



Seminal Plasma Heparin Binding Proteins Improve Semen Quality by Reducing Oxidative Stress during Cryopreservation of Cattle Bull Semen

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ABSTRACT: Heparin binding proteins (HBPs) are produced by accessory glands. These are secreted into the seminal fluid, bind to the spermatozoa at the time of ejaculation, favour capacitation, acrosome reaction, and alter the immune system response toward the sperm. The present study was conducted with an objective to assess the effect of purified seminal plasma-HBPs (SP-HBPs) on cross bred cattle bull sperm attributes during two phases of cryopreservation: Pre freezing and freezing-thawing. SP-HBPs were purified from pooled seminal plasma by heparin affinity chromatography. Three doses of SP-HBPs i.e. 10, 20, 40 µg/mL semen were standardized to find out the optimum dose and 20 µg/mL was found to be an optimum dose. Semen as such and treated with SP-HBPs was diluted with sodium citrate-egg yolk diluter and cryopreserved as per the standard protocol. Sperm parameters i.e. motility, viability, Hypo-osmotic swelling test (HOST), acrosome damage, *in vitro* capacitation and lipid peroxidation were evaluated in SP-HBP treated and untreated (control) semen at both phases of cryopreservation. A considerable variation in percent sperm motility, viability, membrane integrity (HOST), acrosome damage, acrosome reaction and lipid peroxidation was observed at both phases among the bulls irrespective of the treatment. Incubation of neat semen with 20 µg/mL SP-HBP before processing for cryopreservation enhanced the average motility, viability, membrane integrity by 7.2%, 1.5%, 7.9%, and 5.6%, 6.6%, 7.4% in pre-frozen and frozen-thawed semen in comparison to control. There was also an average increase of 4.1%/3.9% in *in vitro* capacitation and acrosome reaction in SP-HBPs-treated frozen-thawed semen as compared to control. However, binding of SP-HBPs to the sperm declined acrosome damage and lipid peroxidation by 1.3%/4.1% and 22.1/32.7 µM/10⁹ spermatozoa in SP-HBP treated pre-frozen/frozen-thawed semen as compared to control, respectively. Significant (p<0.05) effects were observed only in motility, HOST and *in vitro* acrosome reaction. It can be concluded that treatment of neat semen with SP-HBPs before cryopreservation minimized the cryoinjury by decreasing the generation of reactive oxygen species. (**Key Words:** Seminal Plasma, Heparin Binding Protein [HBP], Cryopreservation, Function Tests, Sperm, Cattle Bulls)

INTRODUCTION

Sperm cryopreservation is an important tool for assisted reproductive techniques and is the most efficient way for storing germ plasm (Olaciregui et al., 2014). Cryopreservation has been applied to various species, including humans, swine, cattle, cats, and dogs (Mota Filho et al., 2014). Although sperm cryopreservation preserves sperm motility, metabolic functions, and fertility, but, still the freeze-thawing process causes irreversible damage to

spermatozoa and reduces fertility (Ardon and Saurez, 2013). Even though cryopreservation helps in prolonged storage of semen, but cryo-capacitation is one of the major factors associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract (Watson, 2000), resulting in reduced fertility of frozen-thawed semen. Even with optimized protocols, 40% to 50% of the sperms do not survive after cryopreservation due to irreversible damage (Watson, 2000). Several studies have focused on identifying damages during freezing and thawing, tests to screen sperm quality of frozen-thawed semen, evaluation of alternative cryoprotectants and other additives, and freezing procedures to improve sperm viability and fertility. Recently, Yoon et al. (2015) proposed

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that freezing/thawing steps are the most critical in cryopreservation and may provide a logical ground to understand the process of cryo-damage.

Currently, many diverse proteins are being tested that may increase the fertilization capability of sperm and heparin binding proteins (HBPs) are among these proteins. HBPs have been viewed as favouring capacitation, acrosome reaction, and altering the immune system response toward the sperm. The HBPs are produced by the accessory glands of the male under the control of androgens (Miller et al., 1990) and secreted within the seminal fluid (Nass et al., 1990). The HBPs bind to the epididymal sperm and increase the ability of the acrosome reaction in response to the heparin and other proteins of the zona pellucida (Ax et al., 2002). Absorption of isolated proteins from the seminal plasma can reduce the damage that cold shock can have on the sperm membrane (Barrios et al., 2000). Mogielnicka-Brzozowska and Kordan (2011) mentioned the possibility of adding specific plasma proteins to sperm for the retention of features responsible for the efficient fertilization after storage. HBPs protect sperm from stress of freezing and thawing and maintained intracellular protein homeostasis (Shi et al., 1998). HBPs also protect sperm from lipid peroxidation during cryopreservation (Kumar et al., 2008). Preliminary studies were done by Alvarez-Gallardo et al. (2013) with the recombinant proteins indicated that addition of rFAA and rTIMP-2 to semen before cryopreservation help stabilize the acrosome membrane of the bovine sperm by decreasing post-thaw acrosome damage. Hence the present study was conducted with objective to assess the effect of HBP on sperm function tests at pre-freeze and frozen-thawed phases of cryopreservation.

MATERIALS AND METHODS

Procurement of semen sample

Three ejaculates from each of six healthy cross cattle bulls (Holstein-Friesian×Red Dane×Sahiwal) were procured from GADVASU, Dairy Farm and Semen bank, Bhattian, Khanna, Ludhiana, India. The bulls were kept under loose housing system (covered area, 3.6 m×3.0 m and uncovered area, 7.5 m×3.0 m) and standard feeding schedule along with *ad libitum* green fodder and water availability and standard management conditions. Semen was collected twice a week through artificial vagina method. None of the bulls selected for this study had any preceding physical abnormalities.

A good quality fresh ejaculates (1 to 2 mL) with minimum initial motility of $\geq 60\%$, mass activity of 3+ to 4+, abnormal morphology of $\leq 25\%$ and sperm concentration of $>750 \times 10^6$ sperms/mL of each bull were processed for cryopreservation. Seminal plasma was separated by

centrifuging pooled semen of six bulls at 3,000 rpm for 5 minutes and was stored at -20°C till use.

Purification of HBPs by affinity chromatography

Sepharose (Sigma-Aldrich) was activated and heparin was bound as per the protocol of Farooqui (1980). 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 to 4 h. Thereafter, the column was equilibrated for 1 h with 10 mM Tris HCl (pH 7.4). About 1.0 mL of pooled seminal plasma (filtered through 0.22 μ filter) was loaded and circulated through the column for 15 minutes for absorption of HBPs to the heparin bound resins. The non-HBPs were washed out with 10 mM Tris HCl and the 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 fractions were pooled in agreement with the observed curve, obtained from optical density (280 nm), detected by UV monitor, attached to the fraction collector (Manaskova et al., 2002). The pooled fractions of HBPs were concentrated by spinning through protein concentrators (Millipore, Billerica, MA, USA, 3 kDa) and analyzed for protein content (Lowry et al., 1951).

Conjugation of fluorescein isothiocyanate fluorochrome with purified HBP and confirmation of incorporation of SP-HBP to semen

Conjugation of fluorescein isothiocyanate (FITC) fluorochrome to SP-HBPs was done by using a kit (Life technology, Carisbad, CA, USA) as per the protocol provided with the kit. About 20 μg of purified HBPs was added to 1.0 mL of neat semen and incubated at 37°C for 20 minutes. Semen was washed thrice with phosphate buffered saline (PBS), pH 7.4 by centrifugation at 3,000 rpm for 5 min. Pellet was suspended in 1.0 mL of PBS and a drop of semen on a glass slide covered with coverslip was observed at 400 \times under fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) using blue filter at 480 nm.

Dilution and cryopreservation of semen

Three doses of SP-HBPs i.e. 10, 20, 40 $\mu\text{g}/\text{mL}$ semen were standardized to find out the optimum dose and 20 $\mu\text{g}/\text{mL}$ was found to be an optimum dose. Semen was divided into two fractions: one fraction, supplemented with SP-HBP and other as such (control), were incubated at 37°C for 20 min. After incubation, both fractions were diluted with sodium citrate extender. The pre-freezing sample was kept at 4°C in cold handling cabinet for equilibration and aliquot for freezing was filled and sealed by manual filling and sealing method. The filled and sealed straws were cryopreserved in liquid nitrogen by following standard procedure. Semen at pre-freeze and frozen-thaw phase was analyzed for sperm function (motility, viability and Hypo-

osmotic swelling test [HOST]), acrosome damage, acrosome reaction and lipid peroxidation [LPO]) as detailed below.

Individual motility

About 10 μL of semen was placed on a pre-warmed (37°C) glass slide, covered with a cover slip and placed on the pre-warmed stage (Magnus Mumbai, Maharashtra, India) attached to a phase contrast microscope (Olympus CX21FS1, Japan). Linear progressive motility was assessed by subjective method viewing 10 fields with the aid of closed-circuit television attached to the microscope (400 \times). A total of 200 spermatozoa were observed under phase contrast microscope (Olympus, Japan) at 400 \times in different fields and percentage of motile spermatozoa was calculated.

Sperm viability

Sperm viability was assessed by Syber green/propidium iodide (Life technologies staining kit, USA). A total of 200 live (green) and dead (red) spermatozoa were counted in different fields and percentage of viability was calculated (Figure 1).

Hypo-osmotic swelling test

Briefly, 10 μL of semen was incubated in 100 μL of hypo-osmotic solution at 37°C for 30 min. A total of 150 spermatozoa with coiled and un-coiled tails were counted in different fields (Jeyendran et al., 1984). A control was also run in PBS (pH 7.4). The number of spermatozoa with coiled tailed/swollen heads in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the Hypo-osmotic swelling (HOS)-reactive

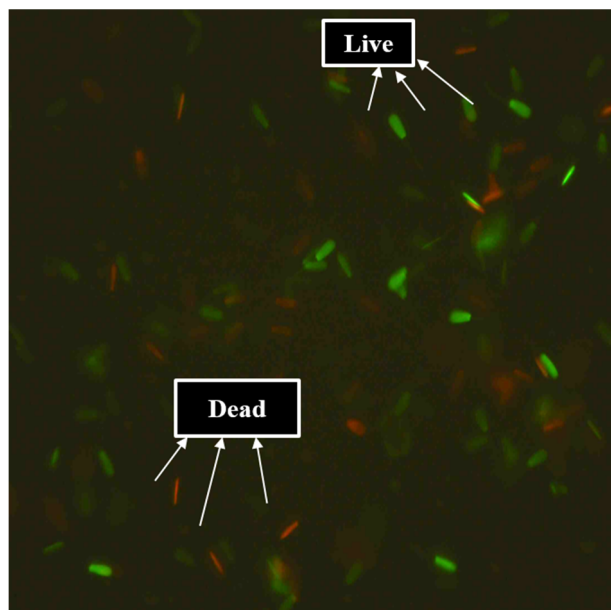


Figure 1. Cattle bull spermatozoa stained with syber green/propidium iodide (400 \times).

spermatozoa.

Acrosomal damage

Acrosome damage was assessed by chlortetracycline cysteine stain (CTC, Figure 2) (Ward and Storey, 1984). Briefly 25 μL of sperm suspension was mixed with 25 μL of 750 mM CTC in 20 mM Tris-HCL, 130 mM NaCl and 5 mM cysteine, incubated at 37°C for 30 min. After adding 100 μL of 12.5% paraformaldehyde and one drop of 0.22 M 1, 4-Diazabicyclo[2.2.2]octane, a drop of sperm suspension was observed under fluorescence microscope (Olympus,

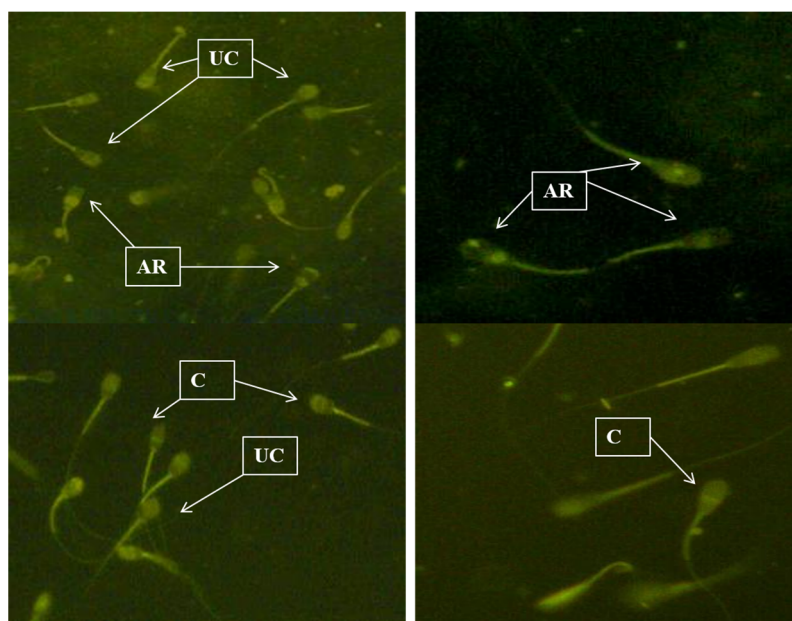


Figure 2. CTC staining of acrosome damage and *in vitro* capacitated/acrosome reacted cattle bull spermatozoa (400 \times). CTC, chlortetracycline cysteine; UC, uncapacitated; C, capacitated; AR, acrosome reacted.

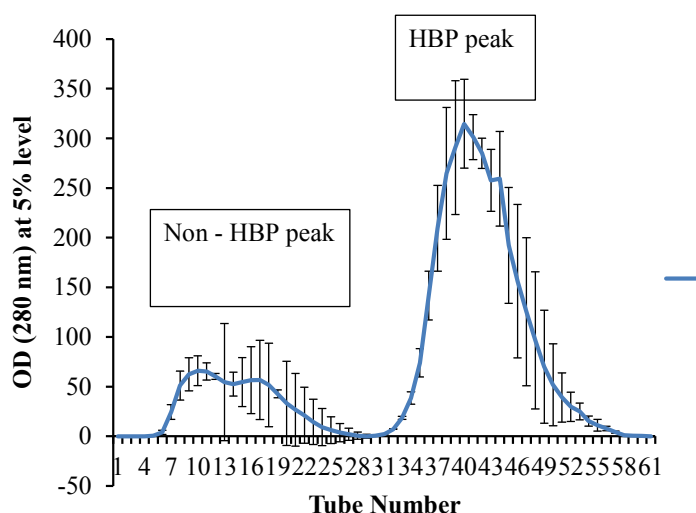


Figure 3. Graphic image of heparin-binding and non-heparin-binding proteins in seminal plasma of cross bred cattle bulls separated on heparin-affinity chromatography.

Japan) at 400× using blue filter at 480 nm. At least 200 spermatozoa with normal (whole sperm head with bright fluorescence) and cryo capacitated sperm (acrosome-intact sperm with fluorescence ring at equatorial segment) and acrosome-reacted (sperm with fluorescence on postacrosomal region) were counted in different fields at 400× and percentage of cryo-capacitated and acrosome-reacted spermatozoa was calculated (Figure 2).

***In vitro* capacitation/acrosome reaction**

Frozen-thawed spermatozoa washed twice with tyrode albumin lactate pyruvate medium (TALP, 92.9 mM NaCl, 4 mM KCl, 25.9 mM NaHCO₃, Na₂HPO₄, 10 mM CaCl₂·2H₂O, 0.5 mM MgCl₂·6H₂O, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid, pH 7.4 were subjected to swim up and only motile spermatozoa were incubated in TALP supplemented with 0.6% bovine serum albumin, 5 mM glucose and 165 units of heparin for 4 hrs at 37°C in an incubator (Fraser et al., 1995). Capacitation/acrosome reaction was assessed by counting 200 spermatozoa in CTC stained slides under fluorescence microscope at 400× (Olympus, Japan).

Lipid peroxidation

Lipid peroxidation was estimated by the end point generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test (Buege and Steven, 1978). Briefly, extended spermatozoa ([40 to 70]×10⁶ cells) in 0.5 mL of cold 10% (wt/vol) chilled trichloroacetic acid to proliferate proteins. The precipitate was pelleted by centrifugation (6,000 rpm for 10 min) and 1 mL of 0.375% (wt/vol) TBA was added to the supernatant and kept in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was read at 532 nm (UV-VIS

spectrophotometer, systronics, Ahmedabad, Gujrat, India).

MDA production was calculated by the formula:

$$[\text{OD at 532 nm} \times \text{volume of assay mixture}] / [\text{molar extinction coefficient} \times \text{volume of sample}]$$

* Molar extinction coefficient = (1.56×10⁵ M⁻¹C⁻¹)

Statistical analysis

The data is presented as mean±standard error of the mean and analyses were performed with CPCS1 program package. One way analysis of variance was applied to evaluate the level of significance at 5% between treatments.

RESULTS AND DISCUSSION

Purification of HBPs from seminal plasma and verification of binding of SP-HBP to spermatozoa

Graphic image of HBP purified from pooled seminal plasma of cross-bred bulls is shown in Figure 3, which indicated two peaks of unbound (non-HBP) and bound proteins (HBP). Addition of 20 µg/mL of SP-HBP to neat semen and incubation at 37°C for 20 min before cryopreservation resulted in its binding mainly to head and mid piece. But binding was more on post-acrosomal cap in majority of the spermatozoa (Figure 4). A study done by Cheema et al. (2015) on natural localization of HBPs in cross-bred bull spermatozoa using anti-AZU-1 revealed that these proteins are mainly localized on acrosomal cap.

Effect of SP-HBP on sperm function tests

Standardization of dose of SP-HBP: Effects of different doses of SP-HBP on percent motility, viability, HOST, acrosome damage, *in vitro* capacitation and LPO in pre-freeze and frozen-thaw semen are given in Table 1. Individual motility (%), viability (%), HOST (%) were

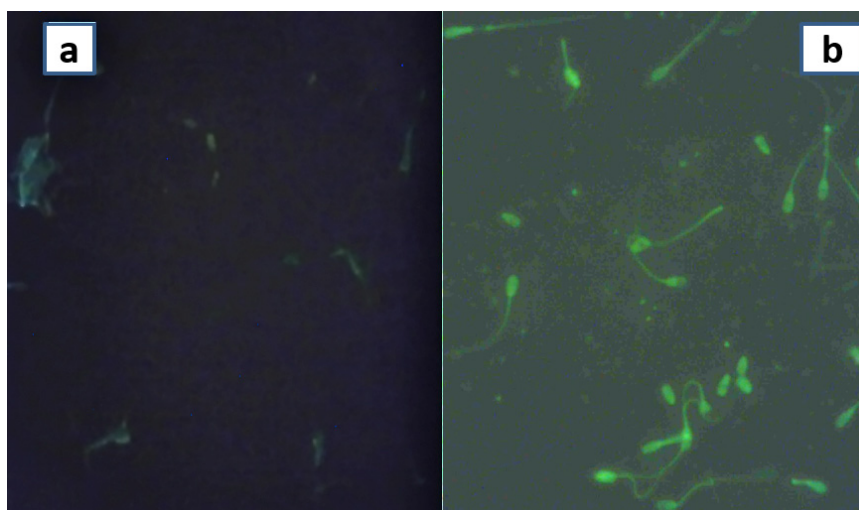


Figure 4. Visualization of binding of SP-HBP to spermatozoa (400 \times). (a) control (Semen incubated without SP-HBP), and (b) treated (Neat semen incubated with SP-HBP). SP-HBP, seminal plasma-heparin binding protein.

found to be higher in pre-freeze and post-thaw semen supplemented with 20 $\mu\text{g}/\text{mL}$ of SP-HBP as compared to 0, 10, and 40 μg of SP-HBP. Percentage of *in vitro* capacitated/acrosome reacted spermatozoa was higher only in frozen-thawed semen supplemented with 20 $\mu\text{g}/\text{mL}$ SP-HBP as compared to 0, 10, and 40 μg . Acrosome damage (%) and MDA ($\mu\text{M}/10^9$ spermatozoa) showed reduction in semen supplemented with 20 $\mu\text{g}/\text{mL}$ of SP-HBP in comparison to 0, 10, and 40 μg SP-HBP at both phases of cryopreservation. Increase in motility, viability, *in vitro* acrosome reaction and decrease in LPO (MDA) in semen supplemented with SP-HBP was statistical significant ($p < 0.05$). Manjunath et al. (2002) also suggested that at a greater concentration of bovine seminal plasma proteins, more cholesterol and phospholipids are removed from the sperm membrane, resulting in its destabilization that decreases sperm resistance to cold shock and freezing.

Harshan et al. (2006) also postulated that higher loss of semen parameters with higher amount of HBP could be due to an increased cholesterol efflux in the spermatozoa which led to more pronounced cryo-damage. Therefore, effects of 20 $\mu\text{g}/\text{mL}$ of SP-HBP on semen cryopreservation of six bulls were evaluated.

Effect of 20 μg SP-HBP on sperm function and fertility tests of six bulls

Motility, Viability and HOST: Mean percentage of motile, live and HOS responsive spermatozoa showed a variation among six cross breed bulls, which ranged from 61.7% \pm 1.7% to 70 \pm 2.9%; 55.7% \pm 2.8% to 73.0% \pm 6.6%; 16.0% \pm 0.7% to 36.1% \pm 8.0%, and 41.7% \pm 4.4% to 58.3% \pm 6.7%; 38.2% \pm 3.1% to 59.5% \pm 2.2%; 8.8% \pm 4.6% to 27.5% \pm 7.5% at the time of pre-freezing and freezing-thawing, respectively (Table 2). Decrease observed in

Table 1. Effect of different doses of affinity purified SP-HBP on sperm function and fertility tests during pre-freeze and frozen-thaw phases of cryopreservation

Sperm parameter	Pre-freeze				Frozen-thaw			
	C ¹	10	20	40	C	10	20	40
Motility (%)	73.3 \pm 1.7 ^a	76.7 \pm 1.7 ^a	83.3 \pm 1.7 ^b	75.0 \pm 0.0 ^a	45.0 \pm 2.9 ^c	46.7 \pm 1.7 ^c	58.3 \pm 4.4 ^d	48.3 \pm 4.4 ^c
Viability (%)	68.7 \pm 3.6 ^{ab}	61.2 \pm 1.2 ^a	76.2 \pm 3.5 ^{ab}	72.4 \pm 3.1 ^b	48.7 \pm 8.7 ^c	47.8 \pm 6.2 ^c	56.0 \pm 7.6 ^c	52.4 \pm 8.2 ^c
HOST (%)	35.3 \pm 2.4 ^a	38.2 \pm 4.3 ^a	42.1 \pm 4.1 ^a	35.72 \pm 2.2 ^a	35.0 \pm 2.2 ^a	12.1 \pm 2.4 ^b	18.6 \pm 3.9 ^b	14.4 \pm 3.0 ^b
Acrosome damage (%)								
Cryo-Cap	22.9 \pm 2.6 ^a	22.1 \pm 1.6 ^a	23.3 \pm 1.5 ^a	24.5 \pm 1.8 ^a	29.2 \pm 2.9 ^b	28.1 \pm 3.4 ^b	28.4 \pm 1.7 ^b	30.1 \pm 2.4 ^b
Cryo-AR	12.1 \pm 4.5 ^a	10.1 \pm 3.8 ^a	10.5 \pm 4.4 ^a	11.5 \pm 4.9 ^a	16.3 \pm 4.3 ^a	13.8 \pm 2.8 ^a	14.6 \pm 3.7 ^a	17.0 \pm 4.2 ^a
<i>In vitro</i> capacitation/acrosome reaction (%)								
Cap	-	-	-	-	39.1 \pm 3.4 ^a	39.4 \pm 2.8 ^a	44.4 \pm 3.0 ^a	42.5 \pm 3.2 ^a
AR	-	-	-	-	27.9 \pm 5.8 ^a	30.1 \pm 6.1 ^{ab}	34.0 \pm 5.1 ^b	33.4 \pm 5.0 ^b
LPO (MDA, $\mu\text{M}/10^9$ spermatozoa)	14.7 \pm 5.7 ^a	12.4 \pm 3.8 ^a	11.9 \pm 3.6 ^a	14.5 \pm 4.6 ^a	50.3 \pm 2.8 ^b	44.3 \pm 4.1 ^{bc}	40.3 \pm 2.6 ^c	45.3 \pm 4.9 ^{bc}

HOST, hypo-osmotic swelling test; Cap, capacitated; AR, acrosome reacted; LPO, lipid peroxidation; MDA, malondialdehyde.

¹ C, control (0), 10, 20, 40 (Dose of HBP in $\mu\text{g}/\text{mL}$).

Superscripts (a, b, ab, bc) indicate the difference at 5% level of significance within the rows.

Table 2. Effect of affinity purified SP-HBP on sperm motility, viability and HOST (mean±SE) during pre-freeze and frozen-thaw phases of cryopreservation

Bull number	Motility (%)				Viability				HOST			
	Pre-freeze		Frozen-thawed		Pre-freeze		Frozen-thawed		Pre-freeze		Frozen-thawed	
	C ¹	T ¹	C	T	C	T	C	T	C	T	C	T
1	70.0±0.0	80.0±0.0	58.3±3.4	66.6±2.9	73.0±6.6	79.6±4.5	59.5±2.2	64.8±2.7	30.6±3.7	36.2±3.3	18.1±3.2	25.1±4.9
2	70.0±2.9	78.3±3.3	55.0±2.9	61.7±1.7	69.1±7.5	76.9±5.8	54.9±7.1	61.6±6.0	32.5±8.1	54.6±2.5	16.9±5.9	27.8±4.8
3	68.3±4.4	73.3±4.4	50.0±5.8	56.6±4.4	65.9±3.8	71.0±3.6	50.4±8.8	56.4±6.1	36.1±8.0	46.3±6.2	27.5±7.5	35.3±5.7
4	61.7±1.7	70.0±2.9	45.0±2.9	50.0±2.9	61.3±2.1	67.0±1.0	43.0±7.2	47.2±7.2	25.3±5.1	30.1±3.3	17.7±1.7	20.7±2.1
5	63.3±3.3	68.3±3.3	41.7±4.4	43.3±3.3	55.7±2.8	59.3±3.3	38.2±3.1	44.9±0.2	16.0±0.7	22.0±2.6	8.8±4.6	12.6±3.2
6	65.0±2.9	71.7±1.7	46.7±6.0	51.7±6.0	65.1±0.5	69.3±1.8	48.5±1.4	59.5±0.7	21.6±4.6	32.5±4.0	12.6±2.0	24.6±1.5
Combination factor mean	66.4 ^a	73.6 ^b	49.4 ^c	55.0 ^d	65.0 ^a	70.5 ^a	49.1 ^b	55.7 ^b	27.0 ^a	36.9 ^b	16.9 ^c	24.3 ^a

SP-HBP, seminal plasma-heparin binding protein; HOST, hypo-osmotic swelling test; SE, standard error.

¹ C, control without HBP; T, supplemented with SP-HBP.

Superscripts a, b, c, d indicate the difference at 5% level of significance within the columns.

percent motility, viability and HOST from pre-freeze to post thaw phase of cryopreservation was significant ($p < 0.05$). Lessard et al. (2000) were of the opinion that sperm viability decrease by 50%, whereas, fertilizing capacity is affected by a factor of sevenfold after cryopreservation. However, incorporation of SP-HBP to the sperm surface resulted in an increase of 5.0% to 10.0%; 3.6% to 7.8%; 4.8% to 22.1%, and 1.6% to 8.3%; 4.2% to 11.0%; 3.0% to 12.0% in motile, live and HOS positive spermatozoa at pre-freeze and frozen-thaw phase of cryo-preservation as compared to control, respectively. However, increase in only motility and HOST due to supplementation of SP-HBP was significant ($p < 0.05$) at both phases of cryopreservation. Harshan et al. (2006) revealed that 40 µg dose of HBP improved the motility, HOST and cervical mucus penetration test at pre-freeze phase of buffalo semen cryopreservation, but they did not observe positive effect of this dose on frozen-thawed semen. Barrios et al. (2000) also demonstrated that the absorption of isolated proteins from seminal plasma may reduce the cold shock effect.

Acrosome damage: Two stages of acrosome damage

were revealed by CTC staining i.e. partial (cryo-capacitated) and complete (cryo-acrosome reacted). These two phases of sperm due to cryo-injury are demonstrated as premature capacitation and spontaneous acrosome reaction (Srivastava et al., 2013). Mean percentage of cryo-capacitated and acrosome reacted spermatozoa showed a variation among the bulls and ranged from 20.1%±1.1% to 30.6%±1.5% and 5.3%±0.3% to 14.3%±4.1% at pre-freeze phase, which was further significantly ($p < 0.05$) increased to 25.6%±4.5% to 37.5%±0.7% and 10.9%±1.3% to 21.1%±0.5% in frozen-thaw phase of cryopreservation in six bulls, respectively (Table 3). Altered mitochondrial function, reduction in motility and failure of chromatin condensation, all of which influence the viability and fertility of sperm have also been reported due to induction of pre-mature acrosome reaction (Watson, 2000; Chaveiro et al., 2006). Acrosomal cap is of utmost importance for fertilization and is related to fertility. Spermatozoa with higher motility cannot be fertile due to acrosomal loss (Raval and Dhama, 2010).

Acrosome is required to undergo biochemical changes in the female reproductive tract before fertilization and

Table 3. Effect of affinity purified SP-HBP on acrosome damage (mean±SE) during pre-freeze and frozen-thaw phase of cryopreservation

Bull number	Pre-freeze				Frozen-thawed			
	Control ¹		Treated ¹		Control		Treated	
	Capacitated	Acrosome reacted	Capacitated	Acrosome reacted	Capacitated	Acrosome reacted	Capacitated	Acrosome reacted
1	20.2±3.4	14.3±4.1	21.2±3.6	11.7±3.7	25.6±4.5	16.8±3.6	23.6±3.4	15.8±3.5
2	25.1±3.8	9.6±2.1	25.2±3.7	8.5±2.8	30.8±3.3	13.3±2.0	21.3±0.8	4.9±0.6
3	20.1±1.1	5.3±0.3	20.3±0.8	4.9±0.6	26.1±1.4	10.9±1.3	26.3±1.4	10.1±0.8
4	22.9±1.0	6.6±0.1	24.4±0.7	5.2±0.1	32.9±0.8	14.0±3.1	33.9±0.4	12.4±2.6
5	30.6±1.5	9.0±0.4	29.6±0.9	10.8±0.7	37.5±0.7	13.5±2.0	35.0±1.2	15.0±2.0
6	21.5±1.0	14.1±2.1	22.1±0.5	12.2±2.6	29.5±0.6	21.1±0.5	29.6±0.2	19.8±1.4
Combination factor mean	23.4 ^a	9.8 ^a	23.08 ^a	8.8 ^a	30.4 ^b	14.9 ^{bc}	28.2 ^{bc}	13.0 ^c

SP-HBP, seminal plasma-heparin binding protein; SE, standard error.

¹ Control, without HBP; Treated, supplemented with HBP.

Superscripts a, b, bc, c indicates the difference at 5% level of significance within column.

premature loss of acrosome and hence loss of enzymes will affect the fertilization process. Supplementation of SP-HBP to cattle bull semen resulted in a non-significant ($p < 0.05$) reduction of 1.0% to 1.4% and 0.6% to 17.9% in acrosome damage at pre-freeze and frozen-thaw phase, respectively. Our study revealed a reduction in cold shock effect on treating the spermatozoa with SP-HBP. Result of a study by Mogielnicka-Brzozowska et al. (2011) also indicated that zona binding proteins of boar seminal plasma have a shielding effect on the plasma membrane and the acrosome of spermatozoa by protecting their structures against the damage caused by cold shock. Mogielnicka-Brzozowska and Kordan (2011) mentioned that addition of specific plasma protein to sperm probably may retain the features responsible for the efficient fertilization after storage.

In vitro capacitation and acrosome reaction: A variation in response of frozen-thawed spermatozoa to *in vitro* capacitation and acrosome reaction was also observed irrespective of treatment among the six bulls. Incubation of frozen thawed spermatozoa of six bulls in TALP energy medium resulted in 36.6%±2.1% to 45.6%±1.8% capacitated and 23.9%±2.2% to 31.7%±1.7% acrosome reacted spermatozoa (Table 4). Incorporation of SP-HBP enhanced the percentage of capacitated (41.9%±1.7% to 48.5%±0.2%) and acrosome reacted (26.6%±2.5% to 33.5%±5.4%) spermatozoa in frozen-thawed semen of six bulls. It indicated a significant ($p < 0.05$) increase of 6.2% to 11.4% in capacitated/acrosome reacted spermatozoa upon binding of SP-HBP to the sperm membrane. Arangasamy (2010) performed *in vitro* capacitation of stallion spermatozoa in the presence of SP-HBP/heparin and found significant difference in treated and control groups. Heparin and heparin like molecules are known to effectively capacitate bovine sperm. High density lipoproteins (HDL) have also been shown to capacitate bull sperm (Therein et al., 1998) and HDL by binding to the bovine seminal plasma proteins (Manjunath et al., 1988). Addition of purified fraction of 15 to 17 kDa, 24 kDa, and 31 kDa HBPs (Miller et al., 1990) to epididymal sperm revealed the effect of heparin. They further conveyed that fraction containing 24 and 31 kDa proteins possessed greatest activity/unit of protein to induce acrosome exocytosis.

Lipid peroxidation: An increase of 35.8 to 108.1 μmoles of MDA/ 10^9 spermatozoa was noticed from pre-freeze to freezing-thawing process of cryopreservation (Table 5). MDA has been used in biochemical assays to monitor the degree of peroxidative damage in spermatozoa and exhibited an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion (Sidhu et al., 1998). Increase in MDA production due to freezing-thawing was non-significantly ($p > 0.05$) less (23.1 to 86.9 $\mu\text{moles}/10^9$ spermatozoa) in semen supplemented with SP-HBP in

Table 4. Effect of affinity purified SP-HBP on *in vitro* capacitation/acrosome reaction (mean±SE) during pre-freeze and frozen-thaw phase of cryopreservation

Bull number	Control		Treated	
	Capacitated	Acrosome reacted	Capacitated	Acrosome reacted
1	41.7±1.6	28.3±5.7	44.1±1.6	33.5±5.4
2	40.9±0.4	24.4±2.2	46.9±0.6	26.6±2.5
3	36.6±2.1	23.9±2.2	41.9±1.7	30.0±2.5
4	45.6±1.8	25.0±3.3	47.1±0.5	30.4±2.9
5	43.0±0.4	27.0±2.9	48.5±0.2	27.7±2.1
6	40.0±2.0	31.7±1.7	44.3±0.5	32.9±3.7
Average±SE	41.4 ^a	26.3 ^b	45.5 ^c	30.2 ^d

SP-HBP, seminal plasma-heparin binding protein; SE, standard error.

¹ Control, without HBP; Treated, supplemented with SP-HBP.

Superscripts a, b, c, d indicate the difference at 5% level of significance within the columns.

comparison to control.

There was a non significant ($p > 0.05$) decline of 5.8 to 40.1 $\mu\text{moles}/10^9$ and 16.3 to 64.7 $\mu\text{moles}/10^9$ spermatozoa in pre-frozen and frozen-thawed semen supplemented with SP-HBP, respectively. Karunakaran et al. (2012) added 25 μg of 28 to 30 kDa HBP to the frozen-thawed bull semen in control and H_2O_2 treated group and concluded that excessive H_2O_2 causes significant increase in LPO levels and 28 to 30 kDa HBP of sperm membrane helped in controlling the oxidative stress of sperm cells. Kumar et al. (2008) were also of the opinion that HBPs protected the buffalo bull spermatozoa from lipid peroxidation stress during cryopreservation.

Seminal oxidative stress develops as a result of an imbalance between reactive oxygen species and scavenging activities. Spermatozoa are particularly susceptible to the oxidative stress induced damage as their plasma membrane

Table 5. Effect of affinity purified SP-HBP on MDA production ($\mu\text{M}/10^9$ spermatozoa, mean ± SE) during pre-freeze and frozen-thaw phase of cryopreservation

Bull number	Pre-freeze		Frozen-thaw	
	Control ¹	Treated ¹	Control	Treated
1	73.5±51.3	51.6±35.0	112.4±38.0	94.9±37.8
2	46.8±19.4	24.9±12.8	83.3±19.4	60.3±15.1
3	22.1±10.3	50.2±33.4	115.2±34.1	98.9±31.6
4	72.9±20.8	32.8±5.4	130.3±47.5	65.6±16.8
5	97.0±24.5	62.3±22.6	132.8±49.6	85.4±31.5
6	51.6±20.7	45.8±20.9	159.7±69.9	132.7±51.1
Combination factor mean	66.7 ^{ac}	44.6 ^a	122.3 ^{bd}	89.6 ^{cd}

SP-HBP, seminal plasma-heparin binding protein; MDA, malondialdehyde; SE, standard error.

¹ Control, without HBP; Treated, supplemented with SP-HBP.

Superscripts a, ac, bd, cd indicates the difference at 5% level of significance within the columns.

contains large quantity of polyunsaturated fatty acids. Cytoplasm of spermatozoa has low concentration of scavenging enzymes (Sharma and Agarwal, 1996). Moreover mature sperm is devoid of cytoplasm and intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds acrosome and tail. Therefore, for their protection spermatozoa depend upon seminal plasma. Small amounts of reactive oxygen species (ROS) have been presented to be required for several functions of spermatozoa, but their excessive levels can negatively impact the quality of spermatozoa and impair the fertilizing capacity (Tvrda et al., 2011). Reactive oxygen species are usually formed during normal enzymatic reaction of inter and intracellular signalling (Agarwal and Allamaneni, 2006). Therefore, declined level of MDA production in the spermatozoa treated with SP-HBP may be due to its interference with enzymatic reaction of inter and intracellular signalling responsible for ROS production.

Oxidative stress affects the fluidity of sperm plasma membrane as well as integrity of DNA in the nucleus (Aitken, 1999). Increase in membrane fluidity leads to sperm capacitation. Therefore, higher level of acrosome damage (cryocapacitation like changes) observed in frozen-thawed spermatozoa as compared to pre-frozen spermatozoa may be due to increase in ROS level during cryopreservation of semen, which was also evidenced from increased level of MDA production. Spermatozoa itself produce small amounts of ROS that are essential to many of physiological process i.e. capacitation, hyperactivation and sperm oocyte fusion (Aitken et al., 2003). Low level of ROS has also been shown to be essential for fertilization, acrosome reaction and motility. Production of higher level of ROS as observed by a rising level of MDA production in frozen-thawed spermatozoa was repressed by addition of SP-HBP. Therefore reduced level of oxidative stress in frozen thawed spermatozoa treated with SP-HBP may be responsible for higher percentage of motile, viable, HOS positive, *in vitro* capacitated/acrosome reacted spermatozoa and spermatozoa with intact acrosomes in pre-frozen and frozen-thawed semen of six cross bred bulls.

It was observed during the present study that cryopreservation of semen of six bulls resulted in reduced motility, viability, HOST and enhanced acrosome damage/oxidative stress with a variation among the bulls. Such a variation may be attributed to varying concentration of membrane cholesterol. Fresh spermatozoa with higher cholesterol content are expected to resist destabilization of membrane following cryopreservation (Srivastava et al., 2013). Since membrane integrity is altered with cryopreservation, spermatozoa with more cholesterol content prior to cryopreservation will also resist membrane damage and effect of membrane damage and

cryocapacitation will be less evident in these bulls (Srivastava et al., 2013). Treatment of semen with SP-HBP improved motility, viability, HOST, acrosome integrity, *in vitro* capacitation/acrosome reaction by diminishing oxidative stress, but with a variation among the bulls. HBPs are acquired by spermatozoa at the time of ejaculation and have been associated with removal of cholesterol from sperm membrane during capacitation (Therien et al., 2001). Therefore, variation in response of spermatozoa to SP-HBP treatments may also be associated to varying concentration of sperm membrane cholesterol.

Our observations revealed that absorption of SP-HBP to the sperm surface reduced the damage that cold shock can have on the sperm membrane during the process of freezing, consequently as pointed out by Lessard et al. (2000), increase in fertilizing capacity of SP-HBP treated cryopreserved semen should be expected.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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