Evaluation of bull semen for fertility-associated protein, *in vitro* characters and fertility

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ABSTRACT

Proteins present in the seminal plasma and sperm influence sperm function and fertilization. The present study was carried out to screen breeding bull semen samples for the presence of fertility-associated 28–30 kDa heparin-binding protein (HPB) and its effect on *in vitro* sperm characters and fertility. Semen samples were collected from 22 breeding bulls and the sperm proteins were extracted by Triton X detergent extraction method. HBPs were eluted, and the molecular weight of the proteins was assessed by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis. Based on the presence/absence 28 kDa HBPs, bulls were categorized into group I and group II. Frozen semen samples were evaluated for *in vitro* sperm characters at immediate post-thaw, 60, 120 and 180 min post-thaw incubation. To assess the field fertility of the bulls, 50 frozen semen straws/bull were used for insemination. Results indicated that only 50% of the bulls screened had 28–30 kDa HBPs in their sperm. Bulls positive for fertility-associated protein had better *in vitro* sperm characters, better protection against oxidative stress, readily underwent capacitation induction by heparin and had 13% higher conception than the bulls lacking the protein. So, it can be concluded that the bulls positive for 28–30 kDa HBPs in sperm had higher chance of fertility and screening for its presence can be included in the regular breeding soundness examination for selection of bulls.

1. Introduction

Currently, breeding soundness examination (BSE) is carried out before introducing a bull into the semen collection programme. BSE involves evaluation of the bull for its health, libido and mating ability. Semen from the bull is evaluated for its volume, sperm cell concentration, motility, viability, morphology and ability to withstand freezing and thawing procedures. Frozen semen samples from bulls that have passed through BSE and possessing quality standards of semen industry were found to yield fertility rates that differed by 20–25% among bulls. The variations in the fertility rate among the bulls were not addressed by the routine semen evaluation parameters (Larson & Miller 2000).

The most accurate method for testing the bull fertility is the insemination of many fertile females, but this method is time consuming, expensive for routine use and only allows a limited number of bulls to be tested at any given time (Barth & Oko 1989). Consequently, it would be of great benefit to the cattle industry to develop a simple, accurate and reliable method of assessing the potential fertility of bulls based on analysis of semen. Subsequently, attention is now being directed towards the assessment of other aspects of semen quality as predictors of bull fertility.

Proteins present in the seminal plasma and sperm have been reported as markers of bull fertility. Seminal plasma, a complex mixture of secretions from testis, epididymis and accessory sex glands contained factors that modulated the fertilizing ability of sperm. Proteins such as osteopontin, prostaglandin D heparin-binding protein (HBP) synthase and bovine seminal plasma proteins (BSP A1, A2, A3) have been reported as indicators of bull fertility (Killian et al. 1993; Bellin et al. 1994; Gerena et al. 1998; Sprotte et al. 2000; Moura et al. 2006). Twenty-eight to 30 kDa HBP of sperm membrane was considered as one of the genetic markers for male fertility and heritable character. Ax (2004) reported that bulls of beef breed positive for the protein in the sperm had 9–40% more conception than the negative bulls upon natural mating. There is no report available regarding the presence/absence of 28–30 kDa HBP among bulls of dairy breed and their effect on conception rate upon artificial insemination (AI). Screening of this protein in dairy bulls which are used as semen donor in frozen stations and its effect on the conception rate upon AI will have future implications for selecting bulls for better conception rate. The present study was therefore undertaken with the following objectives:

(i) To screen semen samples of breeding bulls for the presence of 28–30 kDa HBP.
(ii) To study the effect of presence/absence of 28–30 kDa HBP on the *in vitro* sperm characters of bull semen samples.
(iii) To study the effect of presence/absence of 28–30 kDa HBP on the conception rate upon AI.

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2. Materials and methods

2.1. Experimental animals and source of semen

Semen ejaculates were obtained from 22 breeding bulls maintained by Tamil Nadu Co-operative Milk Producer's Federation Limited, Nucleus Jersey and Stud Farm, Udthagamandalam and Semen Bank, Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai. All the bulls were in regular semen collection programme for insemination purpose and maintained under standard management conditions.

2.2. Collection of semen

Semen was collected by artificial vagina and semen samples that fulfilled the quality criteria of the industry were used in the study. The seminal plasma and sperm cells were separated immediately after collection by centrifugation (560 g for 10 min at 5°C). The sperm cells were washed with 2 ml of Tris Calcium chloride (TC) buffer (40 mM Tris, 2 mM CaCl₂ and 0.01% sodium azide, pH 7.3) by centrifugation (560 g for 5 min at 5°C) to remove the left over seminal plasma, if any. The sperm cells were resuspended with 1 ml of TC buffer containing protease inhibitor (1 mM phenyl methyl sulphonyl fluoride) and washed thrice by centrifugation (560 g for 10 min at 5°C). The sperm pellet and the seminal plasma were transported in ice packed column, Bangalore Genei, India) as per the method described by Manaskova et al. (2002).

2.3. Extraction of sperm proteins

Sperm proteins were extracted as per the method described by Nass et al. (1990). Protein precipitates were separated by centrifugation (8950 g for 10 min at 5°C), air-dried and resuspended in milli-Q water. Protein concentration was estimated using spectrophotometer (Nanodrop, ND-1000, USA) and stored at −80°C until extraction of protein.

2.4. Characterization of sperm proteins by electrophoresis

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). The gel was stained with Coomassie brilliant blue R-250 (0.15%). The apparent molecular mass was determined by using molecular weight markers and Gel Documentation and Analysis System (Gel-Doc. Bio- Rad, UK).

2.5. Bull grouping

Bulls positive for 28–30 kDa HBP in the sperm were categorized into group I and negative bulls into group II.

2.6. Assessment of in vitro sperm characters

Frozen semen samples (French mini straws) were procured for all the 22 bulls for the assessment of in vitro sperm characters. Frozen semen straws were thawed at 37°C for 60 s. The contents were emptied into an eppendorf tube, mixed thoroughly and maintained at thawing temperature in a water bath. In vitro sperm characters were evaluated at immediate post thaw, 60, 120 and 180 min post-thaw incubation (Bollwein et al. 2008). Motility analysis was conducted using a computer-assisted semen analyser (Sperm Class Analyzer, Microptic, Barcelona, Spain) as described by Selvaraju et al. (2008). Functional membrane integrity was assessed using osmotic resistance test (hypo-osmotic swelling test) by incubating an aliquot (100 µl) of semen sample with 1 ml of 150 mM hypo-osmotic and 300 mM iso-osmotic (control) solutions at 37°C for 30 min (Jeyendran et al. 1984). Integrity of sperm DNA was assessed by using acridine orange staining (Chohan et al. 2004). Annexin-V-FITC apoptosis detection kit (Sigma – Aldrich, Saint Louis, USA) was used to detect the translocation of membrane phospholipid phosphatidylserine (PS). The staining procedure was conducted according to the protocol recommended by the manufacturer. Mitochondrial membrane potential was assessed by using JC-1 (5, 5′, 6-`tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide) and plasma membrane integrity was assessed by carboxy fluorescin diacetate (CFDA) and propidium iodide (PI) as described by Selvaraju et al. (2008).

2.7. Assessment of capacitation status

Capacitation status of sperm cells was assessed by chlortetracycline (CTC) fluorescence assay as described previously by Fraser et al. (1995) with modifications. Two numbers of frozen semen straws from each bull were thawed and washed twice with 3 ml of modified Tyreod's solution (mTALP) by centrifugation (400 g for 10 min at room temperature). The sperm pellet thus obtained was resuspended in 400 µl mTALP and incubated in CO₂ incubator (5% CO₂ and 38.5°C) for 180 min. Samples were evaluated for capacitation status at 0, 60, 120, 180 min of incubation. Sperm cells were classified according to their acrosomal staining patterns: Pattern F – bright fluorescence over the entire sperm head and positive mid-piece of the tail-non-capacitated, acrosome-intact sperm; Pattern B – prominent fluorescent positive equatorial segment, mid-piece of the tail and fluorescence free dark band in the post acrosomal region – capacitated, acrosome-intact sperm; Pattern AR – low fluorescent signal thorough out the sperm head, with remaining positive signal in the equatorial segment and mid-piece – acrosome-reacted sperm. Sperm cells with a non-specific or intermediate fluorescent signal status were not selected for analysis.

2.8. Induction of capacitation with heparin

Frozen semen straws were thawed and washed with mTALP as described above and the sperm pellet thus obtained was resuspended in 400 µl mTALP containing heparin (10 µg/ml) and incubated in CO₂ incubator for 180 min to induce capacitation. Samples were evaluated for the capacitation status at 60, 120, 180 min of incubation.

2.9. Estimation of lipid peroxidation

Lipid peroxidation (LPO) level of spermatozoa was estimated in semen samples by measuring the malondialdehyde (MDA)
motility, using thiobarbituric acid as per the method described by Suleiman et al. (1996).

2.10. Al

Fifty numbers of frozen semen straws having more than 50% post-thaw motility were procured for each of the bulls used in the study. AI was carried out by two experienced field workers to assess fertility of bulls. Al workers were unaware about the presence or absence of sperm protein. Total of 1100 cows in second to fifth parity were inseminated at their natural estrum. Pregnancy verification was carried out by rectal palpation of genital organs after 90 days of insemination.

2.11. Data analysis

All statistical analyses were carried out using the Statistical Package for Social Sciences programme (SPSS), version 15.00 software for windows (SPSS Inc. Chicago, IL, USA). Statistical analysis was performed after arcsine transforming the percentage values. Statistical significance was set at 0.05 probability level. If the effect was found significant, comparison of means was done by Duncan Multiple Range Test. Results are expressed as Mean ± Standard Error of Mean. The effect of different duration of incubation on the different in vitro sperm characters and the difference between bull group I and group II at each point of incubation (immediate post-thaw, 60, 120 and 180min post-thaw incubation) were analysed by one-way ANOVA. The following model was used

\[ Y_{ij} = \mu + P_i + e_{ij} \]

where \( Y_{ij} \) is the observation at \( i \)th time of incubation; \( \mu \) the overall mean; \( P_i \) the effect of \( i \)th time of incubation and \( e_{ij} \) the error.

3. Results and discussion

3.1. Electrophoretic profile of HBPs of bovine sperm

Heparin-binding proteins are secreted from the prostate, seminal vesicles and bulbourethral glands into seminal fluid and bind to sperm at ejaculation (Miller et al. 1990; Nass et al. 1990). Protein bands with molecular weight ranging from 15/14 to 205 kDa were observed in the SDS-PAGE of HBPs of bovine sperm membrane. Eleven out of 22 bulls (50.00%) were positive for 28–30 kDa HBP and they were included in the group I, and the 11 bulls negative for the protein were put into the group II. The 28–30 kDa HBP in sperm membrane has been designated as Fertility Associated Antigen (FAA) and it is a heritable trait (Ax 2004). Bellin et al. (1998) reported that the percentage of bulls that were FAA-negative among 44 herds ranged from 0% to 50% (average, 12%; \( n = 2191 \) bulls).

3.2. Evaluation of in vitro sperm characters

3.2.1. Motility and velocity parameters of sperm cells

Motility and velocity parameters of sperm cells assessed by CASA in frozen-thawed semen samples of group I and group II bulls during different periods of incubation are presented in Tables 1 and 2. Bulls in groups I and II did not differ from each other significantly in the motility and velocity parameters during different points of incubation. Per cent of sperm cells with progressive forward motility decreased significantly (\( P < .01 \)) from more than 60% at immediate post thaw to less than 30% at 180 min of incubation in both the group I and II bulls. Similarly, the per cent of total motile cells decreased significantly from more than 90% to around 50% during the incubation. The proportion of static sperm cells increased significantly from 10% to 40% during the incubation period. There was no significant difference observed in the per cent of sperm cells with non-progressive motility during the incubation period. In accordance with the present study, Bag et al. (2004) reported a significant reduction in motile sperm cells during post thaw incubation of ram spermatozoa. The decrease in motility during incubation might be due to a gradual decline in the ability of spermatozoa to generate adenosine triphosphate (ATP) through mitochondrial respiration as a consequence of mitochondrial ageing (Viswanath et al. 1997) or the toxic effect of membrane-bound aromatic amino acid oxidase enzyme released from dead spermatozoa during long storage in ambient temperature (Shannon & Curson 1982).

The curvilinear velocity of the sperm cells in both group I and group II bulls decreased significantly from the initial value of more than 70 µm/s to around 50 µm/s during the 180 min of incubation period. The straight line velocity of the sperm cells was reduced significantly from more than 30 µm/s to less than 20 during the period, while average path velocity was reduced significantly from 40 to 30 µm/s. Linearity of the sperm cell motility varied between 40% and 35%, the straightness of the movement varied between 70% and 65% with the per cent of wobble around 60. Amplitude of lateral head displacement of sperm cells was around 3 µm and the beat cross frequency was 9 Hz, during the incubation period. Though Krzyzosiak et al. (2000) and Bag et al. (2004) reported that velocity parameters did not change during incubation, the reduction in the velocity parameters observed in the present study was well in accordance with the observations of Gil et al. (2000). Variations in the reports on sperm velocity parameters might be due to differences in the settings used in the CASA such as frame rate and frames per field, chamber and time of analysis, sample preparations including thawing temperature, sperm sample concentration and media used for dilution (Contri et al. 2010).

3.2.2. Functional membrane integrity and plasma membrane integrity

In vitro sperm characters such as functional membrane integrity, plasma membrane integrity, mitochondrial membrane potency, DNA integrity and lipid peroxide compound malondialdehyde concentration of semen samples in group I and group II are presented in Table 3. The sperm plasma membrane is the primary site where lesions occur during freezing-thawing of semen (Hammerstedt et al. 1990). Hence, the assessment of structural and functional integrity of plasma membrane is considered useful for predicting the fertilizing ability of sperm (Brito et al. 2003). Structural integrity of plasma membrane was assessed by fluorogenic stain PI and CFDA, while functional integrity was assessed by hypo-osmotic swelling test. Per cent of sperm cells with intact functional membrane and plasma membrane decreased significantly during different periods of incubation. Though the per cent of sperm cells with functional
membrane integrity cells assessed by hypo-osmotic swelling was less than the per cent of structurally intact cells assessed by fluorescent staining, it was not statistically significant. But Selvaraju et al. (2009) reported a significant difference between the per cent of structural intact cells and functional intact cells. This might be because they used eosin-nigrosin staining to estimate the structural integrity of the sperm cells. Woelders (1991) reported that for light microscopic evaluation of membrane integrity, a relatively high concentration of the dye is required; at these concentrations, eosin and many other dyes are toxic, which can lead to underestimation of the proportion of live cells.

Group I bulls had non significantly more number of sperm cells with intact structural and functional membrane than the group II bulls; the presence of fertility-associated proteins in the sperm membrane might have played a role in the protection of membrane integrity. FN-2 domain present in the fertility-associated PSP proteins (BSP/PDC-109) binds to extra cellular matrix and cytoskeleton components and stabilizes the membrane up to the process of capacitation (Juan et al. 2006).

### 3.2.3. Mitochondrial membrane potential of sperm cells
Mitochondria produce ATP by oxidative phosphorylation and provide accessible energy to the tail filaments, thus facilitating efficient propulsion for the sperm both to reach the oocyte and to penetrate through zona pellucida (O’Connell et al. 2002). Mitochondria also provided the required ATP for Na+ / K+ gradient over the plasma membrane. The Na+ / K+ ATPase regulate the chemical and electrical gradient of the plasma membrane. Thus, the functional integrity of mitochondria was important for the sperm survival in the female genital tract.

In the present study, the per cent of sperm cells with high and low mitochondrial membrane potential decreased significantly (P < .01), while the sperm cells with lost mitochondrial membrane potential increased significantly (P < .01) from the immediate post-thaw level during incubation in both the group I and group II bulls. The results obtained in the present study were higher than the value of 6.70% of sperm cells with high mitochondrial membrane potential reported by Selvaraju et al. (2008) in buffalo semen.

Among the groups I and II, group I bulls had significantly (P < .01) more per cent of sperm cells with high mitochondrial membrane potential than the group II bulls during incubation. Caballero et al. (2006) confirmed the protective effect role of fertility-associated PSP proteins by electron microscopic study and reported that these proteins were localized on the acrosomal and post-acrosomal regions of the sperm head and were distributed to other parts during incubation.

### 3.2.4. DNA integrity
Sperm DNA integrity was vital for successful pregnancy and transmission of genetic material to the offspring. The sperm DNA is organized in a specific way that keeps the chromatin compact and stable in the nucleus (Fuentes-Mascorro et al. 2000). Sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis (Saller et al. 1995), defective apoptosis before ejaculation (Sakkas et al. 1999), or excessive production of reactive oxygen species (ROS) in the ejaculate (Moustafa et al. 2004). Sperm cells with fragmented DNA reduced the fertility of bulls (Kasimanickam et al. 2006). The acridine orange staining is a simple microscopic technique to evaluate the DNA integrity (Chohan et al. 2004).

### Table 1. Motility parameters of frozen-thawed bull semen samples.

| Parameter                          | Bull groups | Immediate post thaw | 60 min | 120 min | 180 min |
|------------------------------------|-------------|---------------------|--------|---------|---------|
| Progressive forward motility %     | Group I     | 62.88 ± 2.55p       | 47.61 ± 4.50q | 39.30 ± 5.53r | 29.49 ± 5.03s |
|                                    | Group II    | 63.55 ± 2.08p       | 48.53 ± 3.93q | 37.60 ± 4.45r | 28.61 ± 5.29p |
| Non-progressive motility           | Group I     | 27.60 ± 1.92        | 31.01 ± 1.58 | 28.23 ± 2.68 | 26.83 ± 3.08 |
|                                    | Group II    | 28.02 ± 2.38        | 32.28 ± 2.51 | 30.80 ± 2.32 | 24.55 ± 3.80 |
| Total motility                     | Group I     | 90.48 ± 2.19p       | 78.62 ± 5.22q | 67.53 ± 7.60r | 56.32 ± 7.46s |
|                                    | Group II    | 91.56 ± 2.26p       | 80.81 ± 5.27q | 68.40 ± 6.18q | 53.15 ± 8.68r |
| Static cells                       | Group I     | 9.53 ± 2.19p        | 21.36 ± 5.21q | 32.71 ± 7.66qr | 43.46 ± 7.39r |
|                                    | Group II    | 10.78 ± 4.39p       | 18.69 ± 5.11q | 30.81 ± 6.16qr | 43.68 ± 8.80r |

Notes: Data shown are mean ± SEM (n = 11). Means in a row with different superscripts p, q, r, s differ significantly at P < .01.

### Table 2. Velocity parameters of frozen-thawed bull semen samples.

| Parameter            | Bull groups | Immediate post thaw | 60 min | 120 min | 180 min |
|----------------------|-------------|---------------------|--------|---------|---------|
| Average path velocity VAP (µm/s) | Group I     | 42.89 ± 3.05p       | 39.25 ± 1.92pq | 34.51 ± 3.15qr | 29.95 ± 3.62r |
|                      | Group II    | 46.99 ± 2.69p       | 40.69 ± 2.60p  | 31.37 ± 1.90 q | 30.86 ± 2.59q |
| Linearity %          | Group I     | 41.80 ± 1.32        | 40.30 ± 1.48  | 36.44 ± 1.61  | 35.88 ± 1.82  |
|                      | Group II    | 41.71 ± 1.90        | 39.19 ± 1.86  | 37.71 ± 1.47  | 38.08 ± 1.82  |
| Straightness %       | Group I     | 70.53 ± 1.38p       | 66.71 ± 1.39pq | 64.14 ± 2.01q | 61.81 ± 2.70q |
|                      | Group II    | 69.92 ± 2.14        | 65.97 ± 1.89  | 65.03 ± 1.75  | 65.45 ± 2.40  |
| Wobble %             | Group I     | 59.17 ± 0.96        | 60.28 ± 1.23  | 56.62 ± 1.00  | 57.93 ± 0.94  |
|                      | Group II    | 59.42 ± 1.23        | 59.12 ± 1.20  | 57.84 ± 1.00  | 58.08 ± 1.35  |
| Amplitude of lateral head displacement ALH (µm) | Group I     | 3.36 ± 0.16         | 3.29 ± 0.15   | 3.18 ± 0.18   | 2.82 ± 0.21   |
|                      | Group II    | 3.33 ± 0.25         | 3.34 ± 0.14   | 2.91 ± 0.14   | 2.87 ± 0.18   |
| Beat/cross frequency BCF (Hz)  | Group I     | 9.45 ± 0.45         | 10.11 ± 0.31  | 9.22 ± 0.61   | 8.42 ± 0.76   |
|                      | Group II    | 9.15 ± 0.73         | 9.69 ± 0.50   | 9.61 ± 0.27   | 9.35 ± 0.39   |
| Curvilinear velocity VCL (µm/s)  | Group I     | 72.11 ± 4.29p       | 65.61 ± 3.87pq | 60.99 ± 5.59qr | 51.75 ± 6.29r |
|                      | Group II    | 73.49 ± 6.15p       | 69.07 ± 4.46pq | 54.61 ± 3.76q | 53.39 ± 4.64q |
| Straight-line velocity VSL (µm/s) | Group I     | 30.40 ± 2.42p       | 26.19 ± 2.64pq | 22.09 ± 2.16qr | 18.12 ± 2.00r |
|                      | Group II    | 30.39 ± 2.77p       | 26.87 ± 2.51qr | 24.55 ± 3.80r | 19.74 ± 3.20q |

Notes: Data shown are mean ± SEM (n = 11). Means in a row with different superscripts p, q, r, s differ significantly at P < .01.
In the present study, the loss of DNA integrity during incubation was very low in the group I bulls. The percentages of sperm cells with intact DNA in the group at immediate post thaw, 60, 120 and 180 min of incubation were 94.94 ± 0.61, 94.66 ± 0.56, 93.80 ± 0.66 and 93.64 ± 0.66, respectively. The per cent of cells with intact DNA in group II bulls reduced from the immediate post-thaw level of 93.55 ± 0.70 to 92.17 ± 0.50 at 120 min and to 91.99 ± 0.43 at 180 min of incubation. The per cent of intact DNA cells at immediate post thaw in the present study was in accordance with the reports of Waterhouse et al. (2010). They reported that the proportions of spermatozoa with fragmented DNA increased from 4.80% in fresh semen to 8.90% after freezing and thawing of bull semen. The destabilizing effect of cryopreservation on sperm chromatin may stem from the high ionic strength in frozen nuclei (Courtens et al. 1989) and excessive intracellular influx of free calcium ions (Zhao & Buhr 1995) leading to activation of nucleoprotein-degrading enzymes such as acrosin (Zirkin et al. 1980), endonucleases (Krzyzosiak et al. 2000) and phospholipases with their toxic metabolites, lysocleithins (Upreti et al. 1999). Eveson (1999) suggested that loss of DNA integrity ≥ 20% might announce lower fertility. Loss of DNA integrity during incubation in the present study was less than in the reports of Bollwein et al. (2008) who reported the per cent of DNA fragmentation index increased from 5.70 ± 1.50 at immediate post thaw to 10.90 ± 4.40 at 3 h of incubation. In the present study, group I bulls that were positive for HBP had significantly more number of DNA intact cells than the group II bulls during incubation. The results clearly indicated that fertility-associated proteins preserved the DNA integrity of the sperm cells.

### 3.2.6. Capacitation status of sperm cells

Freshly ejaculated mammalian spermatozoa must undergo a maturation process termed ‘capacitation’ before fertilizing an oocyte (Austin 1952). Capacitation is a continuous biochemical change associated with the functional and structural changes in the sperm. Removal of cholesterol from sperm membrane increases membrane fluidity (Langlais et al. 1988) resulting in increase in calcium influx, cAMP level (White and Aitken 1989) and changes in enzymatic activities such as protein kinase C (Furuya et al. 1993). Capacitation process ended with the acrosome reaction, an essential stage for oocyte fertilization. The physiologic mammalian acrosome reaction was experienced only by sperm that have been previously capacitated. The destabilization of sperm membranes could be evaluated by

| Parameter | Bull group | Immediate | 60 min | 120 min | 180 min |
|-----------|------------|-----------|--------|---------|---------|
| Functional membrane integrity (%) | Group I | 55.61 ± 2.70a | 40.69 ± 2.75b | 25.46 ± 2.95c | 11.59 ± 2.24d |
| | Group II | 49.12 ± 3.98a | 36.29 ± 3.20b | 22.13 ± 3.18c | 11.52 ± 2.07d |
| Plasma membrane (%) | Group I | 58.37 ± 2.79a | 43.30 ± 2.80b | 28.22 ± 3.10c | 15.27 ± 2.11d |
| | Group II | 52.67 ± 4.12a | 38.98 ± 3.48b | 25.96 ± 3.32c | 15.06 ± 2.16d |
| High mitochondrial membrane potential | Group I | 19.65 ± 1.17a | 16.72 ± 1.02b | 12.32 ± 1.04b | 8.31 ± 0.78c |
| | Group II | 19.13 ± 0.99a | 12.71 ± 0.99bii | 8.39 ± 1.02cii | 5.19 ± 1.00dix |
| Low mitochondrial membrane potential (%) | Group I | 52.96 ± 2.50a | 44.35 ± 3.95b | 39.75 ± 5.07bc | 32.03 ± 4.50c |
| | Group II | 55.38 ± 1.73a | 45.68 ± 3.35b | 35.34 ± 3.67c | 33.58 ± 4.24c |
| Lost mitochondrial membrane potential (%) | Group I | 27.39 ± 2.35a | 38.92 ± 3.65b | 47.90 ± 5.05c | 59.79 ± 4.45d |
| | Group II | 25.49 ± 1.35a | 41.62 ± 3.13b | 56.32 ± 3.92c | 61.13 ± 4.56d |
| Intact DNA (%) | Group I | 94.94 ± 0.61a | 94.66 ± 0.56 | 93.80 ± 0.66 | 93.64 ± 0.66 |
| | Group II | 93.55 ± 0.70a | 93.69 ± 0.76ab | 92.17 ± 0.502b | 91.99 ± 0.432b |
| MDA level (µ mol/ml) | Group I | 1.60 ± 0.15a | 2.67 ± 0.19b | 2.79 ± 0.24bii | 3.77 ± 0.41cii |
| | Group II | 1.73 ± 0.13a | 3.04 ± 0.15b | 3.61 ± 0.28bii | 4.84 ± 0.46cii |

Notes: Data shown are mean ± SEM (n = 11). Means with different superscripts a, b, c and A, B in a row differ significantly at P < .01, P < .05, respectively. Means with different superscripts i, ii and 1, 2 in a column for particular treatment differ significantly at P < .01 and P < .05, respectively.

### 3.2.5. Assessment of sperm cell apoptosis

Cell death in general can occur through two distinct ways, necrosis and apoptosis. The externalization of PS was one of the hallmarks of apoptosis. The disturbance of membrane function was detectable as an increase in the cell ability to bind the calcium-dependent binding of annexin-V to the outwardly translocated PS and it could be used as a marker for measuring apoptosis in bovine spermatozoa (Anzar et al. 2002).

Assessment of sperm cell apoptosis using Annexin-V-FITC apoptosis detection kit in bull semen samples during incubation is presented in Table 4. The per cent of viable sperm cells decreased significantly (P < 0.01) during incubation while the per cent of necrotic cells increased in bulls of both groups I and II. The per cent of apoptotic cells also showed a minor increase during incubation. Though it was not significant, group I bulls had less number of apoptotic cells than group II during incubation. Feitosa et al. (2008) reported a significant increase in the per cent of apoptotic cells during incubation over a period of 2 h in frozen-thawed bovine semen samples.

Martin et al. (2007) observed that cryopreservation induces the occurrence of some apoptotic features in bovine spermatozoa and these apoptotic features appeared as ordered events during the cryopreservation process such as decrease in the mitochondrial membrane potential, caspase activation and changes in membrane permeability. The decrease in mitochondrial membrane potential might be facilitated by the fact that bovine spermatozoa contain the pro-apoptotic factor Bax, cytochrome c and flavoprotein apoptosis-inducing factor. The consequence of increased permeability of spermatozoa membrane could lead to early cell death. In accordance with Martin et al. (2007), a significant decrease in membrane integrity and significant loss in mitochondrial membrane potential were observed during incubation in the present study. Januskauskas et al. (2003) suggested that annexin-V-positive cells represent a transitory step between cell viability and necrosis. The incidence of these transitory cells is dependent on the incubation condition and might represent the rate at which sperm cells undergo necrosis in vitro.
tracking the distribution of Ca\textsuperscript{2+} in spermatozoa. Antibiotic CTC, which accumulates in organelles containing high concentrations of Ca\textsuperscript{2+}, was used to evaluate the capacitation status in sperm cells.

In the present study, per cent of sperm cells with F pattern (uncapacitated – acrosome intact) decreased significantly \((P < .01)\) at 60 min of incubation from immediate post-thaw level in both groups I and II (Table 5). Though there was a decrease in the values during further incubation up to 180 min, it was not statistically significant. The per cent of sperm cells with B pattern (capacitated – acrosome intact) was increased significantly \((P < .01)\) at 60 min of incubation from immediate post-thaw level in both e group I and group II bulls (Table 6). Though there was an increase in the values during further incubation up to 180 min, it was not statistically significant. At immediate post thaw, though it was not significant, bulls in group I had low number B pattern sperm cells than bulls in group II (44.12 ± 1.86 vs. 46.30 ± 1.88). But bulls in group I had significantly low \((P < .05)\) number of sperm cells with B pattern at 60 min (50.86 ± 1.82 vs. 55.73 ± 1.76), at 120 min (53.10 ± 1.76 vs. 56.97 ± 1.69) and at 180 min post-thaw incubation (54.79 ± 1.70 vs. 58.43 ± 1.58) than bulls in group II. The per cent of sperm cells with AR pattern (capacitated – acrosome reacted) increased significantly during incubation in both the bull groups (Table 7). In contrast to the present study, Gil et al. (2000) reported slightly more number of F pattern cells (65.60 ± 2.40), less number of B pattern cells (28.60 ± 2.10) and similar number of AR pattern cells (5.80 ± 0.70) at immediate post thaw in bovine semen samples. Kadirvel et al. (2009) reported slightly less per cent of F pattern cells (36.75 ± 1.37), similar per cent of B pattern cells (42.21 ± 2.23) and more per cent of AR pattern acrosome reacted cells (23.34 ± 1.31) at immediate post thaw in buffalo semen samples. All these findings confirmed the presence of a sperm population already capacitated in post-thaw semen, probably induced by the freezing procedures as observed by Watson (1995). Maxwell and Johnson (1997) reported similarities between the changes associated with capacitation and cryoinjury, such as plasma membrane reorganization, fluidization, calcium influx and ability to undergo the acrosomal reaction. This cryo-capacitation is thought to be partly responsible for the reduced fertility of frozen-thawed bull semen (Cormier & Bailey 2003).

3.2.7. Induction of capacitation with heparin

When the semen samples were treated with heparin to induce capacitation, the per cent of F pattern (uncapacitated) cells was reduced significantly, while the per cent of B pattern (capacitated) sperm cells was increased significantly during different points of incubation in both group I and group II bulls when compared to the untreated control. AR pattern (acrosome reacted) cells also showed an increasing trend during incubation with heparin when compared to control. Bulls in group I had significantly \((P < .05)\) low number of F pattern cells and significantly \((P < .05)\) more number B pattern cells at 60, 120 and 180 min post-thaw incubation than the group II.

Glycol amino glycons have been ascribed among a variety of substances within the oviductal fluid as causing sperm capacitation (Tienthai et al. 2004). Heparin stimulates capacitation by binding to and removing seminal proteins associated with sperm membrane (Miller et al. 1990) and calcium uptake. Mehmood et al. (2007) reported that heparin induces capacitation in dose- and time-dependent manner in frozen buffalo semen. HBPs in the sperm cells promote capacitation by increasing the number of heparin-binding sites on the sperm surface and stimulating cholesterol release from the membrane (Theiren 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 (Theiren et al. 1998).

3.2.8. Lipid peroxidation

The mechanism of ROS-induced damage to spermatozoa includes an oxidative attack on the sperm membrane lipids leading to initiation of LPO cascade (Sharma & Agarwal 1996). A simple tool to evaluate the level of LPO in the spermatozoa

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### Table 4. Assessment of sperm cell apoptosis in frozen-thawed bull semen samples during incubation.

| Parameter | Bull group | Immediate | 60 min | 120 min | 180 min |
|-----------|------------|-----------|--------|---------|---------|
| Viable sperm cells (%) | Group I | 60.20 ± 3.93a | 40.99 ± 4.66b | 25.34 ± 5.29c | 13.39 ± 2.68d |
| Necrotic sperm cells (%) | Group II | 50.51 ± 6.89a | 35.78 ± 6.08b | 23.84 ± 5.84c | 15.56 ± 5.67d |
| Apoptotic sperm cells (%) | Group I | 32.30 ± 2.96a | 50.15 ± 4.17b | 64.56 ± 4.24c | 77.02 ± 2.32d |
| | Group II | 39.37 ± 5.27a | 53.65 ± 5.37b | 64.83 ± 6.18b | 73.70 ± 4.06c |

Notes: Data shown are mean ± SEM \((n = 11)\). Means with different superscripts a, b, c, d in a row differ significantly at \(P < .01\).

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### Table 5. Assessment of capacitation status – F pattern (non-capacitated, acrosome-intact) sperm cells in frozen-thawed bull semen samples.

| Treatment | Bull group | Immediate | 60 min | 120 min | 180 min |
|-----------|------------|-----------|--------|---------|---------|
| Control   | Group I    | 51.73 ± 2.11a | 43.19 ± 2.061bp | 40.38 ± 2.011bp | 37.48 ± 2.211bp |
|           | Group II   | 48.19 ± 2.39a | 37.11 ± 2.112bx | 34.48 ± 2.142bx | 31.44 ± 2.282bx |
| Treatment with heparin | Group I | 51.73 ± 2.11a | 31.03 ± 1.841bq | 22.18 ± 1.911cq | 15.76 ± 1.871dq |
|           | Group II   | 48.19 ± 2.39a | 37.43 ± 2.162bx | 27.81 ± 1.962cy | 21.48 ± 1.592dy |

Notes: Data shown are mean ± SEM \((n=11)\). Means with different superscripts a, b, c, d in a row differ significantly at \(P < .01\). Means with different superscripts i, ii and 1, 2 in a column for particular treatment differ significantly at \(P < .01\) and \(P < .05\), respectively. Means of group I bulls with different superscripts p, q in a column differ significantly at \(P < .01\).
is the assay of sperm MDA, a thioobarbituric acid reactive substance (TBARS) that is a stable LPO product (Kasimanickam et al. 2006).

In the present study, the MDA level increased significantly \((P < .01)\) during different periods of incubation from the immediate post-thaw level in both groups I and II. Elevation of MDA level during different periods of incubation suggests that the resumption of metabolic activity of the sperm cells after thawing leads to generation of excessive ROS (Agarwal et al. 2005). Activation of an aromatic amino acid oxidase enzyme from dead sperm cells also might be an additional source of ROS in frozen-thawed semen samples. Among the group I and group II bulls, group I bulls had low level MDA than group II bulls at different points of incubation (Table 3). This clearly indicates that the bulls positive for HBP had significantly better protection against oxidative stress and were able to control the generation of lipid peroxides during incubation.

### 3.3 AI and conception rate

Out of 1100 cows inseminated, 60 cows were sold out during the experimental period and the remaining cows were checked for pregnancy; 57.28% (295 out of 515) cows that were inseminated with semen of group I bulls were pregnant while only 44.38% (233 out of 525) cows that were inseminated with semen from group II bulls were pregnant. The bulls positive for 28–30 kDa HBP had about 13% higher conception rate than those bulls lacking this protein.

Bulls with increased fertility produced sperm with greater affinity to bind heparin-like complex sugars that were commonly found in the reproductive tract of females (Marks and Ax 1985). It has been suggested that seminal proteins mediate the binding of sperm cells to oviductal epithelium and preserve membrane integrity by exerting inhibiting effects on the mitochondrial activity and metabolism to conserve energy needed until fertilization as well as to minimize the production of ROS and LPO of sperm membrane. Proteins would also have activities in anti-apoptosis and cell survival (Rangaswami et al. 2006). Seminal proteins would mediate sperm–oocyte interaction and fertilization (Moura et al. 2006). Thus, a positive effect of HBPs on fertility could be linked to its ability to mediate these events which are crucial for successful fertilization.

### 4. Conclusion

Variations in the electrophoretic profile of sperm proteins were observed among the breeding bulls. Fertility-associated 28–30 kDa HBP was present only in 50% of the breeding bulls screened. Sperm cells positive for fertility-associated protein had better in vitro sperm characters, better protection against oxidative stress and they readily underwent capacitation induction by heparin than the negative cells. Bulls for the protein had 13% higher conception rate than the bulls lacking the protein.

It can be concluded that breeding bulls may be screened for the presence of 28–30 kDa HBP in the sperm in addition to the regular BSE to have a better conception rate after AI.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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