Indian Journal of Animal Sciences **87** (5): 573–578, May 2017/Article

Prediction of buffalo bull fertility based on sperm motion traits, function tests and expression of heparin binding protein

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Received: 3 October 2016; Accepted: 24 October 2016

ABSTRACT

Selection of good fertility bulls is a challenge since large variations exist in the result of tests applied for semen evaluation. Therefore, the present study was undertaken to predict the fertility of buffalo bulls on the basis of sperm motion traits, function tests and expression of heparin binding protein (HBP). Both fresh and frozen semen from breeding Murrah buffalo bulls (30) were procured from 2 government bull farms. The frozen-thawed semen was used to evaluate first service conception rate (FSCR), CASA-based motility (total and progressive) and function tests (HOST, acrosome reaction and DNA integrity). On the basis of FSCR, all bulls were divided into 3 groups, viz. good fertility bulls (GFB, >50%), average fertility bulls (AFB, 30–49.9%) and poor fertility bulls (PFB, <30%). Additionally, fresh and frozen-thawed semen were analyzed for quantification and characterization of HBP through affinity chromatography. CASA based total motility and acrosome reaction were significantly higher in GFB as compared to their counterparts. No significant difference between good and average fertility bulls was observed for percent HOST and progressive motility. The HBP was 6.6±0.2 mg/ml, 0.63±0.02 mg/10^9 sperms and 0.36±0.02 mg/10^9 sperms in seminal plasma, fresh- and frozen-thawed spermatozoa, respectively. Although, there was no significant difference in the quantity of total HBP in semen with respect to bull fertility, a specific trend definitely existed. The HBP peak was separated into proteins of 135, 100, 75, 65, 48, 45, 40, 37, 31, 28, 26, 20, 18, 16 and 11 kDa in seminal fluid. Overall, the purified HBP indicated the presence of 65, 31, 20 and 11 kDa proteins in seminal plasma, fresh- and frozen-thawed sperm extracts of good fertility bulls and seemed to be candidate proteins to predict buffalo bull fertility. It was concluded that CASA-based total motility, acrosome reaction and purified HBP-65, HBP-31, HBP-20 and HBP–11 in buffalo bull sperm could be used for differentiation of good fertility bulls from poor fertility ones.

Key words: Acrosome reaction, Buffalo bull, FSCR, HBP, Heparin binding protein, Semen

Bull fertility depends upon fertilizing ability of the sperm and is best evaluated through fertility trial. However, conducting fertility trial is costly and time consuming. Till date there is no single objective test which could indicate the fertilizing ability of sperm. In the recent past, special emphasis has been laid to investigate the genetic markers for bull and semen quality (Singh *et al.* 2013). Sperm membrane contains approximately 2,000 types of proteins; and heparin binding protein (HBP) and their close associates represent a new superfamily of proteins in the bovine. Three major classes of HBP, viz. 14–27 kDa (sperm adhesins), 24 kDa (cysteine-rich secretory proteins) and 31 KDa (Fn-II type proteins) in seminal plasma of bovines, which interact with the sperm surface and participate in sperm function had been reported (Arangasamy *et al.* 2005). In addition, HBP with molecular weight 18–55 kDa were also named as fertility associated antigen (McCauley *et al.* 2001). They further reported that bulls positive for 31 kDa HBP were 17–19% more fertile than their contemporary herd mates and had been demonstrated as a potential tool for prediction of bull fertility. Moreover, the HBP on spermatozoa indicated its affinity for heparin and subsequent ability to undergo acrosome reaction along with protection from cryodamage (Kumar *et al.* 2008). This indicated that HBP play an important role in the survival of sperm during freezing and thawing. However, there is meager information available on the fertility associated HBP in relation to semen quality and fertility of breeding buffalo bulls. Therefore, keeping in view of the above facts the present study was undertaken to predict fertility of buffalo bulls on the basis of sperm motion traits, function tests and expression of HBP.

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MATERIALS AND METHODS

Semen procurement and preparation of sperm extracts: Both fresh (1–2 ml/bull) and frozen semen (50 straws/bull)

from 30 healthy breeding Murrah buffalo bulls were procured from 2 government semen processing and freezing laboratories in September. All the straws collected and earmarked for further investigations were frozen from same ejaculate and on the same date. The fresh (1–2 ml) and frozen-thawed semen (20 straws/bull) was centrifuged at 3,000 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 remnant sperm pellet obtained from fresh- and frozen-thawed semen was washed thrice with PBS, pH 7.4 to get rid of the 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 sec each) and centrifuged at 15,000 rpm for 30 min. The pellet was discarded and the sodium dodecyl sulphate-sperm extracts (SDS-SE) were stored in 0.5 ml fractions at –20°C till further use.

Evaluation of semen parameters: The frozen-thawed semen was evaluated for per cent HOST, acrosome reaction, DNA integrity and motion traits.

Hypoosmotic swelling test (HOST): Functional integrity of the sperm was evaluated through 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 each under different fields and percentage of spermatozoa positive to HOS test (having coiled tails) was calculated.

Acrosome reaction: The status of acrosome reaction was calculated according to the protocol described by Yanagimachi (1994).

DNA integrity: The method of evaluation of sperm DNA integrity using Acridine Orange (AO) was determined (Lui and Baker 1992).

CASA-based sperm motility: A previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2) was used to denote total and progressive motility (Kathiravan *et al.* 2008). Briefly, 10 µl of frozen-thawed semen from each straw was mounted on a disposable CASA slide (Leja-8; IMV Technologies, France) to analyze motility. Five randomly selected fields were scanned per straw and 5 straws per bull semen were evaluated to denote motility, obtaining 25 scans for each bull. The mean of 25 scans for the total motility (total and progressive) and the mean of 3 replicates for per cent acrosome reaction, HOST and DNA integrity per bull semen were used for the statistical analysis.

Fertility trial: Buffaloes (300) were enrolled for fixed time insemination program ($PGF2_\alpha$-GnRH-$PGF2_\alpha$-GnRH on day –2, 0, 7 and 9, respectively followed by inseminations at 16 and 40 h after last GnRH injection) with frozen semen (Souza *et al.* 2008). All buffaloes were healthy, recently calved (60–80 days earlier), free from physical and genital problems and maintained under identical feeding and management systems. Prior to the start of breeding program, the clinical assessment of genitalia was done ultrasonographically using a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency (AGROSCAN, ECM, France) to visualize a cyclic CL (twice at 10 days apart) and rule out the possibility of reproductive tract infections as well as to minimize the variations in first service conception rate (FSCR) due to fixed time insemination protocol, if any (Mohan *et al.* 2009). The pregnancy diagnosis was done on day 45 post-insemination and confirmed on day 60 using ultrasonography. The FSCR was calculated according to the following formula:

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

On the basis of FSCR, all the bulls were divided into 3 groups, viz. good fertility bulls (GFB, >50%), average fertility bulls (AFB, 30–49.9%) and poor fertility bulls (PFB, <30%).

Purification of HBP by affinity chromatography: The HBP in buffalo semen (seminal plasma, fresh- and frozen-thawed spermatozoa) was purified using heparin-sepharose affinity chromatography Kraus *et al.* (2001) with slight modification. The chromatography glass column (28 mm × 70 mm) was packed with heparin-sepharose media up to the mark at the top of the column and allowed to settle for 3–4 h. Thereafter, the column was equilibrated for 1 h with 10 mM Tris HCl (pH 7.4). Exactly 750 µl of seminal plasma, fresh- and frozen-thawed sperm extracts of each bull was loaded and circulated through the column for 15 min for absorption of HBP to the heparin bound resins. The non-heparin binding proteins were washed out with 10 mM Tris HCl and the HBP were eluted with 1 M NaCl at a flow rate of 1 ml/min in the tubes racked in a fraction collector. The recovered HBP fractions were pooled in agreement with the observed curve, obtained from optical density (280 nm), detected by UV monitor and attached to the fraction collector. The pooled fractions of HBP were concentrated by spinning through protein concentrators and analyzed for protein content (Lowry *et al.* 1951).

Molecular weight determination by SDS-PAGE: Exactly 150 µg of protein was fractionated by SDS-PAGE using 10% separating gel and 4% stacking gel. The gels were run at a constant current of 30 mA and maximum 200 V for 2 h and were stained with commassie brilliant blue. Gel images were captured on Syngene gel doc using Gene Snap image acquisition software (Syngene).

Statistical analysis: The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data were transformed using the arcsine transformation with adjustment to allow for zero values. The mean±SE were calculated using arcsine transformed data in the software.

The independent samples t-test and one-way analysis of variance (ANOVA) were used for comparing the level of significance of different parameters between groups. The minimum significant interaction was considered at 5% level.

RESULTS AND DISCUSSION

Sperm motion traits through computer-assisted semen analysis (CASA): The TM was significantly (P<0.05) higher in GFB as compared to AFB and PFB. The PM was significantly (P <0.05) higher in bulls with good fertility than in poor ones, whereas a nonsignificant (P>0.05) difference was observed between good and average fertility bulls (Table 1). The post-thaw TM indicated a specific decline with decrease in FSCR which could not be appreciated for PM. Sperm motility is considered as an important parameter for the evaluation of post-thaw semen quality in bovines due to its relationship with energy status of sperm (Quintero-Moreno *et al.* 2004). It is presumed that spermatozoa with higher proportion of TM and PM have better chances to reach the site of fertilization and fertilize the oocyte (Muino *et al.* 2008). However, in the present study, good fertility of bulls could be identified only on the basis of TM but not on the basis of PM.

Sperm characteristics in frozen-thawed buffalo bull semen: A considerable variation in membrane integrity (HOST), capacitation/acrosome reaction and DNA integrity was found in the frozen-thawed semen of 30 tested bulls (Table 1). The proportion of HOS-reactive spermatozoa indicated membrane intactness. The percent HOST was significantly (P<0.05) higher in bulls with good and average fertility as compared to those with poor fertility. Similar studies (Swain and Kundu 2009) in cattle bulls also showed that percentage of HOS-reactive spermatozoa ranged between 55–62%. Further, Selvaraju *et al.* (2009) also reported similar observations in buffalo bulls that post-thaw HOS-reactive sperms were 61.25%. Nevertheless, higher percentage of HOST in GFB and AFB suggested that more membrane damage in PFB might be an additional cause for differences in conception rates in 3 groups (Singh *et al.* 2014).

Table 1. Mean (± SE) sperm characteristics and purified HBP in buffalo bull semen with respect fertility

Parameter	GFB (n = 10)	AFB (n = 11)	PFB (n = 9)
Semen fertility and characteristics (%)			
First service conception rate	57.0 ± 2.6^{a}	35.5 ± 1.6^{b}	16.7 ± 1.7^{c}
Total motility	61.3 ± 2.4^{a}	55.8 ± 2.1^{b}	48.6 ± 2.8^{c}
Progressive motility	33.2 ± 1.9^{a}	31.2 ± 1.4^{ac}	23.4 ± 1.9^{b}
Hypoosmotic swelling test	68.3 ± 1.9^{a}	69.1 ± 2.0^{a}	61.4 ± 2.3^{b}
Capacitation / Acrosome reaction	58.7 ± 3.1^{a}	51.5 ± 1.6^{b}	44.3 ± 3.3^{c}
DNA integrity	82.1 ± 3.1^{a}	81.2 ± 3.2^{a}	71.8 ± 3.0^{b}
Purified HBP			
Seminal plasma (mg/ml)	6.6 ± 0.4	6.1 ± 0.3	6.3 ± 0.5
Fresh sperm extracts (mg/10^9 sperms)	0.64 ± 0.05	0.61 ± 0.04	0.63 ± 0.03
Frozen-thawed sperm extracts (mg/10^9 sperms)	0.39 ± 0.04	0.34 ± 0.04	0.34 ± 0.05

Values with different superscripts between groups in the same row differ significantly (P<0.05).

Incubation of frozen-thawed spermatozoa in TALP supplemented with BSA and heparin for 6 h resulted in 58.7±3.1%, 51.5±1.6% and 44.3±3.3% acrosome-reacted spermatozoa in GFB, AFB and PFB, respectively. The capacitated/acrosome-reacted spermatozoa were significantly (P<0.05) higher in good than in average and poor fertility bulls. The capacitation / acrosome reaction of mammalian spermatozoa is prerequisite for successful fertilization (Felipe-Perez *et al.* 2008). Therefore, fertility of a bull can be anticipated on the basis of *in vitro* capacitation/acrosome reaction.

Staining of sperm smears with acridine orange revealed that the percentage of intact DNA was significantly (P<0.05) higher in the bulls with good and average fertility as compared to those with poor fertility. Though, the measurement of intact DNA is one of the sensitive parameters for assessing the semen quality (Agarwal and Said 2003), however, it could not differentiate the good fertility bulls from the average ones in the current study. Hence, complete identification of high fertility bulls could not be predicted merely on the basis of vital semen function tests.

Purification of HBP by affinity chromatography: The elution profile of all the affinity-purified proteins resembled the typical graph of affinity chromatography with a sharp rise in the absorbance and a gradual tailing resembling the non-heparin binding proteins (NHBP) followed by another rise and fall of lower amplitude exhibiting the HBP. The trend was similar for seminal plasma (Fig. 1, A), fresh- (Fig. 1, B) and frozen-thawed semen (Fig. 1, C), although, the difference in the amplitude and duration of the peak for NHBP and HBP was maximum in seminal plasma followed by fresh- and frozen-thawed semen. The overall HBP purified by affinity chromatography was 6.6±0.2 mg/ml, 0.63±0.02 mg/10^9 sperms and 0.36± 0.02 mg/10^9 sperms in seminal plasma, fresh- and frozen-thawed spermatozoa, respectively (Table 1). Although, there was no significant (P>0.05) difference in the quantity of total HBP in seminal fluid vis-à-vis bull fertility, a specific trend definitely existed. Similar studies (Rafiq 2009, Srivastava *et al.* 2012) in seminal plasma of crossbred and Tharparkar bulls recorded total HBP concentration of 22 and 27.9 mg/ml, respectively. Further, Arangasamy *et al.* (2005) also reported total HBP concentration in sperm extracts of buffalo bulls to 2.61 mg/10^9 sperms. At ejaculation, HBP bind to sperm membrane capacitation factors, viz. heparin and glycosaminoglycans, resulting in capacitation, acrosome reaction, sperm oocyte fusion and fertilization (Divyaswetha *et al.* 2008). Higher levels of HBP in good fertility bulls might have contributed towards higher capacitation status

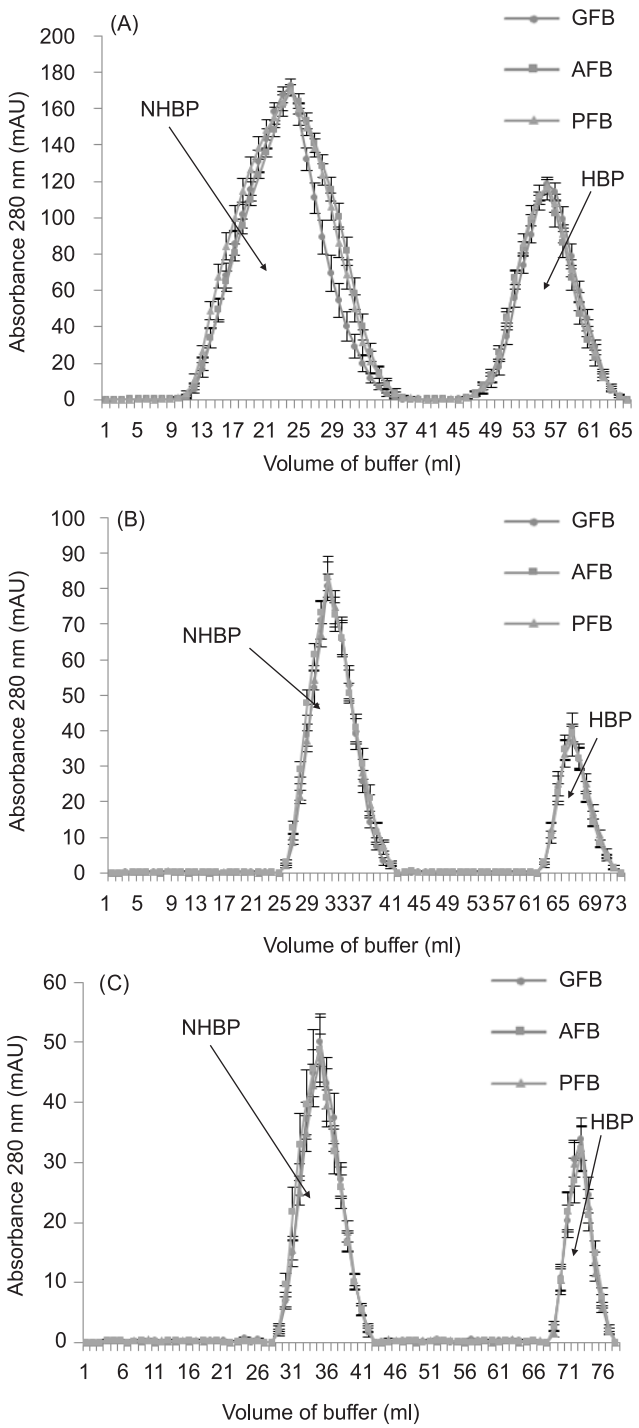

Fig. 1 (A-C). HBP and NHBP concentrations in seminal plasma (A), fresh- (B), and frozen-thawed sperm extracts (C) of buffalo bulls separated by heparin-affinity chromatography.

in the present study.

Electrophoretic characterization of purified HBP by SDS-PAGE: In seminal plasma, the HBP peak was separated into 15 proteins of 135, 100, 75, 65, 48, 45, 40, 37, 31, 28, 26, 20, 18, 16 and 11 kDa (Table 2, Fig. 2). Comparison of purified HBP revealed the presence of 135 kDa and 48 kDa proteins in 40.0% and 100.0% bulls with good fertility, respectively, whereas a minute quantity of 28 kDa and 18 kDa proteins was detected only in the seminal plasma of

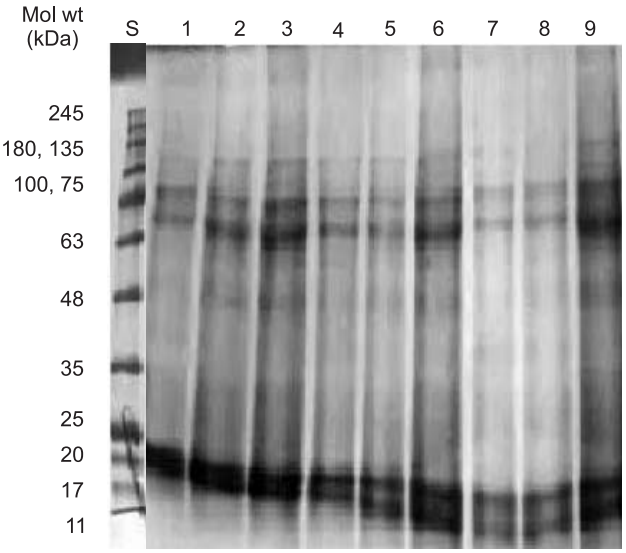

Fig. 2. Electrophoretic pattern of heparin binding proteins (HBP) of good, average and poor fertility semen in buffalo bulls. Lane S, Standard protein marker; Lanes 1, 2 and 3, HBP in seminal plasma; Lanes 4, 5 and 6, HBP in fresh sperm extracts; Lanes 7, 8 and 9, HBP in frozen-thawed semen extracts of poor, average and good fertility bulls.

bulls (77.8%) with poor fertility. A 31 kDa was detected in 90.0 and 81.8% bulls with good and average fertility, respectively, and its quantity was significantly (P<0.05) higher in former than in latter ones. Additionally, 3 proteins of 65, 20 and 11 kDa were detected in all the bulls but their quantity was significantly (P<0.05) higher in the seminal plasma of bulls with good fertility as compared to those with average and poor fertility. The quantity of 16 and 75 kDa proteins was significantly higher (P<0.05) in PFB as compared to AFB and GFB.

Like seminal plasma, purified HBP of fresh spermatozoa also revealed the presence of similar proteins (135, 100, 75, 65, 48, 45, 40, 37, 31, 28, 26, 20, 18, 16 and 11 kDa (Table 2, Fig. 2). Again, the proteins of 135, 48 kDa and 28, 18 kDa were recognized in good and poor fertility bulls, respectively. The concentration of 2 proteins of 65 and 11 kDa was significantly (P< 0.05) higher in GFB than in AFB and PFB. In addition, the concentration of a 20 kDa protein was also significantly (P<0.05) higher in bulls with good fertility as compared to their average and poor contemporary mates. Another protein of 31 kDa was also significantly (P<0.05) higher in good than in average fertility bulls, while undetectable in poor fertility bulls. Corresponding to seminal plasma, the quantity of protein with molecular weight of 16 and 75 kDa was significantly (P<0.05) higher in bulls with poor fertility than in their average and good counterparts. Although nonsignificant (P>0.05), the quantity of a 45 kDa protein was also higher in PFB than in their contemporary mates.

The purified HBP of frozen-thawed sperm extracts resulted in separation of almost similar proteins of molecular weight 100, 75, 65, 48, 45, 40, 37, 31, 28, 26,

Table 2. Mean (± SE) concentration of purified HBP in seminal plasma, fresh- and frozen-thawed semen of buffalo bulls

Mol. Wt. (kDa)	Protein concentration (µg/ml)								
	Seminal plasma			Fresh sperm extracts			Frozen-thawed sperm extracts		
	GFB	AFB	PFB	GFB	AFB	PFB	GFB	AFB	PFB
135	1.0 ± 0.4	—	—	1.0 ± 0.5	—	—	—	—	—
100	1.2 ± 0.6^{ab}	0.8 ± 0.2^{a}	1.6 ± 0.5^{b}	1.5 ± 0.7	1.4 ± 0.3	1.4 ± 0.4	1.2 ± 0.9	0.7 ± 0.3	0.2 ± 0.0
75	1.2 ± 0.6^{a}	13.4 ± 1.9^{b}	11.5 ± 1.6^{b}	1.1 ± 0.7^{d}	8.6 ± 1.2^{e}	11.2 ± 0.3^{f}	0.3 ± 0.1^{g}	6.3 ± 1.2^{h}	10.2 ± 0.2^{i}
65	26.5 ± 2.8^{a}	20.9 ± 1.8^{b}	20.0 ± 3.4^{b}	35.2 ± 3.1^{d}	17.5 ± 1.3^{e}	20.3 ± 2.5^{e}	18.2 ± 2.6^{g}	11.9 ± 1.3^{h}	10.9 ± 1.9^{h}
48	13.7 ± 2.6	—	—	15.0 ± 2.2	—	—	8.6 ± 2.0^{g}	—	—
45	5.4 ± 1.4^{a}	7.1 ± 0.7^{b}	9.0 ± 2.0^{b}	3.9 ± 2.2	5.8 ± 0.8	7.4 ± 1.7	0.8 ± 0.4^{g}	4.0 ± 1.0^{h}	2.6 ± 0.4^{h}
40	2.0 ± 0.7	1.0 ± 0.4	—	1.2 ± 1.1	0.4 ± 0.1	—	—	1.2 ± 0.5	—
37	1.0 ± 0.3^{a}	0.4 ± 0.1^{b}	0.6 ± 0.2^{ab}	1.1 ± 0.6^{d}	3.4 ± 0.8^{e}	0.6 ± 0.1^{d}	1.2 ± 0.3^{a}	1.4 ± 0.4^{gh}	2.2 ± 0.4^{h}
31	1.6 ± 0.6^{a}	0.6 ± 0.1^{b}	—	3.0 ± 0.7^{d}	1.4 ± 0.4^{e}	—	1.8 ± 0.3^{g}	0.9 ± 0.3^{h}	—
28	—	—	0.9 ± 0.4^{b}	—	—	1.5 ± 0.5	—	—	2.4 ± 0.5
26	2.8 ± 1.0	4.7 ± 1.0	3.8 ± 0.6	2.1 ± 0.5^{d}	4.4 ± 1.3^{e}	2.5 ± 0.7^{de}	1.1 ± 0.6^{g}	2.8 ± 1.2^{g}	3.6 ± 0.3^{h}
20	3.6 ± 0.6^{a}	1.7 ± 0.7^{b}	1.5 ± 0.5^{b}	6.8 ± 0.6^{d}	3.8 ± 0.7^{e}	1.6 ± 0.7^{f}	9.0 ± 1.2^{g}	5.4 ± 0.8^{h}	3.9 ± 1.2^{h}
18	—	—	2.1 ± 0.3	—	—	3.0 ± 0.5	—	—	1.6 ± 1.3
16	6.8 ± 2.4^{a}	36.7 ± 4.5^{b}	45.7 ± 3.1^{c}	3.2 ± 0.8^{d}	38.5 ± 3.5^{e}	45.6 ± 1.0^{f}	4.9 ± 0.3^{g}	46.9 ± 1.8^{h}	57.5 ± 1.2^{i}
11	32.8 ± 4.1^{a}	24.7 ± 2.0^{b}	24.2 ± 1.3^{b}	30.4 ± 3.4^{d}	24.5 ± 1.4^{e}	20.5 ± 1.1^{f}	39.0 ± 1.4^{g}	30.2 ± 2.4^{h}	27.5 ± 1.6^{h}

^{a,b,c}Differ significantly (P<0.05) between groups in the same row for protein concentration in seminal plasma. ^{d,e,f}Differ significantly (P<0.05) between groups in the same row for protein concentration in fresh sperm extracts. ^{g,h,i}Differ significantly (P<0.05) between same row for protein concentration in frozen-thowed semen; –, indicates protein not detected.

20, 18, 16 and 11 kDa (Table 2, Fig. 2). Yet again, the 48 kDa protein and 28 and 18 kDa proteins were detected only in spermatozoa of GFB and PFB, respectively. A 31 kDa protein was separated in SDS-SE of bulls with good (80.0%) and average fertility (90.9%), although its quantity was significantly (P<0.05) higher in the former than the latter ones. The proteins of 65 and 11 kDa were significantly (P<0.05) higher in good fertility bulls as compared to their average and poor herd mates. The quantity of a 20 kDa was also significantly (P<0.05) higher in bulls with good fertility than in average and poor fertility. Alternatively, the quantity of proteins with molecular weight of 16 and 75 kDa was significantly (P<0.05) higher in SDS-SE of bulls with poor fertility as compared to those with average and good fertility. Further, the concentration of a 45 kDa protein was significantly (P<0.05) higher in bulls with average fertility than in their good and poor counterparts. Similar studies in seminal plasma of Nellore bulls (Fernandes *et al.* 2009), rams (Martins *et al.* 2013) and human (Kumar *et al.* 2009) identified 8, 13 and 17 bands of affinity purified HBP with molecular weights ranging from 15 to 63 kDa, 10 to 232 kDa and 10 to 90 kDa, respectively. Further, Arangasamy *et al.* (2005) and Kumar *et al.* (2008) observed eight (11, 14, 18, 20, 31, 41, 65 and 71 kDa) and six (14, 20, 24, 31, 41 and 65 kDa) major HBP on SDS-PAGE in buffalo seminal plasma, respectively. Likewise, Singh *et al.* (2013) also recognized 9 protein bands in the range of 10–170 kDa (10, 15, 20, 24, 33, 40, 55, 70 and 170 kDa) in frozen-thawed sperm extracts of buffalo bulls. Furthermore, quantitative differences were also observed in 15 protein bands among the tested seminal fluid of all bulls. The results are in agreement with findings of Marques *et al.* (2000) and McCauley *et al.* (2001) who observed both quantitative and qualitative differences in bovine spermatozoal proteins (31, 29, 25, 21, 20, 18 and 16 kDa). Moreover, the inherent character of the proteins might also contribute toward the difference in number and concentration of bands.

Overall, purified HBP following affinity chromatography revealed that the concentration of 4 proteins (65, 31, 20 and 11 kDa) which were significantly higher in seminal plasma, fresh- and frozen-thawed sperm extracts of good fertility bulls than in their contemporary mates and seemed to be candidate proteins to predict buffalo bull fertility.

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