



Alterations in serum matrix metalloproteinases during different reproductive stages of Murrah buffaloes

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ABSTRACT

A comparative study on serum gelatinase was carried out in Murrah buffaloes. Healthy buffaloes (24), 3–6 year-old were selected and divided into 4 groups, each comprising 6 animals (group 1: pregnant; group 2: estrus; group 3: anestrus; and group 4: regular cyclic). Blood samples were collected in heparinised vacutainer and immediately transported to the laboratory. These samples were centrifuged at 3,000 rpm for 15 min and serum was preserved at –20°C in deep freezer. The serum samples were subjected to gelatin zymography. In group 1, major bands at 220, 92 and 72kDa bands were observed. The 92kDa MMP-9 band was very prominent and its activity was 3 times higher than that of MMP-2 and also 72kDa of MMP-2 band was very prominent as compared to the human marker. The 220 kDa band appeared as doublet of pro and active forms and above the level of 92kDa band. Minor catalytic breakdown products of 135 kDa were also observed. Below the 92 kDa band, 82kDa active form of MMP-9 was also observed as a band. In group 2, bands were observed at 220, 92 and 72kDa. The activity of MMP-9 band was about half a time lesser than that of human marker MMP-9 (92kDa). All the 3 enzymes were found only in their proform. The level of expression of MMP2 (72kDa) was equal to that of human marker. The ratio of MMP-9 and MMP-2 was about 2.5. In group 3, there is a very prominent band of 72kDa MMP-2 and a fainter band of 92kDa MMP-9 and the faintest band of 220kDa MMP-9 were observed. Among the 3 bands, the MMP-2 was very prominent and the ratio of MMP-9 and MMP-2 was 0.25. Finally, in group 4, three bands were observed at 220, 92 and 72 kDa. The level of expression of MMP-9 was greater than MMP-2 and the ratio MMP-9/MMP-2 was about 1.25. It is concluded that MMP2 and MMP 9 was demonstrated in the serum of all the 4 groups. The level of MMP-9 expression was greater in estrus buffaloes as compared to that of regular cyclic buffaloes and it was lowest in anestrus buffaloes.

Key words: Gelatin zymography, MMP-2, MMP-9, Murrah buffalo, Serum

Matrix metalloproteinases (MMPs) are important enzymes in tissue remodelling, a key event for the development of the fetal membranes and placenta, and establishing the fetomaternal interface during early pregnancy (Riley *et al.* 1999). MMPs are a family of enzymes, comprising at least 18 members of enzymes, capable of degrading extracellular matrix (ECM) during several physiological and pathological conditions (Hu *et al.* 2007). Gelatinase activity is an important factor during implantation in cows as well as in various species that have

an invasive type of placenta like humans and rodents (Kizaki *et al.* 2008). MMP-9 plays an important role in tissue remodelling in various physiological processes, including implantation, ovarian and uterine functions during estrus and pregnancy (Smith 1999, Woessner 2002). The organs of the adult reproductive system can undergo an extensive remodelling, experiencing rapid changes in tissue mass and function. Much of this matrix remodelling is attributed to the action of matrix metalloproteinases. Matrix metalloproteinase family members are expressed in a highly-regulated manner in many reproductive processes, including menstruation, ovulation, implantation, uterine, breast, and prostate involution. Metalloproteinase concentrations and activity can be regulated by various reproductive hormones, as well as by growth factors and cytokines that participate in reproductive events.

MMP (matrix metalloproteinase)-9 is routinely assayed for the detection of pregnancy and estrus. MMPs, including

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MMP-2 and MMP-9, play an important role in tissue remodelling in various physiological processes, such as implantation and ovarian and uterine functions during estrus and pregnancy (Takagi *et al.* 2007). MMP-2 is a constitutional housekeeping enzyme and MMP-9 is an inducible enzyme, actively involved in several reproductive processes (Bagavandoss 1998). MMP-2 and also MMP-9, regulated by a range of tissue inhibitors of metalloproteinases (TIMPs), are involved in intra-uterine tissue remodelling during the establishment of pregnancy. The gelatinase B activity of MMP-9 increases in response to the state of the endometrium and may be useful in predicting the time of estrus. The development of such an indicator would be useful for the improvement of conception by the application of artificial insemination at the optimal. Further, perusal of literature revealed no information on serum matrix metalloproteinases during different reproductive stages in any of the domestic animals, viz. cattle, buffaloes, sheep, goat and equines. Hence, this study focuses on the presence of matrix metalloproteinases in serum of estrus, anestrus, pregnant and normal cyclic buffaloes.

MATERIALS AND METHODS

Healthy Murrah buffaloes (24), about 3–6 year-old, were divided into 4 groups (each group consisting 6 animals): group 1 pregnant (P), group 2 estrus (E), group 3 anestrus (A), and group 4 regular cyclic (R) buffaloes. Herein after, the groups 1, 2, 3 and 4 were represented respectively as P, E, A and R. Animals were properly vaccinated and deworming was done. The management and feeding systems were approximately the same at all the farms. The feed received by the buffaloes during the study period was a mixture of roughage and concentrate. Water supplied as *ad lib*. In early morning before feeding the animals, blood samples were collected in a heparinised vacutainer and immediately transported to the laboratory. The blood samples were centrifuged at 3,000 rpm for 15 min. Serum was separated and stored at -20°C until further use. The protein content of the sample was estimated by Lowry method (1951). A standard curve was built using bovine serum albumin (BSA) as standard. The photometric estimation was carried out with spectrophotometer. Gelatin zymography was carried out in the serum of respective group as per Heussen and Dowdle (1980) with some modifications. SDS-PAGE was carried out as per Laemmli (1970). The resolving gel (8%) was co-polymerized with 0.3% gelatin solution (final concentration of gelatin in gel was 0.15%) and the electrophoretic run was carried out at 100V until tracking dye reaches to the bottom. Renaturation was carried out with renaturation solution (2.5% Triton X-100) for 3 h on a mechanical shaker with mild agitation. Then, developing was carried out by incubating the gel in developing buffer (10 mM CaCl_2 , 0.15 M NaCl and 50 mM Tris (pH 7.5) for 18 h at 37°C and stained with 0.25% Coomassie blue for 2 h, followed by destaining for 1 h with destaining solution and further destaining was carried out

with distilled water. Calibration of the gelatin zymogram was carried out with human capillary blood gelatinases as per Makowski and Ramsby (1996). A drop of human capillary blood (15–20 μL) was obtained by fingerstick puncture and placed in a tarred polypropylene tube. The weight of the blood was determined in an analytical balance and 20 volumes of non-reducing Laemmli buffer was immediately added. The sample was then vortex mixed (30s) and aliquots stored at -20°C and the preparation was stable for at least 3 months.

RESULTS AND DISCUSSION

These studies demonstrated the presence of MMP 2 and MMP 9 in the serum samples of all the 4 groups by gelatin zymography (Fig. 1). In group 1 (P), gelatin zymography revealed the presence of major (lane: 1, 5) bands at 220, 92 and 72kDa. The 92kDa MMP-9 band was very prominent; its activity was about 3 to 4 times higher than that of MMP-2. 72kDa of MMP-2 band was very prominent, compared to the human marker (lane 1, 5). The 220kDa band was appearing as doublet of pro and active forms and above the level of 92kDa band. Minor catalytic breakdown products of 135 kDa band were observed. Below the 92 kDa band, 82kDa active form of MMP-9 was also observed as a band.

Our results are in agreement with those of Riley *et al.* (1999) and Novera *et al.* (2002). Riley *et al.* (1999) who demonstrated the gelatinase activity in pregnant women and observed that latent form of MMP-2 (72 kDa) was the predominant gelatinase, with some MMP-9 present in extra-embryonic coelomic fluid. Lesser amounts of active MMP-2 (66 kDa) were detected. Latent form of MMP-2 in coelomic fluid was significantly higher than amniotic fluid. Low levels of gelatinase activity were also detectable in extra-embryonic coelomic fluid at 92 kDa, corresponding to latent MMP-9 with virtually undetectable amounts in the amniotic fluid. In amniotic fluid samples collected in the second trimester MMP-2 activated protein corresponding to the latent (72 kDa) form was the predominant gelatinase activity present. There was a less intense band of activity at 66 kDa, corresponding to the active form of MMP-2. Significantly greater amounts of

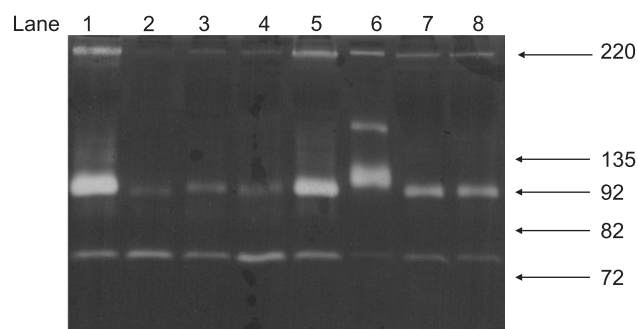


Fig. 1. Gelatine zymography analysis of serum of buffaloes at different reproductive stages. Lane 6, human capillary marker; lane 1, 5, pregnant animals; lane 2, anestrus animals; lane 3, 4, estrus animals; lane 7, 8, regular cyclic animals.

MMP-2 (latent) compared to MMP-9 (latent) were present in the second trimester amniotic fluid. Amniotic fluid in late pregnancy contained predominantly latent forms of MMP-2 and MMP-9 activated protein, there being significantly more MMP-2 than MMP-9, as was the case for second trimester amniotic fluid (Riley *et al.* 1999).

Novera *et al.* (2010) demonstrated an increase in MMP2 activity in uterine extracts in early pregnant rats and found it concentrated at implantation sites. Blastocyst-derived nitric oxide (NO) contributes to the production of uterine-derived MMP2, an essential component of implantation and initiation of placentation.

In group 2 (estrus) buffaloes (lane 7, 8), 3 bands were observed at 220, 92, 72kDa. The activity of MMP-9 band was about half a time lesser than that of human marker MMP-9 (92kDa). All the 3 enzymes were found only in their proform. The level of expression of MMP2 (72kDa) was equal to that of human marker. No catalytic breakdown products were observed except the 3 major bands. The ratio of MMP-9 and MMP-2 was about 2.5. Our results are in accordance with the results of Shia *et al.* (2011) who recorded MMP in endometrium of bitches and Kim *et al.* (2012) in vaginal mucus during estrus and gestation. Kim *et al.* (2012) who demonstrated that MMP-2 and MMP-9 proteins in vaginal mucus were both activated during estrus and gestation.

The activation patterns of these 2 metalloproteinases were similar during this period. The active forms of MMP-2 and MMP-9 were most abundant in vaginal mucus on day 0 of estrus and lowest on day 7 of estrus. The level of active form progressively increased on days 7, 30, and 210 in pregnancy. The gelatinase activity at Mr 72kDa and 92kDa in vaginal mucus zymography corresponded to the latent forms of gelatinase A (MMP-2) and gelatinase B (MMP-9) (Nagase and Woessner 1999), respectively. The increased concentration of MMP-2 and MMP-9 in vaginal mucus might be attributed to the increased concentration of serum gelatinase (MMP-2 and MMP-9).

Further, the ratio of MMP-9 to MMP-2 was 4 in pregnancy, which may be related to gelatinase activity of MMP-9 for the settlement of early foetus into the uterus. Hulboy *et al.* (1997) reported similar results in the invading trophoblast produced MMP-2, MMP-9 and MMP-10, with predominantly greater amounts of MMP-9 in the implantation process of murines.

In anestrus animals, very prominent band of 72kDa MMP-2 (lane 2) and fainter bands of 92kDa MMP-9 and faintest band of 220kDa MMP-9 were observed. Among the 3 bands, the MMP-2 was very prominent and ratio of MMP-9 and MMP-2 was 0.25. In regular cyclic animals (lane 3, 4), 3 bands were observed at 220, 92, 72 kDa. The level of expression of MMP-9 was greater than MMP-2 and the ratio MMP-9/MMP-2 was about 1.25. In none of the samples, 135kDa MMP-9 band was observed compared to that of human marker. Several catalytic products around 135 KDa band were observed. The most prominent band was the 92KDa MMP-9 band.

Our results are in agreement with those of Shia *et al.* (2011) who demonstrated that both latent forms of MMP-2 and MMP-9 were predominant during different phases of canine estrous cycle. The activities of both forms of MMP-2 and MMP-9 increased accompanied by increased progesterone concentrations. This study revealed the association between endometrial MMP-2/MMP-9 activity and sex hormones and suggested that progesterone directly or indirectly regulated the MMP-2 and MMP-9 activity. Kim *et al.* (2012) observed that activities of latent forms of both MMP-2 and MMP-9 were significantly higher in met-estrus (MP) and late diestrus phases (L-DP) than in anestrus (AP) and proestrus/estrus phases (EP). Similarly, activities of the active forms of both MMPs in L-DP were significantly higher than in the other phases of estrous cycle. Significant correlations were found between progesterone and activities of latent and active forms of MMP-9 during MP. However, the specific mechanism still needs further investigation.

In our study, we also observed that level and activity of MMP-9 was greater than that of MMP-2. The ratio of MMP-9 to MMP-2 was greater in estrus (2.5), which was one half in group 4 (R) buffaloes (1.25) and it was very less in anestrus buffaloes (0.25). However, the level of expression of 72kDa MMP-2 was almost constant regardless of various reproductive states of animals. It strongly suggested that MMP plays a major role in estrous and regular cyclic processes of buffalo reproduction.

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