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Effect of heparin binding protein on post capacitation sperm motility and viability of frozen thawed buffalo semen

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Abstract
The present study was conducted to assess the effects of exogenous supplementation of heparin binding protein (HBP) in capacitation medium on sperm motility and viability of frozen thawed buffalo semen. 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 37ºC 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 37ºC for 2 hours and sperm motility and viability were assessed. The post capacitation sperm motility was significantly ($P<0.05$) higher in HBP treatment I (64.00% ± 2.21), II (68.00% ± 1.33) and III (66.00% ± 2.21) as compared to control (55.00% ±2.23). Among treatments, insignificant difference was observed in sperm motility though higher percentages of sperm were motile in HBP treatment II. Sperm viability was significantly ($P<0.05$) higher in HBP treatment I (85.30% ± 0.77), II (86.65% ± 0.92) and III (85.65% ± 0.82) as compared to control (79.80% ±0.73). Among treatments, insignificant difference was observed in sperm viability though more sperm were viable in HBP treatment II. This study revealed that HBP enhanced post capacitation sperm motility and viability which are requisites for successful fertilization.

Keywords: Heparin binding protein, motility, viability, spermatozoa, buffalo semen

Introduction
Cryopreservation of semen is an important tool for artificial insemination (AI) and is the most efficient way for storing germplasm [1]. Freezing of semen is practiced in different domestic animals including human beings [2]. Cryopreservation preserves sperm characteristics, but it causes irreversible damage to spermatozoa and reduces fertilizing capacity of the spermatozoa [3].Standard procedures followed during the frozen semen technology resulted in an overall decrease up to 50% in the level of seminal plasma proteins [4]. The influence of seminal plasma proteins on male reproduction has drawn attention because many studies proved that their expression is associated with fertility scores in cattle [5] and horses [6].
Seminal plasma proteins can potentiate capacitation of bovine epididymal spermatozoa. Sperm-bound seminal plasma proteins binds with heparin present in the female reproductive tract and thereby participate in sperm capacitation and acrosomal reaction [7].
Proteins such as osteopontin (OPN), prostaglandin D synthase, bovine seminal plasma proteins (BSP A1, A2 and A3), heparin binding proteins (HBPs), fertility associated antigen (FAA),phospholipase A2, sperm adhesion Z13, clusterin (CLU) and heat shock proteins (HSPs) have been reported as indicators of fertility [8]. HBPs are accessory gland proteins that may influence fertility. HBP with a molecular weight of 31kDa was named as Fertility Associated Antigen (FAA). Fertility of FAA positive bulls used for natural service was found 9% more than FAA negative bulls [9].
Ejaculated spermatozoa possess more heparin binding sites than cauda epididymal spermatozoa [10]. Some of seminal plasma proteins bind on the sperm surface during ejaculation and protein-coating layers are formed [11].
HBPs on sperm surface bind with heparin in the fallopian tube and thereby participate in sperm capacitation, acrosome reaction, zona binding and fertilization [12-14].
Although HBPs have been identified in bovine [15], equine [16], boar [17], and canine [18] semen, limited studies have been conducted on the potential roles of these proteins on sperm functions. Hence, the present study was undertaken to prospect the role of HBP on post

capacitation sperm motility and viability of frozen 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 the chemicals used in this study were procured from Sigma-Aldrich chemicals Co., USA.

Methods

Capacitation medium/ Sperm TALP was prepared as detailed below.

Table: Capacitation stock solution

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 lactate (60% syrup)	1.86 µl/mL
Sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$)	0.34
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.

Table: 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	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 2 hours incubation, sperm motility and viability were assessed.

Evaluation of sperm motility

The sperm motility was assessed by placing a drop of semen from each group on separate clean grease free glass slide and covered with cover slip. Minimum of three fields were scanned under bright field microscopy to assess the progressive motile spermatozoa and graded in terms of percentage ranging from 0-100 in multiples of 10 [19].

Evaluation of sperm viability

The sperm viability was determined by the supravital Eosin and Nigrosin stain technique. A drop of semen from each group was placed on a separate clean grease free glass slide. One drop of 5% Eosin and two Drops of 10% Nigrosin near the semen drop on either sides were placed by using a blunt glass rod. Eosin was mixed with semen and then Nigrosin was mixed with semen gently. A thin homogenous smear was prepared from the semen stain mixture and dried in air and examined under oil objective of the microscope. Spermatozoa which were unstained (white) classified as live and stained with pink colour classified as dead. At least 200 spermatozoa were observed in each sample [20].

$$\text{Live percentage} = \frac{\text{Live sperm counted}}{\text{Total sperm counted}} \times 100$$

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

Table 1 shows the effect of HBP supplementation to capacitation medium on sperm motility. Sperm motility was significantly ($P<0.05$) higher in HBP treatment I (64.00% ± 2.21), II (68.00% ± 1.33) and III (66.00% ± 2.21) as compared to control (55.00% ±2.23). Among treatment groups, insignificant difference in sperm motility was

observed though higher percentages of sperm were motile in HBP treatment II.

Table 2 shows the effect of HBP supplementation to capacitation medium on sperm viability. Sperm viability was significantly (P<0.05) higher in HBP treatment I (85.30% ± 0.77), II (86.65% ± 0.92) and III (85.65% ± 0.82) as compared to control (79.80% ±0.73). Insignificant difference in sperm viability was observed among treatment groups though more sperm were viable in HBP treatment II.

Discussion

This study revealed that spermatozoa treated with HBP showed a significant increase in sperm motility as compared to control. The findings were in accordance with the earlier studies in which the spermatozoa exhibited an increased motility in presence of heparin and this is possibly mediated through HBP present on the sperm cell surface [21]. HBP expression is associated with sperm capacitation by its localization in the precise region of the sperm head that participate in zona recognition [22]. The involvement of HBP on sperm motility, which is a prerequisite event of the sperm cell's functional maturation [23].

The study also revealed that addition of HBP to capacitation medium significantly improved the viability of spermatozoa. Reasons for better viability of spermatozoa in HBP treatment was evident from the fact that HBP acts as cell survival factor. Similar finding was reported in the previous study in which seminal fluid HBP was found to play a vital role in spermatozoa survival and the overall fertilization process; any alteration in these proteins can directly be related to infertility [24].

Successful fertilization depends on presence of spermatozoa at the site of fertilization, capacitation, acrosome reaction and zona interaction [25, 26, 27]. Sperm capacitation in female reproductive tract is stimulated by binding of heparin with HBPs on sperm plasma membrane [28]. HBPs in seminal plasma attach on the sperm surface, especially with lipids containing the phosphorylcholine group, thus allowing heparin-like glycosaminoglycans in female reproductive tract to induce sperm capacitation [29], which is regulated through a cAMP-dependent pathway in many species [30].

Binding of seminal plasma proteins with mannose in fallopian tube epithelium enables the formation of sperm reservoirs in the female reproductive tract [31]. Presence of HBPs in sperm membranes and its affinity for heparin are related to the potential of sperm to undergo capacitation, acrosome reaction, and subsequently fertilization [32, 33]. HBPs act a receptor for heparin or heparin like-GAGs that required for the sperm capacitation [34].

Phosphorylation of HBP is associated with capacitation of mouse [35], hamster [36], cattle [37], pigs [38], and human [39] spermatozoa, which was confirmed by studies with endogenous protein kinases and radiolabeled (c-32P) ATP. Capacitation induced tyrosine phosphorylation of three proteins (27, 37, and 40 kDa) coincided with an increase in the plasma membrane fluidity, a condition suitable for the sperm cell to gain forward motility [40]. Hence, capacitated spermatozoa attain hypermotility.

Table 1: Effect of HBP supplementation on post capacitation sperm motility of frozen thawed buffalo semen

Groups	Post capacitation sperm motility (% ± SE)
Control	55.00 ^{a} ± 2.23
Treatment I (HBP-25µg/mL)	64.00 ^{b} ± 2.21
Treatment II (HBP-50µg/mL)	68.00 ^{b} ± 1.33
Treatment III (HBP-100µg/mL)	66.00 ^{b} ± 2.21

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

Table 2: Effect of HBP supplementation on post capacitation sperm viability of frozen thawed buffalo semen

Groups	Post capacitation sperm viability (% ± SE)
Control	79.80^{a} ± 0.73
Treatment I (HBP-25µg/mL)	85.30^{b} ± 0.77
Treatment II (HBP-50µg/mL)	86.65 ^{b}± 0.92
Treatment III (HBP-100µg/mL)	85.65 ^{b} ± 0.82

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

Conclusion

This study indicated that addition of HBP in the capacitation medium enhances sperm motility and viability of frozen thawed buffalo semen.

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