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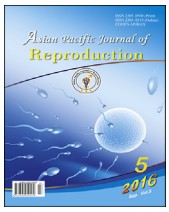

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Heparin binding proteins and their relationship with vital sperm function tests vis-à-vis fertility of buffalo bull semen

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ABSTRACT

Objective: To characterize HBP of seminal plasma, fresh- and frozen-thawed sperm extracts and determine their relationship with post-thaw sperm function tests and bull fertility.

Methods: Both fresh (1–2 ml) and frozen semen (50 straws per bull) were collected from thirty breeding Murrah buffalo bulls and subjected to immunoblotting. Further, frozen-thawed semen was evaluated for first service conception rate (FSCR), percent acrosome reaction, hypoosmotic swelling test (HOST), viability, DNA integrity and total motility and linked to HBP.

Results: Fourteen immunoreactive bands in seminal plasma (135, 75, 70, 65, 60, 55, 45, 37, 33, 31, 28, 24, 18 and 16 kDa), twelve in fresh sperm extracts (75, 70, 65, 55, 48, 37, 31, 28, 24, 20, 16 and 11 kDa) and thirteen in frozen-thawed spermatozoa (135, 100, 75, 70, 65, 55, 48, 45, 37, 31, 28, 24 and 20 kDa) were detected in western blots. In seminal plasma, fresh- and frozen-sperm extracts, bulls positive for 70 and 18 kDa; 55 kDa and 135, 75, 55, 45, 28 and 24 kDa, respectively, had higher ($P < 0.05$) FSCR as compared to their negative counterparts and had also higher ($P < 0.05$) percentages of most seminal parameters in positive ones. The antibody binding was most prevalent in acrosomal and postacrosomal regions of head in majority of spermatozoa.

Conclusion: We have identified buffalo bull seminal HBP that influence semen quality and subsequent fertility of bulls.

1. Introduction

The addition and removal of a variety of proteins at ejaculation play an important role in sperm capacitation and exhibits considerable variation in actual semen fertilization capacity [1]. Heparin binding proteins (HBP) and their homologs are prominent proteins of seminal fluid secreted from accessory sex glands [2]. Binding of HBP to sperm membrane increased the number of heparin binding sites on sperm surface and conveyed the capacitating effects of heparin *in vitro* or other heparin-like glycosaminoglycans *in vivo*, thereby influencing sperm fertilizing ability and success of cryopreservation process [3]. HBP have predominately been linked to bull fertility potential. Five proteins with molecular weight of 18, 31, 33, 48 and 55 kDa have been identified as members of HBP family; referred as fertility-associated antigens and have been used as biochemical markers to predict fertility potential of bulls [4]. These proteins are abundant in seminal plasma than on plasma membrane of spermatozoa and form bulk of HBP group [5]. Analysis of BSP-A1 and BSP-A2 exhibited their identical amino acid composition and binding capacity to heparin and played an important role in buffalo bull fertility [6]. Further, immuno-localization studies have revealed HBP labelling over acrosome and posterior head region of bovine spermatozoa and established their relationship to cellular changes [7]. Characterizing functionally important HBP is a first step toward better understanding the modulating effects of seminal fluid on fertility of buffalo bulls. In addition, fertilization potential of spermatozoa is affected by important semen characteristics viz. acrosome reaction, plasma membrane integrity, viability, DNA integrity and motility [8,9] which involve different signal transduction pathways [10]. Keeping in view of above facts

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and also taken into consideration the deficit knowledge of HBP in buffalo bulls, the present study was designed to characterize HBP in seminal plasma, fresh- and frozen-thawed spermatozoa and determine their relationship with sperm function tests and fertility of buffalo bull semen.

2. Material and methods

2.1. Semen procurement and preparation of sperm extracts

Both fresh (1–2 ml) and frozen semen (50 straws per bull) from thirty breeding Murrah buffalo bulls were procured from two government semen processing and freezing laboratories in the month of September having ambient temperature 30.6 °C and relative humidity 92% for the study. The fresh and frozen-thawed semen (20 straws per bull) was centrifuged at 3000 rpm for 10 min to separate out 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. Sperm pellet from frozen-thawed semen was washed thrice with PBS, pH 7.4 to get rid of dilutor. Sperm extracts (SE) 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 s each) and centrifuged at 15000 rpm for 30 min. The aliquots of sodium dodecyl sulphate-sperm extracts (SDS–SE) were stored at −20 °C till further use.

2.2. Molecular weight determination by immunoblotting

The enzyme linked immuno transfer blot was done as per the method of Towbin *et al.* [11] after electrophoresis of proteins (100 µg) by SDS-PAGE using 10% separating gel and 4% stacking gel. The proteins in seminal plasma, fresh- and frozen-thawed sperm extracts were reacted with anti-HBP (anti AZU-1, Sino Biological) and blot images were captured on Syngene gel doc using Gene Snap image acquisition software and were analyzed for molecular weight and quantity by using Gene Tools gel analysis software (Syngene).

2.3. Fertility trial

The number of females inseminated per bull semen was ten. All the buffaloes (n = 300) were healthy, multiparous (2nd to 5th parity), recently calved (60–80 days earlier) and inseminated 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, during October through April. They were maintained under standard feeding and management systems. Prior to start of breeding program, a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency (EXAGO, ECM, France) was used to visualize a cyclic CL twice at 10 d apart and rule out possibility of reproductive tract infections, if any. The pregnancy diagnosis was done on day 45 post-insemination using ultrasonography. The first service conception rate (FSCR) was calculated according to the following formula:

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

2.4. Evaluation of semen parameters

The frozen-thawed semen was evaluated for acrosome reaction [12], functional integrity by hypoosmotic swelling test [13], viability through Eosin–Nigrosin staining technique, DNA integrity using Acridine Orange [14] and total motility through a previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2). At least 200 spermatozoa were counted in each replicate for different pattern of tests. The number of spermatozoa was converted to percentage.

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

2.5. Immunolocalization of HBP like antigens on buffalo bull spermatozoa

Localization of FAA on sperm cells was determined with anti-HBP as the primary antibody and conjugated goat anti-rabbit-FITC as secondary antibody (Merck).

2.6. Statistical analysis

The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data (acrosome reaction, HOST, viability, DNA integrity, motility and FSCR) were transformed using the arcsine transformation [asin (sqrt (percent/100))] with adjustment to allow for zero values. One way analysis of variance (ANOVA) was used for comparing the level of significance among the group of bulls of different gradients (bulls positive and negative for HBP) for different parameters of tests. The mean ± SE were calculated using arcsine transformed data in the software. The minimum significant interaction was considered at 5% level.

3. Results

3.1. Characterization of HBP in seminal plasma, fresh- and frozen-thawed sperm extracts by immunoblotting

Blot images of protein bands in seminal plasma, fresh- and frozen-thawed sperm extracts of all 30 bulls have been shown in Figures 1–3. Anti-HBP (anti-AZU-1) recognized fourteen proteins in seminal plasma (135, 75, 70, 65, 60, 55, 45, 37, 33, 31, 28, 24, 18 and 16 kDa), twelve proteins in fresh sperm extracts (75, 70, 65, 55, 48, 37, 31, 28, 24, 20, 16 and 11 kDa) and thirteen proteins in frozen-thawed spermatozoa (135, 100, 75,

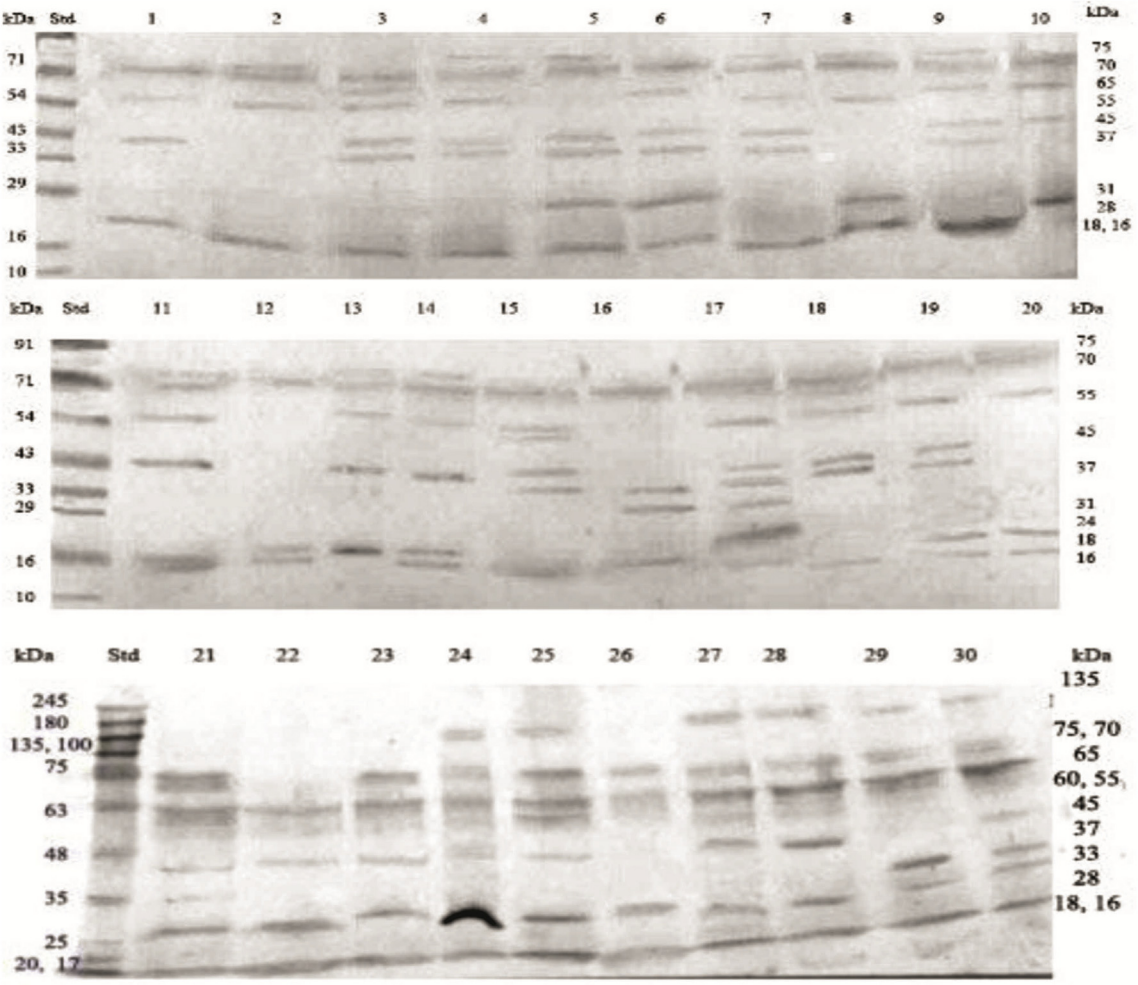

Figure 1. Immunoblotting pattern of HBP in seminal plasma of buffalo bulls.
Lane Std: Standard protein marker; Lanes 1–30: Bull numbers.

70, 65, 55, 48, 45, 37, 31, 28, 24 and 20 kDa) in the present study (Tables 1–3). The electrophoretic profiles showed polymorphism among individual semen samples ranging from 3 to 9 proteins in seminal plasma, 4 to 8 antigens in fresh- and 4 to 10 proteins in post-thaw semen. However, no individual tested bull had all bands in the seminal fluid. While proteins with molecular weight of 135, 75, 70, 65, 60, 55, 45, 37, 33, 31, 28, 24, 18 and 16 kDa were detected in seminal plasma of 7; 14; 12; 9; 11; 25; 24; 3; 7; 21; 5; 3; 24 and 10 bulls, respectively, in SDS–SE of fresh spermatozoa, anti-HBP identified 75, 70, 65, 55, 48, 37, 31, 28, 24, 20, 16 and 11 kDa proteins in 14; 14; 15; 21; 11; 8; 22; 7; 17; 22; 6 and 5 bulls, respectively (Figures 1 and 2). In frozen-thawed spermatozoa, HBP of 135, 100, 75, 70, 65, 55, 48, 45, 37, 31, 28, 24 and 20 kDa were detected in 10; 17; 9; 11; 19; 18; 9; 20; 8; 23; 21; 30 and 7 bulls, respectively (Figure 3). Therefore, qualitative differences (presence or absence of bands) were observed in HBP bands of the 30 bull seminal plasma, fresh- and frozen-thawed sperm extracts.

3.2. Field fertility trial

A field fertility trial with frozen-thawed semen was conducted to determine fertility of 30 bulls and its relationship with HBP in seminal fluid. The results revealed an overall FSCR of 37.0± 3.2% (10%–70%). Based on FSCR, the percentage of tested frozen-thawed semen samples with ≥50% FSCR and those with <50% FSCR were considered as good and poor fertility bulls, respectively; and served as the basis for relationship with various HBP and frozen-thawed sperm traits.

3.3. Relationship of HBP differences with bull fertility in seminal plasma, fresh- and frozen-thawed sperm extracts

The presence or absence of HBP in seminal plasma, fresh- and frozen-thawed spermatozoa was compared with FSCR. In seminal plasma, overall FSCR was higher ($P < 0.05$) in bulls positive for HBP of 70 and 18 kDa as compared to their negative herdmates (Table 1). A difference of about 10.5% and 15.0% in FSCR could be appreciated in bulls positive for HBP of 70 and 18 kDa, respectively than in their negative counterparts. Likewise, a higher percentage of bulls with good fertility (≥50.0% FSCR) was observed in those positive for HBP-70 (50.0%) and HBP-18 (41.7%) as compared to those negative for HBP-70 (22.2%) and HBP-18 (0.0%). Although non-significant ($P > 0.05$), FSCR of bulls positive for HBP with molecular weight of 55, 45 and 24 kDa was higher than in their contemporary mates and had a difference of nearly 6.0, 7.2 and 3.7%, respectively. The proportion of bulls with ≥50.0% FSCR was also higher (36.0, 33.3 and 66.7%) in those positive for HBP of 55, 45 and 24 kDa than in negative ones (20.0, 16.7 and 29.6%). On the other hand, a reverse association with fertility was

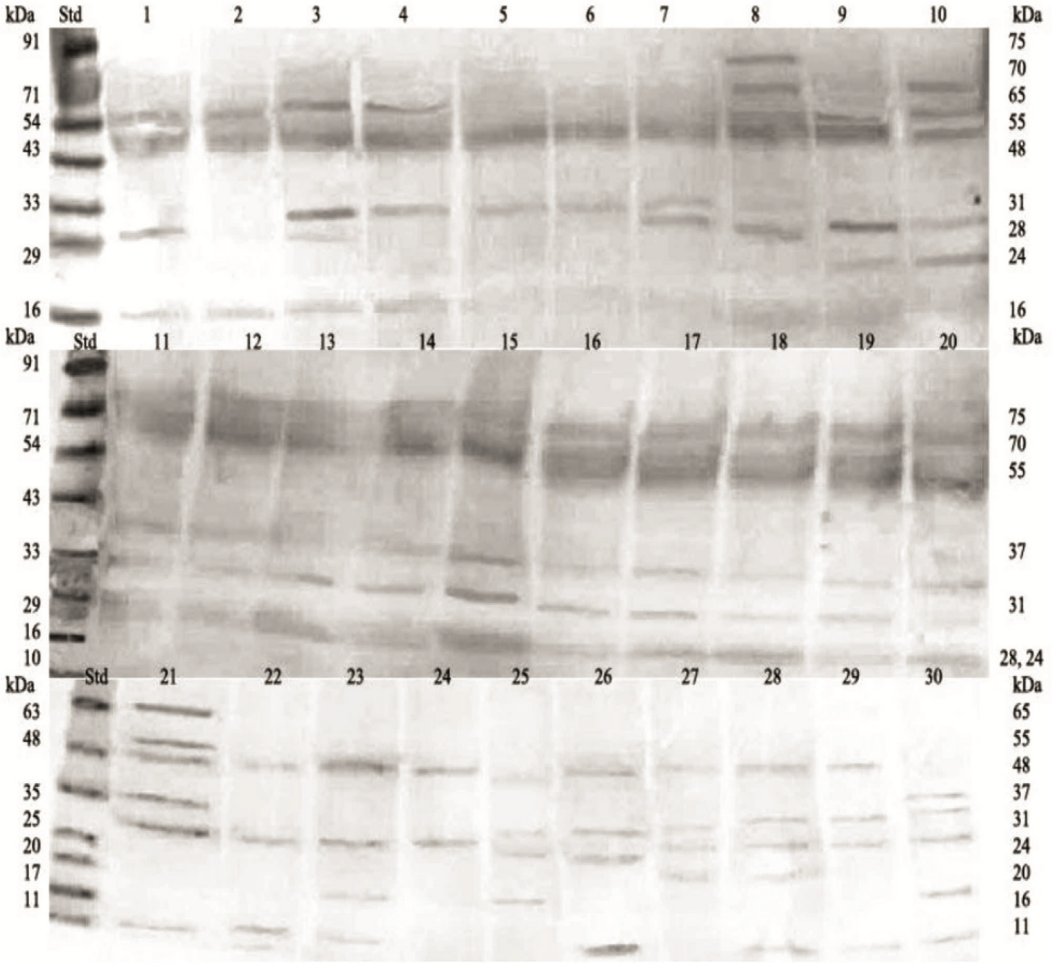

Figure 2. Immunoblotting pattern of HBP in fresh sperm extracts of buffalo bulls.
Lane Std: Standard protein marker; Lanes 1–30: Bull numbers.

observed in bulls with detectable HBP-135 ($P < 0.05$), HBP-65 ($P > 0.05$) and HBP-16 ($P > 0.05$). The percentage of bulls with good fertility was also lower (14.3, 22.2 and 20.0%) in those positive for HBP of 135, 65 and 16 kDa as compared to their negative counterparts (39.1, 38.1 and 35.0%; Table 1).

In fresh sperm extracts, FSCR of bulls positive for 55 kDa HBP was higher ($P < 0.05$) as compared to the negative ones with a difference of 8.4% (Table 2). Similarly, percentage of bulls with ≥50.0% FSCR was higher (38.1%) in those positive for 55 kDa protein than in their negative herd mates (22.2%). The bulls positive for 48, 28, 16 and 11 kDa HBP as compared to negative ones exhibited a non-significant difference in FSCR with an increase of 6.2, 5.7, 10.0 and 6.0%, respectively. Likewise, the percentage of bulls with good fertility was higher (45.5, 42.9, 66.7 and 60.0%) in those positive for 48, 28, 16 and 11 kDa proteins than in their negative herdmates (26.3, 30.4, 25.0 and 28.0%).

In SDS–SE of frozen-thawed spermatozoa, overall FSCR was higher ($P < 0.05$; $P < 0.01$) in bulls positive for 135, 75, 55, 45, 28 and 24 kDa proteins than in their contemporary mates with a difference of approximately 10.5, 10.6, 17.2, 18.0, 11.6 and 37.2% over negative ones (Table 3). The percentage of bulls exhibiting ≥50.0% FSCR was also higher (40.0, 55.6, 44.4, 50.0, 38.1 and 33.3%) in those positive for HBP of 135, 75, 55, 45, 28 and 24 kDa than in their respective counterparts (30.0, 23.8, 16.7, 0.0, 22.2 and 0.0%). HBP-24 was the only protein to be detected in SDS–SE of all bulls. Alternatively, FSCR of bulls with detectable HBP-100, HBP-70, HBP-65 and HBP-48 kDa was 8.0, 5.3, 3.3 and 6.8% lower as compared to those with their respective undetectable HBP. Eventually, the proportion of good fertility was lower in bulls positive for 100, 70 and 65 kDa proteins than in their negative ones (23.5 vs 46.2, 18.2 vs 42.1 and 31.6 vs 36.4%, respectively), while no difference was observed for HBP-48.

3.4. Alterations in HBP during cryopreservation of buffalo bull spermatozoa

The HBP of 16 and 11 kDa were recognized only in fresh sperm extracts whereas HBP-135, HBP-100 and HBP-45 were detected only in frozen-thawed spermatozoa (Tables 2 and 3). The presence of HBP with molecular weight 75, 70, 55, 48 and 20 kDa were identified in fresh spermatozoa of 14, 14, 21, 11 and 22 bulls and frozen-thawed spermatozoa of 9, 11, 18, 9 and 7 bulls leading to alteration in 5, 3, 3, 2 and 15 bulls, respectively. Alternatively, HBP of 65, 31, 28 and 24 were recognized in frozen-thawed spermatozoa of 19, 23, 21 and 30 bulls and fresh spermatozoa of 15, 22, 7 and 17 bulls, thereby causing variation in 4, 1, 14 and 13, bulls respectively. It indicated some alterations in sperm surface during the process of freeze-thawing in these bulls.

3.5. Relationship of semen attributes with different HBP in seminal plasma, fresh- and frozen-thawed sperm extracts

Certain vital semen parameters were used to evaluate post-thaw semen quality of bulls. Measurements of acrosome

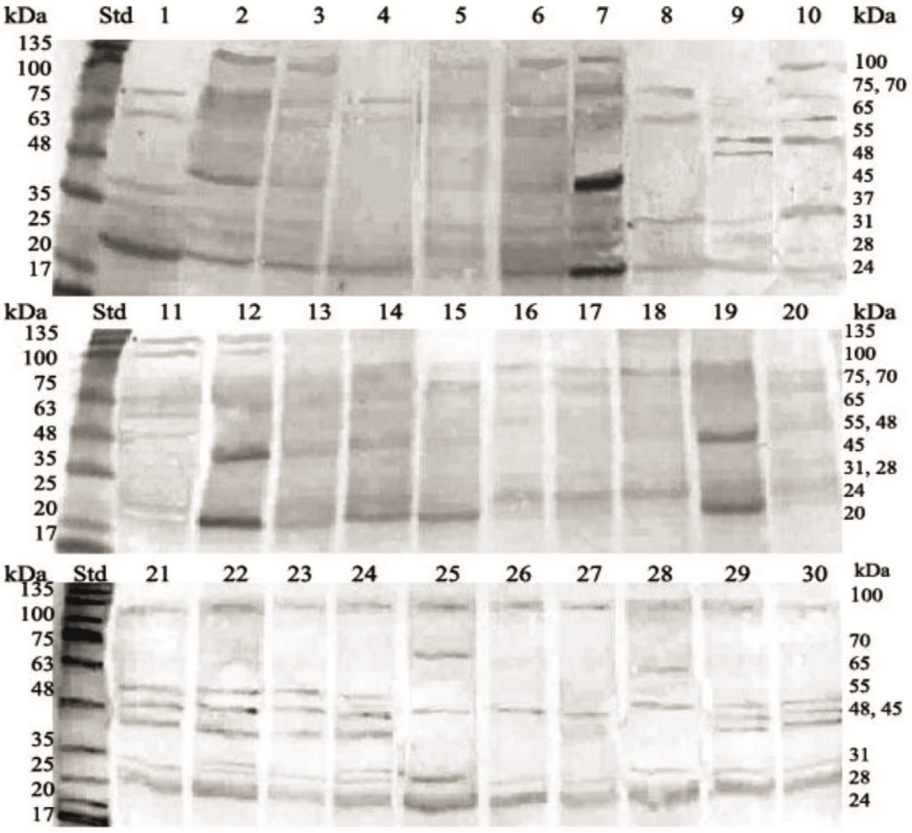

Figure 3. Immunoblotting pattern of HBP in frozen-thawed sperm extracts of buffalo bulls.
Lane Std: Standard protein marker; Lanes 1–30: Bull numbers.

reaction (51.7 ± 1.8%; 32.7–70.6%), HOST (65.1 ± 2.2%; 60.2–80.3%), viability (69.3 ± 1.7%; 55.8–81.3%), DNA integrity (78.7 ± 1.9%; 61.7–95.4%) and total motility (55.5 ± 1.6%; 40.4–72.7%) for frozen-thawed semen exhibited wide variation among 30 tested bulls. In seminal plasma, percent acrosome reaction for HBP-70; viability for HBP-37, HBP-33 and HBP-24 and total motility for HBP-45 and HBP-24 were higher ($P < 0.05$) in bulls positive for HBP than in their negative contemporary mates (Table 1). HBP-55 was the only protein that displayed majority of semen characteristics (percent acrosome reaction, DNA integrity and total motility) to be higher ($P < 0.05$) in HBP-positive bulls. Contrarily, percentage of DNA integrity for HBP-37 and HBP-33; HOST for HBP-24 and viability for HBP-18 were lower ($P < 0.05$) in HBP-positive bulls than in their counterparts. In addition, proteins of 75, 60 and 16 kDa exhibited poor seminal attributes in bulls positive for HBP than in negative ones. In remaining HBP (135, 65, 31 and 28 kDa) a non-significant difference ($P > 0.05$) was noticed for one or the other semen characteristics of bulls positive and negative for HBP. Therefore, most semen parameters exhibited a distinct pattern in detectable and undetectable HBP.

In fresh sperm extracts also, a mixed association of various HBP with semen parameters was observed between HBP-positive and negative bulls (Table 2). While percent total motility for HBP-48 and HBP-31, DNA integrity for 28 kDa, HOST for 20 kDa and acrosome reaction for 16 kDa were higher ($P < 0.05$) in bulls positive for HBP as compared to their negative counterparts, HOST for HBP-48 and total motility for HBP-20 were lower ($P < 0.05$) in the former. Alternatively, a protein of 11 kDa exhibited lower percentages in all but one (acrosome reaction) semen attributes in HBP-positive than in negative bulls. Nevertheless, proteins of 75, 70, 65, 55, 37 and 24 kDa showed no variation in semen parameters of bulls positive and negative for HBP. Viability was the only semen parameter that seemed to exhibit a non-significant difference ($P > 0.05$) in HBP-positive and negative bulls.

At post-thaw stage, of all bands, HBP-24 was the only protein that had higher ($P < 0.01$) percentage of all seminal parameters (acrosome reaction, HOST, DNA integrity and total motility) in bulls positive for HBP than in their negative herdmates (Table 3). In addition, proteins of 75, 55, 45 and 37 kDa showed higher ($P < 0.05$) percentage of acrosome reaction, DNA integrity and total motility in bulls positive for HBP as compared to their contemporary mates. The HBP-70 exhibited a variable response for different semen parameters in HBP-positive and negative bulls. Conversely, an atypical trend ($P > 0.05$) exhibited for most semen characteristics of bulls negative for proteins of 135, 65, 48 and 20 kDa.

3.6. Immuno-localization of HBP to distinct regions of buffalo bull spermatozoa

Localization of anti-HBP on frozen-thawed semen has been depicted in Figure 4. Indirect immunofluorescence of spermatozoa revealed binding of AZU-1 antibody to specific regions of sperm and indicated heterogeneity in the distribution of HBP on surface of buffalo bull spermatozoa. The acrosomal and post-acrosomal segment of head displayed punctate fluorescence in majority of spermatozoa, showing the presence of distinct binding domains for HBP in sperm membranes and representing qualitative assessment of antibody binding. The fluorescence in midpiece and principal piece regions of tail was less punctate in appearance compared with acrosomal staining. However, in few spermatozoa, weak or no signal was also observed on acrosomal

Table 1

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

MW (kDa)		135	75	70	65	60	55	45	37	33	31	28	24	18	16
Bulls positive for HBP	FSCR (%)	27.1 ± 5.6[a]	37.1 ± 5.5	43.3 ± 5.8[a]	32.2 ± 5.2	36.4 ± 4.9	38.0 ± 3.6	37.2 ± 3.2	33.3 ± 14.5	35.7 ± 6.5	37.1 ± 3.7	36.0 ± 8.1	40.0 ± 7.1	40.0 ± 3.6[a]	33.0 ± 5.0
	Bulls (%) with ≥50.0% FSCR	14.3 (1)[#]	35.7 (5)[#]	50.0 (6)[#]	22.2 (2)[#]	27.3 (3)[#]	36.0 (9)[#]	33.3 (8)[#]	33.3 (1)[#]	28.6 (2)[#]	33.3 (7)[#]	20.0 (1)[#]	66.7 (2)[#]	41.7 (10)[#]	20.0 (2)[#]
	AR (%)	49.0 ± 3.9	52.8 ± 2.9	55.6 ± 2.3[c]	50.2 ± 3.8	47.5 ± 2.7[c]	52.8 ± 2.0[c]	51.5 ± 2.1	44.8 ± 6.2	47.6 ± 3.8	51.1 ± 2.3	51.2 ± 1.9	57.8 ± 6.8	52.5 ± 2.0	47.1 ± 2.8[c]
	HOST (%)	64.7 ± 3.1	66.2 ± 1.7	65.7 ± 1.5	64.8 ± 2.4	68.2 ± 2.8	67.4 ± 1.5	67.0 ± 1.5	65.5 ± 6.4	70.1 ± 4.1	66.1 ± 1.5	69.7 ± 2.1	61.9 ± 1.1[e]	67.9 ± 1.3	68.8 ± 3.0
	Viability (%)	67.7 ± 3.8	66.6 ± 2.5[g]	67.6 ± 3.0	70.2 ± 3.2	71.0 ± 2.9	70.3 ± 1.9	70.1 ± 1.8	81.1 ± 0.6[g]	74.0 ± 3.0[g]	71.5 ± 1.9	73.5 ± 4.5	77.7 ± 2.0[g]	68.6 ± 1.8[g]	73.4 ± 2.5
	DNAI (%)	79.3 ± 3.6	78.6 ± 2.7	80.9 ± 3.1	79.1 ± 3.3	77.8 ± 3.6	80.0 ± 2.2[i]	78.8 ± 2.3	71.1 ± 4.7[i]	73.5 ± 4.4[i]	78.2 ± 2.4	78.8 ± 4.7	84.6 ± 7.7	79.1 ± 2.1	75.6 ± 3.4
	TM (%)	54.9 ± 3.3	55.9 ± 2.6	57.9 ± 2.8	57.4 ± 2.8	55.1 ± 2.7	56.4 ± 1.8[k]	57.0 ± 1.7[k]	54.6 ± 4.3	54.0 ± 3.5	55.5 ± 1.9	53.8 ± 4.3	61.2 ± 2.1[k]	56.3 ± 1.9	52.5 ± 2.6
Bulls negative for HBP	FSCR (%)	39.6 ± 3.7[b]	36.9 ± 3.7	32.8 ± 3.4[b]	39.0 ± 4.0	37.4 ± 4.2	32.0 ± 7.3	30.0 ± 9.3	37.4 ± 3.3	37.4 ± 3.7	36.7 ± 6.7	37.2 ± 3.5	36.3 ± 3.5	25.0 ± 4.3[b]	39.0 ± 4.1
	Bulls (%) with ≥50.0% FSCR	39.1 (9)[#]	31.3 (5)[#]	22.2 (4)[#]	38.1 (8)[#]	36.8 (7)[#]	20.0 (1)[#]	33.3 (2)[#]	33.3 (9)[#]	34.8 (8)[#]	33.3 (3)[#]	36.0 (9)[#]	29.6 (8)[#]	0.0 (0)[#]	35.0 (7)[#]
	AR (%)	52.6 ± 2.1	50.8 ± 2.4	49.2 ± 2.5[d]	52.4 ± 2.1	54.2 ± 2.3[d]	46.4 ± 4.2[d]	52.7 ± 3.9	52.5 ± 1.9	53.0 ± 2.0	53.4 ± 3.1	51.7 ± 2.2	51.1 ± 1.9	48.5 ± 4.1	54.1 ± 2.2[d]
	HOST (%)	67.5 ± 1.4	67.4 ± 2.0	67.6 ± 1.9	67.8 ± 1.6	66.1 ± 1.4	64.2 ± 2.1	66.1 ± 3.0	67.0 ± 1.3	65.9 ± 1.2	68.7 ± 2.6	66.3 ± 1.5	67.4 ± 1.4[f]	62.9 ± 3.9	65.9 ± 1.3
	Viability (%)	70.8 ± 1.9	73.1 ± 2.0[h]	71.7 ± 1.9	70.0 ± 2.0	69.5 ± 2.1	68.9 ± 2.8	70.0 ± 4.6	68.8 ± 1.7[h]	68.8 ± 1.9[h]	66.6 ± 3.1	69.4 ± 1.8	69.2 ± 1.8[h]	75.8 ± 3.9[h]	68.4 ± 2.1
	DNAI (%)	78.3 ± 2.4	78.8 ± 2.9	77.2 ± 2.5	78.4 ± 2.5	79.2 ± 2.3	72.3 ± 2.9[j]	78.4 ± 3.3	79.5 ± 2.1[j]	80.3 ± 2.1[j]	79.9 ± 3.4	78.6 ± 2.2	78.0 ± 2.0	76.2 ± 5.4	80.2 ± 2.4
	TM (%)	55.6 ± 1.9	55.1 ± 2.1	53.8 ± 1.9	54.6 ± 2.0	55.4 ± 2.1	51.0 ± 2.5[l]	49.2 ± 3.8[l]	55.5 ± 1.8	56.4 ± 1.9	55.3 ± 3.1	55.8 ± 1.8	54.8 ± 1.7[l]	52.2 ± 2.9	57.0 ± 2.0

Values with different alphabetic superscripts differ significantly ($P < 0.05$) in same column for respective parameter.
Figures in parentheses with symbol [#] indicate the number of tested bulls with ≥50.0% FSCR.
FSCR = first service conception rate; AR = acrosome reaction; HOST = hypoosmotic swelling test; DNAI = DNA integrity test; TM = total motility.

Table 2

Relationship of HBP with FSCR and semen characteristics in fresh semen of buffalo bulls (mean ± SE).

MW (kDa)		75	70	65	55	48	37	31	28	24	20	16	11
Bulls positive for HBP	FSCR (%)	34.3 ± 4.7	37.9 ± 4.6	38.0 ± 4.9	39.5 ± 3.1[a]	40.9 ± 6.5	35.0 ± 5.7	36.8 ± 3.6	41.4 ± 7.0	37.1 ± 4.0	37.3 ± 3.5	45.0 ± 8.8	42.0 ± 10.2
	Bulls (%) with ≥50.0% FSCR	21.4 (3)[#]	42.9 (6)[#]	33.3 (5)[#]	38.1 (8)[#]	45.5 (5)[#]	25.0 (2)[#]	31.8 (7)[#]	42.9 (3)[#]	35.3 (6)[#]	31.8 (7)[#]	66.7 (4)[#]	60.0 (3)[#]
	AR (%)	52.0 ± 2.8	50.0 ± 2.7	51.9 ± 2.4	51.5 ± 2.1	54.1 ± 3.4	48.2 ± 3.4	52.1 ± 2.3	52.7 ± 3.5	49.8 ± 2.5	51.4 ± 1.9	57.3 ± 5.1[c]	60.3 ± 5.3[c]
	HOST (%)	65.1 ± 1.6	67.3 ± 2.0	67.9 ± 1.8	67.7 ± 1.6	64.5 ± 1.8[e]	67.0 ± 2.4	66.9 ± 1.5	65.7 ± 2.4	67.8 ± 2.0	68.2 ± 1.5[e]	65.9 ± 2.5	63.9 ± 1.9[e]
	Viability (%)	68.2 ± 2.8	71.8 ± 2.4	69.0 ± 2.5	68.5 ± 2.7	68.3 ± 3.0	69.5 ± 3.6	69.2 ± 2.0	70.2 ± 3.6	69.6 ± 2.1	70.9 ± 1.8	71.4 ± 4.2	66.1 ± 3.2
	DNAI (%)	80.4 ± 2.8	76.5 ± 2.9	78.8 ± 2.9	78.3 ± 2.4	79.5 ± 2.9	80.6 ± 3.7	77.4 ± 2.1	84.7 ± 4.5[g]	77.8 ± 2.5	78.8 ± 2.4	77.2 ± 3.4	76.2 ± 4.4
	TM (%)	56.3 ± 2.3	54.4 ± 2.4	53.3 ± 2.2	55.2 ± 2.0	59.5 ± 2.5[i]	54.4 ± 3.0	56.8 ± 2.0[i]	55.3 ± 3.7	55.4 ± 2.1	54.0 ± 1.8[i]	56.3 ± 4.1	55.3 ± 1.8
Bulls negative for HBP	FSCR (%)	39.4 ± 4.4	36.3 ± 4.6	36.0 ± 4.2	31.1 ± 3.5[b]	34.7 ± 3.4	37.7 ± 3.9	37.5 ± 7.3	35.7 ± 3.6	36.9 ± 5.4	36.3 ± 7.5	35.0 ± 3.3	36.0 ± 3.3
	Bulls (%) with ≥50.0% FSCR	43.8 (7)[#]	25.0 (4)[#]	33.3 (5)[#]	22.2 (2)[#]	26.3 (5)[#]	36.4 (8)[#]	37.5 (3)[#]	30.4 (7)[#]	30.8 (4)[#]	37.5 (3)[#]	25.0 (6)[#]	28.0 (7)[#]
	AR (%)	51.5 ± 2.5	53.2 ± 2.5	51.6 ± 2.9	52.3 ± 3.9	50.3 ± 2.1	53.0 ± 2.1	50.6 ± 2.8	51.4 ± 2.2	54.2 ± 2.6	52.7 ± 4.7	50.3 ± 1.8[d]	50.0 ± 1.8[d]
	HOST (%)	68.4 ± 2.0	66.5 ± 1.8	65.8 ± 1.9	64.9 ± 2.4	68.3 ± 1.7[f]	66.8 ± 1.6	66.8 ± 2.7	67.2 ± 1.6	65.7 ± 1.6	63.1 ± 2.4[f]	67.1 ± 1.5	67.4 ± 1.5[f]
	Viability (%)	71.7 ± 2.0	68.6 ± 2.4	69.8 ± 2.3	68.2 ± 3.0	71.1 ± 2.0	70.2 ± 1.9	72.5 ± 3.0	70.0 ± 1.9	69.1 ± 2.8	67.8 ± 3.8	68.9 ± 1.8	70.0 ± 1.9
	DNAI (%)	77.2 ± 2.7	80.6 ± 2.6	78.2 ± 2.8	79.5 ± 3.3	78.2 ± 2.6	78.0 ± 2.3	82.1 ± 4.5	76.8 ± 2.0[h]	79.8 ± 3.1	78.4 ± 3.3	79.1 ± 2.3	79.0 ± 2.2
	TM (%)	54.8 ± 2.4	56.4 ± 2.3	57.6 ± 2.2	56.1 ± 2.8	53.1 ± 2.0[j]	55.8 ± 1.9	51.7 ± 2.5[j]	55.5 ± 1.8	55.5 ± 2.6	59.5 ± 3.2[j]	55.2 ± 1.8	56.1 ± 4.5

Values with different alphabetic superscripts differ significantly ($P < 0.05$) in same column for respective parameter.
Figures in parentheses with symbol [#] indicate the number of tested bulls with ≥50.0% FSCR.
FSCR = first service conception rate; AR = acrosome reaction; HOST = hypoosmotic swelling test; DNAI = DNA integrity test; TM = total motility.

Table 3
Relationship of HBP with FSCR and semen characteristics in frozen-thawed semen of buffalo bulls (mean ± SE).

MW (kDa)		135	100	75	70	65	55	48	45	37	31	28	24	20
Bulls positive for HBP	FSCR (%)	44.0 ± 5.2^{a}	33.5 ± 3.4	44.4 ± 7.1^{a}	33.6 ± 4.5	35.8 ± 4.1	43.9 ± 3.7^{a}	32.2 ± 5.7	43.0 ± 3.8^{a}	40.0 ± 5.0	37.8 ± 3.9	40.5 ± 3.7^{a}	37.0 ± 3.2*	41.4 ± 7.4
	Bulls (%) with ≥50.0% FSCR	40.0 (4)$^{\#}$	23.5 (4)$^{\#}$	55.6 (5)$^{\#}$	18.2 (2)$^{\#}$	31.6 (6)$^{\#}$	44.4 (8)$^{\#}$	33.3 (3)$^{\#}$	50.0 (10)$^{\#}$	50.0 (4)$^{\#}$	34.8 (8)$^{\#}$	38.1 (8)$^{\#}$	33.3 (10)$^{\#}$	28.6 (2)$^{\#}$
	AR (%)	52.8 ± 3.2	49.0 ± 2.4^{c}	58.1 ± 1.9^{c}	46.7 ± 2.6^{c}	51.6 ± 1.9	54.3 ± 2.1^{c}	51.0 ± 4.1	53.6 ± 2.3^{c}	59.5 ± 2.3^{c}	50.8 ± 2.2	52.5 ± 2.3	51.7 ± 1.8$^{!}$	55.7 ± 3.7
	HOST (%)	68.2 ± 2.2	65.4 ± 1.7	67.5 ± 1.5	69.3 ± 2.8^{e}	67.5 ± 1.8	68.8 ± 1.8^{e}	64.5 ± 2.1	66.8 ± 1.7	64.5 ± 2.2	64.6 ± 1.7^{e}	68.2 ± 1.6^{e}	65.1 ± 2.2$^{\$}$	67.4 ± 2.7
	Viability (%)	68.2 ± 2.9	70.9 ± 2.1	67.7 ± 3.6	73.9 ± 2.3^{g}	70.4 ± 2.1	70.9 ± 2.2	68.3 ± 3.1	70.1 ± 2.0	69.9 ± 3.7	70.6 ± 2.0	70.9 ± 1.8	69.3 ± 1.7$^{@}$	71.5 ± 4.2
	DNAI (%)	79.1 ± 3.3	77.6 ± 2.1	84.4 ± 3.6^{i}	76.8 ± 3.3	79.5 ± 2.8	79.8 ± 2.4	75.8 ± 2.5	77.1 ± 2.2	86.2 ± 2.3^{i}	78.4 ± 2.2	78.1 ± 2.5	78.7 ± 1.9$^{\hat{}}$	77.8 ± 5.1
	TM (%)	57.8 ± 3.6	55.3 ± 2.1	56.2 ± 3.6	52.4 ± 2.3^{k}	54.9 ± 2.2	57.4 ± 1.9^{k}	55.7 ± 2.6	57.2 ± 2.0^{k}	61.7 ± 2.9^{k}	55.7 ± 1.8	55.6 ± 1.9	55.5 ± 1.6^{+}	54.0 ± 4.1
Bulls negative for HBP	FSCR (%)	33.5 ± 3.9^{b}	41.5 ± 5.7	33.8 ± 3.3^{b}	38.9 ± 4.3	39.1 ± 5.1	26.7 ± 4.3^{b}	39.0 ± 3.8	25.0 ± 3.4^{b}	35.9 ± 4.0	34.3 ± 4.8	28.9 ± 5.6^{b}	0.0 ± 0.0**	35.6 ± 3.6
	Bulls (%) with ≥50.0% FSCR	30.0 (6)$^{\#}$	46.2 (6)$^{\#}$	23.8 (5)$^{\#}$	42.1 (8)$^{\#}$	36.4 (4)$^{\#}$	16.7 (2)$^{\#}$	33.3 (7)$^{\#}$	0.0 (0)$^{\#}$	27.3 (6)$^{\#}$	28.6 (2)$^{\#}$	22.2 (2)$^{\#}$	0.0 (0)$^{\#}$	34.8 (8)$^{\#}$
	AR (%)	51.2 ± 2.3	55.3 ± 2.6^{d}	49.0 ± 2.2^{d}	52.7 ± 2.4^{d}	52.0 ± 3.9	47.9 ± 3.0^{d}	52.0 ± 2.0	48.1 ± 2.8^{d}	49.4 ± 2.2^{d}	54.9 ± 3.2	50.0 ± 3.1	0.0 ± 0.0$^{!!}$	50.5 ± 2.1
	HOST (%)	66.2 ± 1.7	68.7 ± 2.0	66.9 ± 1.8	65.5 ± 1.3^{f}	65.8 ± 1.8	63.9 ± 1.7^{f}	67.9 ± 1.6	66.9 ± 2.3	67.6 ± 1.6	71.4 ± 3.1^{f}	63.7 ± 2.2^{f}	0.0 ± 0.0$^{\$\$}$	66.7 ± 1.5
	Viability (%)	71.0 ± 2.0	69.0 ± 2.8	71.5 ± 1.8	68.0 ± 2.1^{h}	70.1 ± 3.1	68.8 ± 2.7	70.8 ± 2.0	69.9 ± 3.1	70.5 ± 1.9	68.3 ± 3.2	68.2 ± 3.6	0.0 ± 0.0$^{@@}$	69.6 ± 1.8
	DNAI (%)	78.4 ± 2.4	79.7 ± 3.6	76.8 ± 2.2^{j}	79.5 ± 2.5	77.2 ± 2.4	77.0 ± 3.4	79.9 ± 2.5	78.7 ± 3.8	76.7 ± 2.4^{j}	79.6 ± 4.3	79.5 ± 3.2	0.0 ± 0.0$^{\hat{}\hat{}}$	78.7 ± 2.1
	TM (%)	54.3 ± 1.6	55.6 ± 2.6	54.7 ± 1.7	57.0 ± 2.1^{l}	56.4 ± 2.4	52.6 ± 2.7^{l}	55.4 ± 2.1	51.9 ± 2.6^{l}	53.6 ± 1.8^{l}	54.8 ± 3.7	55.1 ± 3.1	0.0 ± 0.0^{++}	55.9 ± 1.8

Values with different alphabetic superscripts differ significantly ($P < 0.05$) in same column for respective parameter.
Values with different symbolic superscripts differ significantly ($P < 0.01$) in same column for respective parameter.
Figures in parentheses with symbol $^{\#}$ indicate the number of tested bulls with ≥50.0% FSCR.
FSCR = first service conception rate; AR = acrosome reaction; HOST = hypoosmotic swelling test; DNAI = DNA integrity test; TM = total motility.

Figure 4. Immunofluorescence of buffalo bull spermatozoa with anti-HBP (40×).

cap. The immunolocalization of HBP on buffalo bull spermatozoa was compared with FSCR; however, it did not exhibit any difference amongst different groups.

4. Discussion

In the present study, characterization of HBP in seminal plasma, fresh- and frozen-thawed sperm extracts by immunoblotting was in agreement to earlier studies by McCauley *et al.* [4] who recognized and named HBP with molecular weight of 18, 31, 33, 48 and 55 kDa as a diagnostic indicator of fertility differences among bulls producing normal semen. While Arangasamy [15] observed eight HBP in the range of 13–71 kDa (13, 14, 16, 20, 36, 41, 56 and 71 kDa); Kumar [16] reported six HBP having molecular weight from 14 to 61 kDa (14, 16, 24, 33, 41 and 61 kDa) in buffalo seminal plasma. Studies using one dimensional polyacrylamide gel electrophoresis identified 32 bands and 35 bands of HBP in seminal plasma of Nellore bulls and rams, respectively [17,18]. A total of nine protein bands in the range of 10–170 kDa (14, 15, 20, 24, 33, 40, 55, 70 and 100 kDa) were observed after SDS-PAGE of buffalo seminal fluid [6,19] leading to qualitative differences in HBP bands of all bulls. Furthermore, the inherent character of proteins may also contribute toward the difference in number of bands.

The relationship of HBP to bull fertility has already been established in bovine [4]. The protein quality of seminal plasma affects bull fertility [20]. A higher ($P < 0.05$) FSCR for 70, 55, 45, 24 and 18 kDa antigens in seminal fluid of bulls positive for HBP was in agreement with the findings of previous workers [21–23] who reported that bulls with detectable HBP on sperm membrane were 11 percentage points more fertile than their contemporary mates. In beef bulls, presence of HBP complexes with greatest affinity for heparin on sperm membrane was positively related to fertility [24]. Alternatively, a negative association of HBP of 135, 65 and 16 kDa with FSCR in seminal plasma than in sperm membrane could be due to modification in sperm surface molecules at the time of ejaculation, since HBP are attached to sperm surface after ejaculation from seminal plasma. Bellin *et al.* [24] observed 82% fertility of bulls, with detectable HBP in sperm membranes but undetectable in seminal fluid and 67% for bulls having HBP both in seminal plasma as well as in sperm membranes. Like seminal plasma, in fresh- and frozen-thawed sperm extracts the findings were in consonance with the observations of Moura *et al.* [25] for 55, 48, 28, 16 and 11 kDa and 135, 75, 55, 45, 28 and 24 kDa proteins, respectively.

Although, 31 kDa HBP could be used to predict fertility of bulls, however, in the present study, no difference was found in FSCR of buffalo bulls with detectable and undetectable HBP-31 in seminal plasma and spermatozoa. Overall, HBP of 70, 55, 45, 24 and 18 kDa in seminal plasma; 55, 48, 28, 16 and 11 kDa in fresh- and 135, 75, 55, 45, 28 and 24 kDa in frozen-thawed spermatozoa exhibited higher fertility of buffalo bulls in the current study.

The alteration in surface proteins during cryopreservation was clearly detected in the current study. This variation in HBP of seminal plasma and spermatozoa may be due to difference in expression of these proteins during the process of cryopreservation of semen of different bulls. Different sperm cells exhibited differences in freezing resistance upon cryopreservation of semen [26]. Freezing results in concomitant coating and decoating of proteins on sperm surface [27]. In bull, sperm surface proteins collected before and after freeze-thawing procedures exhibited differences using comparative SDS-PAGE analysis [28]. Further, qualitative differences in protein patterns between ejaculated (17 bands) and cryopreserved (14 bands) spermatozoa using comparative western-blot analysis [29].

The association of different HBP in seminal fluid has been related with important semen attributes. HBP in seminal plasma attach to sperm surface, allowing heparin-like glycosaminoglycans in female reproductive tract to activate sperm plasma membrane, stimulate sperm capacitation and induce sperm motility [30]. In the present study, HBP of 135, 100, 70 and 18 kDa did seem to activate the functional activity of sperm membrane, *in vitro* acrosome reaction, DNA integrity and sperm motility in relation to higher conception rate in bulls positive for these proteins as compared to their negative counterparts. Marques *et al*. [31] had established a high correlation (r^2 = 0.71) between semen characteristics and HBP (31 and 18 kDa) and presented them as candidate protein markers for fertility. Moreover, the critical role of 18 and 135 kDa proteins in osmotic fragility and acrosome membrane fusion events has also been reported [32]. Therefore, HBP play a vital role in augmenting semen fertility as reflected by higher proportion of acrosome reaction, HOST, DNA integrity and total motility of HBP-positive bulls, in the present study.

The fluorescence was most prevalent in acrosomal and postacrosomal regions in majority of spermatozoa. However, variation in staining pattern of spermatozoa was noticed, since acrosome surface of a few spermatozoa did not show any fluorescence. This could be due to acrosome damage and/or loss of HBP in such spermatozoa owing to freeze-thaw procedures. Previously, it has been reported that HBP are bound to acrosomal and postacrosomal regions of ejaculated sperm with minimal binding to mid piece and principal piece [7].

In conclusion, immunoblots of buffalo bull seminal fluid treated with anti-HBP demonstrated that HBP are present in seminal plasma and on sperm surface and may play a significant role in regulating fertility of buffalo bulls. Higher fertility bulls can be segregated from lower fertility bulls based on presence of HBP variants (135, 75, 70, 55, 45, 28, 24 and 18 kDa) in seminal fluid. Studies are underway to examine and validate the functional relationship between presence of HBP on sperm and increased fertility potential of buffalo bulls.

Conflict of interest statement

The authors report no conflict of interest.

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