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Abstract

The matrix metalloproteinases (MMPs) are a family of enzymes involved in extracellular matrix remodeling. MMPs are secreted in a latent form and activated by local and infiltrating cells. MMP-2 and -9 are the most studied in reproduction and have been detected in bovine, ovine, equine and human placenta. There is only one study on MMPs in the equine amniotic fluid (AF) reporting a decrease in the activity of MMP-2 in case of premature delivery. The aim of this study was focused on MMP-2 and -9 activity in AF collected at parturition from mares with normal or high-risk pregnancy. High-risk pregnancy was defined as a history of premature udder development/lactation, increase of combined thickness of the uterus and placenta, vulvar discharge and/or mare's systemic illness. The diagnosis of placental insufficiency was confirmed retrospectively after macroscopic and histopatologic examination of the placenta. AF was collected by needle puncture of the amnion within 5 min after its appearance through the vulva. The activity of MMP-2 and -9 was analyzed by in-gel zymography allowing the evaluation of both latent and active forms. Twenty mares with normal pregnancy (group 1) and 8 mares with high-risk pregnancy (group 2) were included. All mares in group 2 had a high-risk pregnancy with a diagnosis of placental insufficiency associated with placental villous hypoplasia, placentitis or placental edema. The bands relative to latent and active forms of MMP-2 were clearly visible in both groups and the activity of latent ($P = 0.010$) and active ($P = 0.004$) forms was lower in the AF samples of group 2. The band of the latent form of MMP-9 was visible in 17/20 samples of group 1, while it was completely absent in all samples of group 2. In contrast, the band of the active form was clearly visible and with a greater activity in AF samples of group 2 ($P = 0.002$). A placental disfunction seems to induce a lower MMP-2 activity and a higher MMP-9 activity through the release of pro-inflammatory cytokines. Because fetal pulmonary secretions are a likely source of gelatinases in AF during late gestation, the increased MMP-9 activity could be related to fetal distress. These data provide a starting point to better understand the role of

MMPs in equine pregnancy, although it should be confirmed in a larger and more homogeneous population of mares with high-risk pregnancy.

Keywords: Mare; High-risk pregnancy; Placental insufficiency; Amniotic fluid; Metalloproteinase; Zymography.

1. Introduction

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases involved in extracellular matrix (ECM) remodeling [1]. These enzymes are secreted as zymogens or pro-MMPs which are latent or inactive forms. Latent MMPs need to be activated to be able to hydrolyze ECM proteins [2]. They are regulated by hormones, cytokines and growth factors and excreted by connective tissue and cells producing pro-inflammatory cytokines, including osteoblasts, fibroblasts, endothelial cells, neutrophils and lymphocytes. The most important inhibitors of MMPs are tissue inhibitors of metalloproteinases (TIMPs) [3], which are responsible for the formation of a one-to-one complex with MMPs [4]. One of the most relevant classes of MMPs is the gelatinases. This class includes two members synthesized in the cell and secreted into the extracellular space [5]: i) MMP-2 with molecular mass (MM) for the latent/active forms of 72/66 kDa and ii) MMP-9 with MM for the latent/active forms of 92/86 kDa. Active forms of MMP-2 and -9, expressed by fetal cells during the onset of pregnancy, were detected in the placenta of women [6], mice [7], cows [8], sheep [9] and mares [10]. In mares, it was suggested that MMPs could play a role in early pregnancy due to the production of MMP-2 and -9 by invasive chorionic girdle cells during the establishment of endometrial cup tissue [10].

Few articles identified the presence or determined the activity of gelatinases in equine AF. In particular, MMP-2 and -9, as well as the tissue inhibitor, TIMP-2, were found in the AF of mares during the latter half of pregnancy, as well as in AF collected at parturition by Oddsdóttir et al [11].

The authors reported no change in MMP-9 activity and a decrease in MMP-2 activity in pregnancies resulting in preterm delivery [11]. Matrix metalloproteinase-2 was also identified in AF from mares that delivered live term foals, suggesting that MMPs may have an important role in the development of fetal tissues [12].

Both the maternal and fetal compartments are subject to rapid growth, angiogenesis and tissue remodeling during gestation. These processes, as well as the release of fetal membranes, require proteolytic enzymes and subsequent degradation of ECM components. Matrix metalloproteinases are thought to play a key role in the processes of tissue remodeling and ECM breackdown during placentation and implantation in human, bovine and equine species [13]. In sheep, zymography indicated that MMP-2 was present in increasing amounts in AF from day 70 of gestation to labor and MMP-9 was detectable from day 125 to labor; there was no increase in MMP-2 or -9 during labor [14]. It is unclear whether proteins present in the AF are specifically secreted in the AF for the purpose of degrading basement membrane in fetal membranes. Alternatively, MMPs present in the AF may reflect secretion from adjacent intrauterine tissues, including the placenta, or from the fetus as pulmonary secretions.

Conditions that cause a high-risk pregnancy in the equine species can have a maternal, fetal or placental origin [15]. Most placental conditions pose limited risks to the mare, but significant risks to the fetus [15]. Plancental insufficiency is an ill-defined condition that has been identified in a large retrospective study as responsible for more than 60% of pregnancy losses due to abortion, stillbirth and neonatal death [15,16]. Several factors contribute to placental insufficiency such as premature placental separation, placental villous hypoplasia, placental thickening and especially placentitis [16-17]. The result of this condition is inadequate fetal nutrition resulting in intrauterine growth retardation, premature delivery or abortion [15].

The present study was based on the hypothesis that MMP-2 and –9, reflecting a disruption in utero-placenta balance or a profound change in fetal well-being, may be altered in AF of mares with high-risk pregnancy.The aims of this study were to determine the activity of MMP-2 and -9 in equine AF collected at foaling in mares with normal and high-risk pregnancy, and to establish if these enzymes might provide novel insights on high-risk pregnancy.

2. Materials and methods

2.1. Population

The study was designed as a prospective observational study.

Twenty-eight mares hospitalized at the Perinatology and Reproduction Unit (Equine Clinical Service, Department of Veterinary Medical Sciences) of the University of Bologna during 2017 and 2018 foaling seasons were included in the study. Mares were hospitalized at about 310 days of pregnancy because the owners requested an attended parturition. They were housed in separate wide straw-bedded boxes, fed hay ad libitum and concentrates twice a day and were allowed to go to pasture during the day.

At admission, a complete clinical evaluation, including complete blood count, serum biochemistry, transrectal and transabdominal ultrasonography were performed. Subsequently, mares were clinically evaluated twice a day and by ultrasonography every 5-10 days until parturition.

High-risk pregnancy was defined as a history of premature udder development/lactation, increase of combined thickness of the uterus and placenta [15], purulent/serosanguineous vulvar discharge and/or mare's systemic illness. When an increase in CTUP was observed, a cervical swab was performed to obtain a bacterial colture.

After delivery, macroscopic examination of the placenta was performed in all mares, and samples of placenta were collected for histopathologic examination in mares with high-risk pregnancy. Diagnosis of placental insufficiency was performed retrospectively after macroscopic and histopatologic examination of the placenta [17-19].

The foals were classified as healthy when they had a normal clinical evaluation during the course of hospitalization, including a complete blood count and serum biochemistry at birth and an IgG serum concentration ≥800 mg/dL at 12-24 h of life (DVM Rapid Test II, MAI Animal Health, Elmwood, WI).

Foals were classified as septic in the presence of both infection and systemic inflammatory response [19]. In the present study, infection was confirmed on the basis of positive blood culture. Foals were classified as affected by hypoxic ischemic encephalopathy (HIE) on the basis of clinical history such as abnormal pregnancy and/or parturition, clinical signs such as non-infectious and non-congenital neurological conditions, and laboratory abnormalities [20]. Foals were defined as premature when born prior to 320 days gestation and dysmature when born after 320 days but with immature physical characteristics (e.g. low body weight and inability to maintain body homeostasis) [19,21].

The population was then divided into two groups: mares with normal pregnancy that delivered healthy foals (group 1) and mares with high-risk pregnancy that delivered healthy or sick foals (group 2).

2.2. Clinical data and amniotic fluid collection

The following data were recorded for each mare: breed, age, parity, ultrasound findings, length of pregnancy (days), length of stage II parturition (min), foal's Apgar score [22], placenta and foal's weight (kg), placenta/foal's weight ratio (%) and gross placenta evaluation. In group 2, prepartum treatments [23], mare and foal's diagnosis and histopathologic placenta evaluation were also recorded.

The AF was collected by needle puncture of the amnion within 5 min after its appearance through the vulva, using a 60 mL sterile syringe. The samples were centrifuged at 800 g for 10 min at 4 °C to remove cellular and particulate matter. Aliquots of supernatant were collected, immediately stored at -20 °C and then analyzed within 6 months.

2.3. Identification and measurement of MMP-2 and MMP-9 by zymography

The activity of MMP-2 and -9 was detected using in-gel zymography following manufacturer instructions (NovexTM Zymogram Plus, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Briefly, 16 µg of total protein were loaded, in non-reducing conditions, on 7.5% (W/V) polyacrylamide gel containing 1 mg/mL gelatin. Five µL of molecular mass (MM) marker (BioRad Precision Plus) were loaded for each gel. Matrix metalloproteinases were separated through electrophoretic migration at 125 V in Tris-glycine running buffer with sodium dodecyl sulphate (SDS; 0.1%). After electrophoresis, gels were washed at room temperature with a renaturing buffer (NovexTM Zymogram Renaturing Buffer, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 min and incubated with a developing buffer (NovexTM Zymogram Developing Buffer, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 min. Gelatin gels were then incubated overnight at 37ºC in developing buffer. The buffer was decanted and the gels stained with Coomassie Brilliant Blue G-250 (PageBlu protein staining solution, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 1 h at room temperature on a rotary shaker. Finally, stain was washed out and the gel was then incubated with deionized water for 12 h until transparent bands appeared due to the proteolytic activity of MMPS which degraded the gelatin. The identification of MMP-2 and MMP-9 latent/active forms was performed in accordance with their MM (72/66 kDa and 92/86 kDa, respectively). The gels were digitalized (Chemidoc MP, BioRad, Hercules, California, USA) and the molecular weight, volume and background of each band were determined using a commercial software (ImageLab 5.2.1, BioRad, Hercules, California, USA). The MMP activity

produces the proteolytic bands on the gel and is reported as adjusted volume (AV, volume of the band after background subtraction).

2.4. Statistical analysis

Normality was checked by Shapiro-Wilk test and data reported as mean ± standard deviation (SD). Due to normally distributed data, differences in latent and active forms between group 1 and group 2 were analyzed with Student's t-test. A $P<0.05$ was considered statistically significant. All statistical analyses were carried out using the commercial software Analyse-it, version 2.03 (Analyse-it Software Ltd., Leeds, West Yorkshire, England).

3. Results

3.1. Clinical data

The 28 mares included in the present study were divided into normal (group 1; N=20) and high-risk pregnancy (group 2; N=8).

Mares of group 1 included 19 Standardbred and 1 Westfalia. Mare's mean age was 10 ±4 years (range 6-19). Five mares were primiparous, 15 mares were multiparous and the mean parity was 3 ±3 (range 1-11). Mean gestation length was 342 ±8 days (range 332-359), while mean stage II parturition length was 15 ±7 min (range 4-29). Fourteen/20 foals were female. Foal's mean Apgar score was 9 ±1 (range 6-10). Foal's mean weight was 49 ±6 kg (range 41-59), while mean placenta weight was 5.1 ±0.9 kg (range 3.6-6.4), with a placenta/foal's weight ratio of 10.2 ±1.3 % (range 8.1-12.8 %). No alterations were found during gross placenta evaluation.

Mares of group 2 included 4 Standardbred, 2 Italian Saddlebred, 1 KWPN and 1 Quarter Horse. Mare's mean age was 13 ±4 years (range 7-21). One mare was primiparous, 7 mares were multiparous and the mean parity was 4 ±3 (range 1-9). Clinical and histopathological data collected for group 2 are shown in Table 1. All mares in group 2 had a high-risk pregnancy with a diagnosis of placental insufficiency. Specifically, placental insufficiency was associated to placental villous hypoplasia in

2/8 mares, placentitis in 1/8 mare and placental edema in 5/8 mares. Despite the negative cervical swab and considering the clinical and ultrasonographic findings, 6/8 mares of group 2 received the treatment suggested for placentitis (sulfa-trimethoprim, 30 mg/kg, po, q 12h; altrenogest, 0.088 mg/kg, po, q 24h; flunixin meglumine, 1.1 mg/kg, iv, q 12h; pentoxifylline, 8.5 mg/kg, po, q 12h) [23] during hospitalization.

3.2. Zymograms

The zymograms of group 1 and group 2 are reported in Figure 1 and Figure 2, respectively. The activity of MMPs is reported in Table 2. All zymograms showed lytic bands corresponding to the latent (72 kDa) and the active (66 kDa) MMP-2; the active form accounted for 35% of total MMP-2 activity in group 1 and to 33 % in group 2.

Significant differences were detected for the activity of MMP-2 between group 1 and group 2. In fact, the activities of latent ($P = 0.010$) and active ($P = 0.004$) forms were significantly lower in the AF samples of group 2 respect to group 1.

All the samples analyzed presented the band corresponding to the active (82 kDa) form of MMP-9. The band of the latent form (92 kDa) was present in 17/20 samples of group 1, while it was absent in all the samples of group 2. The active form accounted for 33 % of total MMP-9 activity in group 1 and for 100 % in group 2. A significantly greater activity of the active form of MMP-9 was detected in group 2 than in group 1 ($P = 0.002$).

An additional band of gelatinase activity at an apparent MM of 75 kDa was also present in 15/20 samples of group 1 and 4/8 samples of group 2.

4. Discussion

Data reported in this study confirm the presence and the activity of MMPs in equine AF collected at parturition, as previously reported by Oddsdóttir et al. [11]. The profiles of two gelatinases were

significantly different between normal and high-risk pregnancy, with a decrease of MMP-2 activity and an increase of MMP-9 activity in high-risk pregnancy. To the authors' knowledge, these results have not been reported in other studies.

Matrix metalloproteinase-2 is the gelatinase found with a greater activity in the AF of women [24] and sheep [14]. Accordingly, in healthy mares of group 1, the activity of MMP-2 in AF at parturition was significantly higher than that of MMP-9. It was shown that human endometrial stromal cells are able to produce MMP-2 [25], while amnion epithelial and mesenchymal cells express TIMP-2 [26]. In ovine [14] and bovine [27] placentomes, MMP-2 and –9 are co-localized predominantly in uninucleate, but not binucleate, cells of the fetal compartment. In the mare, the expression of both MMPs is higher in the endometrial than in the allantochorial stroma, and MMP-9 expression is higher than MMP-2 in the stroma of both compartments [28]. From another point of view, an extensive tissue remodeling is necessary during the last days of equine pregnancy, confirmed by maturation of the fetal lung [29] and urinary tract, leading up to a dramatic increase of MMPs activity.

The findings reported in the present study, that pathological conditions such as infection did not increase AF MMP-2 activity in mares with high-risk pregnancy, are consistent with those of a study conducted in human cytotrophoblastic cells, which indicates that pro-inflammatory cytokines such as TNF-α and IL-1 do not increase the activity of MMP-2 and, indeed, in some cases down-regulate its activity [30].

In the present study, the increase of MMP-9 activity determined in mares of group 2 is consistent with data previously reported in women. Despite the differences in anatomy of the placenta, it was demonstrated the induction of MMP-9 expression in the chorioamnion, as well as the increase of MMP-9 concentration in AF during infection [31]. In equine species, it is now recognized that parturition resembles a sterile, inflammatory-like process. Both maternal and fetal parts of the placenta express a wide range of pro-inflammatory mediators, which create an environment that

promotes the remodeling of the placental tissue and the expulsion of the fetus and fetal membranes [32]. Pro-inflammatory cytokines and bacterial proteases are able of inducing the synthesis and secretion of MMP-9 [33]. Considering the role of pro-inflammatory cytokines in parturition, rupture of membranes and microbial invasion of the amniotic cavity [34], an increase of the activity of MMP-9 was not unexpected. Interleukin-1 is mainly produced by monocytes, macrophages and peripheral neutrophil granulocytes. Its β form increases the expression of cell adhesion molecules in the endothelium and appears to be the key cytokine for the induction of MMP-9 expression [35]. The presence of an intrauterine infection also induces the production of TNF-α in the AF, preceding that of both IL-1 and IL-6 [36], which in turns triggers the production of MMP-9 [37]. In the present study, the mare affected by ascending placentitis gave birth to a septic foal immediately after admission, before any treatment, offering a spontaneous model of placentitis, with an increased activity of MMP-9 determined in AF.

Oddsdóttir et al. [11] reported in AF no change in MMP-9 activity and a decrease in MMP-2 activity leading up to premature deliveries and neonatal death, suggesting that these enzymes could originate primarily from the fetus, and so are unchanged or decreased as the fetus becomes distressed or dies, or that the lack of MMPs is involved in fetal demise. These premature foals might have suffered the lack of lung maturity correlated with a lower MMPs activity, and therefore did not survive. Notably, MMP-9 is a major component of the basement membrane of airways [38] and the fetal lung is a likely source of gelatinases in AF during late gestation in an in vivo fetal lamb model [39]. Conversely, in the present study, the mare affected by severe laminitis had a 314 days preterm delivery with an increase in MMP-2 and -9 activity in the AF. In fact, despite the low body weight (23 kg), the foal was completely mature and survived. In the authors' opinion, the mare's systemic illness accelerated the fetal hypothalamic-pituitary adrenal axis maturation and MMPs activity determined the loss of interstitial tissue associated with lung maturation, both necessary for foal's survival.

Interestingly, 3 foals of group 2 included in the present study were born with a red bag delivery, but no differences in MMP-2 and -9 activity were detected. In women, MMP-2 and -9 are known to be involved in the breakdown of the ECM to allow the cervical ripening and the rupture of fetal membranes [40]. Conversely, in sheep no increases in MMP-2 and –9 activity with labor were detected, which may reflect differences in the anatomy of fetal membranes and in placental separation [14]. The equine chorioallantois resists mechanical rupture from day 90 of gestation and a controlled weakening of the membrane is certainly required to rupture it at birth [41]. Further investigation is certainly needed into MMPs role in the rupture of chorioallantois in mares.

As regards the 75 kDa gelatinase identified in many AF samples, data in the literature are scarce. Cheung et al. reported the presence of an unidentified 75 kDa gelatinase in perfused rat heart during aerobic perfusion and reperfusion after ischemia [42]. A 75 kDa band was also evidenced in lung and aorta extracts from mice [43]. Anyway, this gelatinase activity should be related to the latent form of MMP-2.

Some limitations of this design should be noted. First, a larger and more homogeneous population, particularly for group 2, would have improved the role of MMPs activity in equine pregnancy. Second, the quantification of MMPs using gelatin zymography yields the measure of the potential enzyme activity as a result of enzyme activation and MMPs/TIMPs balance. Despite this limitation as a quantitative technique, substrate-zymography still represents the most simple, sensitive, inexpensive, and functional assay for MMPs analysis, which can identify, simultaneously in the same sample, the entire panel of enzymes able to hydrolyze a specific substrate [44]. To increase the resolution, the conventional SDS-PAGE could have been complemented by adding an initial step using isoelectrofocusing. The resulting 2D gel zymography improves the separation of proteins with similar molecular weight but different isoelectric point [45]. Finally, since the owners of mares included in the present study requested an attended parturition, clinicians were not authorized to

perform amniocentesis procedures. Differently, the needle puncture of the amnion at parturition represents a safe technique to collect AF. Due to the presence of a novel transabdominal ultrasound-guided technique to safely perform fetal fluids collection during the last trimester of gestation in mares [46], future research could be conducted on AF MMPs as an early screening test for proper placental function during high-risk pregnancy and specific clinical evaluations of fetal well-being.

5. Conclusions

Despite limitations, this study provides novel information on high-risk pregnancy in equine species, indicating that mares with normal pregnancy differ significantly in terms of MMP-2 and -9 activity in amnion compartment from mares with high-risk pregnancy. The major source of MMPs in AF remains unclear and placental or fetal explanatory mechanisms should be considered. Increased activity of MMP-9 might be implicated not only in placentitis, but also in other pathological conditions, such as placental insufficiency, mare's systemic illness and stillbirths. Taken together, these results provide a starting point to better understand the role of MMPs in equine pregnancy, although they need to be confirmed by further studies in a larger and more homogeneous population of mares with high-risk pregnancy.

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