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Heparin binding proteins of buffalo bulls seminal plasma and their relationship with semen freezability

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ABSTRACT

The present study was conducted on isolation and purification of heparin binding proteins (HBPs) in buffalo bull semen and to establish relationship between level of these proteins and freezability of buffalo semen. Total 40 semen ejaculates were selected from 4 bulls on the basis of mass activity and individual progressive motility. First part of semen ejaculates was used for seminal plasma separation, second part for estimation of cholesterol content in spermatozoa and third part was processed for cryopreservation. Based upon post-thaw motility semen samples were classified into freezable (having individual progressive motility > 40%) and non-freezable (having individual progressive motility < 40%). Seminal plasma of freezable and non-freezable semen samples were pooled separately to isolate HBPs. HBPs were isolated by affinity chromatography. Electrophoretic profile of TSPPs, HBPs was assessed by SDS-PAGE. Out of 40 semen ejaculates, 27 were found freezable and 13 as non- freezable. Seminal parameters like individual progressive motility, per cent live spermatozoa, per cent acrosome intact spermatozoa and cholesterol content of spermatozoa did not differ significantly at fresh stage between freezable and non-freezable semen, however, at post-thaw stage these parameters were significantly higher in freezable as compared to non-freezable semen samples. The level of HBPs (mg/ml) were significantly higher in non-freezable (2.18±0.06) compared to freezable (1.15±0.04) semen. A total of 9 HBPs bands in range of 10 to 170 kDa were recorded on SDS-PAGE. The results showed that higher level of HBPs in non-freezable semen might be responsible for more cryodamage by causing more cholesterol efflux resulting in poor freezability of semen.

Key words: Buffalo bull, Freezability, HBPs, Heparin binding proteins, Semen

Till date there is no single objective test to evaluate the fertility of bull. The search is now on for finding the molecular markers of fertility (Braundmeier and Miller 2001). The development of such markers to identify bulls of high breeding values represents a remarkable way for achieving genetic gain in dairy farming. Cryopreservation of semen has permitted the rapid expansion of reproductive technology such as artificial insemination. However, cryopreservation induces damage to all sperm compartments (Bailey *et al*. 2000, Medeiros *et al*. 2002). Moreover there is also variable degree of morphological, physiological and biochemical alterations in remaining population of live spermatozoa making them unsuitable for optimum fertility (Holt 2000). Even with the best available preservation technique, post-thaw survival of sperm population is approximately 50%. Sperm cryopreservation is a multifactorial problem, where the diluents, the protocol, the species, breed and individual sires within each breed are merely some of the many parameters that need to be included in the evaluation for the success cryopreservation.

Seminal plasma, a complex mixture of secretion originating from the male reproductive tract, contains factors that modulate the fertilizing ability of sperm (Amann *et al*. 1974, Henault *et al*. 1995, Mc Cauley *et al*. 1999). Several factors in seminal plasma were investigated like heparin binding proteins (HBPs), heat shock protein, clusterin and many other unidentified proteins as marker of fertility. HBPs modulate capacitation and zona binding ability of buffalo epididymal spermatozoa (Arangasamy 2003). However, subjecting the HBPs treated buffalo cauda epididymal spermatozoa resulting in significant reduction in motility, viability, acrosomal integrity and response to bovine cervical mucus penetration test (BCMPT) and hypo-osmotic swelling test (HOST) in the HBP treated group when compared to those in control group (Harshan 2007, Kumar 2005).

Major proteins in the bovine seminal plasma constituted by 3 acidic proteins, collectively called as bovine seminal plasma (BSP) proteins (Manjunath 1984). Chandonnet *et al*.

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(1990) later reported the ability of these proteins to bind to heparin. These proteins are secretory products of seminal vesicles (Manjunath *et al*. 1987) and modulate sperm properties (Killian *et al*. 1992, Bellin *et al*. 1994). The BSP proteins stimulate sperm cholesterol and phospholipid efflux in a dose and time dependent manner and prepare the sperm to undergo capacitation after further interaction with either heparin like glycosaminoglycons or high density lipoproteins in the oviduct (Therien *et al*. 1999). Cholesterol is recognized to have stabilizing effect on membranes (Yeagle 1985), hence its efflux provoke reorganization or destabilization of the membrane (Manjunath and Therien 2002). Therefore, keeping in view the above facts and also taken into consideration the deficit knowledge of seminal plasma proteins in buffalos, the present study was designed to investigate the level of heparin binding proteins with semen freezability in buffalo bulls.

MATERIALS AND METHODS

Semen collection and evaluation: The present study was conducted at the Germ-Plasm Centre (GPC), Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). Four Murrah buffalo bulls maintained at the Germ Plasm Centre of IVRI, Izatnagar, were used for the collection of the semen. Semen was collected during morning hour using artificial vagina as per the standard practice. A total of 40 ejaculates, 10 ejaculates from each bull were collected for the experiment on the basis of mass activity as well as individual motility. The mass activity of the semen sample was determined by assessing the motility of the spermatozoa just after semen collection. The individual progressive motility of spermatozoa (%) in semen samples was determined by using sperm quality analyser (SQA-VB) (Johnston *et al.* 1995). The per cent live spermatozoa was determined by adopting differential staining technique using Eosin-Nigrosin stain as described by Campbell *et al*. (1953) and the acrosomal integrity assesses by staining with Giemsa (Watson 1975). The cholesterol content in spermatozoa was estimated as per Bligh and Dyer (1959) with some modification. The semen samples showing mass activity of +3 and individual motility of 70% or above were selected for the study. Each semen ejaculate was divided into 3 parts, first part for seminal plasma protein isolation, second for cholesterol estimation and third for cryopreservation of semen. The part of the ejaculates used for semen cryopreservation was dilute in tris-egg yolk-glycerol dilutor to an extent that each ml of extended semen contains 40 million motile spermatozoa.

Semen freezing: French mini straws (0.5 ml) were filled with the extended semen and sealed with polyvinyl alcohol powder. The straws were subjected to equilibration period at 5 °C for 3 h. After equilibration, the straws were transferred to biological cell freezer for automatic freezing (–140 °C). Then straws plunged into liquid nitrogen (–196 °C) for storage. Semen straws were thawed at 37 °C for 30 sec in water bath and post-thaw motility, viability, acrosomal integrity and cholesterol content of spermatozoa were assessed. Based on the post-thaw motility, semen ejaculates were classified as freezable (having motilitye > 40%) and non-freezable (having motility < 40%). Seminal plasma of freezable and non-freezable semen samples of each bull were pooled separately.

Total proteins in seminal plasma: Total protein concentrations in the buffalo seminal plasma was estimated as per Lowry *et al*. (1951).

Isolation of heparin binding proteins (HBPs): A part of each ejaculate was centrifuged at 4,000 g for 20 min at 4°C to remove the suspended spermatozoa and particulate debris. The cell pellet was discarded and supernatant plasma was collected. It was further centrifuged at 10,000 g for 60 min at 4 °C to clear the seminal plasma and was stored at –20 °C till further isolation of heparin binding proteins.

HBPs in buffalo seminal plasma were isolated using heparin-sepharose affinity chromatography as per Miller *et al.* (1990) with slight modifications. Briefly, clear seminal plasma (2 ml) was loaded into the heparin-sepharose column, pre-equilibrated with 10 mM phosphate buffer, 2 mM EDTA and 0.025% sodium azide. Once the sample entered the HS media, the flow was stopped for 15 min to allow proteins to bind. The column was extensively washed (7 to 8 times) with same equilibration buffer to remove unabsorbed proteins. The adsorbed (or) bound heparin binding proteins (HBP) were eluted in 2 ml fractions with 20 ml elution buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 100 mM choline chloride and 0.025% sodium azide. The optical density (OD) of eluted HBPs fractions was observed in spectrophotometer at 280 nm. Selected fractions were analyzed for protein content by the standard procedure given by Lowry *et al.* (1951).

Molecular weight determination: Electrophoresis was performed (Laemmli 1970) in 15% polyacrylamide gels. Electrophoresed gels were stained with Coomassie Brilliant Blue R–250. The apparent molecular mass was determined by using molecular weight markers gel documentation and analysis-system.

Statistical analysis: The data were analyzed by independent sample t test using SPSS ver 17.

RESULTS AND DISCUSSION

Fresh stage: The mean value for mass activity (0 to +5 scales),% individual progressive motility,% live spermatozoa,% acrosomal integrity and cholesterol content of spermatozoa (μg/50 × 10^6 spermatozoa) in freezable and non-freezable semen samples were presented in Table 1. Seminal parameters were found similar between freezable and non-freezable semen sample. This might be due to selection of those ejaculates having mass activity >3 and individual progressive motility >70%. Mass activity, sperm progressive motility and live sperm percentage in present

Table 1. Seminal parameter of freezable and non-freezable semen at fresh stage (mean±SE)

Freezable/non-freezable	Mass activity	Individual progressive motility (%)	Live (%)	Acrosomal integrity (%)	Cholesterol content (μg/50 × 10^6 spermatozoa)
Freezable (n=27)	3.52±0.13	81.15±1.26	90.3±0.67	84.63±1.14	10.69±0.3
Non-freezable (n=13)	3.31±0.17	80.69±1.90	89.15±1.22	84.04±1.19	11.12±0.32

Table 2. Seminal parameter of freezable and non-freezable semen of buffalo bulls at post-thaw stage (mean±SE)

Freezable/non-freezable	Individual progressive motility (%)	Live (%)	Acrosomal integrity (%)	Cholesterol content (μg/50 × 10^6 spermatozoa)
Freezable (n=27)	50.53±1.55[a]	65.22±1.14[a]	60.08±1.00[a]	7.03±0.17[c]
Non-freezable (n=13)	23.67±2.16[b]	35.92±2.28[b]	31.92±3.31[b]	6.31±0.20[d]

Mean bearing different superscripts in a column differ significantly (ab P<0.01, cd P<0.05).

study supported the earlier findings of Kumar (1989) and Shukla and Misra (2005). Variation in sperm motility could be due to climatic factors (Javed *et al.* 2000), frequency of semen collection (Sayed and Oloufa 1957) and age of bulls (Collins *et al*. 1951). In 2 experiments Lambrechts *et al.* (1999) found intact acrosome percentage to be 89.3±2.3 and 93.5±2.2 in African buffalo epididymal spermatozoa. The cholesterol content of buffalo spermatozoa in present study varied from 8.8 to 13.3 μg/50 million spermatozoa among ejaculates of different bulls. The previous report also showed wide variation (7.87 to 16.75 μg/50 × 10^6 spermatozoa) among ejaculates of different bulls in buffalo (Kadirvel 2006).

Post-thaw stage: Individual progressive motility,% live spermatozoa,% acrosomal integrity and cholesterol content of spermatozoa (μg/50 × 10^6 spermatozoa) in freezable semen sample were 50.93±1.55, 65.22±1.14, 60.08±1.00 and 7.03±0.17 whereas in non-freezable the corresponding values were 22.69±2.16, 35.92±2.28, 31.92±3.31 and 6.31±0.20, respectively, as presented in Table 2. At post-thaw stage individual progressive motility,% live spermatozoa,% acrosomal integrity and cholesterol content of spermatozoa were significantly higher in freezable as compared to non-freezable semen sample. The damage during freezing of buffalo semen resulted in loss of motility as reported by Matharoo and Singh (1980), Kakar and Anand (1981), Kadirvel (2006). Watson (1995) reported that there was 53% reduction in sperm motility after cryopreservation. A significant reduction in post-thaw motility in present study might be due to changes in membrane fluidity because of higher HBPs in non-freezable semen, which makes sperm plasma membrane more prone to damage during freezing and thawing process. HBPs in buffalo seminal plasma caused significantly higher efflux of cholesterol from sperm membrane (freezable 34.23% and non-freezable 43.26%) causing cryodamage to spermatozoa, resulting in poor post-thaw motility. Live percentages of spermatozoa in present study are in agreement with finding of Ansari *et al.* (2011) and Beheshti *et al.* (2011). Per cent acrosomal integrity in buffalo semen as reported by Rasul *et al.* (2001) is in accordance to our finding. More cholesterol efflux from non-freezable ejaculates at post-thaw stage is an indication of adverse effects of HBPs proteins on semen freezability. Higher HBPs in non-freezable semen caused diffusion of cholesterol from plasma membrane which leads to cryoinjury to spermatozoa.

Total seminal plasma protein: The level of total seminal plasma proteins level was found similar in freezable (31.11±2.87 mg/ml) and non-freezable (32.33±1.36 mg/ml) semen. Similar finding for seminal plasma protein was reported by Arangasamy (2003) and Kumar (2005).

Heparin binding proteins (HBPs): The level of HBPs in non-freezable (2.18±0.06) semen was significantly higher than freezable (1.15±0.04) semen. HBPs comprises 3.6% of total seminal plasma protein in freezable and 6.74% in non-freezable samples. Similar finding for HBPs was reported by Arangasamy (2003) and Kumar (2005) in seminal plasma of buffalo bulls. Higher level of HBPs in non-freezable semen might be responsible for more cryoinjury by more cholesterol efflux (Miller *et al.* 1990).

Electrophoretic profile of seminal plasma proteins

Total seminal plasma proteins: On SDS-PAGE total seminal plasma proteins showed 11 bands of molecular weight 10 to 170 kDa range. No difference was observed between electrophoretic profiles of seminal plasma protein present in freezable and non-freezable samples. The major proteins in buffalo seminal plasma were those with a molecular weight less than 25 kDa which supported the earlier findings of Arangasamy (2003).

Heparin binding proteins: A total of 9 protein bands were observed on SDS-PAGE of HBPs in the range of 10 to 170 kDa (around 14, 15, 20, 24, 33, 40, 55, 70 and 100 kDa) as

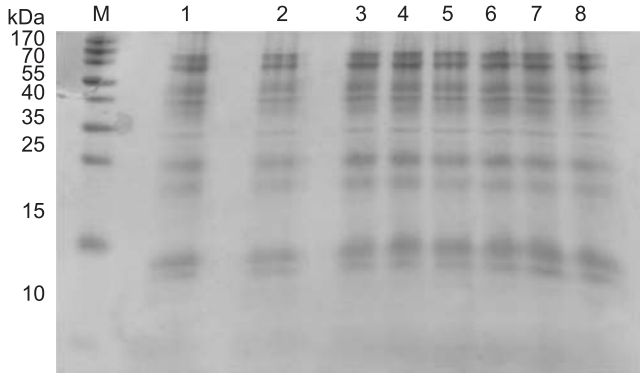

Fig 1. Electrophoretic pattern of heparin binding proteins of freezable and non- freezable semen in buffalo bull. Lane-M: Standard protein marker; lane, 1, 3, 5 and 7: heparin binding protein in freezable sample of four buffalo bulls; lane, 2, 4, 6 and 8: heparin binding protein in non- freezable sample of four buffalo bulls.

shown in Fig 1. Arangasamy (2003) reported 8 and Kumar (2005) reported 6 heparin binding proteins in buffalo bull seminal plasma. The difference in number of bands may be due to inherent character, and also it could be attributed to aggregation products of low molecular weight proteins or a degradation of high molecular weight proteins.

The observations made in the study suggested that the high level of HBPs in semen might be responsible for poor freezability of semen sample. Higher level of HBPs increased the susceptibility of buffalo spermatozoa to stress of cryopreservation process as reflected by lowered post-thaw motility, viability, acrosomal integrity and cholesterol content of spermatozoa. HBPs stimulated cholesterol and phospholipids efflux from spermatozoa membrane in a dose and time dependent manner. The cholesterol and phospholipids efflux resulted in destabilization and increasing membrane fluidity of spermatozoa plasma membrane which results in cryocapacitation of spermatozoa which further resulted in poor freezability of semen.

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