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The presence of heparin binding proteins and their impact on semen quality of Holstein Friesian bulls

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ABSTRACT

Present study was designed to evaluate the semen quality of breeding bulls positive for 24 and 30 kDa heparin binding proteins (HBPs) in the semen. Seventeen Holstein Friesian breeding bulls were screened for 24 and 30 kDa HBPs in semen by heparin-sepharose affinity chromatography and SDS-PAGE, and grouped into three based on presence/absence of HBPs. *In vitro* characteristics of spermatozoa were assessed by standard analysis of sperm abnormalities, functional membrane integrity, acrosome integrity and sperm chromatin integrity in fresh and frozen-thawed semen. Bulls positive for 30 kDa had lowest sperm abnormalities and sperm chromatin fragmentation than bulls positive for 24 kDa and without HBPs in the fresh ejaculates. Further, fresh semen positive for 30 kDa had 8.76% of higher active plasma membrane with 7.63% intact acrosome. The process of cryopreservation of semen increased sperm abnormalities, acrosome damage and chromatin fragmentation in all the semen samples irrespective of presence/absence of HBPs. However, semen samples positive for 24 and 30 kDa HBPs were able to sustain lower percentage of post-thaw sperm abnormalities with higher chromatin integrity in comparison to semen devoid of HBPs. Moreover, semen with 24 and 30 kDa HBPs had higher post-thaw intact acrosome with active plasma membrane than negative semen. The results concluded that bulls positive for 30 kDa had 12.43 and 28.37 percentages of better semen quality than bulls with 24 kDa and devoid of HBPs.

Key words: 24 and 30 kDa, Heparin binding proteins, Quality, Semen

Artificial insemination with cryopreserved or frozen semen is very common to breed the majority of dairy cows in the current scenario of animal reproduction (Ardon and Suarez 2013). The frozen semen quality is utmost important to achieve maximum fertility rate which mainly depends upon the accurate evaluation of the bull fertility. The routine semen analysis used to predict the bull fertility are measurements of sperm concentration, percentage of motility, viability and morphology, but they may not reveal subtle sperm defects. Currently, the attention is being directed towards the assessment of other aspects of semen as predictors of bull fertility. The seminal plasma and sperm membrane proteins contain factors that modulate the fertilizing ability of sperm cells which are related with fertilization process have been reported as markers of male fertility (Kutty *et al.* 2014). The seminal proteins mediate the binding of sperm cells to oviductal epithelium and preserve membrane integrity by minimizing the production of reactive oxygen species and lipid peroxidation of sperm membrane (Karunakaran *et al.* 2012).

In cattle, three bovine seminal plasma (BSP) proteins, BSP1, BSP3 and BSP5 (30 kDa) have been found to play a crucial role in fertilization (Manjunath *et al.* 2009). The heparin binding proteins (HBPs) with 28–30 kDa of sperm membrane are known as fertility associated antigen and considered as one of the genetic marker for male fertility and sperm cryotolerance (Goularte *et al*. 2014). The bulls positive for the fertility associated antigen had 9 to 40% more conception rate than the negative bulls (Ax 2004). Another class of HBP with molecular weight of 24 kDa had significantly correlated with sperm progressive motility in fresh and viability in frozen-thawed semen with 13% higher fertile than negative bulls (Asadpour *et al*. 2007). The inclusion of 24 and 30 kDa HPBs as biomarkers of male fertility along with routine semen analysis of breeding soundness examination may enhance the herd fertility (Ax 2004). Hence, the present study was made to evaluate the semen quality of breeding bulls based on the presence or absence of 24 and 30 kDa HBPs.

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MATERIALS AND METHODS

Experimental animals: Adult Holstein Friesian breeding bulls (17), selected from the Institute, were in regular semen collection programme and maintained under standard managemental practices.

Semen collection and isolation of heparin binding proteins: Semen samples were collected by artificial vagina method and processed immediately after analysis for its quality. Proteins in the seminal plasma were precipitated with ice-cold ethanol at the ratio of 1:9 volumes, incubated at 4°C overnight and protein precipitates were isolated by centrifugation. The sperm membrane extracts were obtained by cell lyses with TC buffer containing triton X-100 (0.1% v/v) and proteins were isolated by precipitation method. Heparin binding proteins in the sperm membrane and seminal plasma proteins were isolated using heparin-sepharose affinity chromatography as per Manaskova *et al*. (2002) with slight modifications. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 12% resolving gel at constant voltage mode. The gel was stained with coomassie brilliant blue R–250 (0.15%) and the apparent molecular mass was determined by using molecular weight markers and gel documentation and analysis system.

The bulls were grouped based on the presence/absence of 24 and 30 kDa heparin binding proteins in seminal plasma as well as in sperm membrane. The bulls were categorized into three groups as follows, group 1 (positive for 30 kDa, n=8), group 2 (positive for 24 kDa, n=5) and group 3 (negative for 24 and 30 kDa; n= 4).

Sperm cell morphology: Morphological evaluation of sperm cells was carried out by using rose bengal stain. Drops of diluted fresh and frozen-thawed semen were taken into an eppendorf tube separately. Two drops of 3% rose bengal stain and 1000 µl of Tris buffer were added to semen samples. The contents were mixed and kept at 37°C for 10 min. Then the sperm cells were washed twice in Tris buffer by centrifugation (560 × g for 5 min) and sperm cells suspended in Tris buffer were placed on a glass slide. A minimum of 500 sperm cells were analyzed at 100× using a microscope.

Functional membrane integrity: Functional membrane integrity was assessed using osmotic resistance test (hypo-osmotic swelling test - HOST) by incubating an aliquot (100 µl) of fresh and frozen-thawed semen with one ml of 150 mOsm hypo-osmotic and 300 mOsm iso-osmotic (control) solutions at 37°C for 30 min (Jeyendran *et al*. 1984). After incubation, one drop of the well mixed sample was placed on a glass slide and covered with cover slip. A minimum of 500 spermatozoa were observed for hypo osmotic response as tail coiling.

Acrosome integrity: A drop of diluted fresh semen (1:10 in 2.9% sodium citrate) and frozen-thawed semen was used to prepare a thin smear and fixed in 5% formaldehyde for 30 min. The slides were washed with running tap water and the air dried smears were stained by immersing in coupling jar containing working Giemsa for 6 h at 37°C. The stains were washed in running water and air dried. A minimum of 500 spermatozoa were counted with a phase contrast microscope (100×) for their normal apical ridge (Watson 1975).

Sperm chromatin integrity: Thin smears of fresh ejaculate and frozen-thawed semen were prepared on pre-cleaned slides and air-dried smears were fixed in freshly made 96% ethanol-acetone (1:1) at 4ºC for 30 min. Hydrolysis was performed with 0.1 N HCl at 4ºC for 5 min and rinsed thrice in distilled water for 2 min each. Smears were stained with toluidine blue (0.05% in 50% McIlvain's citrate phosphate buffer with pH 3.5) for 10 min (Barrera *et al*. 1993). The slides were rinsed briefly in distilled water, lightly blotted with filter paper and dehydrated in tertiary butanol at 37ºC. The stained slides were scored by light microscopy by counting at least 500 sperm heads. Those with good chromatin integrity were light blue and those of diminished integrity were deep violet (purple).

Statistical analysis: All statistical analyses were carried out using the Statistical Package for Social Sciences programme (SPSS), version 16.00 software for windows. Statistical analysis was performed after arcsine transformation of percentage values. The comparison of *in vitro* characteristics of spermatozoa between groups based on the presence or absence of 24 and 30 kDa proteins were analyzed by one way ANOVA. 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. To test the significance of sperm *in vitro* characteristics between fresh and frozen-thawed semen, paired't' test was used. Results are expressed as mean ± standard error of mean.

RESULTS AND DISCUSSION

Sperm cell morphology: The results of sperm cell morphology in fresh and frozen-thawed semen of all the bulls are presented in Table 1. The fresh ejaculates of bulls positive for 30 kDa had 3.01 and 6.54 percentages of lower (P<0.01) sperm cell abnormalities in comparison to bulls positive for 24 kDa and negative for HBPs, respectively. Further, bulls positive for 24 kDa had 3.53% of lower sperm abnormalities than negative bulls. The importance of morphologically normal sperm in the process of fertilization has been widely accepted and the present results are in accordance with the previous findings of Malik *et al*. (2015)

Table 1. Sperm cell abnormalities in the semen of bulls positive for heparin binding proteins

Animal group	No. of animals (n)	Sperm abnormality (%)		't' value
		Fresh semen	Frozen-thawed semen	
1	8	9.36±0.81^{a}	11.56±0.80^{a}	6.09*
2	5	12.37±0.70^{b}	14.94±0.41^{b}	4.46*
3	4	15.90±1.71^{c}	20.14±1.02^{c}	2.92*
'F' value		53.74**	200.75**	

Means with different superscripts in column differ significantly (*, P<0.05; **, P<0.01).

and Zodinsanga *et al*. (2015). The lower percentage of abnormal sperm cells in the bulls positive for HBPs was an indicator of high fertility potential of a male (Malik *et al*. 2015). However, semen of bulls lacking for HBPs had higher (P<0.05) percentage of sperm abnormalities which indicated the association of increased morphological abnormalities of spermatozoa with reduced reproductive efficiency of bull (Zodinsanga *et al*. 2015). The high proportion of abnormal spermatozoa could be of genetically heritable character and have a detrimental impact upon fertilization and embryonic development.

The cryopreservation increased (P<0.01) the sperm abnormalities by 2.39% in frozen-thawed semen of bulls with HBPs and it was 4.24% in case of semen without HBPs in comparison to their fresh ejaculates. However, post-thaw sperm abnormalities were lower (P<0.01) in semen with HBPs. The freezing of living cells can cause unfavourable and damaging effects due to ice crystal formation and/or severe osmotic changes which may affect chromatin structure and sperm morphology (Nandare *et al*. 2011). However, the post-thaw sperm abnormalities were lower (P<0.05) in the semen of bulls positive for HBPs which could be due to the protective effects of HBPs against oxidative damage and mechanical injuries (Karunakaran *et al*. 2012, Bromfield *et al*. 2014). Whereas, the semen negative for HBPs had higher incidence of post-thaw sperm abnormalities as a result of freezing and thawing (Bromfield *et al*. 2014). The proportion of sperm cell abnormalities in the present study was within the accepted level of 20% in all the bulls. These findings were obviously, due to the fact that the bulls involved in the experiment were selected based on breeding soundness examination score and reared as artificial insemination sires which were in the regular production.

Functional membrane integrity: The functional sperm membrane integrity of fresh and frozen-thawed semen of all the bulls is presented in Table 2. The bulls positive for 30 kDa had 3.91 and 8.76 percentages of higher (P<0.01) sperm with active sperm plasma membrane than the bulls with 24 kDa and negative for HBPs, respectively in fresh ejaculates which are in conformity with the findings of Hernandez *et al*. (2015) and Zodinsanga *et al*. (2015). The seminal plasma and HBP proteins may have protective and stabilizing effects on sperm membrane (Karunakaran *et al*. 2012). Further, major BSP proteins (BSP-A1/A2, A3 and BSP-30 kDa) are also known to safe guard and influence capacitation of acrosome by their ability to modulate membrane cholesterol (Moura *et al*. 2007). The fresh ejaculates of bulls devoid of HBPs had higher (P<0.01) percentage of inactive/damaged plasma membrane which might be due to the higher steroid content of sperm membrane which might have reduced their membrane fluidity (Zodinsanga *et al*. 2015). In addition, sperm cells which are not healthy did not respond to hypo-osmotic solution and the reduced HOST response indicates a reduction of membrane/functional integrity in low fertility bulls (Al-Makhzoomi *et al*. 2008).

Table 2. The functional membrane integrity of spermatozoa of semen positive for heparin binding proteins

Animal group	No. of animals (n)	Functional membrane integrity (%)		't' value
		Fresh semen	Frozen-thawed semen	
1	8	84.54±0.62	71.73±2.61	18.40**
2	5	80.63±0.62	67.64±0.53	7.79**
3	4	75.78±1.02	59.88±0.53	77.20**
'F' value		98.99**	89.79**	

Means with different superscripts in column differ significantly (*, P<0.05; **, P<0.01).

The process of freezing and thawing of semen increased (P<0.05) sperm plasma membrane damage in all the semen irrespective of presence or absence of HBPs. However, semen positive for 24 and 30 kDa proteins had an average of 9.81 percentages of higher (P<0.01) sperm with active plasma membrane when compared with the semen devoid of HBPs. The reduction in the post-thaw HOST response indicates mechanical damage which increased the membrane permeability and subsequent loss of plasma membrane integrity associated with osmotic and chemical changes (Ashrafi *et al*. 2013). In addition, cryopreservation also reduces the functional and structural integrity of bovine spermatozoa with increased ROS production (Malik *et al*. 2015). However, Ardon and Suarez (2013) reported that freezing and/or thawing enhances the binding of BSP1, BSP3, and BSP5 to sperm in case of bulls positive for HBPs and protected the sperm membrane. The presence of a 24 kDa band in the seminal plasma of buffalo had improved the membrane integrity of frozen sperm in buffalo (Asadpour *et al*. 2007).

Acrosome integrity: The statuses of acrosome integrity in fresh and frozen-thawed semen of all the bulls are presented in Fig. 1. The fresh ejaculate of bulls positive for 30 kDa HBP had 3.67 and 7.63 percentages of additional (P<0.01) sperm with intact acrosome in comparison to the bulls positive for 24 kDa and lacking for HBPs, respectively. The semen of bulls with 24 kDa also had 3.96 percentage of higher (P < 0.01) intact acrosome than negative bulls. Present results are in conformity of recent reports of Goularte *et al*. (2014) and Odhiambo *et al*. (2014). One of the most important

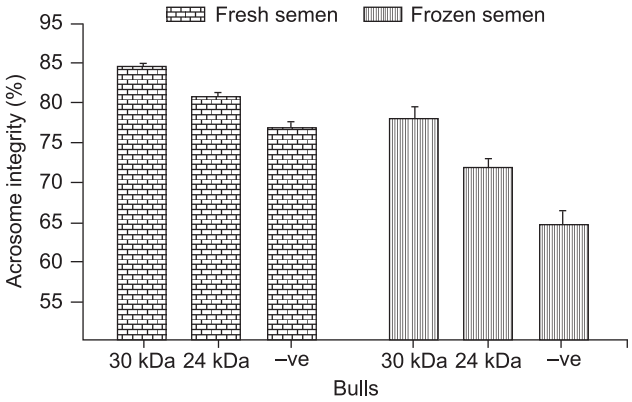

Fig. 1. The status of acrosome integrity of sperm in the semen of Holstein Friesian bulls positive for heparin binding proteins.

consequences of cholesterol efflux from membranes by HBPs is a massive influx of extracellular Ca^{2+}, which is considered a prerequisite for the acrosome reaction process due to changes in the membrane fluidity (Lopez-Ubeda and Matas 2015). In fact, some proteins found in the seminal plasma of bulls are related to prevention of premature acrosome reaction (Goularte *et al*. 2014). Spermatozoa devoid of 30 kDa associated with sperm morphological defects and sperm-surface alterations indicative of acrosomal damage (Odhiambo *et al*. 2014) and reduced viability, acrosomal integrity (Mendoza *et al*. 2013).

The semen of bulls devoid of HBPs had lost around 12.05% of acrosome integrity while freezing and thawing in comparison to their fresh ejaculates. On the other hand, semen with HBPs were able sustain 10% of higher (P<0.01) post-thaw acrosome integrity. Cryopreservation reduces the functional and structural integrity of sperm which resulted in loss of acrosomal integrity with increased ROS production (Ustuner *et al*. 2015). However, frozen-thawed semen of bulls positive for HBPs had higher (P<0.01) number of intact acrosome with active plasma membrane as effect of binding of HBPs with extracellular matrix and cytoskeleton components which stabilizes the membrane (Goularte *et al*. 2014). Mendoza *et al*. (2013) also reported that the FN2 domain of HBPs take part in the protein structure surrounding the spermatozoa which could bind to the sperm surface at ejaculation, stabilizing membrane phospholipids and maintaining the spermatozoa in a decapacitated state and later participating in membrane modification during capacitation. Further, Goularte *et al*. (2014) reported that the presence of 24 and 31 kDa HBPs were improved post-thaw acrosome integrity. The bulls negative for HBPs had lower (P<0.01) per cent of intact acrosome which could be due to severe mechanical damage, cryoinjuries and excess production of lipid peroxides (Ustuner *et al*. 2015). The increase in abnormal sperm morphology after cryopreservation also increased acrosome defects in low fertility bulls (Zodinsanga *et al*. 2015).

Sperm chromatin integrity: The percentages of chromatin integrity of sperm cells in fresh and frozen-thawed semen of all the bulls are presented in Fig. 2. The fresh ejaculates of bulls positive for 30 kDa had significantly (P<0.01) 1.84 and 5.44 percentages of higher intact sperm chromatin in comparison to bulls positive for 24 kDa and negative bulls, respectively. The bulls with 24 kDa had 3.6% of higher (P<0.01) intact sperm chromatin than bulls devoid of HBPs. The sperm chromatin integrity is an important measurement for the prediction of male fertility and the present results coincides with findings of Mendoza *et al*. (2013) and Dogan *et al*. (2015). HBPs might have protected the sperm cells against the oxidative stress which could be the main cause for damage of sperm DNA integrity (Karunakaran *et al*. 2012). Sperm DNA fragmentation of 7 to 10% could be used as indicators of low fertility bulls (Karoui *et al*. 2012) and in our study, it was 7.33% in the bulls lacking for HBPs. Mendoza *et al*. (2013) reported that the lack of HBPs in semen resulted in reduced viability, acrosomal integrity, fertilizing capacity and damaged DNA in ram. In addition to apoptosis and oxidative stress, defective chromatin packaging in spermatozoa is one of the underlying factors of sperm DNA damage that contributes to male infertility (Dogan *et al*. 2015)

The cryopreservation of semen had significantly (P < 0.05) augmented the fragmentation of sperm chromatin by 2.64% in the semen of bulls positive for HBPs when compared with semen lacking for HBPs (3.83%). The main factors assumed to be potentially involved in the damage of sperm DNA during the freezing-thawing process were the osmotic stress and the oxidative stress occurred after thawing (Manjunath *et al*. 2009). However, semen of bulls positive for 24 and 30 kDa retained higher (P<0.01) sperm chromatin integrity in the frozen-thawed semen due to protective effects HBPs. Fragmentation of DNA increased and chromatin maturity reduced in spermatozoa of low fertility bulls or bulls lacking for HBPs (Goularte *et al*. 2014). Consequently, defects of sperm chromatin structure affect sperm function during fertilization and zygotic/ embryonic development (Dogan *et al*. 2015). The destabilizing effect of cryopreservation on sperm chromatin may stem from the high ionic strength in frozen nuclei and excessive intracellular influx of free calcium ions leading to activation of nucleoprotein degrading enzymes. Therefore, the peroxidative damage of membranes and destabilization of deoxyribonucleoprotein complex resulted in the loss of sperm viability, motility and chromatin stability (Waterhouse *et al*. 2010).

The overall result indicated that the fresh ejaculates of bulls positive for 30 kDa had 12.43 and 28.37 percentages of additional semen quality in terms of sperm morphology, active plasma membrane, acrosome and chromatin integrity in comparison to the bulls with 24 kDa and devoid of HBPs, respectively. While freezing-thawing, 24 and 30 kDa proteins might be protect the sperm cells from cryo-injuries and oxidative stress by enhanced coating of these proteins to sperm membrane and stabilization. It was evidenced by 15.70 and 40.35 percentages of better post-thaw semen quality in bulls positive for 30 kDa than 24 kDa and lack of HBPs, respectively. Further, the bulls positive for 24 kDa also had 15.94 and 24.65 percentages of higher semen

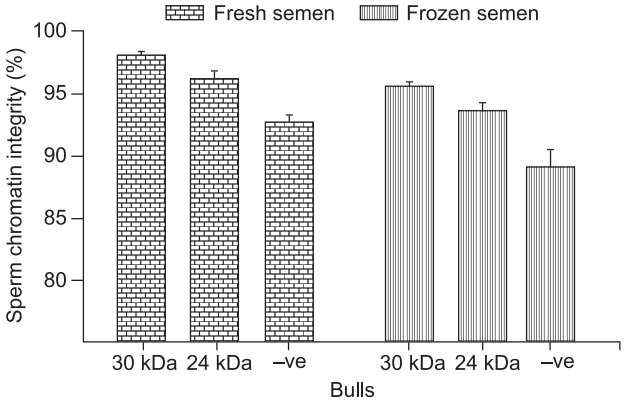

Fig. 2. The chromatin integrity of sperm in the semen of Holstein Friesian bulls positive for heparin binding proteins.

quality against the bulls lacking for the HBPs in fresh and frozen-thawed semen, correspondingly. Therefore, selection of bulls on the basis of 24 and 30 kDa heparin binding proteins for breeding/artificial insemination along with routine semen analysis may enhance the fertility rate. However, further studies may be required to validate the semen quality of bulls positive for HBPs and its field fertility rate in a large number of cows.

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