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Acrosome integrity and mitochondrial membrane potential of frozen thawed buffalo semen treated with heparin binding protein

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Abstract
The experiment was conducted to study the sperm acrosome integrity and mitochondrial membrane potential (MMP) of frozen thawed buffalo semen treated with heparin binding protein (HBP). Buffalo semen straws from 10 bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-560088. The frozen straws were thawed at 37 ºC for 30 seconds and emptied into a 15mL sterile plastic centrifuge tube containing 1mL capacitation medium (control), addition of 25µg/mL (treatment I), 50µg/mL (treatment II) and 100µg/mL (treatment III) of HBP. The contents were incubated at 37 ºC for 2 hours. After incubation, sperm acrosomal integrity was assessed by Giemsa stain method. In control, HBP treatment I, II and III, 51.50% ± 1.29, 46.30% ±0.86, 43.40% ± 0.66and 42.15% ± 0.40 spermatozoa, respectively had intact acrosome. But, 48.50% ±1.29, 53.70% ± 0.86, 56.60% ± 0.66and 57.85% ± 0.40 spermatozoa in control, HBP treatment I, II and III respectively had lost acrosome. Significantly (P<0.05), higher percentages of spermatozoa lost their acrosome integrity in HBP treatments as compared to control. Sperm MMP was determined by JC-1 (5, 5', 6, 6' - tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) stain technique. Significantly (P<0.05) higher percentages of spermatozoa in HBP treatment I (44.05% ± 0.61), II (45.35% ± 0.62) and III (45.05% ± 0.77) showed mitochondrial membrane potential in comparison with control (39.75% ±0.60).Insignificant difference in sperm MMP was observed among treatments. This study suggested that addition of HBP in capacitation medium induces sperm acrosome reaction and enhances mitochondrial membrane potential.

Keywords: Heparin binding protein, acrosome integrity, mitochondrial membrane potential, spermatozoa, buffalo semen

Introduction
Seminal plasma proteins were associated with the fertilizing capacity of sperm. Some of these proteins were found in seminal plasma and others were bound with sperm membrane [1,2,3,4].The role of seminal plasma proteins in the regulation of sperm functions was highly complex and several studies provided direct evidence that seminal plasma proteins were adsorbed to the surface of sperm [5] and affected its functions and properties [6]. The addition of seminal plasma to frozen thawed ram sperm improved motility, viability and mitochondrial respiration [7, 8]. Addition of seminal plasma proteins also increased the resistance of spermatozoa of bull [9], ram [10] and red deer [11] to cryo-injuries.
Some of these proteins are bound to the sperm surface during ejaculation and thus protein-coating layers are formed [12]. In the female reproductive tract, seminal plasma proteins bound to the sperm surface participate first in the formation of the oviductal sperm reservoir [13, 14] ; second, in the control of sperm capacitation by the action of negative (decapacitation factors) and positive regulatory (capacitation-stimulating factors) factors, and finally in central fertilization events such as sperm–zona pellucida interaction and sperm–egg fusion [15,16].
HBP is one of seminal plasma proteins and has been identified in bovine seminal plasma which coats the surface of spermatozoa. HBP is a modulator of sperm capacitation [17, 18, 19]. Successful fertilization depends on the presence of spermatozoa at the site of fertilization and capacitation [20]. Another subsequent critical step is the acrosome reaction, which is normally induced by binding of spermatozoa with the oocyte [21].

Affinity for heparin was found to be a primary feature of seminal plasma proteins in most species of mammals. Proteins that bind polysaccharide regulate capacitation and acrosome reaction processes. Affinity of plasma proteins to mannose of the fallopian tube epithelium enables the formation of sperm reservoirs in the female reproductive tract [22]. Presence of fertility related 31 kDa HBP may be an indication of high fertility of a bull. [23]. The affinity, but not just the presence, of HBP in sperm membranes related to the potential of sperm to capacitate, acrosome react, and subsequently fertilize an oocyte [24]. Eight major heparin binding proteins (HBPs) in the molecular weight range of13-71kDa were observed in buffalo seminal plasma [25]. Thus, seminal fluid HBPs play a vital role in spermatozoon survival and overall fertilization process and any alteration in these proteins can be directly related to infertility. Heparin alone cannot capacitate epididymal spermatozoa. However, when accessory gland proteins that bind heparin are added to epididymal spermatozoa, these spermatozoa are able to undergo capacitation and bind to the ZP with increase in acrosome reaction [26]. HBPs allow spermatozoa to face the challenge of stress by lysophosphatidyl-choline and undergo the acrosome reaction [27].

Although, HBPs have been identified in several species like bovine (Chandonnet *et al*., 1990), equine [29], boar [30], and canine [31], limited studies have been conducted on the potential roles of HBPs on sperm functions. Hence, the experiment was undertaken to study the additive effects of HBP on the sperm acrosome integrity and mitochondrial membrane potential (MMP) of frozen thawed buffalo semen.

Materials and Methods

Materials

All the plasticware used in this study were purchased from Tarson, India. All the glassware used in this study were purchased from Borosil, India. All chemicals used in this study were procured from Sigma-Aldrich chemicals Co., USA.

Methods

Capacitation stock solution

Capacitation stock solution / Sperm TALP was prepared as detailed below.

Components	For 1000 mL (in mM)
Sodium chloride (NaCl)	114
Potassium chloride (KCl)	3.2
Calcium chloride ($CaCl_2.2H_2O$)	2.0
Magnesium chloride ($MgCl_2.6H_2O$)	0.5
Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$)	0.34
Sodium lactate (60% syrup)	1.86 µL/mL
Phenol red	10µg/mL

Pyruvate stock solution

Sodium pyruvate	22mg/10 mL capacitation medium

Heparin stock solution

Heparin	5mg/10mL capacitation medium

Gentamicin stock solution

Gentamicin	50mg/mL in saline

All the stock solutions were filter sterilized (0.2 µm) and stored at 4 ˚C until use.

Capacitation working solution

Components	For 10 mL
Capacitation stock solution	9.5 mL
Pyruvate stock solution	0.1 mL
Heparin stock solution	0.4 mL
Gentamicin stock solution	10 µL
Bovine serum albumin (Fatty acid free)	60 mg

The working solution was prepared freshly, filter sterilized (0.2 µm) and pre-warmed at 37 ˚C for 30 minutes before use. pH and osmolality of the stock and working medium were maintained at 7.6-7.8 and 280-300 mOsm, respectively.

Sperm Preparation

Frozen semen straws from ten buffalo bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-560088. The straws were collected in liquid nitrogen (LN_2 at -196 ºC) container, transported and stored in the semen bank of Madras Veterinary College, Chennai- 600 007. The frozen straws were thawed at 37 ºC for 30 seconds and emptied into 15 mL sterile plastic centrifuge tube containing 1 mL of capacitation medium and treated as below.

Experimental groups and Method of treatment

Experimental groups	Method of treatment
Control	Capacitation medium alone (sperm TALP)
Treatment I	Capacitation medium + Heparin binding protein (HBP-25µg/mL)
Treatment II	Capacitation medium +Heparin binding protein (HBP-50µg/mL)
Treatment III	Capacitation medium +Heparin binding protein (HBP-100µg/mL)

The contents were incubated at 37 ºC for 2 hours. After incubation, the sperm acrosome integrity and mitochondrial membrane potential (MMP) were assessed.

Evaluation of Sperm Acrosome Integrity

A drop of fresh semen was used to prepare a thin smear and fixed in 5 per cent formaldehyde for 30 minutes. The slide was washed with running tap water, air dried and then smear was immersed in working Giemsa stain for 6 hours at 37˚C. Finally, slide was washed in running water and air dried. 200 spermatozoa were counted with a phase contrast microscope (1000X). Acrosomal intact spermatozoa showed acrosomal cap and acrosomal nonintact spermatozoa lost acrosomal cap [32].

Evaluation of Sperm MMP

Mitochondrial membrane potential was assessed by using JC-1 (5, 5', 6, 6' - tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide). 1.53 mM of JC-1 in DMSO, 8.69 mM of CFDA in DMSO and 0.4 mM of PI in PBS were prepared and stored at - 20˚C in dark. 2µl of JC-1 and 10µl of CFDA solutions were added to 100µl of semen sample. The semen samples were incubated at room temperature for 30 minutes in dark.

The sperm nuclei were counterstained by adding 10µl of PI stock solution and incubated in dark for 10 minutes. Then the

sperm cells were washed in PBS by centrifugation at 560 g for 5 minutes. Sperm cells suspended in PBS were placed on a clean grease free glass slide and covered with cover slip and observed under fluorescent microscope. JC-1 existed as a monomer with excitation and emission peaks in the green wavelengths (510-520 nm). However, it also exhibited a second peak in the red-orange range (590 nm). Spermatozoa with high MMP exhibited red- orange fluorescence and those with medium to low MMP exhibited green fluorescence. Spermatozoa without MMP did not exhibit fluorescence. A minimum of 200 spermatozoa were observed [33].

Statistical Analysis

The statistical analysis was carried out by IBM, SPSS version 20.0 for windows. The percentage value of variables was converted into Arsine value before performing one way analysis of variance (ANOVA).

Results

Effect of HBP on Sperm Acrosome Integrity

The sperm acrosomal integrity was assessed by Giemsa stain method. Table 1 shows that 51.50% ± 1.29, 46.30% ±0.86, 43.40% ± 0.66and 42.15% ± 0.40spermatozoa in control, HBP treatment I, II and III respectively had intact acrosome. But, 48.50% ±1.29, 53.70% ± 0.86, 56.60% ± 0.66and 57.85% ± 0.40 spermatozoa in control, HBP treatment I, II and III respectively had lost acrosomal integrity. Significantly ($P<0.05$), higher percentages of spermatozoa lost their acrosomal integrity in HBP treatments as compared to control.

Effect of HBP on Sperm MMP

The sperm MMP was determined by JC-1(5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) stain technique. Table 2 depicts that significantly ($P<0.05$) higher percentages of spermatozoa in HBP treatment I (44.05% ± 0.61), II (45.35% ± 0.62) and III (45.05% ± 0.77) showed mitochondrial membrane potential in comparison with control (39.75% ±0.60). Among treatments, insignificant difference was observed in sperm MMP.

Discussion

Effect of HBP on Sperm Acrosome Integrity

In this study, significantly higher number of spermatozoa lost the acrosome integrity on treatment with HBP. Loss of acrosome integrity is mediated by the acrosome reaction through interaction of HBP with heparin which cause an increase in Ca^{2+} ions in acrosome matrix [34].and results in fusion of the plasma membrane overlying the acrosome with the outer acrosomal membrane, formation of vesicles and time-dependent release of hydrolytic enzymes from the acrosome and finally the disappearance of acrosomal contents. Fusion of the plasma and outer acrosomal membranes coupled with the release of hydrolytic enzymes allows the sperm to penetrate the zona pellucida and fertilize an oocyte [35, 36]. 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 [37]. Acrosin is one of the major sperm acrosomal proteases [38].that modulates protein dispersion during acrosome reaction [39].and sperm penetration into zona pellucida [40].

Effect of HBP on sperm MMP

This study showed a significant increase in the number of spermatozoa that exhibit MMP on treatment with HBP. It is due to interaction of HBP with heparin causes increased influx of Ca^{2+} ions into mitochondria which activates mitochondrial enzymes involved in oxidative phosphorylation to yield energy i.e. ATP required for hyperactivation and successful penetration of spermatozoa into the oocytes [41, 42]. During the process of oxidative phosphorylation, the protons are pumped from inside the mitochondria to the outside, creating an electrochemical gradient called the inner MMP [43]. Oxidation of thiols in sperm proteins by O_2- and H_2O_2 was found to be associated with inhibition of sperm motility and fertilizing ability [44]. Artificially induced oxidative stress by incubation with H_2O_2 has been shown to inhibit sperm motility, decrease ATP levels, and dissipate the MMP [45]. Correlation of MMP results with sperm morphology may provide information as morphologically abnormal spermatozoa with midpiece defects have been linked with excessive production of ROS [46]. Mitochondrial dysfunction has been shown to increase production of ROS [47, 48, 49]. Storage of spermatozoa outside the body cavity can impact availability of oxygen and metabolic processes. Cryopreservation of spermatozoa is associated with both oxidative stress and physical stress [50, 51].Cryopreservation of bull sperm in egg yolk based extenders significantly reduced the intracellular level of thiols and post-thaw treatment of frozen semen with thiols containing antioxidants prevented H_2O_2 -mediated loss of sperm motility [52]. The intracellular concentration of ATP is decreased or lost and the AMP/ADP-rate is increased by the cryopreservation. Sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage [53]. Increased ROS produced by the spermatozoa is associated with mitochondrial injury with a marked decrease in MMP and the measurement of MMP can provide useful information about the fertility potential of an individual [54]. The structural changes produced in the post thaw sperm cell membrane are primarily linked to altered abilities for energy source. This would later influence both cellular metabolism and other sperm functions such as motility [55, 56]. High correlation of MMP with forward motility confirms the strong link between functional status of mitochondria and sperm quality [57]. Energy requirement increases significantly with the onset of activated motility, and becomes even more pronounced when motility is hyperactivated [58,59]. Like many metabolically active body cells, spermatozoa possess the metabolic machinery required for glycolysis, citric acid cycle and oxidative phosphorylation. ATP for spermatozoa is mainly derived either by glycolysis in the cytoplasm or through oxidative phosphorylation in the mitochondria [60].

Conclusion

This study suggested that addition of HBP in capacitation medium induces sperm acrosome reaction and enhances mitochondrial membrane potential.

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Table 1: Effect of HBP Supplementation on Sperm Acrosome Integrity of Frozen Thawed Buffalo Semen

Groups	Acrosome intact spermatozoa (% ± SE)	Acrosome non intact spermatozoa (% ± SE)
Control	51.50 [a] ± 1.29	48.50 [a] ± 1.29
Treatment I (HBP-25µg/mL)	46.30 [b] ± 0.86	53.70 [b] ± 0.86
Treatment II (HBP-50µg/mL)	43.40 [b] ± 0.66	56.60 [b] ± 0.66
Treatment III (HBP-100µg/mL)	42.15[b] ± 0.40	57.85 [b] ± 0.40

Mean with different superscripts (a and b) in a column are significantly different (P<0.05) between groups.
Data are presented as mean % ± SE

Table 2: Effect of HBP Supplementation on Sperm MMP of Frozen Thawed Buffalo Semen

Groups	Spermatozoa with MMP (%±SE)	Spermatozoa without MMP (%±SE)
Control	39.75 [a] ± 0.60	60.25 [a] ± 0.60
Treatment I (HBP-25µg/mL)	44.05 [b] ± 0.61	55.95 [b] ± 0.61
Treatment II (HBP-50µg/mL)	45.35 [b] ± 0.62	54.65 [b] ± 0.62
Treatment III (HBP-100µg/mL)	45.05[b] ± 0.77	54.95 [b] ± 0.77

Mean with different superscripts (a and b) in a column are significantly different (P<0.05) between groups.
Data are presented as mean % ± SE

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