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Characterization of sperm heparin binding proteins (HBPs) using polyclonal antibodies raised against seminal plasma HBPs: Application in buffalo bull fertility

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This study aimed to evaluate rabbit polyclonal antibodies raised against purified seminal plasma sperm membrance extracts (SP) heparin binding protein (HBP) for identifying HBPs in buffalo bull spermatozoa by western blotting. Anti-SP-HBP recognized 11 polypeptides in SDS-sperm membrance extracts (SME) of 31 tested bulls. Thirty one bulls were divided into G-1 (>40%) and G-II (≤40%) based on acrosome reaction. Immunoblotting revealed that HBPs of 24, 30, 38 and 43 kDa were present in 3%, 7.02%, 1.16% and 4.83% more bulls of G-I, whereas, 20 and 46 kDa HBPs were present in 13.2 and 9.65% more bulls of G-II. Immunoblotting of anti-HBP with sperm extracts of 10 bulls (22-31) indicated that 31 kDa positive bulls had 10.9% higher conception rate than 31 kDa negative bulls. Although 24 kDa HBP was detected in 10 bulls, but its expression was very weak in bull number 22, 23 and 26, which had 10.7% lower conception rate than the bulls with strong expression of 24 kDa HBP. In the present study, 17/20 kDa positive bulls exhibited 4.46% and 8.67% low conception rate than 17/20 kDa negative bulls. Mass spectrometry analysis revealed matching of 24, 31, 33 and 38 kDa proteins with MHC class 1 antigen, tRNA methyl transferase 11 homolog partial, parvalbumin alpha-like and cilia- and flagella-associated protein 99. This study suggests that buffalo bull fertility can be predicted from sperm HBP.

Keywords: Buffalo bull fertility, polyclonal antibodies, sperm HBP

Introduction

Seminal plasma is a complex mixture of secretions from the testis, epididymis and accessory sex glands, contains factors that modulate the fertilizing ability of sperm[1]. It contains proteins, which may have either beneficial or detrimental effect to sperm function. These are proteinous or non-proteinous, but nature and characteristics of most of these factors are not well stood[2]. In most mammals, the sperm must reside in the female reproductive tract for about eight hours to undergo capacitation. The final step of capacitation, the 'acrosome reaction' involves attrition of the sperms are held in the oviductal reservoir by binding to the epithelium through binding sperm proteins (BSP) on the head of bull sperm before undergoing capacitation[3]. Many seminally derived proteins have been identified in semen that binds heparin and are known as heparin binding proteins (HBP). HBP are more abundant on the surface of ejaculated spermatozoa than on the plasma membrane of epididymal spermatozoa[1]. HBPs are produced by male accessory sex glands, secreted into seminal fluid and upon ejaculation bind to the sperm. There are three groups of seminal plasma HBPs with molecular weight of 14-17 kDa, 24 kDa and 30 kDa. Proteins of 14-17 kDa had pI of 4.1-6.0 and were associated with sperm membrane and fertility[1]. One of the HBPs that bind to spermatozoa had a molecular mass of 30 kDa which is synthesized by the seminal vesicles and prostate glands[3]. This 30 kDa protein has sequence similarity to DNase 1 and has been termed as fertility associated antigen (FAA). Another 24 kDa HBP was [illegible], which has an amino acid sequence related to tissue inhibitor metalloproteinase-2 (TIMP-2)[4]. Three major classes of HBPs viz. 14-17 kDa (sperm adhesins), 24 kDa (TIMP-2) and 31 KDa (DNase 1 like protein) in seminal plasma of bovines interact with the sperm surface and participate in sperm function[1,4-6]. The 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[7]. The HBPs bind to sperm membrane choline phospholipids, heparin and glycosaminoglycans at ejaculation resulting in efflux

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of phosphatidylcholine and cholesterol which is required mechanism for capacitation, acrosome reaction, sperm oocyte fusion and fertilization[8-10].

HBPs are indicated as biochemical markers to predict fertility of bulls. HBPs purified from buffalo bull seminal plasma by affinity chromatography were also characterized by SDS-PAGE/2-D electrophoresis in number of studies[11-12] and correlated with semen quality/fertility. A commercially available test for a fertility related protein in cow bull semen that is to be used in combination with BSE to help breeders in selecting bulls of high fertility was launched[13]. But, objective markers are not yet available for fertility potential to identify the most fertile buffalo bulls. The aim of this study was to develop polyclonal antibodies against purified HBPs for characterization of HBPs in spermatozoa and their relationship with *in vitro* acrosome reaction and fertility.

Materials and Methods

Reagents

Most chemicals were from Sisco Research Laboratories, Sigma-Aldrich Chemical Company and BR Biochem Life Sciences Private Limited. Millipore (RO/Synergy) purified water was used for the preparation of reagents.

Procurement of Semen Samples

Study was carried out on 31 buffalo bulls. Forty mini straws (0.25 ml) per bull were procured from Animal Husbandry Department, Rauni, Patiala (1-21 bulls), Punjab, India and Semen Freezing Lab, Directorate of Livestock Farms, Guru Angad Dev Veterinary and Animal Science University, Ludhiana (22-31 bulls), Punjab, India. Data for first service conception rate for 22-31 bulls was also obtained from Dairy Farm, Directorate of Livestock Farms, Guru Angad Dev veterinary and Animal Science University. Pooled freshly ejaculated semen of 10 bulls was procured from Semen Bank, Bhattian and seminal plasma was separated by centrifuging semen at 3000 rpm for 5 min at room temperature. Seminal plasma was stored in aliquots at -20^{o}C till further analysis.

Experimental Design

Heparin binding proteins were purified from buffalo bull seminal plasma by affinity chromatography. Polyclonal antibodies were raised against purified HBP and non-HBP in rabbits. Purified IgG fraction from rabbit blood serum was reacted with sperm extracts of 31 bulls on immunoblots to characterize HBP. Polymorphism in HBP among the bulls was related to percentage acrosome reaction and fertility rate in order to find out fertility related HBP in buffalo bulls.

Purification of HBP by Affinity Chromatography[14]

Seminal plasma filtered through 0.2 µ filter was subjected to heparin-sepharose affinity chromatography[12]. A glass column (28 mm x 70 mm) packed with heparin-sepharose media (Thermo-Fisher Scientific Company) was equilibrated for 1 h with 10 mM Tris HCl (pH 7.4). About 500 µl of seminal plasma was loaded and circulated through the column for 15 min for absorption of HBPs to the heparin bound resins. The non-heparin binding proteins were washed out with 10 mM Tris HCl. HBPs 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 and non-HBP (NHBP) fractions were pooled in agreement with the observed curve, obtained from optical density (280 nm), detected by UV monitor attached to the fraction collector.

Raising of Hyper Immune Sera

Rabbits were purchased from Animal House, Institute of Microbial Technology (IMTECH), CSIR, Chandigarh (approved by the Committee for Purpose of Control and Supervision of Experiments on Animals, India) and experiment was performed in accordance with the guidelines approved by Institutional Ethical Committee (Memo No. IAEC/2016/410-435, dated 19/05/1016) Guru Angad Dev Veterinary and Animal Sciences, University, Ludhiana. Six adult male rabbits were divided equally into three groups. G-I and G-II were immunized with HBP and NHBP, respectively. G-III was kept as a control and injected only PBS, pH 7.4. Freund's complete adjuvant (FCA) was used for primary immunization and Freund's incomplete adjuvant (FIA) for subsequent boosters. The rabbits were immunized through intramuscular route on 0, 10 and 20 days with 200 µg of protein mixed with adjuvant in the ratio of 2:1.

Blood was collected from ear vein of immunized and control rabbits before each immunization. Blood was kept tilted for 2 hours and centrifuged at 3000 rpm for 20 min at 4ºC to collect serum. Before storing the serum, it was de-complemented by incubation at 56ºC for 30 min. IgG fraction was purified from rabbit blood serum using purification kit (Mol Bio HiMedia) as per manufacturer's instructions.

Detection/Titration of Anti-HBP and Anti-NHBP Antibodies by Enzyme Linked Immunosorbant Assay

High binding 96 U bottom wells ELISA plate (BR BIOCHEM, Life Sciences) was coated with 100 µl of poly-L-lysine (MP Biomedicals) by incubating at 37°C for 1 h. Plate was washed twice with PBS containing 0.2% Tween 20 (PBS-T) and incubated with 100 µl of antigen (purified HBP and NHBP, 5 µg/100 µl) overnight at 4ºC. Washed the plate thrice with PBS-T and incubated with 300 µl of 2% BSA solution for 2 h at 37ºC. The plate was washed thrice with PBS-T and coated with 100 µl of purified anti-HBP-IgG serially diluted with PBS and incubated at 37ºC for 2.5 h. After washing, 100 µl of conjugate (1:10000) goat anti rabbit HRPO conjugate (Genexbio) was added in all the wells and incubated for 3 h at 37ºC. After washing the plate with PBS-T, 100 µl of o-phenylenenediamine dihydrochloride hydrogen peroxide was poured and incubated for 20 minutes in dark. The reaction was stopped with 50 µl of 5 N H_2SO_4. Absorbance was measured using an ELISA reader (Tecan) at 492 nm. Antibody titer was calculated from the maximum absorbance value at 492 nm[15]. Percent positivity was also calculated by the formula:

$$\% \text{ positivity} = \frac{\text{Mean OD of treated IgG} - \text{Mean OD of control IgG}}{\text{Mean OD of control IgG}} \times 100$$

Extraction of Sperm Proteins

Sperm membrane proteins were extracted with sodium dodecyl sulphate (SDS)[16]. Ten frozen semen straws / bull were centrifuged at 3000 rpm for 10 min to separate out seminal plasma and dilutor. Sperm pellet was suspended in 0.5 ml of 2% SDS in 62.5 mM Tris-HCl (pH 6.8) containing protease inhibitors (Cocktail, SERVA). Sperm suspension was sonicated at 20 Watts for 3 × 20 sec, centrifuged at 10000 rpm for 15 min. Pellet was discarded and sperm extract was concentrated through 3 kDa protein concentrators (Millipore) and concentrated protein extracts were stored in aliquots at -20°C till further use.

Confirmation of Anti-HBP and NHBP, Detection of HBP in Sperm Extracts by Western Lotting

Purified HBP and NHBP were reacted with anti-HBP-IgG and anti-NHBP IgG to confirm the presence of antibodies against HBP and NHBP in immunized rabbit's blood serum[17]. Purified HBP and NHBP were also cross-reacted with NHBP and HBP to confirm the specificity of purified HBP. Sperm extracts of 31 bulls were reacted with ani-HBP-IgG to characterize HBP in spermatozoa. HBP and NHBP were also reacted with control IgG.

The proteins resolved on 10% SDS-PAGE were transferred electrophoretically to nitrocellulose membrane (NCM, Pall Corporation) using Pierce G2 fast blotter (Thermo Scientific). The complete stack of two absorption pads, gel, NCM, two absorption pads was kept on the G2 fast blotter between cathode and anode. Blotting was carried out for 15 min at 25 volts and 1.3 A. The membrane, after transfer was incubated in 2% BSA for 2 h at room temperature on platform rocker for blocking the nonspecific binding sites. The membrane was washed thrice with PBS-T (each washing for 15 min) and incubated with 1:500 diluted purified IgG at 4°C for overnight. Subsequently, the membrane was washed thrice with PBS-T and incubated for 45 min at 37°C with 1:10000 diluted goat anti-rabbit HRPO conjugate (Bangalore Genei). After washing, the protein antibody reaction was detected by incubating the membrane with substrate containing 0.06% 3,3 diaminobenzidine tetrahydrochloride (DAB) and 0.06% H_2O_2 for 5 min. The reaction was terminated by washing the membrane with distilled water (DW). The image analysis and molecular weight determination was done by using GeneSnap image acquisition software (Syngene).

Mass Spectrometry

Bands of 24, 31, 33 and 38 kDa of sperm extract separated on acrylamide gels were cut and submitted to Central Instrumentation Facilities (CIF), University of Delhi, South Campus, New Delhi for mass spectrometry analysis by MALDI-TOF. Identified peptide sequences were aligned and compared with the sequences of different proteins using BLAST search engine.

In-vitro Capacitation and Acrosome Reaction

(i) *Basic TALP medium* (92.9 mM NaCl, 4 mM KCl, 25.9 mM $NaHCO_3$, Na_2HPO_4, 10 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES). All ingredients were dissolved in DW, pH was set at 7.4 and final volume was made to 100 ml.

(ii) *Energy medium* (Basic TALP medium supplemented with 0.09% glucose, 0.6% bovine serum albumin, 50 µg / ml gentamycin and 10 µg

/ ml heparin). Three straws per bull were thawed at 37°C in 5 ml microfuge tube and washed twice with the basic TALP medium by centrifuging at 1000 rpm for 5 min. The sperm suspension was re-suspended in the energy medium (0.5 ml) to a final concentration of 200X 10^6/ml spermatozoa and incubated at 37°C for 4 h. A smear was prepared, stained with Giemsa and assessed for acrosome reaction. At least 200 spermatozoa were counted from each slide and percentage of acrosome reacted spermatozoa was calculated.

First Service Conception Rate

First service conception rate for 10 bulls (22-31) was obtained from the Dairy Farm, Directorate of Livestock Farms, GADVASU, Ludhiana. It was calculated based on 20 inseminations per bull.

Analysis of Data

Thirty one bulls were grouped based on capacitation/acrosome reaction status and compared for the presence of HBP. Bull numbers 22-31 were also grouped based on conception rate and compared for the presence of HBP. Significant differences among the two groups were tested by paired t sample test using SPSS 16 software. A linear regression model was applied to the relationship among semen traits. $P < 0.05$ was considered statistically significant.

Results and Discussion

Purification of HBP and Characterization by SDS-PAGE

Graphic image of HBP purified from seminal plasma (SP-HBP) of buffalo bulls is shown in Figure 1, which indicated two peaks of unbound (NHBP) and bound proteins (HBP). SDS-PAGE analysis of the pooled eluted HBP peak identified 13 bands, with molecular weights ranging from 11 to 125 kDa (Fig. 2a). NHBP were separated into 15 bands ranging from <10 kDa to >250 kDa. Electrophoretic analysis of affinity purified HBP fractions revealed 11 bands ranging from 16-130 kDa in seminal plasma of cross-bred cattle bulls[18]. HBP were separated into 13 (10-232.2 kDa) and 8 bands (15-63 kDa) on 1-D gels in seminal plasma of Nellore bulls and ram, respectively[19-20]. Variation in number of bands and molecular weights of seminal plasma HBP fractions was also observed within buffalo i.e. eight bands of 13-71 kDa, six bands of 14-61 kDa, nine

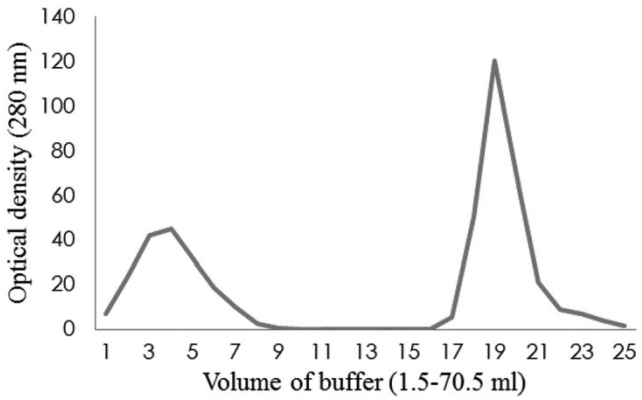

Fig. 1 — Graphic image of heparin-binding and non-heparin-binding protein fraction purified from seminal plasma of buffalo bulls by heparin-affinity chromatography.

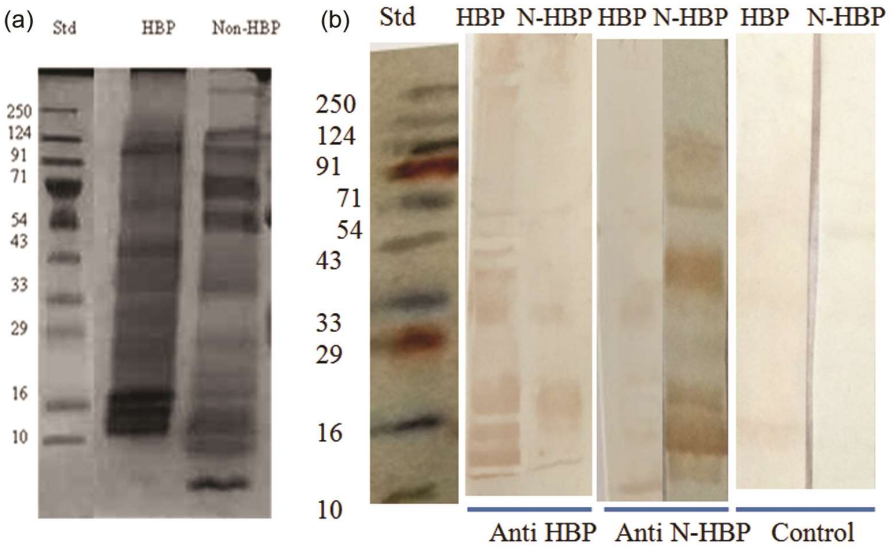

Fig. 2 — a) Separation of purified SP-HBP and SP-Non-HBP on 10% acrylamide gels under reduced - denatured conditions; b) Immunoblotting of anti HBP IgG, anti- NHBP-IgG and control IgG with HBP and NHBP. Proteins separated on 10% acrylamide gel-b were reacted with antibodies.

bands of 10-170 kDa[6,20-21]. Variation in number and molecular weight of HBP fractions may be attributed to species and individual variation. Immunoblotting of purified HBP and NHBP with anti-SP-HBP and anti-NHBP-SP confirmed the purity of antibodies raised against HBP and NHBP. It further revealed the immunogenicity of only 9 HBPs and 6 NHBPs.

Raising of Anti-HBP and Confirmation by Immunoblotting

First immunization with SP-HBP and NHBP resulted in 3.1 and 5.5% positivity of raised antibodies, respectively. It was further increased to 28.13% and 20.9% after 3rd immunization for anti-HBP and NHBP, respectively. An antibody titer of 6400 and 3200 was obtained for anti-HBP-IgG and NHBP-IgG after 3rd Immunization, respectively.

Purified IgG from non-immunized serum did not show reaction with SP-HBP and SP- NHBP (Fig. 2b). Among 13 SP-HBP separated on SDS-PAGE, only 9 HBP of 11, 13, 17, 25, 31, 35, 38, 42 and 46 kDa reacted with anti-HBP-IgG on immunoblots. Anti-HBP IgG also reacted non-specifically with three NHBP. Anti-NHBP-IgG reacted with 6 NHBP of 12, 17, 26, 36, 55 and 72 kDa on immunoblots, whereas, a non-specific response was obtained with four HBPs. Non-specific reaction of anti-HBP-IgG with NHBP and vice versa may be due to some similarity in the sequence of HBP and NHBP.

Characterization of HBPs in Sperm Extracts with Anti-HBP

Eighteen peptides with molecular weight of 11, 15, 20, 24, 30, 33, 38, 43, 46, 50, 57, 68, 72, 80, 90, 105, 150 and 293 kDa were separated in SDS-sperm membrance extracts (SME) of buffalo bulls on 10% acrylamide gels (Fig. 3). Anti-SP-HBP-IgG recognized 11 polypeptides of 80, 72, 50, 46, 43, 38, 33, 30, 24, 20 and 15 kDa in SDS-SME of 31 tested bulls (Fig. 4). However, 11 polypeptides were not detected in all the tested bulls but ranged from 3-10. HBP of 80, 72, 50, 46, 43, 38, 33, 31, 24, 20 and 17 kDa were detected only in SDS-SME of 20, 18, 18, 23, 19, 20, 20, 13, 29, 26 and 26 bulls, respectively. Difference in intensity of HBP positive bands indicated qualitative as well as quantitative difference in HBP among the bulls. However, 13 HBP of 20-135 kDa were detected in frozen-thawed spermatozoa of 30 buffalo bulls[12]. They further observed polymorphism in HBP among bulls ranging from 4-10, which is near our observations (3-10). Difference in number and molecular weight range of HBP between previous and present studies may be due to the use of indigenous antibodies to identify HBP during the present study. Studies using one dimensional polyacrylamide gel electrophoresis detected 32 bands and 35 bands of HBP in the seminal plasma of Nellore bulls and rams, respectively[3,22].

Relationship of HBP with *In Vitro* Acrosome Reaction

Thirty one bulls were divided into G-I (> 40% AR) and G-II (≤ 40% AR). Comparison of HBP among the bulls based on rate of acrosome reaction revealed that HBP of 24, 31, 38 and 43 kDa were present in 3%,

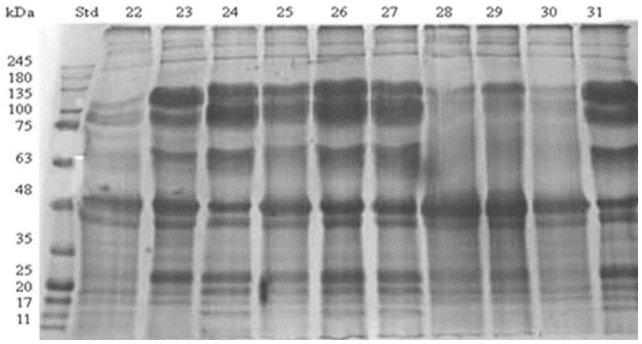

Fig. 3 — Represents SDS-PAGE of buffalo bull sperm proteins extracted with SDS on 10% acrylamide gels. Std (standard) and 22-31 (bull numbers).

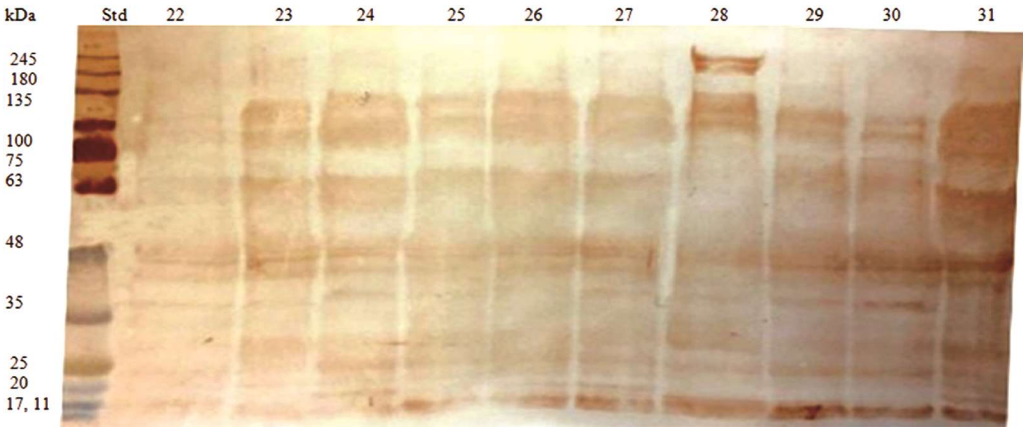

Fig. 4 — Represents immunoblotting of anti-SP-HBP-IgG with SDS-SME of buffalo bulls. Std (standard), 22-31 (bull numbers).

7.02%, 1.16% and 4.83% more bulls of G-I, whereas, 20 and 46 kDa HBP in 13.2 and 9.65% more bulls of G-II. Heparin and heparin-like molecules bind to the bovine spermatozoon through a ligand-receptor interaction and promotes capacitation without inducing acrosome reaction. It is possible that 24 / 31 / 38 / 43 kDa HBP may persuade capacitation mechanism in buffalo but with a variation among the bulls. Since bovine seminal plasma also has decapacitating factors, therefore, 20 and 46 kDa HBP may have role in declining the capacitation mechanism in some of the buffalo bulls.

Relationship of HBP with Conception Rate

HBPs in ten bulls (22-31) were also compared based on conception rate and it was found that 31 kDa HBP positive bulls had 10.9% higher conception rate than 31 kDa HBP negative bulls. Bulls which contained a 31 kDa protein in sperm extract were 17% more fertile as compared to their contemporary mates[23] under natural-mated conditions. Likewise, when the semen of fertility associated antigen (FAA) positive and negative bulls was inseminated artificially, the FAA positive bulls were 16% more fertile than FAA-negative artificially inseminated (AI) sires[24]. Although 24 kDa HBP was detected in all 10 bulls, but during the present study its expression was very weak in bull number 22, 23 and 26 which had 10.7% lower conception rate than the bulls with strong expression of 24 kDa HBP. A monoclonal antibody generated against purified HBP from seminal fluid was designated as M1[25] and has been used to detect FAA and TIMP-2 in bovine semen[5]. Western blot analysis, with the M1 antibody determined that bulls which had the antigen in sperm extracts were 14% higher in fertility than those lacking the antigen[7]. Like FAA, the bulls which possessed TIMP-2 in detergent extracts of sperm were 13% more fertile than their counterparts[26]. In the present study, 17 and 20 kDa positive bulls also exhibited 4.46% and 8.67% low conception rate than 17 and 20 kDa negative bulls, respectively. Killian *et al*[27] found that two proteins of 26 kDa, pI 6.2 and 55 kDa, pI 4.5 predominated in higher-fertility bulls and two proteins of 16 kDa, pI 6.7 and 16 kDa, pI 4.1 predominated in lower-fertility dairy Holstein bulls. It can be concluded from these observations that 31 kDa / 24 kDa HBP and 17 / 20 kDa HBP may be related to higher and low fertility of buffalo bulls, respectively. Singh *et al*[12] identified HBPs in buffalo bull frozen-thawed spermatozoa using anti-AZU-1 and exposed higher ($P < 0.05$) first service conception rate (FSCR) in bulls positive for 135, 75, 55, 45, 28 and 24 kDa as compared to their negative counterparts.

Mass Spectrometry Analysis of Sperm HBPs

Mass spectrometry (MS) analysis revealed matching of 24, 31, 33 and 38 kDa proteins with MHC class 1 antigen, tRNA methyltransferase 11 homolog partial, parvalbumin alpha-like and cilia- and flagella-associated protein 99 with a top score of 81, 53, 56 and 60, respectively (Table 1). A total of 15, 10, 8 and 11 peptides of 24, 31, 33 and 38 kDa bands matched to identified proteins from database, which had coverage of 65%, 35%, 30% and 33% of whole protein sequence, respectively. Based on previous data 24 kDa and 31 kDa proteins have been designated as TIMP-2 and FAA in bovine. But 24 and 30 kDa proteins of buffalo bull sperm did not match to TIMP-2 and FAA (DNase 1 L3) in MS analysis. pI of TIMP-2 and FAA are reported as 7.46 and 7.5-8.0[4], whereas that of MHC class 1 antigen and tRNA methyltransferase 11 as 6.10 and 8.62, respectively. It is possible that TIMP-2 / MHC class 1 antigen and tRNA methyltransferase 11/FAA shares same molecular weight but has different P^I in buffalo bull sperm. The presence and abundance of TIMP-2 in bovine semen and its ability to bind heparin[3] suggests that it has an important relationship to reproduction in bulls. This 31 kDa heparin-binding protein found on sperm membranes has been correlated to increased fertility among bulls[7].

MHC class 1 expression appears to be required during spermatogenesis to produce sperm cells capable of taking up foreign DNA[28] and its presence on sperm played an important role in the recognition between sperm and egg in fertilization[29]. Transfer RNA methyletransferase has a role in methylation of RNA[30]. Main function of this enzyme is to assure tRNA integrity and function[31]. Many tRNA fragments are gained during post-testicular maturation in the epididymis and regulate retroelements upon delivery to the embryo[31]. Therefore, integrity of tRNA maintained by tRNA methyletransferase is important in sperm fertility. Parvalbumin is a calcium-binding albumin protein. Calcium binding proteins like parvalbumin play a role in many physiological processes[32]. It has been concluded in many studies that Ca^{2+}, HCO^{3-}, and cAMP are the key secondary signaling molecules that regulate sperm capacitation. Calcium exerts its effect on sperm capacitation

Table 1 — Mass spectrometry analysis of buffalo bull sperm proteins

S. No.	Mol wt (kDa)	Sequence detected	Sequence matched to	Score	Protein sequence coverage (%)
1	24	M.APRTLLLVLSGALALTQTR.A + Deamidated (NQ), R.AGSHSMRYFYTAVSRPGR.W R.FIAVGYVDDTQFVRFDSDAESQR.M + 2 Deamidated (NQ, R.FDSDAESQRMEPR.A, R.YMKTATQMAPVDLR.N + Deamidated (NQ), R.NLRGYYNQSEAGSHTLQTMYGCDLGPDGR.L + 2 Deamidated(NQ),R.GYFQDAYDGKDYIALNQDLR.S + 3 Deamidated (NQ), R.SWTAADMAAQNTQR.K R.SWTAADMAAQNTQRK.W, R.SWTAADMAAQNTQRK.W + 3 Deamidated (NQ), R.AYLEGTCLEWLRR.H K.THVTHHPVSDQEATLRCWALGFYPAEITLTWQR.D + 2 Deamidated (NQ), R.DGEDQTQDTELVETRPAGDGTFQK.W R.KGGSYSQAASSDSAQGSDVSLTACK.V + 2 Deamidated (NQ), K.GGSYSQAASSDSAQGSDVSLTACKV.- + 2 Deamidated (NQ)	MHC class I antigen (*Macaca mulatta*)	81	65
2	31	-.MAPPGILNRYLLLMAQEHLEFR.L, M.APPGILNR.Y R.YLLLMAQEHLEFRLPEIK.S + Deamidated (NQ) K.SPFWILSIPSEDIAR.N, R.TVCAKSIFELWGHGK.C K.SIFELWGHGK.C,K.KPQHVFSILEDYGLDPNHIPENPHNIYFGR.W + 3 Deamidated (NQ), K.RHFIGNTSMDAGLSFIMANHGK.V + 2 Deamidated (NQ), R.QYGLEKYYLDVLVSDASKPCWR.K + Deamidated (NQ), K.YYLDVLVSDASKPCWRK.G	tRNA methyltransferase 11 homolog partial (*Bubalus bubalis*)	53	35
1	33	R.AQDLSILSTLAGRPRPGHR.L,R.AQDLSILSTLAGRPRPGHRLSAR.S + Deamidated (NQ), K.NADQQIWNPGLVLGWIQR.L + 5 Deamidated (NQ), R.RAVPVSPGAVLAEGDWGQNSPER.G, R.GPSTLSMGSGQILSPSR.S + Deamidated (NQ), R.SLRPSVPHDTRER.K, K.FFQTSGLAKMSASQVK.D + Deamidated (NQ), K.SLMAAADNDGDGKIGADEFQEMVHS.- + 2 Deamidated (NQ)	Parvalbumin alpha-like (*Bos taurus*)	56	30

through Ca^{2+}-binding proteins that undergo conformational changes upon interaction with the divalent cation[33]. Cilia- and flagella-associated protein 99 may play a role in ciliary/flagellar motility by regulating the assembly and the activity of axonemal inner dynein arm. Therefore, 24, 31, 33 and 38 kDa proteins, which are characterized as MHC class 1 antigen, tRNA methyltransferase, parvalbumin and cilia- and flagella-associated protein 99 are also important in sperm function.

This study concluded that sperm HBP of 31 / 24 kDa and 17 / 20 kDa may be associated with high and low fertility of buffalo bulls, respectively. It can also be inferred that TIMP-2 / MHC class 1 antigen and FAA / tRNA methyltransferase may have interaction for a similar sperm function in buffalo bull.

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