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Heparin Binding Proteins of Black Bengal Buck Semen and Their Correlation with Sperm Characters and Freezability

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ABSTRACT

This experiment was conducted to study the electrophoretic characters of heparin binding proteins (HBP) of Black Bengal buck semen and their correlation with sperm characters and cryo-survivability. Semen ejaculates (n=20/buck) were collected from nine bucks and *in vitro* sperm characters were evaluated at collection, after equilibration and after freeze - thawing. HBP were isolated through heparin column and discontinuous Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to assess molecular weight. Significant difference (P<0.01) were observed among the bucks in sperm characters and freezability. Eight protein bands of 17 to 180 kDa in seminal plasma and 7 bands in sperm were found. 180 -136 kDa HBP of seminal plasma and 134-101 kDa HBP of sperm had showed high correlation with *in vitro* sperm characters. Further studies on identification of these proteins and their correlation with *in vivo* pregnancy are needed to find their role as marker for buck selection.

Key words: Buck, Freezability, Heparin binding proteins, *In vitro* characters, Semen.

INTRODUCTION

Black Bengal breed of goats, famous for its meat quality and adaptability are found in Eastern and North Eastern states of India and Bangladesh. Males are castrated and slaughtered at an early age for meat purpose and results in drastic reduction in the availability of matured breeding bucks. The ratio of breeding buck and doe was reduced to 1.13: 88.7 against the recommended 1:20 in farmers' field (Nandi *et al*., 2011) and does were mated by available scrub bucks of nondescript/ mixed breed which resulted in dilution/ loss of valuable germplasm (Khandoker *et al*., 2011). Collection, preservation of semen from elite Black Bengal bucks and its distribution for artificial insemination (AI) could help in protection of this valuable germplasm from indiscriminate breeding. AI costs less when compared to keeping breeding buck(s) in small flocks of less than 10 goats and the farmers will have access to quality elite buck semen. AI in goat is gaining popularity for the last few years in India (Konyak *et al*., 2018 and Karunakaran *et al*., 2019). While adapting AI technology, the buck selected as semen donor should have acceptable fertility, as it influences the reproductive potential of large female population. Proteins present in the seminal plasma and sperm have been reported as markers of fertility (Karunakaran *et al*., 2012). Proteins such as osteopontin, prostaglandin D synthase, bovine seminal plasma proteins (BSP A1, A2, A3) and HBP have been reported as indicators of bull fertility (Karunakaran *et al*., 2016 and Krishnan *et al*., 2016). HBP promote capacitation of sperm cells by increasing the number of heparin binding sites on the sperm surface and stimulating cholesterol release from the membrane (Therein *et al*., 1998). Thus, a positive effect of HBP on fertility could be linked to its ability to mediate these events which are crucial for successful fertilization. Although a lot of works have been carried out on seminal proteins in bovine and other species, only few studies have been carried out in buck semen. Considering the present demand and future scope of preserved goat semen, the current study was carried out to study the *in vitro* sperm characters and freezability of Black Bengal buck semen, to study electrophoretic profile of HBP of seminal plasma and sperm proteins, to find the correlation between HBP and *in vitro* sperm characters and semen freezability.

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MATERIALS AND METHODS

The present experiment was carried out at Eastern Regional Station of ICAR- National Dairy Research Institute, Kalyani, West Bengal, India. Nine Black Bengal bucks (*Capra hircus*) were used in the study. Semen ejaculates (20 ejaculates from each bucks) were collected using artificial vagina. Neat semen samples were evaluated for volume (ml), sperm concentration (millions per ml; Haemocytometer method), mass activity (0 to +5), abnormal count (Rose Bengal staining method), individual motility (percentage of

progressive forward motility) and functional membrane integrity (Hypo osmotic swelling test). After initial evaluation, semen ejaculates were diluted 1: 5 with Tris buffer and equilibrated at refrigeration temperature for three hours and frozen by conventional freezing method in liquid nitrogen (Karunakaran *et al*., 2017). Semen samples were evaluated for *in vitro* characters such as individual motility, functional membrane integrity and concentration of malondialdehyde (MDA; thiobarbituric acid- trichlor acetic acid method) after completion of the equilibration and post freeze thawing of sperm cells.

For protein studies, seminal plasma and sperm cells were separated immediately after collection by centrifugation (560g for 10 min at 5°C). The sperm pellet and the seminal plasma were stored at – 20°C until extraction of protein. Proteins in the seminal plasma were precipitated by ice-cold ethanol method (Asadpour *et al*., 2007) and the sperm proteins were extracted as by Triton X detergent extraction method (Nass *et al*., 1990). HBP in the sperm and seminal plasma proteins were isolated using heparin-sepharose affinity chromatography (Heparin- CL agarose prepacked column, Biolinkk, India) as per Manaskova *et al*. (2002). SDS-PAGE was performed to characterize the proteins based on molecular weight (Laemmli, 1970). Correlation between *in vitro* sperm characters and protein bands obtained was determined by Pearson's correlation coefficient. Two-way ANOVA was applied to analyze the effect of buck on *in vitro* sperm characters at different stages of semen preservation.

RESULTS AND DISCUSSION

In vitro sperm characters

In the present study, an average of 397.40 µl semen ejaculate volume, 2517.50 millions/ml sperm concentration, 3.20 mass motility, 59.60% forward motility, 61.80% functional membrane integrity and 4.80% abnormal count were recorded in the neat semen of Black Bengal bucks (Table 1). Significant differences (P<0.01) among the bucks were observed in the *in vitro* sperm characters. It was noticed that sperm motility and functional membrane integrity of the sperm cells were drastically reduced from their initial values after freeze thawing (Table 2). 34.30±3.78% post-thaw motility was recorded in the present study. In

Table 1: *In vitro* sperm characters (mean ± SEM) of neat semen samples in Black Bengal bucks.

Buck number	Volume (µl)	Concentration (millions/ml)	Mass motility	Individual motility (%)	Functional membrane integrity (%)	Abnormal count (%)
46	490±16.33^{a}	2533.10±76.60^{bc}	4.60±0.16^{a}	72.40±1.06^{a}	65.30±3.11^{bc}	3.70±0.25^{b}
48	485.20±36.55^{a}	2594.10±31.48^{ab}	3.40±0.26^{b}	62.40±3.18^{bc}	58.00±2.32^{de}	5.00±0.26^{ab}
51	485.20±56.29^{a}	3020.10±80.86^{a}	3.10±0.31^{bc}	60.90±4.46^{bcd}	73.20±3.38^{a}	4.70±0.30^{ab}
52	415.20±22.42^{a}	2339.60±20.00^{c}	2.90±0.23^{bc}	49.90±3.57^{e}	61.40±2.79^{bcd}	4.90±0.31^{ab}
53	425.20±13.43^{a}	2417.40±36.54^{c}	2.70±0.21^{c}	49.90±2.38^{e}	62.60±2.35^{bcd}	4.40±0.40^{ab}
55	455.20±8.97^{a}	2406.60±65.10^{c}	3.80±0.20^{ab}	67.90±2.36^{ab}	71.70±2.61^{ab}	4.70±0.47^{ab}
57	290.20±12.47^{b}	2486.60±73.41^{bc}	2.80±0.20^{c}	63.40±1.83^{ab}	60.70±3.00^{cd}	4.80±0.33^{ab}
59	270.20±8.16^{b}	2329.60±10.43^{c}	2.70±0.15^{c}	54.40±1.38^{de}	51.50±1.20^{e}	5.70±0.16^{a}
67	260.20±6.66^{b}	2530.10±12.42^{bc}	2.80±0.13^{c}	55.40±1.17^{cde}	51.80±0.92^{e}	5.70±0.26^{a}
Mean	397.40±32.25	2517.50±69.63	3.20±0.21	59.60±2.60	61.80±2.53	4.80±0.20

Means in a column with different superscripts a, b, c, d and e differ significantly at P < 0.01.

Table 2: *In vitro* sperm characters after completion of equilibration and post freeze thaw in Black Bengal bucks.

	After completion of equilibration period			Post freeze and thaw		
Buck number	Individual motility (%)	Functional membrane integrity (%)	Concentration of MDA (µ mol/ml)	Individual motility (%)	Functional membrane integrity (%)	Concentration of MDA (µ mol/ml)
46	56.50±1.42^{a}	52.40±2.83^{a}	0.28±0.08^{a}	41.50±1.23^{a}	42.30±1.60^{a}	0.57±0.03^{a}
48	45.50±1.74^{b}	43.20±1.76^{ab}	0.17±0.01^{b}	30.00±1.67^{b}	27.70±1.98^{b}	0.32±0.03^{a}
51	44.00±2.77^{b}	48.90±2.59^{ab}	0.31±0.06^{ab}	26.50±1.83^{b}	27.20±1.35^{b}	0.28±0.04^{a}
52	38.50±1.83^{b}	39.10.±1.90^{ab}	0.18±0.04^{b}	28.50±1.30^{b}	25.30±1.11^{b}	0.38±0.03^{a}
53	38.50±1.98^{b}	46.60±2.26^{b}	0.20±0.06^{b}	33.00±2.00^{ab}	30.80±1.57^{ab}	0.45±0.04^{b}
55	49.50±2.63^{a}	51.10±2.98^{a}	0.30±0.03^{b}	40.50±1.74^{a}	36.40±2.10^{a}	0.63±0.06^{a}
57	49.00±1.25^{a}	44.30±1.45^{b}	0.26±0.03^{c}	34.50±1.17^{ab}	27.10±0.65^{b}	0.13±0.04^{b}
59	45.56±1.74^{b}	41.90±1.31^{b}	0.50±0.05^{c}	39.00±2.08^{ab}	34.30±1.76^{ab}	0.13±0.07^{b}
67	46.00±1.80^{b}	39.00±1.15^{b}	0.56±0.61^{c}	35.50±1.89^{ab}	29.20±4.36^{ab}	0.13±0.09^{b}
Mean	45.89±1.85	45.00±1.64	0.31±0.04	34.30±3.78	31.10±1.84	0.33±0.06

Data shown all mean ± SEM (n = 10) (Means in a column with different superscripts a, b, c differ significantly at P < 0.01).

concurrence to this observation, Apu *et al*., (2008) reported 38.33% and 6.00% post thaw motility of goat semen in Triladyl and Tris diluents, respectively. Under the best experimental conditions, about half the population of motile spermatozoa survives the freeze–thaw process. Buck sperm cells were not well adapted to cooling to low temperatures and there was drastic reduction in post thaw survivability, as a consequence of accumulated cellular injuries that arise during cryopreservation (Teixeira *et al.,* 2002).

Electrophoretic profile of heparin binding proteins of buck seminal plasma

Eight protein bands of molecular weight 180-136 kDa, 134-101 kDa, 75 kDa, 62-49 kDa, 47-36 kDa, 35-25 kDa, 20 kDa and 17 kDa were found in the HBP of seminal plasma. The protein of molecular weight 180-136 kDa was present in 55.55% bucks screened, while 134-101 kDa was present in 77.77% bucks. 75 kDa, 62-49 kDa, 20 kDa and 17 kDa proteins were present in all the nine bucks (100%), while 47-36 kDa and 35-25 kDa proteins were present in 88.88% and 22.22% bucks, respectively. 16 protein bands of 14 to 97 kDa were reported in seminal plasma proteins of Anglo-Nubian goats (Yue *et al*., 2009) and 15 protein bands with molecular weight ranging from 15.13 kDa to 116.20 kDa were recorded in ram seminal plasma (Deori *et al.,* 2018). Karunakaran (2011) identified protein bands in the molecular weight ranging from 15/14 to 205 kDa in the SDS- PAGE of heparin binding proteins of bovine seminal plasma. The variations in the number of bands might be due to aggregation of products of low molecular weight proteins or degradation product of high molecular weight or due to species/breed variations (Arangasamy *et al*., 2005).

Electrophoretic profile of heparin binding proteins of buck sperm

A total of seven protein bands such as 134-101 kDa, 100 kDa, 75 kDa, 62-49 kDa, 47-36 kDa, 20 kDa and 17 kDa were noticed in HBP of sperm. Protein bands of 17 kDa and 20 kDa were present in all the bucks (100%). Proteins of 134-101 kDa, 100 kDa, 75 kDa, 62-49 kDa and 47-36 kDa were present in 33.33%, 55.55%, 66.66%, 88.88% and 33.33% bucks, respectively. Sperm membrane proteins in Assam Hill Goat (AHG) revealed 20 different protein bands with molecular weight ranging from 10 kDa to 75 kDa and six bands such as 10, 14, 16, 49, 57 and 60 kDa were consistently present in all 8 bucks (Deori *et al*., 2018). Further, the protein with molecular weight 22, 30 and 38 kDa showed frequency distribution of 87.50%, 28, 45 and 47 kDa proteins had frequency distribution of 75.00% in AHG. Karunakaran (2011) reported protein bands with molecular weight ranging from 15/14 to 205 kDa in the SDS- PAGE of HBP of bovine sperm membrane.

Correlation between heparin binding proteins of seminal plasma and *in vitro* sperm characters

Eight protein bands with molecular weight 17 to 180 kDa were found in the SDS-PAGE of HBP of seminal plasma. In neat semen samples (Table 3), 180-136 kDa protein showed

Table 3: Correlation between heparin binding proteins of buck seminal plasma and *in vitro* sperm characters.

Protein molecular weight (kDa)	Neat semen						After equilibration				Post freeze thawing			
	Volume	Mass Motility	Indiv. motility	Functional membrane integrity	Sperm concen tration	MDA level	Abn. count	Individual Motility	Functional memb. Integrity	MDA level	Individual Motility	Functional membr. Integrity	MDA level
180-136	0.491	0.711	0.581	0.699	0.29	0.207	-0.334	0.518	0.707	-0.825	0.37	0.532	0.192
134-101	0.407	-0.154	-0.06	0.551	0.321	-0.202	-0.074	-0.421	0.082	-0.525	-0.473	-0.463	-0.36
75	-0.266	0.085	-0.103	0.321	-0.26	-0.213	0.122	0.045	-0.055	0.035	0.354	0.311	0.401
62-49	-0.11	-0.128	-0.122	0.078	-0.04	0.372	0.119	-0.067	-0.128	0.037	-0.036	-0.491	-0.066
47-36	-0.209	-0.274	-0.282	-0.343	-0.282	-0.055	0.071	-0.209	-0.122	0.101	0.03	0.058	0.021
35 - 25	0.371	0.660	0.572	0.444	-0.191	0.149	-0.318	0.547	0.547	0.033	0.5	0.565	0.418
20	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0	0

a significant positive correlation with mass motility (0.711) and functional membrane integrity (0.699) while 35 - 25 kDa HBP showed significant positive correlation with mass motility (0.660). The 180-136 kDa HBP showed significant positive correlation with functional membrane integrity (0.707), significant negative correlation with MDA level (-0.825) and moderate positive correlation with individual motility (0.518) in semen samples after completion of equilibration period. In post freeze thaw evaluation (Table 3), protein band of 180-136 kDa showed moderate positive correlation with functional membrane integrity (0.532) and non-significant positive correlation with individual motility (0.37). Singh *et al*. (2016) found 14 bands in heparin binding seminal plasma protein, 12 in fresh sperm extracts and 13 in frozen-thawed spermatozoa in western blots. They found that in seminal plasma, fresh- and frozen-sperm extracts, bulls positive for 70 and 18 kDa; 55 kDa and 135, 75, 55, 45, 28 and 24 kDa proteins, respectively, had higher ($P < 0.05$) percentages of most seminal parameters ($P < 0.05$) as compared to their negative counterparts. Further they suggested that heparin binding proteins of 135, 100, 70 and 18 kDa influenced the functional activity of sperm membrane, *in vitro* acrosome reaction, DNA integrity and sperm motility. McCauley *et al.*, (2001) recognized the HBP with molecular weight of 18, 31, 33, 48 and 55 kDa as a diagnostic indicator of fertility.

Correlation between heparin binding proteins of sperm and *in vitro* sperm characters

The HBP of sperm revealed seven bands *viz* 134-101 kDa, 100 kDa, 75 kDa, 62-49 kDa, 47-36 kDa, 20 kDa and 17 kDa. In neat semen samples (Table 4), the 134-101 kDa protein showed significant positive correlation with mass motility (0.741) and moderate correlation with individual motility (0.490). After completion of equilibration period, the protein of 134-101 kDa showed a significant positive correlation with individual motility (0.653) and moderate positive correlation with functional membrane integrity (0.485). In post freeze thaw evaluation of semen samples, the protein band of molecular weight 134-101 kDa showed significant positive correlation with functional membrane integrity (0.675) and moderate positive correlation with individual motility (0.44). Asadpour *et al*. (2007) found that 24 kDa protein was significantly correlated with sperm progressive forward motility in neat semen and live count in frozen-thawed semen. The 28-30 kDa heparin binding protein in sperm membrane has been designated as Fertility Associated Antigen (FAA) and it is a heritable trait (Ax, 2004). Bull semen with the presence of FAA in sperm membranes had increased fertility by 9 to 40 per cent under natural service and artificial breeding in beef cattle (Sprott *et al*., 2000). Bellin *et al*. (1998) reported that the per cent of bulls that were FAA negative among 44 herds ranged from zero to 50. Heparin stimulates capacitation by binding to and removing seminal proteins associated with sperm membrane (Miller *et al*. 1990) and calcium uptake. HBPs in the sperm cells promote capacitation by increasing the number of

Table 4: Correlation between heparin binding proteins of buck sperm and *in vitro* sperm characters.

Protein molecular weight (kDa)	Neat semen							After equilibration			Post freeze thawing		
	Volume	Mass Motility	Indiv. motility	Functional membrane integrity	Sperm conc.	MDA level	Abn. count	Individual Motility	Functional memb. Integrity	MDA level	Individual Motility	Functional membr. Integrity	MDA level
134-101	0.332	0.741	0.491	0.158	0.01	0.048	-0.462	0.653	0.485	-0.041	0.44	0.675	0.239
100	0.153	0.081	-0.108	0.038	0.33	0.315	-0.054	-0.096	-0.179	0.016	-0.478	-0.318	-0.165
75	-0.132	0	-0.065	-0.05	-0.63	0.037	-0.353	-0.003	-0.024	-0.181	0.371	0.266	0.172
62-49	0.306	0.139	-0.097	0.097	0.435	0.152	-0.139	-0.103	-0.137	-0.123	-0.554	-0.258	-0.135
47-36	0.209	0.581	0.353	0.082	0.041	0.097	-0.527	0.522	0.33	-0.6	0.299	0.594	0.192
20	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0	0

heparin-binding sites on the sperm surface and stimulating cholesterol release from the membrane (Therein *et al*. 1998). In female reproductive tract, HBPs-bound sperm interacted with oviductal components like high-density lipoproteins which stimulated a second cholesterol efflux resulting in capacitation (Therien *et al*. 1998). It is concluded that variations exist among the bucks in their *in vitro* sperm characters, seminal plasma and sperm heparin binding protein profiles and their ability to withstand freezing injury. HBPs influence the *in vitro* sperm characters and could be used as a supplementary tool in addition to breeding soundness examination for selection of breeding bucks.

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