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A comparative gelatinase expression study between Indian hilly (Toda and Bargur) and plain (Murrah) bubaline species

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Abstract

A comparative study was conducted to find out the existence of gelatinases in hilly breeds of buffalo (Toda and Bargur) with plain breed of Murrah buffalo. Buffaloes with similar age (2-3 years) group were divided into six groups and each group comprised of eight animals (n=8) in both male and female. Group I: Toda he-buffalo, Group II: Toda she-buffalo, Group III: Bargur he-buffalo, Group IV: Bargur she-buffalo, Group V: Murrah he-buffalo, and Group VI: Murrah she-buffalo. Blood samples were collected in clot activator in the morning prior to concentrate feeding, centrifuged and the separated serum samples were subjected to gelatin zymography. These three buffalo breeds exhibited major bands at 220 kDa, 135 kDa and 92 kDa of Matrix metalloproteinase (MMP)-9 and at 72 kDa of MMP-2. In Toda, Bargur and Murrah buffaloes, the intensity of latent form of MMP-2 (72 kDa) was 1.5 folds higher in he-buffaloes as compared to she-buffaloes whereas the intensity of latent form of MMP-9 was lower in he- as compared to she- buffaloes. She-buffaloes had thicker bands at 135 and 220 kDa of MMP-9 as compared to he-buffaloes indicated that the expression of pro-enzymatic forms of MMP-9 was very clear and they were lytic bands of MMP-9. Thus, expression of MMP-2 was higher in he-buffaloes compared to she-buffaloes whereas the expression of MMP-9 was higher in she- than in he-buffalo groups. The intensity of MMP-2 was 3-4 folds higher in Toda and 2-3 folds higher in Bargur buffaloes than the latent form of MMP-9 as compared to Murrah buffaloes.

Key words: *Matrix metalloproteinase; gelatin zymography; serum; buffaloes.*

Introduction

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that can break down all of the extracellular matrix's (ECM) constituents. The ECM's deterioration is highly essential as it affects angiogenesis and embryonic development. MMPs play a pivotal role in tissue remodeling and cell healing (Cabral-Pacheco *et al*., 2020). The MMPs and their associated endogenous inhibitors act together to regulate the location and degree of ECM turnover all over the body. The MMP system regulates various biological processes such as embryonic advance, organ morphogenesis, angiogenesis, cartilage renovation, bone growth, wound curing, periodontal integrity and corneal restoration. Inequity among MMPs and tissue inhibitors of metalloproteinases (TIMPs) leads to pathological conditions in humans and animals (Asawakarn and Asawakarn, 2012).

The MMP system also controls various functions of the reproductive system including the structural changes that occur throughout the menstrual or estrous cycle. These structural changes are regulated by various hormones, growth factors and cytokines and are crucial for normal ovarian and uterine physiology (Hulboy *et al.,* 1997). Among the family of MMPs, gelatinases including gelatinase A (MMP-2) and gelatinase B (MMP-9) belong to this group, readily digest the denatured collagens i.e. gelatins. These enzymes have three repeats of a type II fibronectin domain interleaved in the catalytic domain, which they fix to gelatin, collagen, and laminin. This suggests that MMP-2 is important for osteogenesis. MMP-2 and MMP-9 are equally significant regulators of vascular and uterine remodeling in a healthy pregnancy (Nikolov and Popovski, 2021).

Buffaloes are vital to the Indian livestock segment followed by other ruminant species. India possesses 199.1

million cattle and 105.3 million buffaloes and ranks first in the milk production in the global record and mostly backed by buffaloes. But milk productivity is much lower owing to many reproductive and non-reproductive diseases. The major reproductive problems affecting the buffaloes are anestrus, repeat breeding and post-parturient disorders. In India, Murrah buffalo is one of the most valuable breeds of buffalo based on its milking capacity and its potential towards genetic improvement. The reproductive efficiency of buffalo is badly affected by certain constraints such as late maturity, poor expression of the estrous signs, irregular estrous cycle, silent heat, seasonality in breeding, less conception rate, early embryonic mortality and prolonged inter-calving interval. Since, gelatinases such as MMP-2 and MMP-9 have significant roles in various reproductive functions, to ascertain the existence of gelatinases between different breeds of Indian buffaloes were undertaken in this study. Specifically, in hypoxic conditions, MMP-9 is essential for controlling the angiogenesis and basement membrane (BM) degradation (Huang, 2018). However, no study was conducted on the expression pattern of gelatinases in different breeds of bubaline species raised at different geographical altitudes. Hence, this study hypothesized that expression pattern of gelatinases could be differing between hilly and plain breeds and between the sexes in buffaloes. Therefore, the objective of the present study was to examine the expression pattern of MMPs (MMP-2 and MMP-9) in the serum of different breeds of Indian hilly buffaloes as compared with plain Murrah buffalo.

Materials and Methods

The present investigation was carried out at the Department of Veterinary Physiology and Biochemistry, TANUVAS-Veterinary College and Research Institute, Orathanadu, Thanjavur, Tamil Nadu, India. The institute is located 30 meters above mean sea level (MSL) with a latitude of 10.6°N and 79.3°W.

Experimental animals

Experimental buffaloes were selected from the organized farms in Namakkal, Erode and Nilgiris Districts of Tamil Nadu, India with similar age group (2-3 years). These experimental animals were divided into six groups and each group comprised of eight animals (n=8) in both male and female. Group I: Toda he-buffalo, Group II: Toda she-buffalo, Group III: Bargur he-buffalo, Group IV: Bargur she-buffalo, Group V: Murrah he-buffalo and Group VI: Murrah she-buffalo. These experimental buffaloes were dewormed, vaccinated, fed and watered as per the farm schedule. Toda buffaloes were reared in Nilgiris District and the experimental station was situated at an elevation of 900 to 2636 meters above MSL. Its latitudinal and longitudinal location is 130 km (Latitude: 11°12 N to 11°37 N) by 185 km (Longitude: 76°30 E to 76°55 E). Bargur buffaloes were reared in the deciduous forest from 11.40 N to 12. N latitude and between 77.2 E and 77.7 E longitude. Bargur hills are located with an elevation 1000 meters above MSL. Murrah buffaloes were reared in Namakkal District situated at an elevation of 218 metres above MSL and located between 11.23°N and 78.17°E.

Collection of blood samples

Blood samples were collected by jugular venipuncture from experimental buffaloes with clot activator once in a week prior to concentrate feeding in the morning. Blood samples were immediately centrifuged at 4 °C at 3000 rpm for 15 min to separate the serum and serum was labelled and stored at −80 °C for further analysis. Protein concentration of serum samples was estimated according to the standard procedure of the Lowry method (Lowry *et al*., 1951) with use of a spectrophotometer (Thermoscientific, Germany).

Gelatin zymography

Serum samples were subjected to gelatin zymography by modified SDS-PAGE [a modified method of Laemmli (1970)] as performed by Heussen and Dowdle (1980). In this method, gelatin (0.3%) was added as a copolymerization substrate to obtain (final concentration 0.15%) the resolving gel (8%). The samples were electrophoresed at 100 V for 20 min. Renaturation was performed with 2.5% Triton X-100 for 3 h on a mechanical shaker with gentle shaking. The gel was then incubated in 10 mM $CaCl_2$, 0.15 M NaCl and 50 mM Tris, pH 7.5, for 18 h at 37 °C. The gel was stained with 0.25% Coomassie brilliant blue for 2 h, followed by destaining

with a destaining solution for 1 h. Finally, the gel was washed with distilled water.

Analyzing the results of gelatin zymogram

Human capillary blood gelatinase served as the standard marker for evaluating the zymogram bands, following the protocol outlined by Makowski and Ramsby (1996). By performing a finger stick, blood was obtained from a capillary and measured using a precise analytical balance in a tarred polypropylene tube. Afterward, the samples were combined with 20× volume of Laemmli buffer and thoroughly blended. These aliquots remained stable for a duration of 3 months at -20° C.

Results and Discussion

Serum samples of six groups from three buffalo breeds were subjected to gelatin zymography (Figure 1). Serum samples were proteolytically active as they completely degraded the gelatin. Major bands at 220 kDa, 135 kDa and 92 kDa of MMP-9 and 72 kDa of MMP-2 were observed in six experimental buffalo groups in all three breeds. In Murrah breed, two prominent bands at 92 kDa and 72 kDa were observed and they represented the latent forms of MMP-9 and MMP-2, respectively. Further, two lytic bands were observed at 220 kDa and 135 kDa and they represented as the proforms of MMP-9 in each experimental group. Below the latent form of MMP-9, the active form of 87 kDa of MMP-9 was also observed. However, the active form of MMP-2 was not observed in the serum samples of different experimental groups in these three buffalo breeds. The intensity of MMP-2 (72 kDa) was two to three folds higher than the latent form of MMP-9 (92 kDa) which was more prominent than the human marker (lane 7).

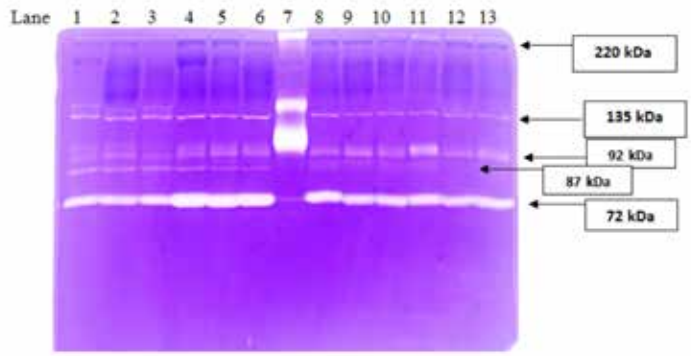

Fig. 1. Comparative Gelatin Zymogram of hilly (Toda and Bargur) and plain (Murrah) bubaline species

Lane	Breed	Sex	Group	Age (Years)
2-3	Toda	Male	I	2-3
1	Toda	Female	II	2-3
4-5	Bargur	Male	III	2-3
6	Bargur	Female	IV	2-3
7	Human capillary blood MMP marker			
8, 12-13	Murrah	Male	V	2-3
9-11	Murrah	Female	VI	2-3

The results of present study were in agreement with the results of Prakash Krupakaran *et al*. (2016) and Balamurugan *et al*. (2023) in bovine, Prakash Krupakaran *et al*. (2015) in bubaline and Balamurugan *et al*. (2017) in ovine species. Similarly, Prakash Krupakaran *et al*. (2016) reported that the latent form of MMP-2 (72 kDa) was more prominent as compared to MMP-9 monomer (92 kDa) in the serum of a Jersey crossbred bull. Similar to our results, Belo *et al*. (2009) reported in human plasma gelatin zymography that plasma samples showed all forms of MMPs including the homodimer of the pro-MMP-9 form (225 kDa), the pro-MMP-9 complexed

with neutrophil gelatinase-associated lipocalin form (130 kDa), the pro-MMP-9 form (92 kDa) and the pro-MMP-2 (72 kDa) form.

Effect of sex on the expression of gelatinase activity

Gelatin zymogram of he- and she- buffaloes in hilly breeds of Toda and Bargur buffaloes were compared to Murrah buffaloes raised in plain region to find out the association between the MMPs expression with sex. In both Toda and Bargur buffaloes, the intensity of latent form of MMP-2 (72 kDa) was 1.5 folds higher in he-buffalo groups (Toda Gr I: lane 2-3; Bargur Gr III: lane 4-5) as compared to she-buffalo groups (Gr IV: lane 6; and Gr II: Lane 1). The intensity of latent form of MMP-9 was lower in he-buffalo (Toda Gr I: lane 2-3; Bargur Gr III: lane 4) as compared to she- buffaloes (Gr IV: lane 5-6; and Gr II: lane 2-3). The she-buffalo (Gr IV: lane 6; and Gr II lane 1) had thicker bands at 135 kDa and 220 kDa as compared to he-buffalo groups indicated that the expression of pro-enzymatic forms of MMP-9 was very clear and they were lytic bands of MMP-9.

Similarly, in Murrah buffaloes, the intensity of latent form of MMP-2 (72 kDa) was 1.5 folds higher in he-buffalo (Gr V: lane 8, 12–13) than in she-buffalo (Gr VI: lane 9-11). The intensity of latent form of MMP-9 was lower in he- (Gr V: lane 8,12-13) than in she-Murrah buffaloes (Gr VI: lane 9–11). The she-buffaloes (Gr VI: lane 9-11) exhibited thicker bands at 135 kDa and 220 kDa than the he-buffaloes, which indicated that the expression of pro-enzymatic forms of MMP-9 was prudent and clear and they were the lytic bands of MMP-9. Similar pattern of MMP-2 and MMP-9 expressions was noticed in different studies by various authors (Bonnema *et al*., 2007, Belo *et al*., 2009, Kusnierova *et al*., 2015, Cancemi *et al*., 2020).

Similar to the present study results, Palei *et al*. (2008) reported that pregnant women with uncomplicated pregnancies, gestational hypertension, preeclampsia and healthy non-pregnant women had all forms of MMPs including the homodimer of the pro-MMP-9 form (225 kDa), the pro-MMP-9 complexed with neutrophil gelatinase-associated lipocalin (NGAL) form (130 kDa), the pro-MMP-9 form (92 kDa) and the pro-MMP-2 (72 kDa) form by gelatin zymography. On the contrary to the present study results, Belo *et al.* (2009) reported that non-significant difference was observed between boys and girls on different MMPs (MMP-8, pro-MMP-9, MMP-9, MMP-2, TIMP-1 and TIMP-2) and the concentration of Pro-MMP-9 (A.U) in girls (0.99) than boys (0.76) but the concentration of MMP-2 was equal in both sexes (1.59). Similarly, Bonnema *et al*. (2007) reported that women had a non-significantly lower value (MMP-2: 1352 ± 58 *vs* 1300 ± 49; MMP-9: 16.5 ± 6.3 *vs* 19.4 ± 3.80) as compared to men with respect to MMPs (ng/mL). Correspondingly, higher MMP concentration alteration was observed in male patients as compared with female patients (Singh Yadav *et al*., 2018). However, there was a non-significant difference exist in MMP expression (MMP-1, MMP-2, MMP-7, MMP-8, MMP-9 and MMP-13) in plasma and serum samples of men and women (Jonsson *et al*., 2016).

The expression of MMPs is also dependent on the hormonal status in female (Berg *et al*., 2014). Similarly, in human beings, Kusnierova *et al*. (2015) reported that MMP-2 and MMP-3 levels were found to be age dependent and MMP-3 and MMP-9 levels were gender dependent. Further, they concluded that plasma MMP-9 level was not age dependent; it was associated with gender, showed lower concentration of MMP-9 in women. Further, Sathyamoorthy *et al*. (2015) found that plasma MMP-8 concentration was 1.51-folds higher in men than women with tuberculosis (TB) and this difference was not due to greater disease severity in men. This might be due to men mount a greater and often more damaging inflammatory response to infection compared to women of reproductive age (Guerra-Silveira and Abad-Franch, 2013).

It was inferred that the expression of MMP-2 was higher in male groups compared to female buffalo groups. But the expression of MMP-9 was higher in female than in male buffalo groups. Thus, the expression of MMP-9 was more in female than in the male animals which might be due to female sex hormones which are protective (Marriott and Huet-Hudson, 2006) and female neutrophils showed to express decreased MMP-9 during the period of the menstrual cycle when oestrogen levels are higher (Smith *et al*., 2007). Thus, it might be inferred that the levels of gelatinases expression may be correlated with individual physiological status within the sex.

Effect of breed on the expression of gelatinase activity

Gelatin zymography was performed to find out the influence of different breeds raised in different geographical altitude on MMPs expression. Toda buffalo's (Gr I: lane 2-3 and Gr II: lane 1) gelatinase expression was compared with Murrah buffaloes raised in plain region (Gr V: lane 8, 12–13 and Gr VI: lane 9-11). The major bands at 220 kDa, 135 kDa and 92 kDa of MMP-9 and 72 kDa of MMP-2 were observed in both breeds. Since they completely degraded the gelatin, all the forms of MMP proteins were proteolytically active in both breeds. The latent forms of MMP-9 and MMP-2 observed as clear bands at 92 kDa and 72 kDa in both Toda and Murrah breed buffaloes, respectively. Similarly, two lytic bands were observed at 220 kDa and 135 kDa indicates the proforms of MMP-9 were prevalent in both breeds. The intensity of MMP-2 (72 kDa) was two to three times higher than the latent form of MMP-9 as compared to human marker (lane 7). However, the intensity of MMP-2 (72 kDa) was three to four times higher than the latent form of MMP-9 as in Murrah buffaloes as compared to Toda buffaloes. Interestingly, the active form of MMP-9 (87 kDa) was clearly observed in Toda buffaloes (Gr I: lane 2-3 and Gr II: lane 1) as compared to Murrah buffaloes (Gr V: lane 8, 12–13 and Gr VI: lane 9-11). However, the active form of MMP-2 (67 kDa) was not observed in serum of both the breeds.

Bargur buffaloes were reared about 1000 meters above MSL. In this breed, the major bands (lane 4-6) were observed at 220 kDa, 135 kDa, 92 kDa and 87 kDa of MMP-9 and 72 kDa of MMP-2 in both sexes. The latent bands at 92 kDa of MMP-9 and MMP-2 and 72 kDa were observed as stronger bands than the active forms. The intensity of MMP-2 (72 kDa) was three to four times higher than the latent form of MMP-9 as compared to human marker (lane 7). However, the intensity of MMP-2 (72 kDa) was two to three times higher than the latent form of MMP-9 in Bargur buffaloes as compared to Murrah buffaloes. The active form of 87 kDa of MMP-9 was also observed as a clear band below the latent form of MMP-9. But, the active form 67 kDa of MMP-2 was not observed in serum sample of both the sexes in these breeds. As compared to Murrah buffaloes raised in plain region (mean sea level 218 metres above MSL), the activity of MMP-2 was four times higher in Bargur breed buffaloes followed by Toda buffaloes. Our results were in agreement with the results of earlier reports from the same laboratory Prakash Krupakaran *et al*. (2015), Prakash Krupakaran *et al*. (2016), Balamurugan *et al*. (2017) and Balamurugan *et al*. (2023).

In our laboratory, gelatin zymography was performed in different domestic animal's serum including cattle and buffalo. Prakash Krupakaran *et al*. (2016) reported that major bands were observed at 220 kDa, 135 kDa and MMP-9 monomer (92 kDa) and latent form of MMP-2 (72kDa) in Jersey bull serum. Further, to accord with the present results, the intensity of 72 kDa of MMP-2 band was two to three times more prominent than 92 kDa of MMP-9. In the earlier study of Prakash Krupakaran *et al*. (2015), the intensity of the latent form of MMP-2 (72 kDa) in cattle was matched with the intensity of the same band in regular cyclic buffaloes which was in agreement with the present results. Similarly, to agree with the present study, Balamurugan *et al*. (2017) observed that both latent forms of MMP-2 and MMP-9 were exhibited and the latent bands were observed as thicker bands than the active form in sheep. Further, the intensity of 72 kDa of MMP-2 was 3-5 times higher than 92 kDa of MMP-9 in sheep. Recently, Balamurugan *et al*. (2023) observed the similar pattern of 72 kDa of MMP-2 was 3-5 times higher than 92 kDa of MMP-9 in Umblachery cattle breed as compared to Jersey cattle breed.

In hilly breeds, the expression of MMP-2 and MMP-9 was higher as compared to plain breed of Murrah buffaloes. In hilly breeds, the expression of MMP-2 was higher which might be due to these buffaloes were raised in hill region more than 1000 to 3000 meters above MSL. MMP's are derived from neutrophils; macrophages which are the primary sources among different immune cells responsible for MMP production (Lopez-Otin and Hunter, 2010). Chronic hypoxia in hilly breeds may be the cause of increased gelatinase expression. To agree with the observation, in the heart ventricles of fetal guinea pigs, prolonged hypoxia raises the levels of inducible nitric oxide synthase (iNOS) protein and mRNA expression.

Overproduction of nitric oxide (NO) can cause nitrosative stress, which in turn can produce peroxynitrite and increase MMP expression (Evans *et al*., 2012).

Zhu *et al*. (2022) found that acute hypoxia upregulates the MMP-9 expression in bone marrow in rats. Rat bone marrow microvascular BM was degraded by prolonged hypoxia and this was strongly correlated with elevated MMP-9 expression (Huang, 2018). Similarly, MMP-2, which is produced when ECs experience hypoxia and is involved in autocrine processes that control EC migration and apoptosis (Ben-Yosef *et al*., 2005).

Conclusion

The present study concluded that expression of MMP-2 was higher in he-buffaloes as compared to she-buffaloes whereas the expression of MMP-9 was higher in she- than in he-buffalo groups. The intensity of MMP-2 was 3-4 folds higher in Toda and 2-3 folds higher in Bargur buffaloes than the latent form of MMP-9 as compared to Murrah buffaloes.

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