Supplementation of Seminal Plasma-Heparin Binding Proteins to Capacitation Medium Increases *In Vitro* Acrosome Reaction Percentage in Beetal Bucks

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ABSTRACT

This study was focused evaluating the effect of heparin binding proteins (HBP) on *in vitro* capacitation and acrosome reaction (AR) in Beetalbucks. HBP were purified from pooled seminal plasma of six bucks to evaluate their effect on *in vitro* capacitation. Semen, washed twice with TALP medium was incubated in TALP, supplemented with glucose, BSA, heparin and HBP for 6 hrs at 37°. A control was also run without HBP. Incubated samples were evaluated for motility, viability, acrosome reaction and inner mitochondrial membrane potential (IMMP) after every 2 hrs. Standardization of HBP concentration on *in vitro* acrosome reaction revealed 150 µg/ml HBP as an optimum dose. Based on sperm parameters, six bucks were divided into two groups i.e. G-I (> 80% motility) and G-II (< 80% motility).There was a significant ($p<0.05$) decrease in individual motility, viability and high IMMP from zero to six hrs of incubation in both control and HBP treated samples of both groups. A significant ($p<0.05$) increase in acrosome reaction and medium IMMP was noticeable in control and HBP treated samples with an increase in incubation time. *In Vitro* induced AR was also significantly ($p<0.05$) higher in HBP treated samples compared to control in both groups at 4 hrs and 6 hrs of incubation. However, Effect of buck on *in vitro* capacitation and AR was noticeable in both control and HBP treated samples of G-I and G-II. A non-significant ($p>0.05$) increase in LPO during capacitation and AR was also observed. This study concluded that SP-HBP enhanced the rate of *in vitro* AR in beetal bucks.

Keywords: Beetal bucks, Spermatozoa, Heparin binding proteins, Capacitation, Acrosome reaction

Seminal plasma (SP) includes many proteins that bind to heparin and related to glucosaminoglycans (GAGs). Heparin binding proteins (HBPs), synthesized in male accessory sex glands,transferred into secretions, bind to spermatozoa at ejaculation and play a vital role during fertilization processes. They interact with GAGs present in the female genital tract and enhance the subsequent zona pellucida-induced acrosome reaction (AR) and are correlated with fertility in some species. Heparin binds to spermatozoa of several mammals in a pH-, Ca^{+2}, and temperature-dependent manner which is needed for the initiation of *in vitro* capacitation, and also enhances sperm motility and AR (Cormier *et al.*, 2003; Pons-Rejraji *et al.*, 2009). There are studies that indicate that heparin alone cannot capacitate epididymal spermatozoa and regulate other steps of fertilization. However, when accessory glands proteins (HBPs) are added to epididymal

spermatozoa, they undergo capacitation and zona pellucida induced AR is potentiated (Miller *et al.*, 1990). SP proteins that bind heparin may be directly involved in sperm capacitation and AR (two essential steps for mammalian fertilization). HBPs also help spermatozoa to face the stress challenge by lysophosphatidyl-choline and undergo the AR (Lane *et al.*, 1999). HBPs play pivotal role in spermatozoa survival and overall fertilization process, and any impairment in these proteins might be directly or indirectly related to infertility. Sperm capacitation is pre-requisite for acrosome reaction (AR) and potentially enhanced by glycosaminoglycans (GAG) and heparin. Heparin binding proteins in seminal plasma (SP-HBP) are supposed to attach themselves to the sperm surface, especially lipids containing the phosphoryl-choline group, thus allowing heparin-like GAGs in the female reproductive tract to activate sperm capacitation (Miller *et al.*, 1990). Addition of HBP to epididymal -, ejaculated spermatozoa induced heparin-stimulated acrosome reaction (Miller *et al.*, 1990; Singh *et al.*, 2016). Keeping in mind the importance of HBP in fertilization processes including capacitation in mammals, this study was aimed to evaluate the effect of SP-HBP on *in vitro* capacitation and AR in Beetal bucks.

MATERIALS AND METHODS

Procurement of semen samples

All the procedures were approved by Institutional ethical committee (GADVASU/2018/IAEC/46/06). Semen was collected twice a week using goat artificial vagina (IMV technologies) from six bucks, maintained at Goat Research Farm, Department of Livestock Production and Management, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, Punjab, India.

Experimental design

HBP were purified from pooled SP of six bucks. In the first experiment, optimum dose of HBP was established. In the second experiment, effect of optimum dose of HBP was evaluated in *in vitro* capacitation of six bucks.

Purification of HBP by affinity chromatography

HBP from SP were purified by affinity chromatography (Manaskova *et al.,* 2002) using cyanogen bromide activated heparin bound sepharose (Farooqui, 1980). A clean and dry column (28 mm × 70 mm) was packed with heparin-sepharose up to the mark at the top of the column and allowed to settle for 3-4 hrs. Thereafter, the column was equilibrated for 1 hr with 10 mM Tris HCl (pH 7.4). About 0.5 ml of SP was loaded and circulated through the column for 15 min for absorption of HBP to the heparin bound resins. HBP peak was eluted with 1M NaCl at a flow rate of 1 ml/min in the tubes racked in a fraction collector. The recovered HBP fraction was pooled in agreement with the observed curve, obtained from optical density (280 nm) detected by UV monitor attached to the fraction collector (Fig. 1).
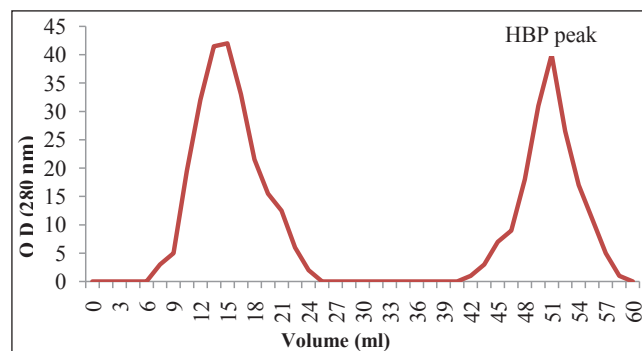
Fig. 1: Heparin-Sepharose affinity chromatography of seminal plasma.

The pooled HBP fraction was concentrated by spinning through protein concentrators (Millipore, 3 kDa) and analyzed for protein

content (Lowry *et al.*, 1951). Concentrated HBP fraction was mixed with glycerol @ 25% and stored at -20° till further use.

Semen analysis

Three ejaculates of six bucks were analyzed for initial motility, viability, plasma membrane integrity and acrosome integrity prior to induction of *in vitro* acrosome reaction (Dhillon *et al.*, 2019). Therefore, based on these parameters, bucks were divided into two groups i.e. G-I (> 80%) and G-II (< 80%).

Effect of HBP on capacitation/acrosome reaction

Basic TALP medium: 4.78 mM NaCl, 25 mM NaHCO$_3$, 5.9 mM Na$_2$HPO$_4$, 1 mM CaCl$_2$.2H$_2$O, 1.19mM MgSO$_4$, 1 mM sodium pyruvate, 25 mM sodium lactate. All ingredients were dissolved in DW, and pH was set at 7.4 and final volume was made to 100 ml. Energy medium: Basic TALP medium supplemented with 5.56 mM glucose, 20 mg/ml BSA, 50 µg/ml gentamycin and 10 µg /ml heparin.

In first experiment, fresh semen was divided into four fractions (200 µl each); Ist fraction was kept as control and 100 µg, 150 µg and 200 µg /ml of HBP were added to the IInd, IIIrd and IVth fractions, respectively. Samples were incubated at 37ºC for 10 min. Energy TALP was added to the sperm suspensions, mixed gently and centrifuged at 3000 rpm for 3 min. This step was repeated twice and finally pellet of each fraction was gently mixed with energy TALP and energy TALP+ respective HBP dose to get a final sperm concentration of 200 × 10^6 sperms /ml. Control as well as HBP treated semen was incubated at 37°C for 6 hrs. Semen was checked for motility pattern every 2 hrs. A smear was also prepared at 0 hr, 4 hrs and 6 hrs, stained with giemsa and assessed for AR. In the second experiment semen of each buck was divided into two fractions (control and HBP treated) and processed as in experiment I. In addition to motility and acrosome reaction,

viability (Eosin-Nigrosin stain) and inner mitochondrial membrane potential (IMMP) (JC-1 stain kit, Sigma –Aldrich) were also evaluated at 0 hr, 4 hrs and 6 hrs.

Lipid peroxidation in capacitated spermatozoa (LPO, Buegeand Steven, 1978)

Capacitated spermatozoa after 6 hrs were also subjected to LPO estimation. Sperm suspension was incubated with 0.1 ml of 150 mM Tris HCl (pH 7.1) at 37°C for 20 min. After incubation, 0.5 ml of 10 % Trichloroacetic acid and 1.0 ml of 0.375% Thiobarbituric acid were added and kept for 20 min in the boiling water bath. Thereafter, mixture was centrifuged for 15 min at 5000 rpm and absorbance of supernatant was taken at 532 nm. The molar extinction coefficient for (Malondialdehyde) MDA is 1.56 × 10^5 M^{-1}.cm^{-1}.

$$MDA\ (\mu M) =$$

$$\frac{OD \times \text{Volume of assay mixture} \times \text{Extinction coefficient}}{\text{Volume of sample}}$$

STATISTICAL ANALYSIS

The mean and standard error were calculated using Microsoft excel program. Significant differences in control and HBP treated groups were tested by ANOVA using SPSS16 program (Student version for windows, SPSS Inc.233 South Wacker Drive, 11th floor Chicago, IL 60606-6412). Normality of the data was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using the Levene test.

RESULTS AND DISCUSSION

Standardization of SP-HBP concentartion for *in vitro* capacitation /AR

Acrosome reaction (vessiculation + shedding) was significantly (p<0.05) higher in the presence of 150 µg/ml HBP as compared to control and other doses (Table 1). However, motility was

non-significantly (p>0.05) less in the presence of 200 µg/ml HBP as compared to control and other doses. Therefore, effect of 150 µg /ml HBP was analyzed on *in vitro* capacitation/AR of six bucks.

Table 1: Effect of different doses of SP-HBP on motility and acrosome reaction after six hours of incubation at 37°.

Attribute	Control	100	150	200
Acrosome Reaction	46.4±11.5	34.6±5.8	56.4±1.3	46.5±2.0
Motility	55.1±2.7	53.9±5.1	48.2±4.6	40.9±8.4

**Effect of seminal plasma heparin binding proteins on *in vitro* capacitation and acrosome reaction**

Spermatozoa exhibiting different stages of AR and IMMP are shown in Figs. 2 and 3, respectively. Motility, viability and high IMMP were significantly (p<0.05) high in G-I as compared to G-II at zero hr of incubation (Fig. 4-7).
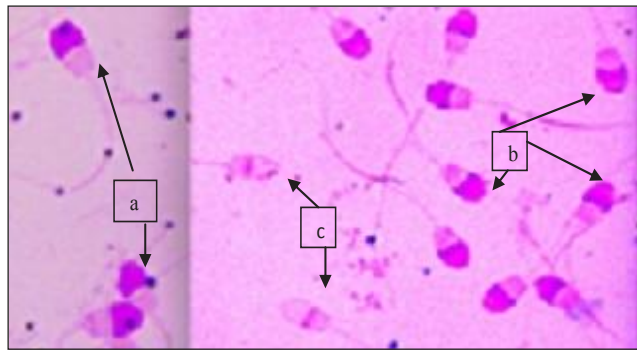

Fig. 2: Showing vesiculation and shedding of acrosome in beetal buck spermatiozoa. Spermatozoa with **(a)** intact, **(b)** vesiculted and **(c)** shedded acrosome

On the contrary, acrosome damage and medium IMMP were significantly (p<0.05) high in G-II as compared to G-I at zero hour of incubation. There was a significant (p<0.05) decrease in individual motility, viability and high IMMP from zero to six hrs of incubation in both control and HBP treated samples of both groups (Fis. 4-7). Decrease in motility and viability was higher in HBP treated (36.66 to 58.34% and 27.17 to 33.38%) compared to control (25.66 to 53.34% and 32.07 to 39.38%).
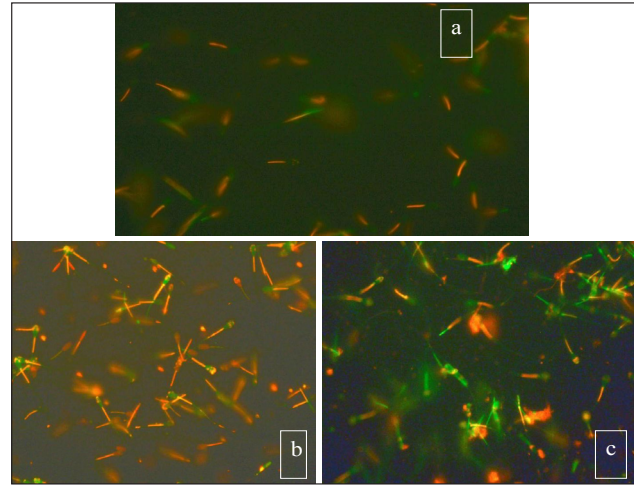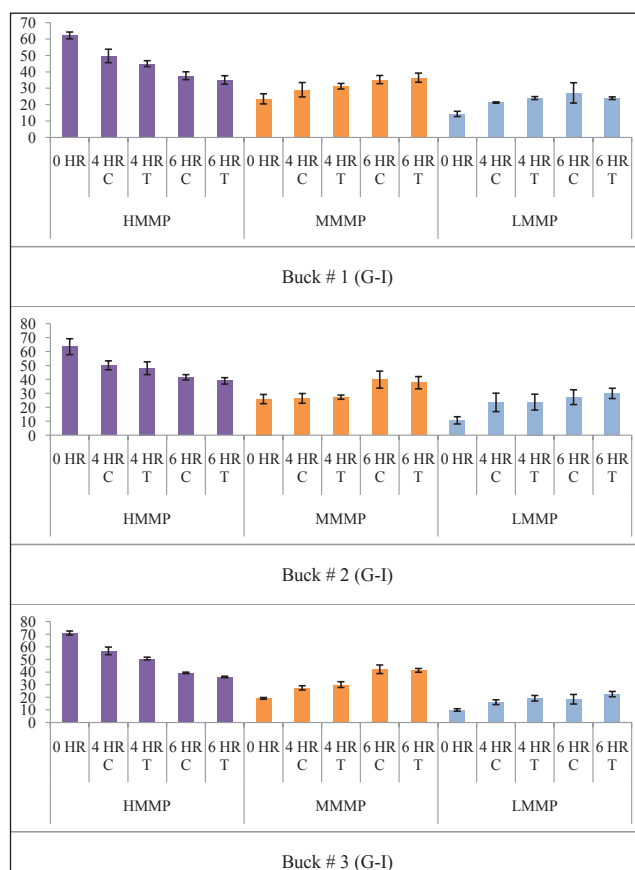

Fig. 3: Showing inner mitochondrial membrane potential of spermatozoa during *in vitro* capacitation and acrosome reaction. (a) 0 hr, (b) 4 hrs and (c) 6 hrs of incubation. Mitochondria with orange, yellow and green fluoresence indicate high, medium and low mitochondrial membrane potential
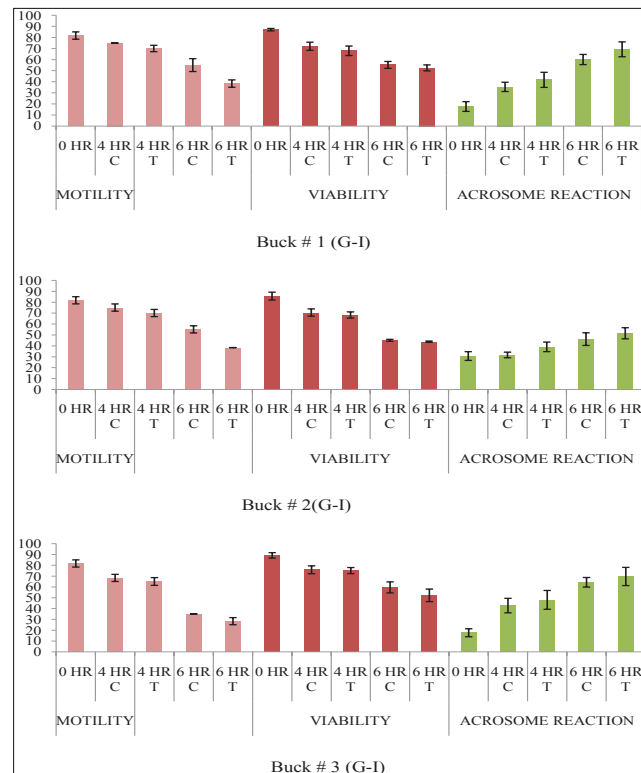
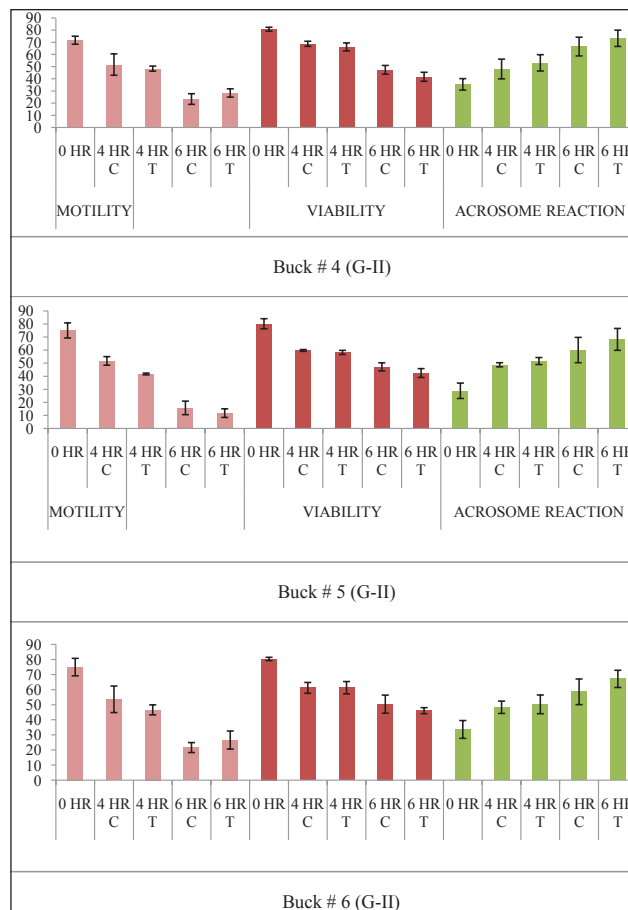**Fig. 4:** Effect of SP-HBP on motility, viability and

acrosome reaction during incubation of spermatozoa of buck # 1, 2 and 3 (exhibiting > 80% initial motility) at 37°C for 6 hrs

Fig. 5: Effect of SP-HBP on Inner mitochondrial membrane potential during incubation of spermatozoa of buck # 1, 2 and 3 (exhibiting > 80% initial motility) at 37°C for 6 hrs

The more rapid decrease in motility and viability was also observed in bovine spermatozoa with FF and LD-FF fraction (Therien *et al.,* 2001) compared to control. Decrease in motility was higher than viability in HBP treated as well as control samples. It may be hypothesized that once the spermatozoa are acrosome reacted, they lose the motility. Corresponding to motility and viability reduction, there was also decline in the high IMMP with increase in time of incubation. Mitochondrial function is usually highly correlated with viability assays (Kasai *et al.,* 2002 and Marchetti *et al.,* 2004).
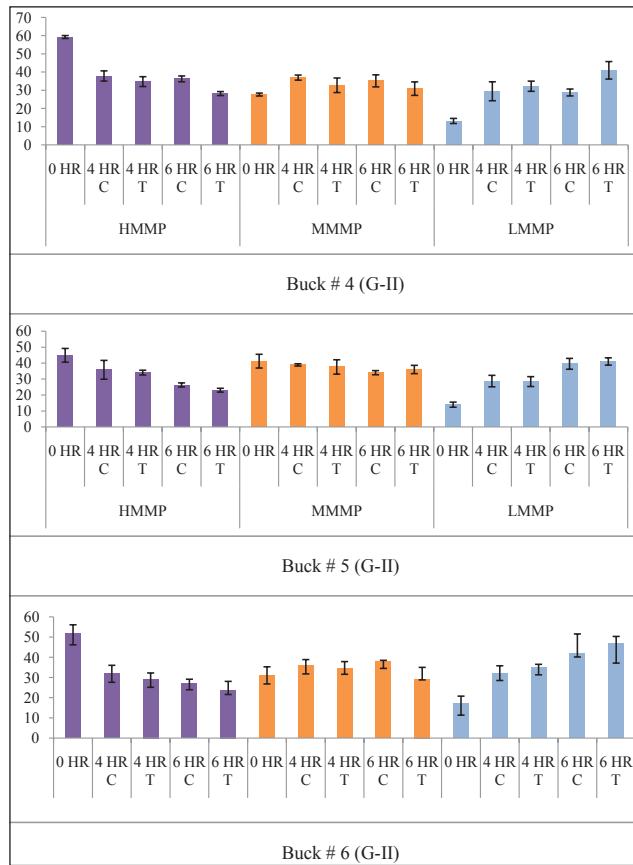

Fig. 6: Effect of SP-HBP on motility, viability and acrosome reaction during incubation of spermatozoa of buck # 4, 5 and 6 (exhibiting < 80% initial motility) at 37°C for 6 hrs

Mitochondrial respiration would be predominant source of ATP required for flagellar movement. Hence, sperm motility would indirectly reflect the ability of mitochondria to propel the sperm and represent a way to detect the ATP production. Determination of IMMP was done by JC-1 probe. Several reports suggest that analysis of IMMP may be also a mean to assess sperm motility and changes in IMMP could be a good indicator of sperm's functional status. A highly significant correlation between high IMMP and motility has been described in stallion and humans (Love *et al.*, 2003 and Marchetti *et al.,* 2002). In other words, it can be said that due to decrease in IMMP, there was

a decrease in motility of spermatozoa during *in vitro* induction of capacitation/AR of goat buck spermatozoa. However, it was higher in the presence of SP-HBP.

Fig. 7: Effect of SP-HBP on Inner mitochondrial membrane potential during incubation of spermatozoa of buck # 4, 5 and 6 (exhibiting < 80% initial motility) at 37°C for 6 hrs.

A significant (p<0.05) increase in acrosome reaction (vesiculated and acrosome reacted spermatozoa) and medium IMMP was noticeable in control and HBP treated samples with an increase in incubation time (Figs. 4-7). Percentage of spermatozoa with vesiculated and acrosome reacted spermatozoa was higher in two bucks of G-I compared to G-II in control as well as HBP treated samples. It indicated that higher percentage of initial motility does not result in high rate of AR. Rather, buck effect was noticeable on *in vitro* capacitation and AR

in both groups. *In vitro* induced AR was also significantly (p<0.05) higher in HBP treated samples compared to control in both groups at 4 hrs and 6 hrs of incubation. Therefore, observations indicated that induction of capacitation and AR results in decrease in motility, viability and high IMMP.

During the present study, supplementation of HBP in the presence of 10 µg/ml heparin enhanced the *in vitro* AR rate. It has been determined in both *in vivo* and *in vitro* that heparin or GAGs capacitate spermatozoa, probably due to their ability to sequester coating proteins (Parrish *et al.*, 1994). Due to this reason, these proteins are called as HBP. HBP are produced by the accessory glands under the control of androgens (Miller *et al.,* 1990) and secreted within the SP (Nur *et al.*, 2005). It is well documented in bovines that HBP bind the epididymal sperm and increase the ability of AR in response to heparin and other proteins of zona pellucida (Ax *et al.*, 2002). Similar to our observation, significant difference in *in vitro* capacitation of SP-HBP treated and control group was observed in buffalo semen (Arangasamy *et al.*, 2005).

Effect of SP-HBP on *in vitro* capacitation and AR of buck spermatozoa was revealed by a significant (p<0.05) increase of 6.79% and 7.90% in G-I and G-II, respectively. Patel *et al.* (2016) also revealed 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. It indicated that incorporation of HBP to the buck spermatozoa also resulted in high rate of capacitation/AR. In bovine spermatozoa, capacitation is also modulated by phophatidyl choline binding protein of SP in the presence of heparin (Amman *et al.,* 1999). Therien *et al.* (2001) reported that FF and FF-HDL induced capacitation was increased by a pre-incubation with BSA – A1/A2 proteins in bovine epididymal spermatozoa. BSP proteins can modulate the capacitation process by two different mechanisms using either heparin or high-density lipoprotein (HDL) as capacitating

agent (Lane *et al.*, 1999). BSP proteins are reported to bind spermatozoa and also interact with a polipoprotein A1 which is a major component of HDL (Lane *et al.*, 1999). Moreover, the HDL has been identified as a cholesterol acceptor and implicated in sperm capacitation and the AR of human sperm (Hamdi *et al.*, 2010). Upon ejaculation, the BSP proteins interact with the sperm surface via their membrane choline phospholipids and induce sperm cholesterol efflux (Therien *et al.*, 1995; Moreau *et al.*, 1999; Lusignan *et al.*, 2007). This leads to the sperm capacitation following subsequent interaction with heparin. BSP proteins bind to the sperm membrane and act as heparin receptors. This binding increases the heparin-binding sites on the sperm membrane. The binding of Heparin to the BSP proteins-coated sperm membrane induce a series of intracellular events such as an increase in pH, Ca^{+2}, and cAMP (Pons-Rejraji *et al.*, 2009). Since, both heparin and SP-HBP were incorporated in the capacitation medium, therefore, the above proposed mechanism may seem responsible for enhanced rate of *in vitro* AR in SP-HBP treated semen of Beetal bucks.

Literature indicating effect of HBP on capacitation/AR of goat buck spermatozoa is lacking. However, a few reports indicated successful induction of *in vitro* capacitation/AR of goat buck spermatozoa. The plasma membrane alterations accompanying *in vitro* capacitation and AR of goat buck spermatozoa were studied, which revealed fusion of plasma membrane and outer acrosomal membrane at multiple foci (Bawa *et al.,* 1993). During the present study, 20.86% to 52.01% and 15.52% to 46.67% AR was induced in HBP treated and control samples after 6 hrs of incubation. Incubation of mature goat spermatozoa in BSA free medium containing of β-cyclodextrin resuted in 35% AR (Ibbora *et al.*, 2000).

Effect of HBP on lipid peroxidation during *in vitro* capacitation/AR

Average MDA production ($\mu M/10^9$ spermatozoa) was 28.83 ± 3.41, 32.30 ± 2.24 and 23.71 ± 1.43 in freshly ejaculated, HBP induced and without HBP induced acrosome reacted spermatozoa, respectively (Table 2). It indicated a non-significant (p>0.05) increase in LPO during capacitation and AR of buck spermatozoa. Spermatozoa itself, produce small amounts of ROS that are essential to many of physiological processes i.e. capacitation, hyper-activation and oocyte fusion (Aitken *et al.,* 1998). Low levels of ROS are also shown to be essential for fertilization. Therefore, it can be revealed that higher levels of MDA in capacitated and acrosome reacted spermatozoa was essential for the induction of capacitation and AR. MDA level was non-significantly (p>0.05) less in HBP treated spermatozoa as compared

Table 2: Effect of heparin binding proteins on lipid peroxidation (MDA, μM / 109 spermatozoa) during *in vitro* capacitation and acrosome reaction of goat buck spermatozoa

Buck No.	Spermatozoa		
	Ejaculated	**Capacitated /AR**	**HBP treated, Capacitated /AR**
1	25.24 [a] ± 2.89	32.75 [a] ± 2.11	24.76 [a] ± 2.40
2	15.27 [a,b] ± 3.74	29.58 [a] ± 6.47	22.41 [a] ± 7.07
3	24.58 [a] ± 2.17	35.58 [a] ± 6.99	24.10 [a] ± 2.91
4	33.00 [a] ± 3.45	27.69 [b] ± 3.54	20.34 [b] ± 2.57
5	27.80 [a] ± 3.98	30.61 [a] ± 3.12	23.31 [a] ± 3.72
6	47.11 [a,c] ± 16.02	37.59 [a] ± 10.12	27.34 [a] ± 2.49
Total	28.83 [a] ± 3.41	32.30 [a] ± 2.24	23.71 [a] ± 1.43

Figures in superscripts indicate significant (a, b, c) and non-significant differences (a, a) among the bucks and treatments.

to control samples. However, rate of AR was significantly (p<0.05) higher in HBP treated spermatozoa compared to control. Although, small amounts of ROS are required for several sperm functions, but excessive levels may have negative impact on the quality of spermatozoa and impair the fertilizing capacity (Tvrda*et al.,* 2011). Therefore, reduced levels of ROS in HBP treated spermatozoa may be responsible for higher rate of AR in comparison to control. These observations also reveal that LPO can be an indicator of *in vitro* capacitation and AR of buck spermatozoa.

CONCLUSIONS

Study highlighted significance of SP-HBP on *in vitro* capacitation / AR of buck spermatozoa. Since, capacitation/AR is pre-requisite for fertilization, therefore, roll of HBP in higher fertility rate of goat buck semen may also be predicted in goat bucks.

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