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Heparin binding proteins in seminal plasma of breeding buffalo bulls and their relation with semen freezability and *in vivo* fertility

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ABSTRACT

The present study was conducted to determine the concentrations of heparin binding proteins (HBP) in seminal fluid of breeding buffalo bulls and establish their relationship with frozen-thawed semen characteristics and fertility. Both fresh and frozen semen were collected from same ejaculate of 30 breeding buffalo bulls. Based on HBP concentrations in seminal plasma obtained from affinity chromatography, all the bulls were divided into 2 groups, viz. high fertility bulls (HFB, ≥ 6.5 mg/ml; n=15) and low fertility bulls (LFB, < 6.5 mg/ml; n=15) for further comparisons. The frozen-thawed semen of same ejaculate was evaluated for per cent CASA-based sperm kinetics, viability, Hypo-osmotic swelling test (HOST), acrosome integrity, *in vitro* acrosome reaction and first service conception rate (FSCR). The overall HBP concentrations were significantly higher in seminal plasma (7.31±0.14 vs 5.33±0.19 mg/ml) and fresh (0.66±0.03 vs 0.59±0.02 mg/10^9 sperms) sperm extracts of HFB than LFB. The frozen-thawed semen characteristics, viz. total motility, per cent acrosome reaction and FSCR were also significantly higher in bulls with high fertility than in lower ones. No significant difference was observed in sperm viability, HOST and acrosome integrity in the 2 groups of bulls. Total 12 HBP bands in range of 11 to 135 kDa were recorded on SDS-PAGE. Higher levels of HBP in seminal plasma of HFB might be responsible for their better semen quality and fertility.

Keywords: Buffalo bull, Breeding, Fertility, FSCR, HBP, 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. Consequently, attention is now being directed toward the assessment of molecular markers of semen quality as predictors of bull fertility. Development of markers to identify bulls of high breeding values represents a remarkable way for achieving genetic gain in dairy farming. Several factors in seminal plasma and/or spermatozoa have been investigated like heparin binding proteins (HBP), heat shock protein, clusterin, sperm adhesion, osteopontin and many other unidentified proteins that modulate the fertilizing ability of spermatozoa (Asquith *et al.* 2005). Among these, HBP and their close associates represent a superfamily of proteins in bovine. Five major HBP proteins with molecular weight of 18–55 kDa (18, 20, 24, 31 and 55 kDa) have been identified in buffalo seminal fluid which participate in sperm function and have been named as fertility associated antigen-5-complex (McCauley *et al.* 2001). Further, 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. For optimum fertility, a semen sample should have 6.5 mg/ml HBP (Chacur *et al.* 2010). Moreover, HBP on spermatozoa indicate its ability to undergo acrosome reaction along with protection from cryodamage (Kumar *et al.* 2008, Singh *et al.* 2017). However, studies on buffalo seminal plasma HBP and their relation with freezability and *in vivo* fertility are still meager and not fully understood. Keeping in view the above facts, the present study was undertaken to determine the HBP concentrations in seminal plasma of breeding buffalo bulls and their relation with semen freezability and *in vivo* fertility.

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MATERIALS AND METHODS

Semen procurement and preparation of sperm extracts: Healthy breeding buffalo bulls (30) maintained under identical conditions of feeding and management at government semen processing and freezing laboratory were used for collection of semen. Both fresh (1–2 ml per bull) and frozen semen (50 straws per bull) of same ejaculate were collected from all the bulls and earmarked for further investigations in September having 30.6°C ambient temperature and 92% relative humidity. The fresh (1–2 ml per bull) 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. 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 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 15000 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.

Purification of HBP by affinity chromatography: The HBP in seminal plasma, fresh- and frozen-thawed semen was obtained using heparin-sepharose affinity chromatography. The chromatography glass column (28 mm × 70 mm) was packed with heparin-sepharose media upto 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). Briefly, 750 µl of seminal plasma/fresh/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 extensively washed (7 to 8 times) 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). The molecular weight and quantity of HBP were confirmed by SDS-PAGE using 10% separating gel and 4% stacking gel (Laemmli 1970). The gels were run at a constant current of 30 mA and maximum 200 V for 2 h and stained with commassie brilliant blue. Gel images were captured on Syngene gel doc using Gene Snap image acquisition software (Syngene). On the basis of HBP concentration obtained from seminal plasma, all bulls were divided into 2 groups, viz. high fertility bulls (HFB, ≥ 6.5 mg/ml; n=15) and low fertility bulls (LFB, < 6.5 mg/ml; n=15) for further comparisons (Chacur *et al.* 2010).

Evaluation of post-thaw sperm functional assays: The frozen-thawed sperm of same ejaculate were evaluated for CASA-based motility, viability, hypo-osmotic swelling test (HOST), acrosome integrity and *in vitro* acrosome reaction. The numbers of sperm were converted to percentage.

Sperm motility: A previously validated computer assisted semen analysis (CASA; version Hamilton-Thorne IVOS 12.2) was used to denote total motility. Immediately after thawing in water bath (37°C for 30 sec), 10 µl semen was mounted on a pre-warmed CASA slide (Leja-8; IMV Technologies, L'Aigle, France). For each semen sample, 5 aleatory fields with atleast 150 sperm per field were considered and 5 semen samples (straws) per bull were evaluated to denote motility, obtaining 25 scans for each bull.

Viability: The live sperm count was determined through Eosin-Nigrosin staining technique. Briefly, a 10 µl aliquot of semen was thoroughly mixed with 10 µl of stain at 37°C and a thin smear was prepared on a clean and grease free glass slide from the semen stain mixture. The slides were observed under oil immersion at 100× of light microscope. About 200 live (white head), partial dead (light pink head) and dead (dark pink head) spermatozoa were counted in different fields and per cent sperm viability was calculated.

Hypo-osmotic swelling test (*HOST*)*:* Functional integrity of the sperm was evaluated by HOST using hypo-osmotic solution (HOS; 100 mosm/L). Frozen-thawed semen (20 µl) was mixed with 100 µl of HOS and incubated at 37°C for 30 min. Simultaneously, 20 µl of semen was incubated in 100 µl of phosphate buffer saline (PBS) under similar conditions. Incubated semen (10 µl) both from HOS and PBS was placed on separate glass slides and covered with a cover slip. The semen was examined under bright field microscope (100×) for curled tail spermatozoa. About 200 coiled and uncoiled spermatozoa were counted separately in PBS and HOS in different fields. The number of curled tail spermatozoa in PBS was deducted from that in HOS and the resultant figure was taken as the HOS-reactive sperm.

Acrosome integrity: Acrosome integrity of spermatozoa was assessed using Giemsa stain. Briefly, a smear (10 µl) of washed semen was prepared on a clean glass slide, air dried and fixed in methanol for 30 min. After drying, the smear was stained in Giemsa working solution for 4 h. The slides removed from the stain were rinsed quickly in DDW, air dried and examined under oil immersion (100×) of the bright field microscope. At least 200 spermatozoa with intact acrosome and damaged acrosome (partially or completely) from each slide were counted in different fields. The percent acrosome integrity was calculated as:

$$\text{Acrosome integrity (\%)} = \frac{\text{No of sperm with intact acrosome}}{\text{Total sperm counted}} \times 100$$

Acrosomal status: Frozen-thawed semen (10 straws per bull) was taken in 15 ml graduated tube and washed twice with the basic TALP medium (100 mM NaCl, 31 mM KCl, 25 mM $NaHCO_3$, 21.6 mM Na lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2.4H_2O$, 10 mM HEPES, 1 mM Na pyruvate, 0.6% BSA, 5 mM glucose and 10 µg/ml heparin) by centrifuging at 1,000 rpm for 5 min. The sperm suspension was then re-suspended in the energy rich TALP medium (0.5 ml), at 37°C for 6 h. After 6 h, a 10 µl sperm suspension was removed from the aliquot, smear was prepared, stained with Giemsa stain as mentioned for acrosome integrity and assessed for acrosome reaction using Giemsa stain. Evaluation of smear was carried under bright field microscope (100×). About 200 spermatozoa showing swelling, vesciculation and shedding of acrosome were counted in different fields.

The mean of 25 scans for sperm motility and mean of three replicates for per cent sperm viability, HOST, acrosome integrity and *in vitro* acrosome reaction were used

for the statistical analysis.

In vivo fertility rate: Inseminations (300) were performed under field conditions (pooled frozen-thawed semen of HFB: 150; pooled frozen-thawed semen of LFB: 150). All the buffaloes (300) enrolled for fixed time insemination program were healthy, recently calved (60–80 days earlier), multiparous (2nd to 5th parity), free from physical problems, vaginal discharge and maintained under standard feeding and management systems. Prior to start of breeding program, the clinical assessment of genitalia was done ultrasonographically twice at 10 days interval using a B-mode linear array trans-rectal transducer with 5/7.5 MHz interchangeable frequency (AGROSCAN, ECM, France) to visualize a cyclic CL. The buffaloes were synchronized using doublesynch protocol ($PGF2_{\alpha}$-GnRH-$PGF2_{\alpha}$-GnRH on day –2, 0, 7 and 9, respectively) followed by FTAI at 16 and 40 h after last GnRH injection, respectively. The pregnancy diagnosis was done on day 60 using ultrasonography. The first service conception rate (FSCR) was calculated on non-return basis according to the following formula:

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

Statistical analysis: The statistical analysis was performed with Statistical Package for Social Sciences (SPSS, version 16.0) program. The proportionality data (acrosome reaction and FSCR) were transformed using the arcsine transformation [asin (sqrt (percent/100))]. The mean±SE were calculated using arcsine transformed data in the software. Duncan's multiple range test and one way analysis of variance (ANOVA) was used for comparing the level of significance among the group of bulls of different gradients (HFB and LFB). The minimum significant interaction was considered at 5% level. When F-ratio was found significant ($P < 0.05$), least significant difference test was used to compare the means. The data on *in vivo* fertility rates were analyzed using Chi-square test.

The area under HBP curve was calculated by Simpson's 1/3rd rule (Jain *et al.* 1993).

RESULTS AND DISCUSSION

HBP concentrations in buffalo bull semen: HBP levels in seminal plasma and fresh sperm extracts were significantly ($P < 0.05$) higher in HFB than in LFB (Table 1; Fig. 1). Average HBP concentrations seen in this study was similar to an earlier report in buffalo bulls (Arangasamy *et al.* 2005; 6.61 mg/ml), but are much lower than those in cattle semen as reported by Srivastava *et al.* (2012; 27.9 mg/ml). This difference in HBP levels could merely be a species variation and/or inherent character. Moreover, lower concentrations of HBP in buffalo semen could also be attributed to lower levels of blood testosterone in buffalo than cattle bulls (Kulkarni *et al.* 1995). Similarly, the area under HBP curve was significantly greater ($P < 0.05$) in HFB (1035.7±30.0 mm^2) than in LFB (879.3±30.2 mm^2). Furthermore, it was also significantly ($P < 0.05$) more in HFB as compared to LFB for fresh sperm extracts (Fig. 2). Limited studies in buffalo bulls have revealed that HBP concentrations were drastically suppressed in bulls with sub-fertility (Singh *et al.* 2014). The overall HBP concentration was much higher ($P < 0.05$) in seminal plasma than in fresh sperm extracts in both the groups. Seminal plasma appeared to further intensify the release of HBP. Previous studies (McCauley *et al.* 2001) have shown that HBP are primarily produced from accessory sex glands, secreted into seminal fluid and bound to sperm at ejaculation. Therefore, a greater concentration in seminal plasma could possibly be due to the site of HBP secretion at ejaculation (D'Amours *et al.*

Table 1. HBP concentrations, post-thaw sperm characteristics and *in vivo* fertility of buffalo bulls (Mean±SEM)

Parameter	HFB (n=15)	LFB (n=15)
HBP-SP (mg/ml)	7.31±0.14^{aX}	5.33±0.19^{bX}
HBP-FSE (mg/10^9 sperms)	0.66±0.03^{aY}	0.59±0.02^{bY}
Area under HBP-SP curve (mm^2)	1035.7±30.0^{a}	879.3±30.2^{b}
Total motility (%)	58.7±1.9^{a}	52.2±2.4^{b}
Progressive motility (%)	31.7±2.6	27.4±2.8
VAP (μm/s)	108.6±5.0	103.7±3.6
VSL (μm/s)	85.3±3.7	85.8±2.8
VCL (μm/s)	170.3±6.8	166.1±4.3
ALH (μm)	6.5±0.6	6.7±0.4
BCF (Hz)	35.3±0.6	34.4±0.7
STR (%)	82.8±1.0	82.9±1.1
LIN (%)	53.0±1.8	54.8±1.6
Viability (%)	68.8±3.3	69.8±2.4
HOST (%)	66.0±1.5	67.1±3.2
Acrosome integrity (%)	75.3±1.6	75.7±1.4
Acrosome reaction 0 h (%)	6.3±0.8	5.3±0.6
Acrosome reaction 6 h (%)	59.2±2.9^{a}	51.9±2.4^{b}
First service conception rate (%)	42.7±4.6^{a}	31.3±4.0^{b}

[a,b]Values with different superscripts differ significantly (P<0.05) in the same row. [X,Y]Values with different superscripts differ significantly (P<0.05) in the same column. HBP-SP, Heparin binding proteins in seminal plasma; HBP-FSE, Heparin binding proteins in fresh sperm extracts; VAP, Velocity Average Path; VSL, Velocity Straight Line; VCL, Velocity Curvilinear; ALH, Amplitude of Lateral Head displacement; BCF, Beat Cross Frequency; STR, Straightness; LIN, Linearity.

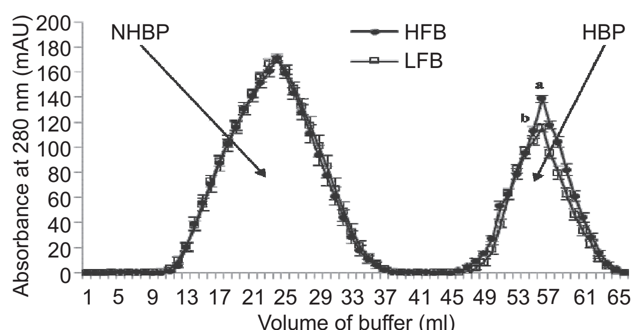

Fig. 1. HBP and NHBP concentrations in seminal plasma of high and low fertility buffalo bulls separated by heparin-affinity chromatography. a differs from b (P<0.05).

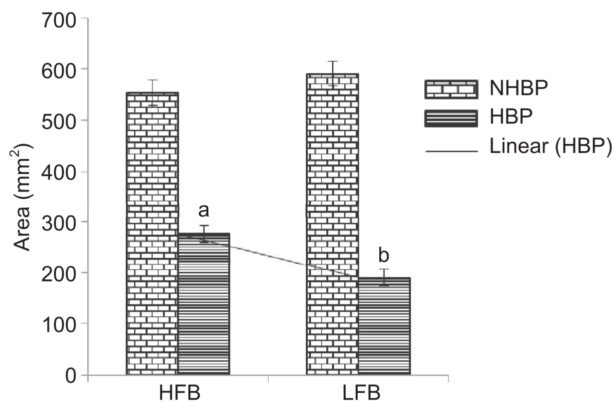

Fig. 2. HBP and NHBP concentrations in seminal plasma of high and low fertility buffalo bulls separated by heparin-affinity chromatography. a differs from b (P<0.05).

2010) which gives a more legitimate picture.

Post-thaw semen characteristics of buffalo bulls: In frozen-thawed semen, CASA-based motility, *in vitro* acrosome reaction and FSCR were significantly (P<0.05) higher in HFB as compared to LFB semen samples (Table 1). The progressive motility was also considerably (P>0.05) higher in bulls with high fertility than in their counterparts. The sperm kinetic traits, viz. VAP, VSL, VCL, ALH, BCF, STR and LIN differed non-significantly (P>0.05) in high and low fertility bulls. Nevertheless, the differences, if any, in kinetic traits may be due to variation in level of freezing damage to the sperm (Andrabi 2009). A significant increase in post-thaw motility in the semen of HFB might possibly be due to higher levels of HBP which results in greater ability to maintain intracellular sperm homeostasis, minimizes the production of reactive oxygen species and lipid peroxidation and makes spermatozoa less prone to damage from stress of freezing and thawing process (Kadirvel 2006). There was no significant (P>0.05) difference in the percentage of sperm viability, HOST and acrosome integrity in present study. Similarly, Harshan *et al.* (2006) observed no difference in functional integrity of membrane (72.0±0.93 vs 73.33±0.87%) and acrosome integrity (80.42±0.82 vs 80.58±0.90%) in control and HBP treated buffalo semen. The assessment of inducibility of acrosome reaction remained similar (P>0.05) in bulls of both groups at 0 h and was significantly (P<0.05) higher after 6 h of incubation in HFB which is in accordance to an earlier report by Patel *et al.* (2016). 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).

In vivo *fertility of cryopreserved buffalo sperm:* The FSCR was significantly (P<0.05) higher in HFB than in their counterparts. The HFB had about 11% higher conception rate than LFB. Determination of fertility rate after artificial insemination could help predict the quality of cryopreserved semen accurately (Mirmahmoudi and Prakash 2012). Previous studies (Sprott *et al.* 2000) in cattle bulls have recorded 15–17% higher pregnancy rates in females inseminated with HBP-positive spermatozoa than those inseminated with HBP-negative spermatozoa. Further, Karunakaran and Devanathan (2017) also obtained 13% higher fertility from HBP-positive bulls as compared to their negative counterparts. Bulls with higher HPB produced sperm with greater affinity to bind heparin-like complex sugars that were commonly found in the reproductive tract of females. HBP mediate sperm binding to oviductal epithelium and exert inhibiting effects on the mitochondrial activity and metabolism to conserve energy needed until fertilization. Thus, higher concentrations of HBP could be linked to its ability to mediate these events which are crucial for successful fertilization.

Electrophoretic characterization of HBP by SDS-PAGE: In seminal plasma, fresh- and frozen-thawed sperm extracts, total 12 protein bands were observed on SDS-PAGE of HBP in the range of 11 to 135 kDa (11, 16, 20, 26, 31, 37, 40, 45, 65, 75, 100 and 135 kDa). A qualitative difference (presence or absence of bands) was observed between electrophoretic profiles of HBP present in HFB and LFB semen samples. The results are in agreement with the findings of Arangasamy *et al.* (2005) and Kumar *et al.* (2008) who observed 8 (11, 14, 18, 20, 31, 41, 65 and 71 kDa) and 6 (14, 20, 24, 31, 41 and 65 kDa) major HBP on SDS-PAGE in buffalo seminal plasma, respectively. Further, Singh *et al.* (2013) 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 with differences between freezable and non-freezable semen samples. The difference in number of bands may be due to inherent character, and also it could be attributed to aggregation products of low molecular weight proteins or degradation of high molecular weight proteins.

It is concluded that, higher levels of HBP in semen of bulls with high fertility might be responsible for its better freezability and fertility as reflected by increased post-thaw sperm motility, *in vitro* acrosome reaction and FSCR.

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