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Matrix Metallo Proteinases modulates the semen quality profiles of Kangeyam bull

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Abstract

A study was conducted to find out the existence of Matrix Metallo Proteinases (MMP) in the semen of native cattle of Tamil Nadu 'Kangeyam'. Sixteen (n=18) apparently healthy Kangeyam bulls of approximately 4 to 6 years of age with good body condition (score 5-6) were selected. Semen samples were collected from the experimental animals during early morning before concentrate feeding and routine semen quality parameters were analysed. Based on the semen characters, bulls were divided into two groups Group I Normospermic (n=9) and Group II Oligospermic (n=9). Semen samples were subjected to gelatin zymography. It was confirmed that the major bands were observed at 72 kDa of MMP-2 and 92 kDa of MMP-9 in all the experimental animals in both the groups. In normospermic groups, the major bands were observed at 72 kDa and 92 kDa and it represents the latent forms of MMP-2 and MMP-9, respectively. Further, in each group, two lytic bands were observed at 220 kDa and 135 kDa and it represents the proforms of MMP-9. The intensity of 72 kDa of MMP-2 was 2-3 times higher than the marker. In oligospermic groups, both latent (92 kDa) and active (87 kDa) forms of MMP-9 was observed. Further, the intensity of latent form of (92 kDa) MMP-9 was 1.5 times higher than that the normospermic groups. Similarly, in oligospermic groups, two more prominent bands were noticed at 220 kDa and 135 kDa revealed that they were the lytic bands of MMP-9 indicating the proenzymatic forms of MMP-9. It was concluded that the expression of latent and active forms of MMP-9 and latent form of MMP-2 were observed in all experimental animals. But the expression of MMP-9 was positively associated with low sperm count as it was strongly expressed in oligospermic groups whereas the expression of MMP-2 was positively associated with high sperm count as it was strongly expressed in normospermic groups. Further, more studies required to ascertain the enzymatic activity of MMP-2 and MMP-9 which may serve as an alternative biomarker in determining semen quality.

Key words: Matrix Metallo Proteinase; gelatin zymography; semen

Introduction

Matrix metalloproteinase (MMP) family consists of at least 20 structurally related zinc metallo endopeptidases capable of degrading the extracellular matrix components. These enzymes participate in embryonic development, morphogenesis, blastocyst implantation, angiogenesis and tissue resorption, and in diseases such as arthritis, cancer cell invasion and metastasis (Nagase, 1996). This group of enzymatic proteins is classified into collagenases, gelatinases, stromelysins, and membrane-type MMPs (Nagase and Woessner, 1999). MMPs have primary roles in cellular functions such as cell proliferation, apoptosis, migration, differentiation, and angiogenesis. They are also effective in physiological processes such as reproduction, fetal growth, tissue reconstruction, wound healing, and bone development (Klein and Bischoff, 2011). MMP-2 and MMP-9 (also known as gelatinases A and B) particularly degrade gelatin, elastin, as well as various types of collagens. MMPs are capable of degrading nearly all kinds of proteins of the extra cellular matrix (ECM). At the same time, MMPs are also involved in the processing of signaling proteins such as cytokines and chemokines, thereby, modulating their release and/or activity (Bonnans *et al.,* 2014).

Seminal plasma contains many proteinases originating either from testicular cells or from prostate and other accessory sex glands (Yin *et al.,* 1990). MMP-2 takes part in penetration of sperm into oocyte and functions as acrosin. Thus, probably this enzyme is located on the inner acrosomal membrane of sperm (Ferrer *et al*.,

2012). In male reproductive system, MMP-2 and MMP-9 are detected in seminal plasma. MMP-2 and MMP-9 are released from prostate and seminal vesicles; however, there is less information about the function of this enzyme in male reproduction compared to female reproductive system (Baumgart *et al.,* 2002). MMP-2 and MMP-9 help the movement of germ cells in spermatogenesis by digesting of extracellular matrix (Chen, 2011). MMP-2 and MMP-9 are involved in the breakdown of the oocyte membranes to allow sperm to enter the oocyte. MMPs along with other proteins in seminal plasma regulate spermatogenesis, sperm motility, antioxidant protection, and retain lipid stability in sperm membrane (Dietrich *et al.,* 2014).

Most of the studies were carried out in female animals and there were limited studies in male domestic animals. Several studies evaluated the association among alterations of genes and proteins of MMPs with sperm parameters, semen quality, and normospermic infertility (Baumgart *et al.,* 2002, Mohagheghi *et al.,* 2015, Kurzawski *et al.,* 2017, Mohagheghi *et al.,* 2018). Till date, the existence of gelatinase in native bulls of India was not carried out, and their relationship with semen parameters was not attempted. Kangeyam cattle is a recognized and registered breed of cattle (dual purpose breed for draught and milk) in the home tract of Erode and Coimbatore districts of Tamil Nadu. Hence, the present study was carried out to find out the expression of MMP-2 and MMP-9 in seminal plasma samples and to assess the association between MMP-2 and MMP-9 expression and semen quality parameters in native cattle breed of Tamil Nadu, Kangeyam.

Materials and Methods

The proposed study was conducted at the Department of Veterinary Physiology and Biochemistry, Veterinary College and Research Institute (TANUVAS), Orathanadu, Tamil Nadu, India. The institute is located 30 meters above sea level at latitudes 10.6°N and 79.3°W.

Experimental animals

Eighteen (n=18) apparently healthy Kangeyam bulls of approximately 4 to 6 years of age with good body condition (score 5-6) were selected from the herd of organized farms, Salem, India. All these animals were fed with standard basal diet consisting of concentrate mixture and roughage (wheat straw) to meet their nutrient requirements according to NRC (1985) throughout the study period. All the animals were maintained under uniform feeding, housing and managemental conditions as per the management of farm. Further, all the bulls were vaccinated and feeding and watering were done as per the feeding standard of the farm.

Collection of semen

Semen samples were collected once a week from all the bulls for 6 weeks with use of standardised artificial vagina method. The collections were obtained by exposing the bulls to the oestrous cow. Immediately after collection, the tubes containing semen were placed in a small thermo-flask having water at 37 ˚C for further processing. The ejaculates were analysed and accepted for evaluation as tested for routine seminal parameters and accepted for evaluation after meeting the minimum standard protocol (MSP) standards like-wise concentration: >500 million/mL; mass activity: >3+, individual motility: >70% and overall abnormality not more than 10%. A total of six ejaculates from each bull (18 x 6 = 108) were collected; after screening, 72 of 108 ejaculates were selected and assessed.

Semen analysis

The volume of ejaculated sperm was measured and recorded in milliliters (mL) straight from the graded sperm collection tube. A small drop of freshly collected semen was put on a clean, grease-free, pre-warmed glass slide at 37°C and viewed without a cover slip using a low power (100×) phase contrast microscope (Nikon, Eclipse 80i) to assess the mass activity of the semen sample. The appearance of waves and swirls was used to rate the mass activity on a scale of 0 to 5. The concentration of sperm ($\times 10^6$/mL) in neat sperm was determined using a haemocytometer and the red blood cell counting process. Special care was taken when diluting the sample with 1% formal saline, which was then cross-checked by diluting neat semen at a ratio of 1:200 with a micro pipette. The homogeneity of the sperm samples was ensured by hand

shaking the tube containing the sperm samples as well as charging the haemocytometer.

Seminal parameters, viz. sperm motility (Salisbury *et al.,* 1985), viability and total sperm morphological abnormalities by Eosin–Nigrosin staining (Agarwal *et al.,* 2016), acrosomal integrity by Giemsa staining (Watson, 1975) and plasma membrane integrity by hypo-osmotic swelling test (Jeyendran *et al.,* 1984) were determined with standard procedures.

Separation and preparation of seminal plasma

One mL of semen was centrifuged at 4000 rpm for 20 min at 4°C to separate sperm pellets and seminal plasma. Seminal plasma was stored at -80°C for further analysis. The supernatant containing seminal plasma proteins was separated for SDS- PAGE analysis.

Gelatin zymography

Seminal plasma samples were subjected to gelatin zymography by modified SDS-PAGE (modified from the method of Laemmi, 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%) a separating 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 colorless solution for 1 h. Finally, the gel was washed with distilled water.

Analyzing the results of gelatin zymogram

Human capillary blood gelatinase served as a standard marker for the evaluation of zymogram groups according to the protocol described by Makowski and Ramsby (1996). Blood was drawn from a capillary by pricking a fingertip and an accurate analytical weight was measured in a tarred polypropylene tube. Samples were then combined with a 20 × volume of Laemmli buffer and mixed thoroughly. These lots were stable for 3 months at -20°C.

Results and Discussion

The semen samples were subjected to gelatin zymography from both groups. All the semen samples were proteolytically active, since all completely degraded the gelatin. The major bands were observed in both groups at 72 kDa of MMP-2, 92 kDa, and 87 kDa of MMP-9. Both latent (92 kDa) and active (87 kDa) forms of MMP-9 and latent form of (72 kDa) MMP-2 was observed in both groups. Further, two more lytic bands were observed at 220 kDa, and 135 kDa indicate the proforms of MMP-9. The intensity of latent form of MMP-2 (72 kDa) was 2-3 times higher than that the latent form of 92 kDa of MMP-9 and four times higher than marker in all the experimental animals. However, the active form of (72 kDa) MMP-2 was not observed in both groups. It was clearly visible that the latent forms of MMP-9 and MMP-2 were dominant than the active forms. Various authors reported the existence of gelatinases in seminal plasma of human (Shimokawa *et al.,* 2002, Warnick *et al.,* 2015, Baumgart *et al.,* 2002, Kratz *et al.,* 2014) and canine (Saengsoi *et al.,* 2012). Similarly, in human seminal plasma, both latent and active forms of MMP-2 and MMP-9 were detected by Kratz *et al.* (2014) and Tentes *et al.* (2007) and concluded that the latent forms were the predominant ones. Gelatin zymogram reveals the existence of gelatinases in both the groups as shown in Fig. 1.

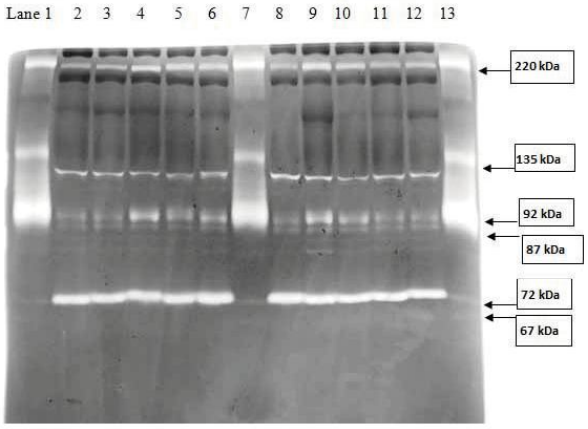

Fig.1: Gelatin zymography of Kangeyam bull Semen

Lane 1, 7 & 13 - Human capillary blood MMP marker

Lane 2, 3, 8, 11, 12 - Kangeyam Normospermic animals

Lane 4, 6, 9, 10 - Kangeyam Oligospermic animals

Lane	Group
1.	Human capillary blood MMP marker
2.	Normospermic
3.	Normospermic
4.	Oligospermic
5.	Normospermic
6.	Oligospermic
7.	Human capillary blood MMP marker
8.	Normospermic
9.	Oligospermic
10.	Oligospermic
11.	Normospermic
12.	Normospermic
13.	Human capillary blood MMP marker

In normospermic groups (G I: Lane 2, 3, 5, 8, 11, 12), the major bands were observed at 72 kDa and 92 kDa indicating the latent forms of MMP-2 and MMP-9, respectively. Further, one minor band was observed at 87 kDa indicating the active form of MMP-9 and two more lytic bands observed at 135 kDa, and 220 kDa indicating the proforms of MMP-9. The intensity of latent form of MMP-2 (72 kDa) was 2-3 times higher than marker (Lane 1, 7, 3). The intensity of latent form of (92 kDa) MMP-9 was lighter than the latent form of MMP-2 in all the experimental animals in this group. However, the active form of (67 kDa) MMP-2 is not observed in all the animals. The intensity of (72 kDa) MMP-2 was 1.5 times higher in normospermic group than oligospermic groups (G II: Lane 4, 6, 9, 10). Further, one faint band was observed at 80 kDa indicated the proform of MMP-2 in normospermic groups. It was obviously indicated that the expression of MMP-2 higher than MMP-9. It might be inferred that the expression of MMP-2 was associated with sperm concentration and characteristics. Our results were in agreement with the results of Baumgart *et al.* (2002), Shimokawa *et al.* (2002), Tentes *et al.* (2007), Saengsoi *et al.* (2012) and Warnick *et al.* (2015). Similar to these results, Warnick *et al.* (2015) observed that gelatinase

activities between normal and abnormal semen samples showed a significant and six-fold higher in proMMP-2 and MMP-2 activity in high than low sperm concentration samples ($p < 0.001$).

In oligospermic groups (G II: Lane 4, 6, 9, 10), the major bands observed at 92 kDa and 72 kDa indicating the latent forms of MMP-9 and MMP-2, respectively. Further, one minor band was observed at 87 kDa indicating the active form of MMP-9 and two more lytic bands observed at 135 kDa, and 220 kDa indicating the proforms of MMP-9. The intensity of latent form (92 kDa) of MMP-9 was two times higher than normospermic groups (G I: Lane 2, 3, 5, 8, 11, 12) but lower than the marker. As compared to normospermic groups, the oligospermic groups (G II: Lane 4, 6, 9, 10) showed thicker bands at 135 kDa and 220 kDa indicating the expression of proenzymatic forms of MMP-9 was very clear. The present study results were in agreement with results of various authors (Shimokawa *et al.,* 2002, Warnick *et al.,* 2015, Baumgart *et al.,* 2002). To concurrence with these results, in human subjects, Baumgart *et al.* (2002) observed that ProMMP-9 and MMP-9 levels were significantly elevated in samples with low sperm counts compared to those with high sperm density ($p < 0.001$). Further, High level of proMMP-2 and MMP-2 was associated with high sperm motility (≥70%, $p < 0.001$).

From these results, it was inferred that the expression of MMP-9 was higher in oligospermic groups. It might be associated MMP-9 expression increased with low sperm concentration. Similarly, in human seminal plasma Shimokawa *et al.* (2002) observed that three major bands of gelatinase activity at 72 kDa, 67 kDa and 52 kDa and minor bands at 92 kDa, 84 kDa and 45 kDa in gelatin zymography. These results indicate that two kinds of pro-form and active-form matrix metalloproteinases, MMP-2 and MMP-9, and their degradation products, are present in human seminal plasma. Further, these proteinases were all recognized by the polyclonal antibodies for MMP-2 or MMP-9. These activities were the metalloproteinases proMMP-9 (92kDa), proMMP-2 (72kDa) and MMP-2 (67kDa), and that their degradation products were present at lower molecular weights. Hence, the expression of MMP-9 was higher in low sperm concentration animals than high sperm concentration animals. To harmony with these results, Tentes *et al.* (2007) observed that ProMMP-9 levels were higher in semen samples with abnormally low concentration ($\leq 19\times10^6$/ml) compared with semen samples with concentration $\geq 50\times10^6$/ml.

Relationship of Semen characters with expression of MMP-9 and MMP-2

The basic semen characteristics of both groups were analyzed and presented in the Table 1. In both groups, the major bands at 72 kDa, 92 kDa, and 87 kDa and major bands at 220 kDa, and 130 kDa were observed. Both latent (92 kDa) and active (87 kDa) forms of MMP-9 and latent form of (72 kDa) MMP-2 were observed in both groups. But the active form of (72 kDa) MMP-2 was not observed in both groups. The expression of latent form of (72 kDa) MMP-2 was higher in normospermic groups (G I: Lane 2, 3, 5, 8, 11, 12) than the oligospermic groups (G II: Lane 4, 6, 9, 10). But in oligospermic groups, the latent and active forms of (92 kDa and 87 kDa) of MMP-9 were higher than the normospermic groups. It was clearly understood that in olgospermic groups, the expression of MMP-9 was higher is due to low sperm count in the respective group. Likewise, the expression of MMP-2 was higher in normospermic groups is due to high sperm count and sperm without abnormality. Though, the active form of MMP-2 was not present in this group, one minor band was observed at 80 kDa indicated the proform of MMP-2. Our results were in agreement with the results of Baumgart *et al.* (2002), Shimokawa *et al.* (2002), Tentes *et al.* (2007), Saengsoi *et al.* (2012) and Warnick *et al.* (2015). In human, MMP-2 concentration in seminal plasma was significantly associated with sperm count in a linear trend (Baumgart *et al.,* 2002). Similarly, Buchman-Shaked *et al.* (2002) conducted a study on human subjects and found that the major bands were observed at 92 kDa, 72 kDa, 62 kDa, and 28 kDa molecular-weight bands exhibiting gelatin-degrading activity in both normal and abnormal sperm samples. The 92 kDa, 72 kDa, and 62 kDa bands with gelatinolytic activity were consistent with pro-MMP-9, pro-MMP-2, and active MMP-2, respectively. Further, they concluded that a higher 28 kDa activity and a lower 92 kDa MMP activity in normal sperm samples relative to abnormal samples were detected.

Table 1. Descriptive statistics of basic semen parameters and MMPs in two group

Parameters	Group I Normospermic (*n*=9)	Group II Oligospermic (*n*=9)
Semen volume (ml)	4.15±0.57[a]	4.52±0.36[a]
Sperm concentration (×10^6/mL)	1058.85±4.87[a]	624.34±6.86[b]
Total motility (%)	76.54±0.87[a]	32.75±0.76[b]
Viability (%)	80.74±1.21[a]	38.98±0.65[b]
Total sperm abnormality (%)	5.72±0.32[a]	16.83±0.87[b]
Acrosomal Integrity (%)	86.46±0.78[a]	41.65±0.43[b]
Plasma membrane Integrity (%)	81.34±0.98[a]	39.12±0.67[b]

Values are presented as mean±standard error.
*Values indicated are significantly different ($p<0.05$).

On contrary, Atabakhsh *et al.* (2018) observed in human subjects that MMP-2 and MMP-9 activities in seminal plasma have a positive effect on sperm count and motility. Further, direct correlation between activity of MMP-2 and MMP-9 in follicular fluid with oocyte quality and fertilization rate was reported. Similarly, in stallion, Kareskoski *et al.* (2021) observed that latent pro-MMP-2, active MMP-2 and total MMP-9 were present in all fractions of the stallion's ejaculate, with higher relative activity levels of the latent than active forms and the highest relative activity in the high fraction. Because these MMPs are associated with sperm concentration and total number of sperm, and they are emitted into the first sperm-rich fractions of the ejaculate, the glands contributing to these fractions are probably their main source.

To conclude, MMP-2 expression was higher in normospermic groups and MMP-9 expression was higher in oligospermic groups. To harmony with our results Kratz *et al.* (2014) observed that seminal MMP-9 expression was higher in childless men than in fertile subjects, whereas there were no significant differences in MMP-2 expression between the analysed seminal groups. Tentes *et al.* (2007) observed that MMP-2 and MMP-9 were both present in human semen, and low sperm concentration semen samples have higher MMP-2 and lower MMP-9. Semen samples with a normal sperm count, semen samples with a low sperm count (≤ 19 × 10^6/ml) showed reduced sperm viability, a reduced percentage of Grade A sperm, a reduced percentage of morphologically-normal sperm, and lower proMMP-9 and MMP-9 but higher proMMP-2 and MMP-2 levels ($P < 0.05$). There were correlations between MMP-2 and MMP-9 expression and the percentage of Grade A sperm and morphologically-normal sperm ($P < 0.05$).

Conclusion

It was concluded that the expression of latent and active forms of MMP-9 and latent form of MMP-2 were observed in all the experimental animals. But the expression of MMP-9 was positively associated with low sperm count as it was strongly expressed in oligospermic groups. Whereas the expression of MMP-2 was positively associated with high sperm count as it was strongly expressed in normospermic groups. Further, more studies are required to ascertain the enzymatic activity of MMP-2 and MMP-9 which may serve as an alternative biomarker in determining semen quality.

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