Indian Journal of Animal Sciences **83** (8): 788–790, August 2013/Short communication

Effect of heparin binding proteins on the *in vitro* sperm characters and lipid peroxidation status of frozen thawed bull semen*

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Received: 25 January 2013; Accepted: 6 March 2013

Key words: Bull, Heparin binding proteins, Semen, Sperm characters

Fertility associated proteins such as osteopontin, bovine seminal plasma proteins (BSP A_1, A_2, A_3), heparin binding proteins (HBP) and others are reported as indicators of bull fertility (Moura *et al*. 2006). HBPs were secreted from the prostate, seminal vesicles and bulbo-urethral glands into seminal fluid and bind to sperm at ejaculation. HBPs with molecular weight of 28 to 31 kDa in the sperm membrane were named as fertility-associated antigen (FAA) and reported as marker for bull fertility. The present study was carried out to find whether addition of HBPs to the frozen thawed bull semen samples will affect the vital *in vitro* sperm characters such as sperm motility, functional membrane integrity, mitochondrial membrane potential and also to know whether these proteins had any effect on controlling the oxidative stress and lipid peroxidation of bull semen samples during incubation.

Frozen semen samples were procured for 22 bulls and the *in vitro* sperm characters namely, sperm motility, functional membrane integrity, mitochondrial membrane potency and lipid peroxidation status were assessed using standard procedures at immediate post thaw, 60 min, 120 min and 180 min post thaw incubation (Bollwein *et al*. 2008). To assess the effect of HBP on *in vitro* sperm characters, 25 µg of HBP isolated from neat semen of bulls positive for 28– 30 kDa protein in the sperm membrane was added to the thawed semen and *in vitro* sperm characters were assessed at 60, 120 and 180 min of incubation. All statistical analyses were carried out using the Statistical Package for Social Sciences programme.

Per cent of sperm cells with progressive forward motility decreased significantly ($P < 0.01$) at different periods of incubation 60 min, 120 min and 180 min post thaw in both control and treatment group (Table 1). Percent of sperm cells with progressive forward motility was significantly lower ($P < 0.01$) in HBP treated group than the control at each incubation points 60 min, 120 min and 180 min post thaw. Percent sperm cells with non progressive motility in HBP treated group was significantly ($P < 0.01$) higher than control during post thaw incubation. Total motility in sperm cells decreased significantly ($P < 0.01$) during different periods of incubation 60 min, 120 min and 180 min post thaw in both control and treatment groups. Total motility in HBP treated group and control did not differ during different points of incubation. Curvilinear velocity of the sperm cells (µm/sec) reduced significantly ($P < 0.01$) at 120 min and at 180 min incubation from the initial immediate post thaw level in control group. Similarly in HBP treated group velocity significantly reduced at 60 min and at 180 min of incubation. There was no significant difference between control and HBP treated groups in the curvilinear velocity of sperm cells during different points of incubation. The results indicated that the addition of fertility associated protein reduced the progressive forward motility of the sperm cells but it did not cause a total loss of sperm motility. Einspanier *et al*. (1971) and Schoneck *et al*. (1996) observed that higher concentration of acidic seminal proteins inhibited the motility of sperm cells to conserve their energy. Dostalova *et al*. (1994) suggested that removal of adhered proteins from sperm membrane in the female reproductive tract restores the motility of the sperm cell. In the present study, the retention of potential for motility was clearly indicated by maintenance of high mitochondrial membrane potential in sperm cells treated with HBP than the untreated control group.

*Part of P.hD. thesis of first author submitted to TANUVAS, Chennai.

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Table 1. Motility patterns, functional membrane integrity, mitochondrial membrane potential and lipid peroxidation status in frozen thawed bull semen samples - control and treated with heparin binding proteins (HBP) during different period of incubation

Parameter	Treatment	Immediate post thaw	60 minpost thaw	120 minpost thaw	180 minpost thaw
Progressiveforward motility %	Control	63.21±1.61^{a}	48.07±2.92^{b1}	38.45±3.47$^{c\ 1}$	29.05±3.59$^{d\ 1}$
	HBP treatment		38.15±2.01$^{b\ 2}$	28.25±2.74$^{c\ 2}$	18.56±2.83$^{d\ 2}$
Non progressive Motility %	Control	27.81±1.50^{a}	31.65±1.45^{1}	29.51±1.75	25.69±2.40^{1}
	HBP treatment		41.25±1.53$^{b\ 2}$	34.71±1.80^{c}	36.31±1.65$^{c\ 2}$
Total motility %	Control	91.02 ± 1.54^{a}	79.71 ± 3.62^{b}	67.96 ± 4.77^{c}	54.74 ± 5.56^{d}
	HBP treatment		79.40 ± 1.89^{b}	62.96 ± 1.82^{c}	54.88 ± 2.01^{d}
Static cells %	Control	10.15±2.40^{a}	20.03±3.58^{ab}	31.76±4.81^{bc}	43.57±5.61c
	HBP treatment		20.60±1.47^{ab}	37.94±1.99^{bc}	45.13±2.05c
Curvilinear Velocity VCL	Control	72.80 ±3.67^{a}	67.34±2.91^{a}	57.80±3.36^{ab}	52.57±3.82^{b}
(µm/sec)	HBP treatment		57.40±1.82 ^{b}	53.90±1.73^{bc}	48.10±2.22^{c}
Functional membrane integrity %	Control	52.37± 2.45^{a}	38.49± 2.12^{b1}	23.80± 2.15^{c1}	11.55± 1.49^{d1}
	HBP Treatment		43.16± 2.11^{b2}	31.28± 1.95^{c2}	22.15± 1.51^{d2}
High MMP %	Control	19.39±0.75^{a}	14.72±0.7^{b1}	10.36±0.74^{c1}	6.75± 0.63^{d1}
	HBP treatment		15.54±0.60^{b2}	12.06±0.48^{c2}	9.02±0.52^{d2}
Lipid peroxidation levels	Control	1.67± 0.1^{a}	2.85± 0.12 ^{b1}	3.20± 0.20 ^{b1}	4.31± 0.32^{c1}
(MDA µ mol/ml)	HBP treatment		1.95± 0.11^{b2}	2.40± 0.12^{c2}	2.99±0.15^{d2}

Data shown all mean (n = 22) ± SEM. Means with different superscripts (a, b, c, d) in a row and (1, 2) in a column differ significantly (P<0.01).

Sperm cells with functional membrane integrity (%) significantly (P < 0.01) reduced during different points of incubation in both control and HBP treated semen samples (Table 1). Sperm cells with functional membrane integrity were significantly higher (P < 0.01) in HBP treated semen sample than untreated control at each incubation points 60 min, 120 min and at 180 min post thaw. Barrios *et al*. (2000) reported that plasma membrane damages in ram membrane were controlled by the addition of seminal proteins P14 and P 20. The adsorption of proteins by sperm cells reverted the damage as confirmed by the results of electron microscopy. Sperm cells with high mitochondrial membrane potency (%) was significantly (P < 0.01) higher in HBP treated semen sample than control semen at each incubation points (Table 1). In the present study sperm cells treated with HBP retained mitochondrial membrane potential than the untreated control group. Centurion *et al*. (2003) reported that addition of fertility associated protein sperm adhesin preserved mitochondrial activity of spermatozoa up to 5 h of incubation, while most of the sperm cells lost their mitochondrial activity in the untreated control group during the incubation period. MDA level increased significantly (P < 0.01) during different periods of incubation in both control and treatment groups (Table 1). MDA level was significantly lower (P < 0.01) in HBP treated semen sample than control semen during incubation. Elevation of MDA level during different periods of incubation suggested that the resumption of metabolic activity of the sperm cells after thawing leads to generation of excessive ROS (Agarwal *et al*. 2005). In HBP treated group, though the MDA level showed an increase during incubation, it was significantly (P < 0.01) lower than the untreated control samples at different points of incubation. The results indicated that HBP helped in combating oxidative stress of sperm cells. Marti *et al*. (2008) reported that seminal proteins accounted for an increase in the sperm enzyme activity levels even after freezing and thawing.

SUMMARY

The addition of heparin binding proteins to frozen thawed bull semen samples were able to preserve the vital *in vitro* sperm characters essential for successful fertilization such as sperm motility, functional membrane integrity and mitochondrial membrane potential. The proteins were also able to reduce the oxidative stress to the sperm cells during incubation as indicated by less production of lipid peroxide compounds.

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