.* 1999, Hasegawa *et al.* 2005, Sawada *et al.* 2006, Giunta *et al.* 2012).

Figure 7 Immunolocalization of LTF, SCGB1A1 and MUC4 in endometrium. Immunohistochemical localization of LTF, SCG and MUC4 in estrus (A, B, C), diestrus (D, E, F) and late pregnant (G, H, I) endometrial samples. Magnification of 200 $\times$.

SERPINA14 and *ACP5* were expressed at relatively low levels during estrus and diestrus, but concentrations increased dramatically during pregnancy in both the endometrium and the cervical mucosa (Figs 3 and 4). Both genes are known to be progesterone responsive, accounting for their increased expression during pregnancy (Joshi *et al.* 1980, Roberts & Bazer 1988, Padua & Hansen 2010, Padua *et al.* 2012). Estrous changes in *SERPINA14* were not noted in the ewe, and *ACP5* was not identified in either cervical mucus or uterine fluid (Soleilhavoup *et al.* 2016).

SERPINA14 is a highly expressed immunosuppressive agent and is able to inhibit lymphocyte proliferation by preventing the upregulation of IL2 (Peltier *et al.* 2000). It has also been shown to form complexes with both IgA and IgM as well, potentially tempering their action against the fetus (Hansen & Newton 1988). *ACP5* plays a well-established role in iron

metabolism and is involved in transporting iron to the fetus (Roberts & Bazer 1988, Young *et al.* 1989). It is also intricately involved in the immune system and serves as a marker for macrophage activation (Janckila & Yam 2009).

Mucins also appear to be independently regulated between the cervical mucosa and endometrium, with both *MUC4* and *MUC6* exhibiting significantly different expression patterns between these tissues (Fig. 5). Within the endometrium, mucins are believed to play a role in modulating signal transduction processes, as well as facilitating cell attachment and implantation (Constantinou *et al.* 2015). In the cervix itself, mucins have a number of very different roles, including retention of positively charged molecules, inhibiting the diffusion of bacteria and inhibiting viral replication (Hollingsworth & Swanson 2004, Mall 2008). Given this information, perhaps it should not

be surprising to see differential regulation between tissues within the reproductive tract.

The CMP provides protection to the fetus on multiple levels. It provides not only structural barrier but also numerous antimicrobial components to prevent placental infection and preterm birth. Changes in its composition can be detrimental to pregnancy outcomes (Critchfield *et al.* 2013). The structural framework is primarily provided by mucins, which also provide a steric inhibition of bacterial invasion (Becher *et al.* 2009) as well as inhibiting viral replication (Habte *et al.* 2008). The CMP is rich in bactericidal proteins including LTF and DMBT1. These proteins are produced by the cervical mucosa itself, where it appears that the majority of the CMPs are manufactured. Contrary to previous beliefs, the CMP does not appear to be primarily composed of extraneous endometrial and fetal proteins, although we cannot rule out their presence. Additionally, many of the proteins we selected for additional study appear to be differentially regulated between the cervical mucosa and the endometrium, suggesting that they are under tightly regulated local control. Overall, the CMP is a very deliberate antimicrobial structure, which provides a formidable barrier against bacterial invasion of the uterus. Expanding our knowledge of equine cervical mucus will provide important information about its role in the maintenance of pregnancy, as well as in the prevention of infectious disease such as placentitis and chorioamnionitis.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0396>.

Declaration of interest

The authors declare that there is no conflict of interest which could be perceived as prejudicing the impartiality of the research reported.

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