

## Comparative study of heparin-binding proteins profile of Murrah buffalo (*Bubalus bubalis*) semen

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### Abstract

**Aim:** The experiment was conducted to study the total seminal plasma protein (TSPP) and heparin-binding proteins (HBPs) in relation to initial semen quality of buffalo bull.

**Materials and Methods:** Semen from two Murrah buffalo bulls (bull no. 605 and 790) with mass motility of  $\geq 3+$  were used for the study and categorized into three groups (Group I- Mass motility 3+, Group II- Mass motility 4+ and Group III- Mass motility 5+). Seminal plasma from semen was separated by centrifugation. HBPs was isolated and purified from heparin-agarose affinity column by modified elution buffer. TSPP and isolated HBPs concentration was estimated by Lowry's method. The purified HBPs were resolved on Sodium dodecyl sulfate polyacrylamide gel electrophoresis to check the protein profile of two bulls.

**Results:** The mean values of TSPP concentrations in bull no. 605 and 790 in Group I, II and III were  $30.64 \pm 0.12$ ,  $31.66 \pm 0.09$ ,  $32.53 \pm 0.19$  and  $28.51 \pm 0.09$ ,  $29.49 \pm 0.15$ ,  $30.45 \pm 0.17$  mg/mL, respectively. The mean values of HBPs concentrations in bull no. 605 and 790 in Group I, II and III were  $3.11 \pm 0.07$ ,  $3.32 \pm 0.06$ ,  $3.46 \pm 0.08$  and  $2.51 \pm 0.08$ ,  $2.91 \pm 0.05$ ,  $3.10 \pm 0.03$  mg/mL, respectively. Both the values of TSPP and HBPs were significantly higher ( $p < 0.01$ ) in bull no. 605 when compared to 790 in all the three groups. 31 kDa HBP was more intensely present in bull no. 605, thus may indicate its superiority over bull no. 790 in relation to fertility potential.

**Conclusion:** TSPP and HBPs shows variation in concentration with respect to initial semen quality. Furthermore, presence of fertility related 31 kDa HBPs in one of the bull may be an indication of high fertility of a bull. In future, *in-vivo* and *in-vitro* correlative study on larger basis is needed for the establishment of fertility-related HBPs in semen which might establish criteria for selection of buffalo bull with high fertility potential.

**Keywords:** buffalo bull, heparin-binding proteins, semen, seminal plasma.

### Introduction

Traditional semen quality tests in routine use to evaluate semen quality provide limited information about the potential fertility of bulls and do not provide high correlation or even consistent results of bull fertility. An alternate approach to testing semen quality with more accuracy is the protein fertility marker. A correlation between seminal plasma proteins and fertility of the male have been reported in some of the domestic animals such as cow bull [1], stallion and boar [2], goat [3], and ram [4]. Specific seminal plasma proteins have been identified as potential markers of human male fertility/infertility [5].

Bovine seminal plasma (BPS) contains factors that may have either beneficial and/or detrimental effect to sperm function. Some of these factors are

contributed by accessory sex glands. These are proteinous/non proteinous, but the nature and characteristics of most of these factors are not well-understood [6]. Over the past few decades, special attention has been paid to proteins present in seminal plasma and to their potential roles in sperm maturation events [7].

BPS constitutes of a number of proteins referred as heparin-binding proteins (HBPs) responsible for heparin binding ability of the spermatozoa [8]. These peptides are testosterone dependent and produced by seminal vesicles, prostate and Cowper's glands, binds to sperm during ejaculation as sperm traverse the male reproductive tract [9]. Seminal plasma from a number of mammalian species contains HBPs, which mediate sperm capacitation [10], were associated with fertility due to their modulatory role during the acrosomal reaction [11]. Presence of HBPs in sperm membranes was indicative of the fertility potential of bulls [12]. HBPs modulate capacitation and zona binding ability of buffalo cauda epididymal spermatozoa [13].

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The total seminal plasma protein (TSPP) concentration in buffalo bulls was ranged from 28 to 36 mg/mL [14,15] and concentration of buffalo HBPs reported in the range from 1.47 to 2.61 mg/mL [15-17]. Eight major buffalo HBPs were reported in the molecular weight range of 13-71 kDa [13]. Until date, the study on buffalo HBPs in terms of their concentration and numbers in relation with initial semen quality is meager.

In the present study, TSPP and HBPs were studied among the ejaculate of a bull and ejaculate between the bulls with the different mass motility. The present study may be helpful in buffalo bull HBPs proteomic research, and characterization - identification of fertility related protein in future.

## Materials and Methods

### Climate and experimental animals

Bareilly is located at 28°10' North latitude and 78°23' East latitude at an altitude of 172 m above the mean sea level, known to have moderate climate. Summer temperature goes up to 40°C while winter's goes down up to 8°C. The rainy season is from June to September with moderate humidity. Two healthy adult Murrah buffalo bulls maintained at Germ-Plasm Center of Animal Reproduction Division, IVRI, Izatnagar, Bareilly, UP, India were utilized for the study.

### Chemicals

All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck, India.

### Experimental animals and semen collection

Two healthy adult (4-6 years) Murrah buffalo bulls maintained under standard management conditions were used for semen collection for the entire period of study. Semen was collected during morning hours following standard procedure using an artificial vagina.

### Semen evaluation

Semen samples were evaluated for mass motility immediately after collection. Ejaculates having mass motility 3+ (0-5 point scale) and above were selected for study and grouped in three groups (Group I- mass motility 3+, Group II -mass motility 4+ and Group III- mass motility 5+). Semen samples were aliquoted in 2 mL microcentrifuge tubes supplemented with protease inhibitor cocktail (P2714, Sigma Aldrich, USA).

### Seminal plasma separation

Each ejaculate was centrifuged initially at 4000 g for 20 min at 25°C and supernatant was collected. Supernatant was again centrifuged at 10000 g for 60 min at 4°C to remove suspended spermatozoa and debris, if any. Clear seminal plasma (supernatant) was then stored at -20°C until further processing.

### Purification of buffalo HBPs

Heparin-agarose (HA) matrix (H6508, Sigma Aldrich, USA) was packed in a clean pre-washed

plastic column (Bio-Rad, USA) up to 10 mL level mark. HA column regeneration was done alternately passing 50 mL each of citrate buffer (0.1 M citrate buffer, 1.5 M NaCl, 0.025% sodium azide (NaN<sub>3</sub>), pH-5.5) and Tris-HCl buffer (0.1 M Tris-HCl, 1.5 M NaCl, 0.025% NaN<sub>3</sub>, pH-8.8). Thereafter, HA column was equilibrated with 50 mL of Tris-HCl buffer (equilibration buffer; 50 mM Tris-HCl, 150 mM NaCl, 0.025% NaN<sub>3</sub>, pH-7.4). One mL of clear seminal plasma was loaded on to the pre-equilibrated HA column. Once the sample entered HA media, flow was stopped for 10 min to allow binding of HBPs. Column washing was done 5-10 times of bed volume with equilibration buffer to remove unabsorbed proteins, followed by elution of adsorbed HBPs in 2 mL fractions with modified elution buffer, (50 mM Tris-HCl, 800 mM NaCl, 0.025% NaN<sub>3</sub>, pH 7.4). The absorbance of eluted proteins was taken at 280 nm on spectrophotometer (Spectramax M5, Molecular Devices, USA). The fractions containing proteins were pooled and concentrated by protein concentrator tube (Amicon, Ultra 15, 10K, Merck-Millipore, India). The concentrated proteins was then dialyzed against Tris buffer (20 mM Tris, 20 mM NaCl, pH 7.4) and stored at -20°C until further use.

### Estimation of protein concentration

TSPP and purified HBPs concentration were estimated as per the method given by Lowry [18].

### Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The isolated and purified HBPs were analyzed by 18% SDS-PAGE as described [19] using Coomassie Brilliant Blue G-50 stain. The apparent molecular mass was determined by using molecular weight marker (PG-PMT2922, Genetix Biotech Asia Ltd., India) Gel documentation and analysis system (Gel-Doc<sup>XR</sup>, Bio-Rad, USA).

### Statistical analysis

To minimize the variation in the subjective evaluation of the present study, each sample was evaluated in duplicate and average was analyzed using SPSS version 18.0, (IBM Software Company, USA). Analysis of variance (ANOVA) was performed for statistical analysis of data, using PROC GLM of SAS 9.3 software (SAS Institute Inc., USA) for estimation of mean and standard error of variables under investigation. ANOVA and t-test were used for the comparison between the bulls for the variables viz., TSPP and respective HBPs.

## Results and Discussion

### TSPP

The mean value of TSPP concentrations in bull no. 605 and 790 in Group I, II and III were 30.64±0.12, 31.66±0.09, 32.53±0.19 and 28.51±0.09, 29.49±0.15, 30.45±0.17 mg/mL, respectively (Table-1). The TSPP concentration was

significantly ( $p < 0.01$ ) higher in Group III when compared to Group I and II in both the bulls. TSPP levels of all the groups in bull No. 790 were significantly ( $p < 0.01$ ) lower than bull no. 605, which might be due to lower level of plasma testosterone [20] and health status of accessory sex glands [21]. The TSPP values in present study ranged from 28.51 to 32.53, which were in agreement (28-33 mg/mL) with previous researcher [14-16]. Variation in the concentration of TSPP in split ejaculates from the same Holstein bull and within the bull was also observed [22]. The variation in the buffalo TSPP concentration might be due to individual bull variation, age, inherent character, size, season and health status of seminal vesicle [21] and other factors.

#### HBP's concentration and electrophoretic profile

Overall mean value of HBPs concentrations in bull no. 605 and 790 in Group I, II and III was  $3.11 \pm 0.07$ ,  $3.32 \pm 0.06$ ,  $3.46 \pm 0.08$  and  $2.51 \pm 0.08$ ,  $2.91 \pm 0.05$ ,  $3.10 \pm 0.03$  mg/mL, respectively (Table-1). The bull no. 605 showed significantly ( $p < 0.01$ ) higher HBPs concentration when compared to bull no. 790 in all the three groups. HBPs level was significantly ( $p < 0.01$ ) higher in Group III for bull no. 605 and also significantly ( $p < 0.01$ ) higher in both Group II and III than Group I for bull no. 790.

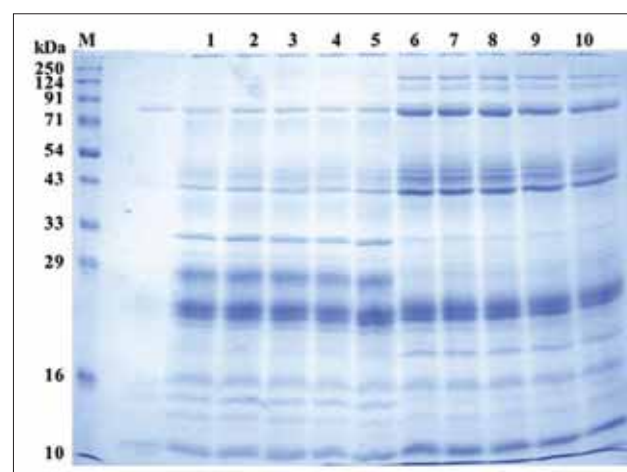
In the present study, the overall mean values of HBP ranged from 2.84 to 3.30 mg/mL and found greater than the previously reported concentrations 2.61 mg/mL [13], 2.01 mg/mL [17] and 1.15-2.18 mg/mL [23,24]. Variation in the concentration of BPS in split ejaculates from the same Holstein bull and within the bull was also observed [22]. HBP in seminal plasma may positively influence fertility [1].

SDS-PAGE analysis of isolated HBPs was done to assess the number of purified HBPs (Figure-1) in individual bull. In the present study HBPs showed 11-14 protein bands on SDS-PAGE in the molecular weight range of 11-129 kDa (11, 14, 15, 16, 20, 28, 29, 31, 43, 48, 53, 71, 92 and 129) and of varying intensity in the Coomassie stained gel. Previous reports indicated 6 to 9 buffalo HBP bands [13,17,24]. In bull no. 605 there was the absence of three HBP bands at 20, 92 and 129 kDa positions, however, in bull no. 790 only one HBP band was absent at 29 kDa positions. Variation in the intensity at 14, 15, 16, 31

and 71 kDa HBP was also observed in bull no. 605 and 790 (Figure-1), which shows the variation in their respective concentration in the seminal plasma. Absence or presence of a particular HBP in the seminal plasma may be associated with fertility potential of a bull. Presence of 31 kDa HBP (fertility associated antigen) showed better conception in cattle bull has been reported [25]. In the present study also, a 31 kDa HBP was more intensely present in bull no. 605, thus might indicate its superiority over bull no. 790 in relation to fertility potential.

The variation in concentration and number of HBPs within and between the bulls might be due to testosterone dependent secretory activity of accessory sex glands [9], health status of accessory sex glands [26] and inherent character of an individual bull. Furthermore, aggregation product of low molecular weight proteins or degradation product of high molecular weight proteins [13] may alter the HBPs profile of a bull.

Variation in TSPP and HBPs concentration and number of HBPs between the bulls may be an indicative of low or high fertility potential of a bull. To the author's best knowledge, no literature is available in this regard. To establish any such correlation would require screening of a large number of ejaculates from number of bulls. Thus, further study is needed on a



**Figure-1:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of heparin binding proteins (HBPs) of buffalo bull 605 and 790. Lane M: Marker; Lane 1,2,3,4 and 5: HBPs of bull number 605; Lane 6,7,8,9 and 10: HBPs of bull number 790.

**Table-1:** Concentration of TSPP and HBPs from semen of buffaloes (mean $\pm$ SE).

Groups	TSPP (mg/mL)		HBPs (mg/mL)	
	Bull number 605 (n=4)	Bull number 790 (n=4)	Bull number 605 (n=4)	Bull number 790 (n=4)
Group I (MM- 3+)	30.64 $\pm$ 0.12 <sup>Ca</sup>	28.51 $\pm$ 0.09 <sup>Cb</sup>	3.11 $\pm$ 0.07 <sup>Bx</sup>	2.51 $\pm$ 0.08 <sup>By</sup>
Group II (MM- 4+)	31.66 $\pm$ 0.09 <sup>Ba</sup>	29.49 $\pm$ 0.15 <sup>Bb</sup>	3.32 $\pm$ 0.06 <sup>ABx</sup>	2.91 $\pm$ 0.05 <sup>Ay</sup>
Group III (MM- 5+)	32.53 $\pm$ 0.19 <sup>Aa</sup>	30.45 $\pm$ 0.17 <sup>Ab</sup>	3.46 $\pm$ 0.08 <sup>Ax</sup>	3.10 $\pm$ 0.03 <sup>Ay</sup>
Overall mean	31.61 $\pm$ 0.24 <sup>a</sup>	29.48 $\pm$ 0.25 <sup>b</sup>	3.30 $\pm$ 0.06 <sup>x</sup>	2.84 $\pm$ 0.08 <sup>y</sup>

Means bearing different superscripts (a, b and x, y) in a row differ significantly ( $p < 0.01$ ), Means bearing different superscripts (A, B and C) in a column differ significantly ( $p < 0.01$ ). n=number of ejaculates, MM=Mass motility, TSPP=Total seminal plasma protein, HBPs=Heparin binding proteins, SE=Standard error

larger basis, which might be helpful in the selection of buffalo bull with high fertility.

### Conclusion

In the present study, one of a bull (No. 605) showed significantly ( $p < 0.01$ ) higher concentration of TSPP and HBPs in relation to initial semen quality. Furthermore, more intensity of 31 kDa HBP in the same bull might reflect its better fertility potential. In the future, *in-vivo* and *in-vitro* correlative study on a larger basis is needed for the establishment of fertility related HBPs in semen that might establish the criteria for selection of buffalo bull with high fertility potential.

### Authors' Contributions

The present study is a part of the Ph.D. thesis program of SSR under approved Institutional research project "isolation and characterization of HBP with special reference to PDC-109 as a fertility marker of buffalo bull" sponsored by Indian Veterinary Research Institute, Izatnagar. JKP is the principal investigator of the project and supervisor of Student Advisory Committee, JKP, SKG, SKB, GKD and SSR planned and designed the research work. Experimental semen collection, grading of semen, seminal plasma separation was done by JSR, MP and NCB. Isolation of HBPs, estimation of TSPP, HBPs and SDS-PAGE analysis was done by SSR and AMS. The manuscript was prepared by SSR and was supervised, reviewed and edited by JKP, SKG, SKB and GKD. All authors have read and approved the final manuscript.

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### Competing Interests

The authors declare that they have no competing interests.

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