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Differences in extracellular matrix remodeling in the placenta of mares that retain fetal membranes and mares that deliver fetal membranes physiologically

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ABSTRACT

Introduction: In mammals, placenta separation at term may involve degradation of the extracellular matrix by matrix metalloproteinases (MMPs). The activity of MMPs is modulated by TIMPs. We hypothesized that the placentas of mares that deliver fetal membranes physiologically and those that retain fetal membranes (FMR) differ in terms of histology; mRNA expression of MMP-2 and MMP-9; protein expression of MMP-2, MMP-9, and TIMP-2; and the potential activity of both MMPs.
Methods: Placenta biopsies were taken from mares (n = 9; 4 FMR, 5 controls) immediately after foal expulsion. Retention was defined as failure to expel all fetal membranes within 3 h of expulsion. All mares were monitored for time of expulsion. The degree of allantochorial/endometrial adhesion was determined in FMR mares, and biopsies from all mares were histologically examined.
mRNA expression, protein immunolocalization, protein amount and potential enzyme activity were determined with RT-PCR, immunohistochemistry, Western Blotting and zymography, respectively.
Results: FMR mares had strong to extremely strong allantochorial/endometrial adhesion, and significantly more connective tissue in the allantochorial villi than controls. The range of MMP-2 mRNA expression levels was more than 13 times greater in FMR mares than in controls. Protein content of both MMPs and TIMP-2 differed significantly between groups. The range of potential MMP-2 and MMP-9 activity was larger in FMR mares, and MMP-2 potential activity was 1.4 times higher in controls ($P = 0.02$).
Discussion: These results indicate differences in extracellular matrix remodeling in FMR mares and controls, and suggest dysregulation of MMP expression and activation in FMR mares.

1. Introduction

The failure to release fetal membranes, known as fetal membranes retention (FMR), can be a life-threatening condition. Its incidence is up to 10% in light, warmblood horses and ponies, and 35–54% in heavy draft horses [1–3]. In heavy draft horses, FMR is associated with adhesion between the allantochorion and the endometrium, and this adhesion depends on fibrosis, overdevelopment of connective tissue, and less-branched allantochorial villi [4]. In general, mares should expel fetal membranes 30 min after foal delivery [5], but the time at which FMR is diagnosed is somewhat arbitrary, ranging from 3 to 12 h after foal delivery [1,6].

Unlike humans and rodents, mares have a diffuse, non-invasive (epitheliochorial) placenta with 6 layers of tissues (3 in the endometrium, 3 in the allantochorion), which separate maternal and fetal blood [7,8]. The allantochorion creates highly-vascularized villi that fit into corresponding crypts in the endometrium to improve exchange between maternal and fetal blood [8,9] and

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anchor the allantochorion [10]. Although the molecular details are unknown, the electron dense nature of the material between the maternal and fetal epithelia appears to indicate an adhesive contact between them [10].

To enable the release of fetal membranes, it has been hypothesized that the allantochorion shrinks to loosen the villi [11]. When such tissue remodeling takes place, changes in the extracellular matrix (ECM) involving matrix metalloproteinases (MMPs) are known to be involved [12]. Also, if cell-adhesion molecules connect the allantochorion and the endometrium, they would likely need to be broken down by proteases like MMPs. Moreover, the above mentioned histological abnormalities that are associated with FMR are known to involve ECM remodeling [12].

MMP-2 and MMP-9 degrade ECM components, including fibronectin and laminin, which connect cells to the ECM, and various collagens, such as collagen IV, a major component of basement membranes under epithelial cells [12]. To control the destructive potential of MMPs, the time and place of their expression and activation are tightly regulated at many levels [12–15]. MMPs are activated by moving their inhibitory pro-domain away from their active site, either allosterically, by interactions with other molecules that induce conformational changes, or proteolytically, by removal of the pro-domain by another protease [14,15]. MMPs can be inhibited by various molecules, including tissue inhibitors of metalloproteinases (TIMPs) [12,15–17]. Although MMP-2 and -9 have numerous activators and inhibitors [12,16,17], the interactions of MMP-2 with MT1-MMP and TIMP-2 are especially complex: some TIMP-2 must be present for activation via a trimolecular complex (pro-MMP-2–TIMP-2–MT1-MMP), but too much TIMP-2 inhibits the process by inhibiting all free MT1-MMPs [18].

Active forms of MMP-2 and MMP-9 have been found in the placenta of humans [19–22], mice [23,24], cows [25] and mares [26]. Both MMPs have been associated with the process of forming the placenta and its growth in humans [27–29], cows [25], and mares [30]. These enzymes appear to participate in the degradation of the extracellular matrix, helping the release of fetal membranes during the placenta delivery stage of parturition in women [27] and cows [31–33]. Differences in the activity of MMP-2 and MMP-9 may lead to FMR in cows [31–33], but this has never been studied in mares. Therefore, we asked whether the allantochorion and endometrium of mares that retain fetal membranes differ from those of mares that deliver these membranes physiologically in terms of histology, mRNA expression of MMP-2 and MMP-9, protein expression of MMP-2, MMP-9 and TIMP-2, and the potential activity of MMP-2 and MMP-9.

2. Materials and methods

2.1. Animals and samples collection

Polish heavy draft mares were monitored during physiological labor (n = 9; average age 8.4 ± 4 years; average weight approximately 850 kg). Although it would have been ideal to have more horses in the study, such small numbers are common in studies with these large, expensive animals [7,8,10].

To gain insight into the processes leading up to fetal membranes retention, which is determined 3 h after foal delivery [1], placenta biopsies were taken immediately after delivery of the foal with the largest available alligator-type equine biopsy punch. The size of the biopsy was approximately 0.5 × 1 cm. All biopsies were taken from the same place in the body of the uterus, where the allantochorion was firmly attached to the endometrium. Due to the relatively small jaws of the biopsy punch and the structure of the equine placenta (diffuse epitheliochorial [8]), it was impossible to take biopsies of connected endometrium and allantochorion. After many attempts, we were forced to manually separate both parts of the placenta (still inside of the uterus) just before sampling. We took 8 biopsies per mare (4 biopsies of the endometrium and 4 biopsies of the allantochorion) and immediately preserved them for RT-PCR, zymography, Western Blotting and light microscopy.

All of our mares were monitored for time of fetal membranes expulsion (none of these fetal membranes had any macroscopic changes). If this time was ≥3 h, then mares were classified as having FMR [1]. In the 3rd hour of parturition, mares were obstetrically examined and the strength of the connection between the endometrium and the allantochorion was graded in a scale from 0 to 5+ (Table 2). We had 4 FMR mares and 5 control mares.

2.2. Histopathology

Because biopsies were taken without visual guidance, all samples underwent histopathological examination to confirm that the biopsy contained either the endometrium or the allantochorion, so that there would be no cross contamination. A pathologist who was blinded to the clinical history of the mares performed this examination. 6-point scales were used to determine the histological characteristics of the placenta tissues (Table 3). The scales were defined as follows: 0, a complete lack or very slight development; 3 or higher; overdevelopment; 5, very extensive development; and physiological blood perfusion, 3; mild hyperemia, 4; severe hyperemia, 5. The same scales were used by Rapacz et al. [4].

All samples were stained according to a routine procedure. Briefly, the biopsies were fixed in 10% buffered formalin (24 h), then embedded in paraffin. The sections were stained with hematoxylin (Sigma–Aldrich, HTS32) and eosin (Sigma–Aldrich, HT110132). Previously, we found that placental fibrosis is common in heavy draft mares [4], so we performed Mallory's trichrome staining to check the collagen content (Bio- Optica, 04-020802).

2.3. Real time RT-PCR

To determine the mRNA expression of the MMPs, RT-PCR was performed (2 replicates per sample according to [34]). Biopsies were fixed in RNA-later, stored (4 °C, 24 h), then transferred to −80 °C and stored until assay. Primers were designed using GenBank (Primer-BLAST Tool) (Table 1). 3 reference genes were used that were previously described as good reference genes: GAPDH, B-actin and SDHA [35]. All these genes showed similar CT levels. mRNA expression of the MMPs was calculated according to the formula[1]: $1/2^{(CT^{MMP\ gene} - CT^{average\ of\ our\ 3\ reference\ genes})}$.

Total RNA was isolated using a Total RNA Mini Kit (A&A Biotechnology). For quality control, RNA was examined with a NanoVue spectrophotometer. 3 μg of total RNA were subjected to reverse transcription of cDNA with a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) in a Mastercycler (Ependorf) according to the manufacturer's instructions. RT-PCR was performed with a Fast Start Universal Sybr Green Master Rox Kit (Roche) in a Fast Real-Time PCR System thermocycler (Applied Biosystems).

2.4. Immunocytochemistry

Both frozen and paraffin fixed tissues were stained. Frozen

[1] CT – cycle treshold.