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The expression of mucin genes and the presence of mucin gene products in the equine endometrium

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ABSTRACT

In the equine reproductive tract, little is known about mucin gene expression and the role of mucins in barrier function and host-cell interaction. The aims of the study were to identify equine orthologs of mammalian mucin genes using available equine sequence data, to profile expression of equine orthologous mucin genes in the endometrium using reverse transcriptase polymerase chain reaction (RT-PCR), to determine spatial expression patterns of mucin genes using *in situ* hybridisation, and to confirm the presence of mucin gene products using Western blotting and equine-specific mucin antibodies during oestrus and dioestrus. While the mucin gene expression pattern in equine endometrium is similar to that of other mammals, several mucins appear to be uniquely expressed in this tissue (*eqMUC3B, 7, 18*, and *20*) and one is hormonally regulated (*eqMUC3B*).

1. Introduction

The lining epithelium of the equine uterus is comprised of mucus-secreting and ciliated cells. The mucus-secreting cells are found both in the surface endometrium and also within endometrial glands, located in the underlying lamina propria. The secretions of these cells lubricate and hydrate the mucosal surface, and protects it against infection (Causey, 2007). The endometrium also plays an important role during embryo implantation and development, and acts as a habitat for commensal bacteria when the cervix is open (Lagow et al., 1999; Rutllant et al., 2005). Mucus forms a semi-adhesive, enzyme-resistant barrier against pathogens and toxins (Lagow et al., 1999; Hattrup and Gendler, 2008; Thornton et al., 2008). In acute and sub-acute endometritis, mucus production by the endometrium increases, and changes in the production, elasticity or viscosity of this mucus may interfere with mucociliary clearance and hence fertility (Causey, 2007; LeBlanc, 2010). Consequently, the characterisation of equine endometrial mucin genes will provide an understanding of the composition of mucus in normal, healthy mares, and the basis for further studies dealing with the role of mucus in reproductive diseases.

The dry matter of mucus is composed largely of mucin glycoproteins. Due to their high content of O-linked oligosaccharides (Lagow et al., 1999; Hebbar et al., 2005), they bind substantial amounts of water upon secretion to generate a hydrated biopolymeric mucus gel (Andersch-Bjorkman et al., 2007). This gel is a dynamic and responsive part of the mucosal barrier. Mucin core proteins contain domains comprised of variable numbers of tandem repeat (VNTR) sequences that contain proline and are rich in serine and/or threonine (Lagow et al., 1999; Rose and Voynow, 2006; Andersch-Bjorkman et al., 2007), which are linked to O-glycans. These heavily glycosylated domains are flanked by unique, non-repetitive NH_2- and COOH-terminal domains, which are unique in sequence and length for each mucin (Hebbar et al., 2005; Rose and Voynow, 2006; Hattrup and Gendler, 2008).

Mucins occur as secreted gel-forming mucins, secreted non-gel-forming mucins, and membrane-bound mucins (Linden et al., 2008). The combination and relative amount of individual mucins in mucus are cell and tissue-specific. Their localisation within the epithelium also differs (Thornton et al., 2008). The cysteine-rich regions of the N- and C-termini of the large secreted gel-forming mucins MUC2, MUC5AC, MUC5B, MUC6, and MUC19 allow polymerisation through inter-molecular disulphide bonds, accounting for their gel-forming ability (Gipson et al., 1997; Lagow et al., 1999; Rose and Voynow, 2006; Linden et al., 2008). Membrane-bound mucins consist of two subunits connected via sodium dodecyl sulphate (SDS)-labile bonds (Hattrup and Gendler, 2008).

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However, they may form part of secreted mucus via shedding from epithelial surface membranes or direct secretion. MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20 are membrane-bound mucins (Linden et al., 2008). MUC1, MUC4, MUC8, and MUC16 are expressed in the human endometrium (Gipson et al., 1997, 2008; Lagow et al., 1999; Hebbar et al., 2005; Koscinski et al., 2006), whereas MUC2, MUC5AC, and MUC6 are expressed at very low levels (Hebbar et al., 2005).

Mucins have diverse roles in the reproductive tract. It has been suggested that the expression of MUC1 in the epithelium of the human uterine tube may prevent ectopic pregnancy (Lagow et al., 1999). In the uterine luminal epithelium, MUC1 is locally downregulated by the blastocyst in rabbits and humans and extensively downregulated in mice during the phase of embryo implantation. This enables ligand–receptor interactions at the cell surface that are essential for embryo attachment and implantation (Braga and Gendler, 1993; Surveyor et al., 1995; Hoffman et al., 1998; Meseguer et al., 2001; Brayman et al., 2006).

Studies on rat MUC4 have shown that it is expressed on the epithelium of the uterine luminal but not the glandular epithelium, and that its expression decreases during the influence of progesterone. This suggests that its expression in the luminal surface of the rat endometrium varies during the oestrous cycle and that MUC4 needs to be lost from the endometrial surface before blastocyst implantation (Carraway and Idris, 2001). In humans, it is hypothesised that MUC4 may play a similar role inhibiting implantation attributable to steric hindrance resulting from its extended conformation (Koscinski et al., 2006). In the sow, MUC4 is present in the surface and deep glandular endometrium, with no difference in its expression between pregnant and non-pregnant animals (Østrup et al., 2010). MUC8 may prevent sperm adhesion during travel through the male and female reproductive tracts, particularly the cervical canal (Hebbar et al., 2005).

MUC16, another membrane-spanning mucin expressed in the uterine epithelium, is downregulated during the receptive phase in humans, to facilitate adhesion of the trophoblast. This indicates that MUC16, like MUC1, may prevent cell adhesion in the human endometrium (Gipson et al., 2008). Mucin gene expression is known to be influenced by sex hormones, such as progesterone and oestrogen (Lagow et al., 1999) with variations between mucins and species. For example, MUC1 levels in the rabbit cervix only show a 2 to 3-fold variation in response to steroid hormones, compared to alterations in excess of 10-fold in the uterus (Hewetson and Chilton, 1997).

The lack of studies describing the spatial mucin gene expression and presence or absence of mucin gene products in the healthy equine endometrium during the oestrous cycle led to this study. We hypothesised that the equine endometrium has a similar spatial mucin gene expression profile to those described in other species, and that their mucins fulfill analogous roles with regard to barrier function and host-cell interaction. We identified equine orthologs to mammalian mucins using available sequence data, profiled expression of equine mucin orthologs in the equine endometrium using RT-PCR, determined spatial expression patterns of mucin genes using *in situ* hybridisation, and confirmed the presence of mucin gene products using Western blotting and equine-specific mucin antibodies.

2. Materials and methods

2.1. Tissue collection

Endometrial tissue samples were collected at a local abbatoir from 18 mares within 30 min of slaughter. Blood samples were taken from each animal for hormone analysis, to determine the stage of the oestrous cycle. The reproductive history of the mares was unknown. These tissue samples were divided in two. One was fixed immediately in 4% paraformaldehyde in phosphate buffered saline (PBS) for histology and *in situ* hybridisation. The other tissue sample was stored in RNAlater (Qiagen, Crawley, UK) for 24 h at 4 °C and subsequently (after RNAlater removal) stored at −80 °C until RNA extraction.

2.2. Stage determination of oestrous cycle

Endometrial tissue samples were collected in April and May to ensure that the mares were fully cycling and not in anoestrus or in the transition period. The stage of the oestrous cycle of these 18 mares was determined by measuring the progesterone (P_4) concentration of jugular vein blood samples, which were collected in uncoated tubes. Progesterone levels of >1 ng/mL were considered to be indicative of dioestrus, while those with levels below 1 ng/mL were considered to be indicative of oestrus (Samper et al., 2007). Gross anatomical assessment of the ovaries was also performed. Ovaries of mares in oestrus showed a preovulatory follicle, and no corpus luteum was detected. A corpus luteum was visible on the ovaries of mares in dioestrus.

2.3. Reverse transcriptase PCR (RT-PCR)

Equine genomic DNA was used to test primer quality. A jugular blood sample was collected from a healthy adult mare into a heparinised blood tube. DNA was isolated from the sample using a DNeasy DNA extraction kit (Qiagen) according to the manufacturer's instructions. Reverse-transcriptase PCR was carried out using the genomic DNA and the relevant primers. Primers that had abnormal bands, as defined by size, were redesigned.

2.4. RNA extraction and cDNA synthesis

A maximum of 30 mg of tissue was dissected, snap-frozen in liquid nitrogen and ground thoroughly with a mortar and pestle. RNA was then extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen), following the manufacturer's instructions. Eluted RNA was checked for quality on a 1% agarose gel (Sigma–Aldrich, Tallaght, Dublin 24, Ireland) and stored at -80 °C. cDNA was made with an Omniscript Reverse Transcription Kit (Qiagen) using the manufacturer's protocol. Master Mix (18.5 μL) and 1.5 μL (400 ng/μL) of RNA template were added to obtain a final reaction volume of 20 μL. The tubes were incubated at 37 °C for 2 h and stored in the freezer at −20 °C.

2.5. Primer design

At the time of designing the original primers, the equine genome had not been fully annotated. Therefore, in order to identify equine mucin orthologs, human mucin sequences were compared using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the equine genome. Equine mucin orthologs were identified. Primer sets (Sigma–Aldrich) were generated for the following equine mucin (*eqMUC*) genes: *eqMUC1, 2, 3B, 4, 5AC, 5B, 6, 7, 13, 15, 16, 17, 18, 20* (Table 1), and GAPDH, which was used as a housekeeping gene and to verify the integrity of the RNA samples.

2.6. Polymerase chain reaction (PCR) and DNA visualisation

Platinum Taq High Fidelity Polymerase kit (Invitrogen, Bio-Sciences, Dun Laoghaire, Co. Dublin, Ireland) and a Peltier Thermal Cycler Bio-Rad DNA Engine Dyad (Bio-Rad, Alpha Technologies, Blessington, Wicklow, Ireland) were used for all PCR reactions. Reactions were carried out in a final volume of 25 μL, containing 1 μL of cDNA. One μL of cDNA was used per reaction, and each