

Case Study

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## Sperm Morphology and DNA Integrity of Frozen Thawed Buffalo Semen Treated with Heparin Binding Protein

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### ABSTRACT

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The present study was conducted to assess the sperm morphology and DNA integrity 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, Bangalore-560088. The frozen straws were thawed at 37°C 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°C for 2 hours. After incubation, the sperm morphology was determined by Rose Bengal stain technique. Morphologically normal sperm in control, HBP treatment I, II and III were 86.70% ± 1.52, 87.00% ± 1.21, 87.00% ± 1.28 and 86.55% ± 1.59 respectively. There was no significant difference between control and HBP treatments with respect to sperm morphology. The sperm DNA integrity was assessed by acridine orange stain method. There was no significant difference among control (90.15% ± 1.14), HBP treatment I (90.25% ± 1.25), II (89.85% ± 1.05) and III (90.40% ± 1.16) with respect to sperm DNA integrity. This study indicated that HBP supplementation in capacitation medium did not alter the sperm morphology and DNA integrity.

### Introduction

Cryopreservation of semen is an important tool for assisted reproductive techniques (ARTs) and is the most efficient way for storing germ plasma (Olaciregui *et al.*, 2014).

Cryopreservation has been applied to various species, including human, cattle, swine, dogs and cats (Mota Filho *et al.*, 2006). Although sperm cryopreservation preserves sperm motility, metabolic functions and fertility, still the freeze-thawing process causes irreversible

damage to spermatozoa and reduces fertility (Ardon and Saurez, 2013). Reduced efficiency of AI in buffaloes has necessitated to find out the factors that contribute to the infertility. Several studies have been conducted in the past decades in search of exact potential fertility markers in bulls. The influence of seminal proteins on male reproduction has always drawn attention because many studies proved that their expression is associated with fertility scores in dairy cattle (Cancel *et al.*, 1997), beef cattle (Parent *et al.*, 1999) and horses (Brandon *et al.*, 1999). Further studies in this field may help to design accurate measures in AI technology for augmenting the reproductive performance.

Seminal proteins mediate the binding of sperm cells to oviductal epithelium and preserve membrane integrity by exerting the inhibitory effects on the mitochondrial activity and metabolism to conserve energy needed until fertilization as well as to minimize the production of reactive oxygen species (ROS) and lipid peroxidation (LPO) of sperm membrane (Schoneck *et al.*, 1996). Seminal proteins also have activities in anti-apoptosis and cell survival (Chakraborty *et al.*, 2006 and Rangaswami *et al.*, 2006). A number of seminal plasma proteins have been investigated which act as molecular markers of fertility in different species (Jonakova *et al.*, 2010). Interestingly, proteomics has identified large number of proteins that are differentially expressed in sperm membrane, seminal plasma, accessory sex gland fluid and epididymal fluid in case of both fertile and sub-fertile bulls (Amours *et al.*, 2010). 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 and heat shock proteins (HSPs) have been reported as indicators of fertility (Fouchecourt *et al.*,

2000; Sprott *et al.*, 2000; McCauley *et al.*, 2001 and Moura *et al.*, 2006).

Bovine spermatozoa that have been exposed to seminal plasma possess more binding sites for heparin than the cauda epididymal fraction of sperms, which have not exposed to seminal plasma. A common feature of most seminal plasma proteins is their ability to interact with different types of inorganic and organic materials present in seminal fluid (Cameron *et al.*, 2007). Some of these proteins are bound to the sperm surface during ejaculation and thus protein-coating layers are formed (Varilova *et al.*, 2006). Seminal fluid HBPs are supposed to attach themselves to the sperm surface, especially lipids containing phosphoryl-choline group, thus allowing heparin-like glycosaminoglycans in the female reproductive tract to activate the sperm capacitation (Miller *et al.*, 1987). 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 (Miller *et al.*, 1990). HBPs allow spermatozoa to face the challenge of stress by lysophosphatidyl-choline and undergo the acrosome reaction (Lane *et al.*, 1999). HBPs were also found to modulate capacitation and zona binding ability of buffalo epididymal spermatozoa (Arangasamy *et al.*, 2005).

Ejaculated bovine sperm have more heparin binding sites than epididymal sperm and exposure of epididymal sperm to seminal plasma increases the number of heparin binding sites on the sperm (Lee *et al.*, 1985). Sperm surface properties are

dramatically altered during epididymal maturation and at ejaculation by the removal or addition of a variety of proteins and lipid constituents, and sperm surface components play an important role in capacitation of sperm (Yanagimachi, 1988 and Saling, 1989). HBPs protect sperm from stress of freezing and thawing and maintained intracellular protein homeostasis (Shi *et al.*, 1998). HBPs themselves bind to spermatozoal membrane to make it stable (Manjunath and Therien, 2002). Mogielnicka and Korden, 2011 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.

## 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 <sub>2</sub> .2H <sub>2</sub> O)	2.0
Magnesium chloride (MgCl <sub>2</sub> .6H <sub>2</sub> O)	0.5
Sodium dihydrogen orthophosphate (NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O)	0.34
Sodium lactate (60% syrup)	1.86 µL/mL
Phenol red	10µg/mL

#### Pyruvate stock solution

Sodium pyruvate	22mg/10 mL capacitation medium
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#### Heparin stock solution

Heparin	5mg/10mL capacitation medium
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#### Gentamicin stock solution

Gentamicin	50mg/mL in saline
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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, Bangalore-560088. The straws were collected in liquid nitrogen (LN<sub>2</sub> at -196 °C) container, transported and stored in the semen bank of Madras Veterinary College, Chennai- 600 007. The frozen straws were thawed at 37°C 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
<b>Control</b>	Capacitation medium alone (sperm TALP)
<b>Treatment I</b>	Capacitation medium + Heparin binding protein (HBP-25µg/mL)
<b>Treatment II</b>	Capacitation medium +Heparin binding protein (HBP-50µg/mL)
<b>Treatment III</b>	Capacitation medium +Heparin binding protein (HBP-100µg/mL)

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

### Evaluation of sperm morphology

Sperm morphology was assessed by Rose Bengal stain. 250µl semen was emptied into a separate eppendorf tube containing 500µl of Tris buffer and then 3 drops of Rose Bengal stain was added. The contents were centrifuged at 2000-3000 rpm for 3 minutes.

Then 1 mL Tris buffer was added. Again, the contents were centrifuged at 2000-3000 rpm for 3 minutes. Supernatant was removed. Finally 100µl Tris buffer was added. One drop of the well mixed sample was placed on a clean grease free glass slide and covered with cover slip. Spermatozoa were observed under 40X. Spermatozoa showing head, mid-piece and tail abnormalities were included in total abnormalities. A minimum of 200 spermatozoa were observed (Enciso *et al.*, 2011).

**Evaluation of sperm DNA integrity**

DNA integrity of sperm was assessed by using acridine orange staining. Semen samples were smeared on glass slides and air-dried. Then, the smears were fixed with Carnoy's solution (1 part glacial acetic acid: 3 parts methanol) for 2 hours. After fixation, smears were air-dried and stained with freshly prepared acridine orange stain (0.19 mg/mL) for 5 minutes in the dark. After staining, smears were washed with distilled water and immediately evaluated under a fluorescent microscope at excitation wavelength of 450-490 nm. An average of 200 sperm were counted on each slide and the duration of evaluation was 40 seconds per field. Sperm with normal DNA content showed green fluorescence whereas sperm with damaged DNA content appeared as a spectrum of yellow-green to red (Chohan *et al.*, 2004).

**Statistical analysis**

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

**Results and Discussion**

**Effect of HBP on sperm morphology**

The sperm morphology was determined by Rose Bengal stain technique. Table 1 indicates that effect of HBP supplementation to sperm TALP on sperm morphology. Morphologically normal sperm in control, HBP treatment I, II and III were 86.70% ± 1.52, 87.00% ± 1.21, 87.00% ± 1.28 and 86.55% ± 1.59 respectively. There was no significant difference between control and HBP treatments with respect to sperm morphology.

**Effect of HBP on sperm dna integrity**

The sperm DNA integrity was assessed by Acridine orange stain method. Table 2 shows that effect of HBP supplementation to sperm TALP on DNA integrity.

There was no significant difference among control (90.15% ± 1.14), HBP treatment I (90.25% ± 1.25), II (89.85% ± 1.05) and III (90.40% ± 1.16) with respect to sperm DNA integrity.

**Table.1** Effect of HBP supplementation on sperm morphology of frozen thawed buffalo semen

Groups	Normal spermatozoa (% ± SE)	Abnormal spermatozoa (% ± SE)
Control	86.70 ± 1.52	13.30 ± 1.52
Treatment I (HBP-25µg/mL)	87.00 ± 1.21	13.00 ± 1.21
Treatment II (HBP-50µg/mL)	87.00 ± 1.28	13.00 ± 1.28
Treatment III (HBP-100µg/mL)	86.55± 1.59	13.45 ± 1.59

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 dna integrity of frozen thawed buffalo semen

Groups	Spermatozoa with DNA integrity (%±SE)	Spermatozoa without DNA integrity (%±SE)
Control	90.15 ± 1.14	9.85 ± 1.14
Treatment I (HBP-25µg/mL)	90.25 ± 1.25	9.75 ± 1.25
Treatment II (HBP-50µg/mL)	89.85 ± 1.05	10.15 ± 1.05
Treatment III (HBP-100µg/mL)	90.40± 1.16	9.60± 1.16

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

### Effect of HBP on sperm morphology

HBP supplementation has no direct effect on sperm morphology. This study was in accordance with the results of Enciso *et al.* (2011) who stated that major sperm abnormalities might have a genetic origin or the result of an abortive apoptotic mechanism. There may be some other protein which may be a useful indicator for predicting the sperm abnormality. HBPs themselves bind to the spermatozoal membrane to make it stable (Manjunath and Therien, 2002). Mogielnicka-Brzozowska *et al.* (2011) indicated that boar seminal plasma proteins have a shielding effect on the plasma membrane and the acrosome of spermatozoa by protecting their structures against the damage caused by cold shock.

### Effect on sperm dna integrity

There was no positive influence of HBP supplementation on sperm DNA integrity as observed in sperm morphology. This study showed a clear relationship between sperm morphology and DNA integrity. In particular, major sperm abnormalities appear to be closely associated with the presence of a highly damaged DNA molecule. Morphologically abnormal spermatozoa are more prone to DNA damage. A failure to address DNA damage in morphologically normal spermatozoa leads to misdiagnosis of male reproductive potential and an

underestimate of male factor infertility (Enciso *et al.*, 2011).

Exogenous addition of fertility associated proteins had shown to protect the sperm cells against the oxidative stress, which in turn helped to maintain DNA integrity (Maxwell and Johnson, 1999).

However, Saravia *et al.* (2009) carried out experiments on the influence of seminal plasma proteins on boar spermatozoa DNA integrity. They could not establish any significant effect on DNA stability of post thaw semen after treatment with seminal plasma.

In conclusion from this study, it is evident that HBP supplementation in capacitation medium did not alter the sperm morphology and DNA integrity. Extensive studies have to be carried out in determining the influence of HBP as such on sperm morphology and DNA integrity.

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