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Characterization of tissue inhibitor metalloproteinases in semen and their relationship with vital sperm function tests vis-à-vis fertility of breeding buffalo bulls

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ABSTRACT

The present study was undertaken to separate and compare the tissue inhibitor metalloproteinases **(**TIMP) of seminal plasma and frozen-thawed sperm extracts from 30 buffalo bulls by immunoblotting and determine a relationship between various TIMP with post-thaw sperm function tests vis-à-vis bull fertility. Seven immunoreactive bands in seminal plasma (65, 55, 48, 33, 31, 24 and 11 kDa) and 5 in frozen-thawed spermatozoa (75, 65, 55, 24 and 16 kDa) were detected in Western blots following incubation (TIMP–140) and subsequent washing *in vitro*, indicating that TIMP is bound to sperm membranes. The frozen-thawed semen was evaluated for first service conception rate (FSCR), per cent HOST, acrosome reaction, viability, DNA integrity and total motility and linked to TIMP. In seminal plasma, the bulls positive for 48, 33 and 24 kDa TIMP had significantly higher FSCR (57.0 ± 2.6 vs 27.0 ± 2.4%, 55.7 ± 3.0 vs 31.3 ± 3.2% and 45.0 ± 3.8 vs 32.8 ± 4.7%, respectively) as compared to their negative counterparts. Except per cent viability, almost all seminal parameters (acrosome reaction, per cent HOST, DNA integrity and total motility) were significantly higher in bulls positive for TIMP of 48, 33, 31 and 24 kDa than in their negative contemporary mates. In frozen-thawed sperm extracts, the bulls positive for TIMP–24 had significantly higher FSCR (51.7 ± 3.7 vs 27.2 ± 3.0%), higher percentage of acrosome-reacted (55.9 ± 2.8 vs 48.9 ± 2.2%) and HOS-positive (69.2 ± 1.5 vs 65.3 ± 1.9%) spermatozoa in comparison to their negative herd mates. These results suggested that TIMP influences semen quality and subsequent fertility of buffalo bulls through inhibition of metalloprotease activity in semen.

Key words: Buffalo bull, FSCR, Semen, TIMP

Sub-optimal fertility of males has a significant negative economic impact on dairy farming. Bulls producing semen that meets the standards evaluated during a breeding soundness examination differ widely in actual fertility (Sylla *et al.* 2007). However, till date, there is no single objective test to evaluate the fertility of bull. Therefore, there is a need to identify the molecular markers of fertility. The development of such markers to identify bulls of high breeding values represents a remarkable way for achieving genetic gain in dairy productivity. Seminal plasma contains multiple proteins that modulate the fertilizing ability of sperm and have been determined as marker of fertility factors (Dai *et al*. 2009). In bovine, tissue inhibitor metalloproteinases (TIMP) and their close associates represent a new superfamily of proteins. They originate from accessory sex glands (bulbourethral, prostate and seminal vesicular glands); play a key role in a number of reproductive processes including modulation of sperm binding events, capacitation, fertilization, implantation and embryonic development and serve as molecular markers of bull fertility (Belleanneea *et al.* 2011). They migrate from posterior head to acrosomal region that regulates the rate of membrane proteolysis or fusion, and determines their role in sperm capacitation and/or acrosome reaction (Newton *et al.* 2010). Four distinct TIMP protein isoforms, viz. TIMP-55, TIMP-48, TIMP–33 and TIMP-24 were recognized in immunoblots of bull seminal plasma and sperm extracts and their presence on sperm was shown to be an indicator of increased fertility of bulls based on artificial insemination field fertility trials (Manjunath *et al.* 2002, Rueda *et al.* 2013). When the semen of TIMP-positive and negative bulls was inseminated artificially, the bulls which contained the TIMP in sperm extract were 13% more fertile as compared to their contemporary mates (Dawson *et al.* 2002). Thus, correlative data and direct evidence in practical applications have demonstrated that an increasing number of apparently diverse seminal molecules play an important role in modifying fertility. Keeping in view the above facts and taking into consideration the deficit knowledge of TIMP in buffalo bulls, the present study was

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designed to characterize TIMP in seminal plasma and frozen-thawed spermatozoa and determine their role in sperm function tests in relation to fertility of breeding buffalo bulls.

MATERIALS AND METHODS

Semen procurement and preparation of sperm extracts: Both fresh (1–2 ml) and frozen semen (50 straws/bull) from 30 breeding Murrah buffalo bulls were procured from 2 government semen processing and freezing laboratories in september having ambient temperature 30.6°C and relative humidity 92% for the study. The fresh and frozen-thawed semen (20 straws/bull) was centrifuged at 3000 rpm for 10 min to separate out seminal plasma and dilutor, respectively. The seminal plasma from fresh semen was transferred to cryovials for storage at –20°C until analysis. The dilutor from frozen-thawed semen was discarded. The sperm pellet from frozen-thawed semen was washed thrice with PBS, pH 7.4 to get rid of the dilutor. Sperm extracts were prepared by suspending 1×10^9 spermatozoa in 2.0 ml of 62.5 mM Tris-HCl (pH 6.8, 2% SDS, 1 mM PMSF, 25 mM benzidine), ultrasonicated (3 bursts of 20 sec each) and centrifuged at 15,000 rpm for 30 min. The aliquots of sodium dodecyl sulphate-sperm extracts (SDS-SE) were stored at –20°C till further use.

Molecular weight determination by immunoblotting: Exactly 100 µg of protein was fractionated by SDS-PAGE using 10% separating gel and 4% stacking gel. After electrophoresis, enzyme linked immuno transfer blot was done as per Towbin *et al*. (1979) with slight modifications. The frozen-thawed sperm extracts were reacted with anti-TIMP to correlate such sperm specific proteins of buffalo bull spermatozoa with semen function tests and bull fertility. The blot images were captured on gel doc using image acquisition software and were analyzed for molecular weight and quantity by using gel analysis software.

Fertility trial: The number of females inseminated per bull semen was ten. Therefore, 10 mini straws from each bull were used for the field fertility trial. Buffaloes (300) enrolled for fixed time insemination program (Oct to Apr) were healthy, multiparous (second to fifth parity), recently calved (60–80 days earlier), free from physical problems, vaginal discharge, condition of genitalia and maintained under standard feeding and management systems. The buffaloes were synchronized using double ovsynch protocol ($PGF2_\alpha$-GnRH-$PGF2_\alpha$-GnRH on day –2, 0, 7 and 9, respectively) followed by fixed time inseminations at 16 and 40 h after last GnRH injection, respectively. The pregnancy diagnosis was done on day 45 post-insemination and confirmed on day 60 using ultrasonography. The first service conception rate (FSCR) was calculated according to the following formula:

$$\text{FSCR (\%)} = \frac{\text{Number of buffaloes conceived after first insemination}}{\text{Total number of first services}} \times 100$$

Evaluation of semen parameters: Frozen-thawed semen was evaluated for FSCR (%), acrosome reaction (%), hypo-osmotic swelling test (HOST; %), viability (%), DNA integrity (%) and total motility (%). Ten straws of each bull were used to determine *in vitro* acrosome reaction. The frozen-thawed semen was mixed with double the volume of TALP (100 mM NaCl, 31 mM KCl, 25 mM $NaHCO_3$, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2.4H_2O$, 10 mM HEPES, 1 mM Na pyruvate, 0.6 % BSA, 5 mM glucose and 10 µg/ml heparin) and centrifuged at 1,000 rpm for 5 min to allow removal of seminal plasma and extender. Sperm pellet was washed twice with TALP and finally suspended in 2 ml TALP (100×10^6 sperms/ml) and incubated at 37°C for 6 h. The smears were prepared every 2 h until 6 h and stained with Giemsa. About 200 sperms were evaluated under the light microscope at 400× for various stages of acrosome reaction, viz. swelling of acrosome, vesiculation and acrosome shedding.

Functional integrity of the sperm was evaluated by HOST using hypo-osmotic solution (100 mosm/L). Frozen-thawed semen (20 µl) was mixed with 100 µl of HOS solution and incubated at 37°C for 30 min. A drop of semen on a slide covered with cover slip was observed at 400× under light microscope. A total of 200 sperms each were counted under different fields and percentage of spermatozoa positive to HOS test (having coiled tails) was calculated.

Sperm viability was established by analyzing the slide stained through nigrosin-eosin staining method. Briefly, a semen sample (1 straw) was washed twice in phosphate buffer solution. One drop of semen was mixed with one drop of stain and a thin smear was prepared using a pre-warmed, clean and grease free glass slide from the semen stain mixture and examined under oil immersion lens of light microscope to determine sperm viability. About 200 spermatozoa were counted under different fields and classified into two categories viz. live sperms with clear bright head and dead sperms with stained and partially stained head and percent live sperm was calculated.

The method of evaluation of sperm DNA integrity using Acridine Orange (AO) was modified from the method used for evaluation of human spermatozoa (Lui and Baker 1992). The AO staining stock solution was prepared by adding 6 mg of AO in 1 ml of DDW and stored in the dark at 4°C. Washed frozen-thawed semen (200 µl) was added to 400 µl of solution A (0.1% Triton-X-100, 0.08 N HCl, 0.15 M NaCl and 3 µl of AO stock solution) and mixed gently for 30 sec. Then 1.2 ml of ice cold solution B (1 mM sodium EDTA, 0.15 M NaCl, 0.3 M $Na_2HPO_4.7H_2O$ and 0.1 M citric acid at pH 6.0) was mixed gently and allowed to equilibrate for 15 min. Finally, 10 µl of AO mixed semen was gently placed on a glass slide and covered with coverslip. About 200 spermatozoa were evaluated under an epifluorescent microscope (40×). The heads of the sperm cells with normal DNA integrity (double stranded) emitted green fluorescence, whereas those with denatured or single stranded DNA had orange, yellow and/or red fluorescence. The slides were evaluated within one hour after staining.

A previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2) was used to evaluate sperm motion trait. Briefly, 10 µl of frozen-thawed semen from each straw was mounted on a disposable CASA slide to analyze the total motility. Five randomly selected fields were scanned per straw and five straws per bull semen were evaluated to denote the total motility, obtaining 25 scans for each bull.

The mean of 25 scans for the total motility and the mean of three replicates (straws) for percent HOST, acrosome reaction, viability, DNA integrity and total motility per bull semen was used for the statistical analysis.

Based on FSCR, the percentage of tested frozen-thawed semen samples with > 50% FSCR and those with < 50% FSCR were considered as high fertility and low fertility semen samples, respectively for further comparisons.

Statistical analysis: The statistical analysis was performed with Statistical Package for Social Sciences program. Duncan's multiple range test, independent sample 't'-test and one way analysis of variance (ANOVA) were applied to determine mean, standard error and level of significance. The data were presented as mean ± SE. The minimum significant interaction was considered at 5% level.

RESULTS AND DISCUSSION

Characterization of TIMP in seminal plasma and frozen-thawed sperm extracts by immunoblotting: Blot images of protein bands in seminal plasma and frozen-thawed sperm extracts of all 30 bulls are shown in Figs 1 and 2. The anti-TIMP (TIMP-140) recognized 7 proteins (65, 55, 48, 33, 31, 24 and 11 kDa) in seminal plasma and five proteins (75, 65, 55, 24 and 16 kDa) in frozen-thawed spermatozoa in the present study (Tables 1, 2). The electrophoretic profiles showed polymorphism among individual semen samples ranging from 1–4 proteins in each tested seminal plasma and 2–4 proteins in each post-thaw semen sample. However, no individual tested bull seminal plasma and frozen-thawed semen had all bands. These bands arranged themselves from the starting line solely in the direction of the negative pole. This was in agreement to earlier studies by Correa *et al.* (2000) who named proteins with molecular weight of 24, 33 and 48 kDa as tissue inhibitor metalloproteinases-3-complex (TIMP-3-complex) in sperm extracts and recognized them as a diagnostic indicator of fertility differences among bulls producing normal semen. While Dawson (2005) observed 6 major TIMP in the range of 24–55 kDa (24, 31, 33, 48, 52 and 55 kDa); Moura *et al.* (2007) reported 9 TIMP having molecular weight from 11–97 kDa (11, 16, 24, 48, 55, 65, 75, 84 and 97 kDa) in Holstein bull seminal plasma. In the present study, the proteins of 65, 55, 48, 33, 31, 24 and 11 kDa were detected in seminal plasma of 17; 25; 10; 7; 3; 12 and 8 bulls, respectively, while in SDS-SE of frozen-thawed spermatozoa, anti-TIMP identified 75, 65, 55, 24 and 16 kDa proteins in 4; 4; 25; 12 and 6 bulls, respectively (Figs 1 and 2). Therefore, qualitative differences (presence or absence of bands) were observed in TIMP bands of the 30 bull seminal plasma and

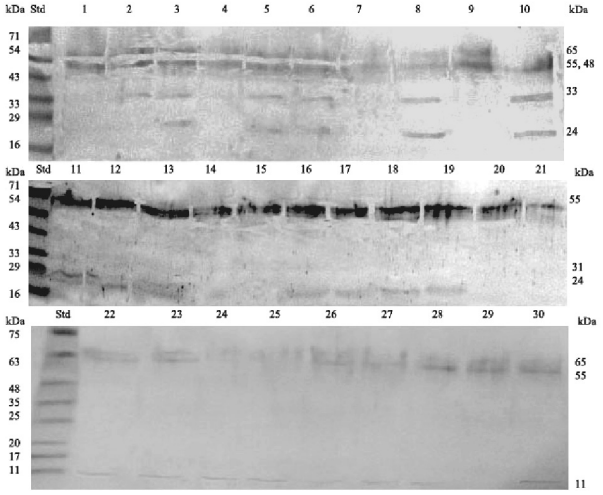

Fig. 1. Immunoblotting of seminal plasma proteins of buffalo bulls with anti-TIMP. Bull # 1–30 exhibited variable fertility; ≥50.0% (1–10), 30.0–49.9% (11–21) and < 30.0% (22–30).

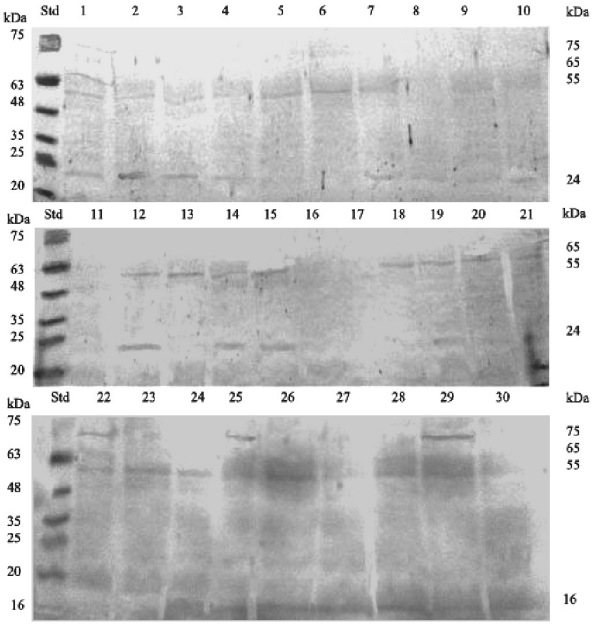

Fig. 2. Immunoblotting of frozen-thawed sperm extracts of buffalo bulls with anti-TIMP. Bull # 1–30 exhibited variable fertility; ≥50.0% (1–10), 30.0–49.9% (11–21) and < 30.0% (22–30).

frozen-thawed sperm extracts. The results were in consonance with findings of Manjunath *et al.* (2002) who observed qualitative differences in TIMP with molecular mass of 55, 48, 33 and 24 kDa in cattle bull spermatozoa. The inherent character of the proteins may also contribute toward the difference in number of bands.

Field fertility trial: A field fertility trial with frozen-thawed semen was conducted to determine the fertility of 30 bulls and its relationship with TIMP. The results revealed an overall first service conception rate (FSCR) of 37.0 ± 3.2% (10–70%). Bull fertility varied widely even after using good quality semen (Kuhn *et al.* 2008). The FSCR in estrus

Table 1. Relationship of TIMP with FSCR and semen characteristics in seminal plasma of buffalo bulls (mean ± SE)

Mol. Wt. (kDa)	Bulls positive for TIMP							Bulls negative for TIMP						
	FSCR (%)	Bulls with > 50.0% FSCR	Acrosome reaction (%)	HOST (%)	Viability (%)	DNAI (%)	TM (%)	FSCR (%)	Bulls with > 50.0% FSCR	Acrosome reaction	HOST (%)	Viability (%)	DNAI (%)	TM (%)
65	31.2 ± 3.5[a]	5.9 (1)[#]	51.4 ± 2.1	67.9 ± 1.7	68.1 ± 2.3	77.8 ± 2.4	53.4 ± 2.0[i]	44.6 ± 5.1[b]	69.2 (9)[#]	52.2 ± 3.1	65.6 ± 2.1	70.9 ± 2.3	79.7 ± 3.3	58.7 ± 2.6[j]
55	37.6 ± 3.7	36.0 (9)[#]	52.6 ± 2.0	67.2 ± 1.5	69.9 ± 1.9	79.3 ± 2.1	56.6 ± 1.8[i]	34.0 ± 6.0	20.0 (1)[#]	47.6 ± 4.9	65.2 ± 2.4	66.4 ± 3.7	75.4 ± 5.5	49.6 ± 2.5[j]
48	57.0 ± 2.6[*]	100.0 (10)[#]	58.7 ± 3.1[c]	68.3 ± 1.9	69.7 ± 2.9	82.1 ± 3.1	61.3 ± 2.4[i]	27.0 ± 2.4[**]	0.0 (0)[#]	48.3 ± 1.8[d]	66.1 ± 1.7	69.2 ± 2.1	77.0 ± 2.4	52.6 ± 1.8[j]
33	55.7 ± 3.0[a]	100.0 (7)[#]	59.7 ± 3.0[c]	66.0 ± 1.2	71.5 ± 3.2	83.4 ± 3.1[g]	62.3 ± 2.8[i]	31.3 ± 3.2[b]	13.0 (3)[#]	49.8 ± 1.9[d]	67.1 ± 1.7	68.7 ± 1.9	77.2 ± 2.3[h]	53.4 ± 1.7[j]
31	40.0 ± 0.0	0.0 (0)[#]	50.7 ± 2.9	72.1 ± 3.7[e]	64.3 ± 4.3	80.0 ± 8.2	58.4 ± 6.2	36.7 ± 3.5	37.0 (10)[#]	51.8 ± 2.0	66.3 ± 1.4[f]	69.9 ± 1.8	78.5 ± 2.0	55.6 ± 1.6
24	45.0 ±3.8[a]	41.7 (5)[#]	53.4 ± 2.2	68.6 ± 1.6	70.0 ± 2.7	80.2 ± 3.0	59.1 ± 2.3[i]	32.8 ± 4.7[b]	27.8 (5)[#]	50.6 ± 2.7	65.7 ± 1.9	68.9± 2.2	77.6 ± 2.6	53.0 ± 2.1[j]
11	16.3± 1.8[*]	0.0 (0)[#]	45.7 ± 3.3[c]	61.0 ± 2.5[e]	68.4 ± 3.3	72.0 ± 3.4[g]	47.0 ± 2.5[i]	44.5 ± 2.9[**]	45.5 (10)[#]	53.9 ± 2.0[d]	69.0 ± 1.3[f]	69.7 ± 2.0[d]	81.1 ± 2.1[h]	58.5 ± 1.6[j]

[a,b] differ significantly ($P < 0.05$) in the same row for overall FSCR; [*,**] differ significantly ($P < 0.01$) in the same row for overall FSCR; [c,d] differ significantly ($P < 0.05$) in the same row for percent acrosome-reacted spermatozoa; [e,f] differ significantly ($P < 0.05$) in the same row for percent HOST; [g,h] differ significantly ($P < 0.05$) in the same row for percent DNA integrity; [i,j] differ significantly ($P < 0.05$) in the same row for percent total motility; figures in parentheses indicate the range; figures in parentheses with [#] indicate the number of tested bulls with > 50.0% FSCR.

synchronized buffaloes depends upon semen handling, semen quality, number of sperms deposited, site of insemination, season of breeding, fertilization status, embryo quality, bull effect and time of AI (Dalton *et al.* 2012). In the present study, all the buffaloes were inseminated by same person to minimize the variations due to the insemination technique, time of insemination, semen handling and site of semen deposition. Hence, the difference in the FSCR might probably be due to variation in semen quality of the selected bulls.

Relationship of TIMP differences in seminal plasma and frozen-thawed sperm extracts with bull fertility: The presence or absence of TIMP in seminal plasma and frozen-thawed spermatozoa was compared with FSCR. The overall FSCR was significantly ($P < 0.05$) higher in bulls positive for 48, 33 and 24 kDa proteins as compared to their negative counterparts (Table 1). A difference of about 57.0, 42.7 and 17.2% in FSCR could be appreciated in bulls positive for TIMP of 48, 33 and 24 kDa, respectively than in their negative contemporary mates. Likewise, the percentage of bulls with good fertility (≥50.0% FSCR) was higher (100.0, 100.0 and 41.7%) among the bulls positive for TIMP-48, TIMP-33 and TIMP-24 kDa proteins as compared to their negative herdmates (0.0, 13.0 and 27.8%). Although non-significant ($P > 0.05$), the FSCR of bulls positive for TIMP-55 and TIMP-31 kDa was higher than in their counterparts and had a difference of 3.6% and 3.3%, respectively. On the other hand, a significant ($P < 0.05$) reverse association with fertility was observed in the bulls with detectable TIMP of 65 and 11 kDa. The percentage of bulls with good fertility was also lower (5.9 and 0.0%) in those positive for TIMP 65 and 11 kDa as compared to their negative counterparts (69.2 and 45.5%), respectively. The results were similar to that reported by Liberda *et al.* (2001) who purified and characterized 48, 33 and 24 kDa proteins from bovine seminal fluid and demonstrated their 90% similarity with TIMP. A negative association of TIMP-65 and TIMP-11 with FSCR in the present study was in accordance with findings of Moura *et al.* (2007) who found that the intensity of 75, 65, 16 and 11 kDa proteins in accessory sex gland fluid exhibited an inverse relationship with bull fertility.

In SDS-SE of frozen-thawed spermatozoa, the overall FSCR was significantly ($P < 0.05$) higher among the bulls positive for 24 kDa protein in comparison to their negative herdmates (Table 2). A difference of nearly 24.5% was observed in the FSCR of bulls positive as compared to negative for 24 kDa TIMP. The percentage of bulls exhibiting ≥50.0% FSCR was also higher (66.7%) among the bulls positive for 24 kDa protein as compared to their contemporary mates (11.1%). The relationship of TIMP to bull fertility was already established in bovine (Dawson *et al.* 2002). They further reported that the bulls who possessed TIMP-24 were 13% more fertile than the negative ones. Previously, TIMP was identified as a major seminal plasma protein in bovine (Liberda *et al.* 2001). Alternatively, the FSCR of bulls with detectable TIMP–75, TIMP-65 and TIMP-55 and TIMP-16 kDa was 5.2, 5.2, 8.4 and 27.5% lower, respectively as compared to that with undetectable

Table 2. Relationship of TIMP with FSCR and semen characteristics in frozen-thawed sperm extracts of buffalo bulls (mean ± SE)

Mol. Wt. (kDa)	Bulls positive for TIMP							Bulls negative for TIMP						
	FSCR (%)	Bulls with > 50.0% FSCR	Acrosome reaction (%)	HOST (%)	Viability (%)	DNAI (%)	TM (%)	FSCR (%)	Bulls with > 50.0% FSCR	Acrosome reaction	HOST (%)	Viability (%)	DNAI (%)	TM (%)
75	32.5 ± 12.5	100.0 (1)#	50.4 ± 8.8	66.6 ± 3.0	61.1 ± 2.6g	69.8 ± 2.5i	55.8 ± 5.2	37.7 ± 3.2	31.0 (9)#	51.9 ± 1.7	66.9 ± 1.5	71.4 ± 1.7h	80.0 ± 2.1j	55.4 ± 1.7
65	32.5 ± 12.5	25.0 (1)#	50.4 ± 8.8	66.6 ± 3.0	61.1 ± 2.6g	69.8 ± 2.5i	55.8 ± 5.2	37.7 ± 3.2	34.6 (9)#	51.9 ± 1.7	66.9 ± 1.5	71.4 ± 1.7h	80.0 ± 2.1j	55.4 ± 1.7
55	35.6 ± 3.6	32.0 (8)#	51.8 ± 2.1	66.1 ± 1.4e	69.5 ± 1.7	78.4 ± 2.1	55.3 ± 1.9	44.0 ± 6.8	40.0 (2)#	51.3 ± 2.6	70.6 ± 2.9f	72.7 ± 5.3	80.1 ± 5.9	56.4 ± 2.6
24	51.7 ± 3.7a	66.7 (8)#	55.9 ± 2.8c	69.2 ± 1.5e	70.9 ± 2.4	80.2 ± 2.9	57.9 ± 1.9	27.2 ± 3.0b	11.1 (2)#	48.9 ± 2.2d	65.3 ± 1.9f	69.5 ± 2.3	77.6 ± 2.7	53.8 ± 2.4
16	15.0 ± 2.2*	0.0 (0)#	42.4 ± 3.7c	59.8 ± 2.2e	64.1 ± 3.9g	72.8 ± 3.7i	51.5 ± 3.6	42.5 ± 3.0**	41.7 (10)#	54.1 ± 1.8d	68.6 ± 1.3f	71.5 ± 1.8h	80.2 ± 2.2j	56.4 ± 1.8

[a,b] differ significantly ($P < 0.05$) in the same row for overall FSCR; [*,**] differ significantly ($P < 0.01$) in the same row for overall FSCR; [c,d] differ significantly ($P < 0.05$) in the same row for percent acrosome-reacted spermatozoa; [e,f] differ significantly ($P < 0.05$) in the same row for percent HOST; [g,h] differ significantly ($P < 0.05$) in the same row for percent total motility; [i,j] differ significantly ($P < 0.05$) in the same row for percent DNA integrity; figures in parentheses indicate the range; figures in parentheses with # indicate the number of tested bulls with > 50.0% FSCR.

TIMP. The percentage of good fertility was also lower (25.0, 32.0 and 0.0%) in the bulls positive for TIMP-65 and TIMP-55 and TIMP-16 kDa as compared to those with undetectable contemporary mates (34.6, 40.0 and 41.7%). Nevertheless, a higher proportion of good fertility (100.0%) in bulls positive for TIMP-75 than in their negative counterparts could merely be due to less number (one) of bulls with > 50.0% FSCR. Like in seminal plasma, the findings were in agreement with the observations of Moura *et al.* (2007).

Alterations in TIMP during cryopreservation: The presence of TIMP with molecular weight 55 and 24 kDa were identified in seminal plasma of 11 and 22 bulls and frozen-thawed spermatozoa of 9 and 7 bulls leading to alteration in spermatozoa of 2 and 15 bulls, respectively.

While TIMP of 48, 33, 31 and 11 kDa showed their expression in seminal plasma, the TIMP-75 and TIMP-16 were recognized in frozen-thawed spermatozoa only. This difference in TIMP of seminal plasma and spermatozoa may be due to variation in expression of these proteins during the process of freeze-thawing of semen of different bulls. Leahy and Gadella (2011) reported that different sperm cells exhibited differences in freezing resistance upon cryopreservation of semen. Freezing results in the concomitant coating and decoating of proteins of the sperm surface (Druart *et al.* 2009). Zigo *et al.* (2013) observed qualitative differences in protein patterns between ejaculated (17 bands) and cryopreserved (14 bands) spermatozoa using comparative western-blot analysis.

Relationship of different TIMP with semen attributes in seminal plasma and frozen-thawed sperm extracts: Certain vital semen parameters were used to evaluate the post-thaw semen quality of the bulls. A considerable variation in HOST (65.1±2.2; 60.2–80.3%), viability (69.3±1.7; 55.8–81.3%), acrosome reaction (51.7±1.8; 32.7–70.6%), DNA integrity (78.7±1.9; 61.7–95.4%) and total motility (55.5 ± 1.6; 40.4–72.7%) was found in the frozen-thawed semen of 30 tested bulls. Currently, the data was in agreement with the observations of Peixoto *et al.* (2012) who reported that semen characteristics viz. HOST, acrosome reaction, DNA integrity and motility are most valuable indicators of semen quality in bulls. Therefore, some merit still lies in determining the semen quality based on vital semen function tests.

In seminal plasma, except viability, almost all seminal parameters (acrosome reaction, percent HOST, DNA integrity and total motility) were found to be significantly ($P < 0.05$) higher in bulls positive for TIMP of 48, 33, 31 and 24 kDa than in their negative contemporary mates. Alternatively, the TIMP with molecular weight 65 and 11 kDa exhibited poor seminal attributes in TIMP positive bulls than in negative ones (Table 1). The seminal fluid TIMP attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing glycosaminoglycans in the female reproductive tract to activate sperm plasma membrane, stimulate sperm capacitation and induce sperm motility (Newton *et al.* 2010). In the present study, the TIMP of 48, 33, 31 and 24 kDa did seem to activate the functional activity of sperm membrane, *in vitro* acrosome reaction and sperm motility vis-à-vis higher conception rate in bulls positive for these proteins as compared to their negative counterparts.

At post-thaw stage, of the 5 bands, only 1 protein with molecular weight of 24 kDa had significantly ($P < 0.05$) higher percentage of acrosome-reacted (55.9±2.8 vs 48.9±2.2%) and HOS-positive (69.2±1.5 vs 65.3±1.9%) spermatozoa in bulls positive for TIMP than in their negative herdmates (Table 2). Marques *et al.* (2000) established a high correlation ($r^2 = 0.71$) of TIMP-24 with percent HOST,

percent acrosome reaction and percent motility and presented it as a candidate protein marker for fertility. Further, Rueda *et al.* (2013) reported the critical role of 24 kDa protein in osmotic fragility, DNA fragmentation and acrosome membrane fusion events. On the other hand, percent HOST (59.8±2.2 vs 68.6±1.3%), acrosome reaction (42.4±3.7 vs 54.1±1.8%) and DNA integrity (72.8±3.7 vs 80.2±2.2%) were significantly ($P < 0.05$) lower in bulls positive for TIMP-16 as compared to their negative counterparts. The relationship of 75, 65 and 55 kDa TIMP with seminal characteristics exhibited a nonsignificant ($P > 0.05$) difference in bulls positive for TIMP than in their negative contemporary mates (Table 2) which was in consonance with the results demonstrated by Amours *et al.* (2010) in cross-bred cattle bull. Therefore, high percentages of seminal parameters as reflected by higher proportion of acrosome reaction, HOST, DNA integrity and total motility of spermatozoa in bulls positive for TIMP might be responsible for improved quality of semen sample.

In the present study, immunoblots of extracts from frozen-thawed sperm treated with anti-TIMP demonstrated that TIMP binds spermatozoa *in vitro*. Higher fertility bulls can be segregated from lower fertility bulls based on presence of TIMP variants (TIMP-48, TIMP-33 and TIMP-24) on sperm. To our knowledge, these are the first data to suggest that TIMP is associated with sperm membranes and may play a significant role in regulating buffalo bull fertility. However, further experimentation will be needed to elucidate how TIMP interacts with sperm to establish a transduction signal(s) which might contribute to differences in fertility potential of sperm cells.

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