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Sperm mitochondrial membrane potential and motility pattern in the Holstein bull semen positive for heparin binding proteins

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ABSTRACT

Present study was aimed to evaluate sperm motility and mitochondrial membrane potential (MMP) in relation to the presence of heparin binding proteins (HBPs) in semen. Semen was collected from 17 Holstein Friesian breeding bulls. Seminal plasma and sperm membrane proteins were isolated by precipitation method; HBPs were isolated using heparin-sepharose affinity chromatography. SDS-PAGE was performed and bulls were categorized into 3 groups based on presence/absence of 24 and 30 kDa HBPs. Computer-assisted semen analysis revealed that fresh ejaculates of bulls positive for 30 kDa HBP had 4.24 and 14.18% of higher total motile sperm than semen with 24 kDa and negative for HBPs. Semen with 30 and 24 kDa had 18.72 and 12.34% of higher number of sperm with progressive motility and velocity than semen devoid of HBPs. Simultaneous assessment of plasma membrane integrity (PMI) and MMP by fluorescence staining revealed that post-thaw sperm of semen with 30 kDa HBP had 6.28 and 12.79% of higher intact PMI. Further, sperm of semen with 30 kDa HBP had 4.61 and 10.50% of higher MMP, which had travelled an extra distance of 9.71 and 4.95 mm through cervical mucus than semen with 24 kDa and devoid of HBPs. The mucus penetration distance showed a strong positive correlation with high MMP, PMI, sperm motility and velocity. It is to be concluded that HBPs were able to sustain higher percentage of motile sperm with progressive motility and MMP which was depicted by more mucus penetration distance.

Key words: Heparin binding proteins, Mitochondrial membrane potential, Motility, Plasma membrane integrity, Velocity

Cryopreservation of semen plays a major role in genetic improvement, economization of breeding programs in the livestock industry, preservation of endangered species and is clinically valuable in the management of infertility (Yoon *et al*. 2015). Motility is one of the most important factors in assessing sperm quality and essential for transporting the spermatozoa to the site of fertilization (Ardon and Suarez 2013). On the other hand, motion kinematics assessed by computer assisted semen analysis (CASA) provides an accurate representation of sperm movements objectively (Najjar *et al*. 2013). Sperm motility and the velocity parameters mainly rely on the sperm mitochondrial membrane potential (MMP) of the sperm cell. The reduction in fertility of frozen-thawed semen can be attributed to changes in temperature during the freezing process as well as osmotic and toxic stress owing to exposure to the cryoprotectant and the formation of ice crystals (Papa *et al*. 2015). Further, the field fertility rates differ among bulls even after standard semen evaluation (Parisi *et al*. 2014).

Hence, the attention is being directed towards the assessment of other aspects of semen quality as predictors of bull fertility (Kutty *et al*. 2014). Proteins present in the seminal plasma and sperm membrane were reported as markers of male fertility (Asadpour *et al*. 2007). It was suggested that seminal proteins mediate the binding of sperm cells to oviductal epithelium and preserve membrane integrity by exerting inhibition effects on the mitochondrial activity and metabolism to conserve energy needed until fertilization as well as to minimize the production of reactive oxygen species and lipid peroxidation of sperm membrane (Karunakaran *et al*. 2012). In cattle, 3 bovine seminal plasma (BSP) proteins, BSP1, BSP3 and BSP5 (30 kDa) play a crucial role in fertilization (Manjunath *et al*. 2009). The heparin binding protein (HBP) with 28–30 kDa of sperm membrane is known as fertility associated antigen and considered as one of the genetic markers for male fertility and sperm cryotolerance (Goularte *et al*. 2014, Ramteke *et al*. 2014). Another class of HBP of 24 kDa have significantly correlated with sperm progressive motility in

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fresh and viability in frozen-thawed (Asadpour *et al*. 2007). Hence, the present study was attempted to evaluate sperm motility pattern and mitochondrial membrane potential in relationship with the presence or absence of 24 and 30 kDa HBPs in semen.

MATERIALS AND METHODS

Experimental animals: Adult Holstein Friesian breeding bulls (17), selected from Central Frozen Semen Production and Training Institute, Hessarghatta, 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 with the quality control criteria of the institute. The semen samples were immediately processed 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 tris calcium chloride 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 (heparin- CL agarose pre-packed column) 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 3 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).

Evaluation of sperm motility: Sperm motility analysis was conducted using a computer-assisted semen analysis (CASA). Analysis was based on examination of 25 consecutive digitalized images per sec using a 5 X negative phase contrast objective. The parameters set were cell size (range 5–70 μm^2), spermatozoa concentration ($5–8\times10^6$ / ml), motile cells (> 10 µm/s at 37ºC), progressive forward motility (> 60 % straightness index, STR); circular (< 50 % linearity index, LIN), the straight-line velocity (VSL, in µm/s is the average path velocity of the spermatozoa head along a straight line from its first to last position), curvilinear velocity (VCL, in µm/s is the average path velocity of the spermatozoa head along its actual trajectory), the percentage of linearity (LIN, is the ratio between VSL and VCL) and the lateral head displacement (ALH, in µm/s is the average value of the extreme side-to-side movement of the spermatozoa head in each beat cycle).

Simultaneous assessment of plasma membrane integrity and mitochondrial membrane potential: Mitochondrial membrane potential was assessed by using JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl benzimidazolyl carbocyanine iodide) and plasmalemma integrity was assessed by carboxy fluoresin diacetate (CFDA) and propidium iodide (PI). 1.53 mM of JC-1 in DMSO, 8.69 mM of CFDA in DMSO and 0.4 mM of PI in phosphate buffer were prepared and stored at –20°C in dark. 2 µl of JC-1 and 10 µl of CFDA solutions were added to 100 µl of semen sample. The semen sample was incubated at room temperature for 30 min in dark. The sperm nuclei were counterstained by adding 10 µl of PI stock solution and incubated in dark for 10 min. Then the sperm cells were washed in PBS by centrifugation (560 × g for 5 min). Sperm cells suspended in PBS were placed on a glass slide and covered with cover slip. A minimum of 200 cells were analyzed at 400X using an epifluorescence microscope equipped with a CFDA filter set (excitation filter 510–560 nm, barrier filter 505 nm). Sperm cells exhibiting orange to red fluorescence in the mid piece were considered as those having high mitochondrial membrane potential and yellowish fluorescence considered as low potential. Cells showing complete green fluorescence in the plasma membrane were considered plasmalemma intact and those showing partial or complete red nuclei were classified as dead (Selvaraju *et al*. 2009).

Bovine cervical mucus penetration test: Cervical mucus penetration test was performed as per the method described by Murase and Braun (1990) by collecting cervical mucus from normal cyclic cow in estrus. Mucus was filled in a pre-warmed micro capillary tube by capillary action and one side of capillary tube was sealed with polyvinyl alcohol powder. The frozen-thawed semen was taken in an eppendorf and capillary tube with its open end was placed in the frozen-thawed semen. After 30 min of incubation at 37°C, the capillary tube was fixed on scaled glass slide and examined under microscope at 400X to measure sperm penetration distance (mm/30 min).

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 transforming of the percentage values. The comparison of total motility, progressive motility, sperm velocity parameters, plasma membrane integrity, mitochondrial membrane potential and mucus penetration distance of sperm cells 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. Significance of sperm characteristics between fresh and frozen-thawed semen was analyzed by paired 't' test. Further, to establish the correlation between sperm motility, mitochondrial membrane potential and other parameters Pearson's correlation coefficient (two-tailed) was performed. Results are expressed as mean ± standard error of mean.

RESULTS AND DISCUSSION

Sperm motility: Motility and progressive motility of sperm cells assessed by CASA in fresh and frozen-thawed semen of all the bulls are presented in Table 1. The fresh semen of bulls positive for 30 kDa HBP had 4.24% and 14.18% of higher ($P < 0.05$) total motile sperm than semen positive for 24 kDa and negative for HBS, respectively. The sperm progressive motility was significantly ($P < 0.05$) higher (6.38%) in semen with 30 kDa HBP in comparison with semen positive for 24 kDa HBP. However, semen positive of 30 and 24 kDa HBPs had 18.72% and 12.34% of higher ($P < 0.01$) number of sperm cells with progressive motility than the HBPs negative semen. The results of total sperm motility and progressive motility are in agreement with the previous reports of Najjar *et al*. (2013) in Holstein bulls and Naseer *et al*. (2014) and Kumar *et al*. (2015) in buffalo. Bulls, positive for HBPs had higher per cent of motility due to the action of HBPs, which have been reported to bring about an initial cholesterol efflux along with release of phospholipids from the spermatozoal membrane (Karunakaran *et al*. 2013, Naseer *et al*. 2014). Further, changes in cholesterol level in the plasma membrane over the mid piece and principal piece increased the lateral mobility of membrane components (Kim *et al*. 2013). The bulls negative for HBPs had lower ($P < 0.05$) per cent of motility which could be due to lack of HBPs in the semen and high lipid content of sperm membrane might have prevented the sperm mobility. Consequently, the high content of cholesterol resulted in lipid peroxidation and oxidative stress. The motility is a function of intracellular adenosine triphosphate (ATP) content which was highly correlated with progressive motility of spermatozoa of fresh and cryopreserved bull semen (Reis *et al*. 2014). Whereas, Jobim *et al*. (2004) and Asadpour *et al*. (2007) reported that 24 kDa protein fraction of semen was significantly correlated with sperm progressive motility in fresh semen.

The per cent of total motile sperm with progressive motility had decreased ($P < 0.01$) in all the frozen-thawed semen when compared with fresh semen irrespective of presence or absence of HBPs. However, frozen-semen of bulls positive for HBPs were able to sustain higher ($P < 0.01$) per cent of total motile sperm with progressive motility than semen lacking HBPs. Moreover, the total motile sperm and progressive motility showed higher ($P < 0.01$) correlation with the mitochondrial membrane potential, plasma membrane integrity and mucus penetration distance (Table 5). The BSP proteins, in particular the HBPs had protected the sperm cells against the cryo-injuries and oxidative stress by coating of these proteins while freezing (Ardon and Suarez 2013, Goularte *et al*. 2014). However, Kim *et al*. (2013) reported that the initial cholesterol and phospholipid efflux from sperm membrane could trigger a decreased stability of the spermatozoa to a series of stress factors to which spermatozoa are exposed during cryopreservation and thawing. Further, it was suggested that localization of BSP proteins in the mid piece close to the mitochondria was indicative of the fact that BSP proteins controlled sperm motility (Souza *et al*. 2006). The reduced sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage as a result of mechanical damage, oxidative stress and lipid peroxidation (Parisi *et al*. 2014). The reduction in motility in our study

Table 1. Per cent of sperm total motility and progressive motility in fresh and frozen-thawed semen of Holstein Friesian breeding bulls

Group (no. of bulls)	Sperm total motility (%)		't' value	Progressive motility (%)		't' value
	Fresh semen	Frozenthawed semen		Fresh semen	Frozenthawed semen	
1 (8)	85.45±0.36^{a}	79.22±0.64^{a}	8.13**	61.48±0.50^{a}	54.71±0.38^{a}	9.13**
2 (5)	81.21±0.68^{b}	72.84±0.76^{b}	19.23**	57.04±0.68^{b}	48.18±0.55^{b}	9.84**
3 (4)	71.27±1.61^{c}	60.50±0.77^{c}	8.88*	39.09±0.73^{c}	31.24±0.67^{c}	8.24*
F value	79.27	157.82		325.37	535.18	

Means with different superscripts in the same column differ significantly. *, a b ($P < 0.05$); **, a c and b c ($P < 0.01$).

Table 2. The amplitude of lateral head displacement and beat frequency in fresh and frozen-thawed semen of Holstein Friesian breeding bulls

Group (no. of bulls)	Amplitude of lateral head displacement (μm)		't' value	Beat frequency (Hz)		't' value
	Fresh semen	Frozenthawed semen		Fresh semen	Frozenthawed semen	
1 (8)	8.02±0.24^{a}	6.48±0.21^{a}	5.82**	27.71±0.23^{a}	24.09±0.35^{a}	7.53**
2 (5)	5.50±0.26^{b}	4.50±0.32^{b}	4.14*	22.39±0.26^{b}	16.67±0.54^{b}	10.21**
3 (4)	4.40±0.20^{c}	3.37±0.13^{c}	7.82*	15.15±0.47^{c}	10.09±0.36^{c}	8.96*
F value	56.51	42.72		439.96	268.85	

Means with different superscripts in the same column differ significantly. *, a b ($P < 0.05$); **, a c and b c ($P < 0.01$).

Table 3. Straightness and linearity of sperm cells in fresh and frozen-thawed semen of Holstein Friesian breeding bulls

Group (no. of bulls)	Straightness (%)		't' value	Linearity (%)		't' value
	Fresh semen	Frozenthawed semen		Fresh semen	Frozenthawed semen	
1 (8)	90.84±0.24[a]	81.50±0.29[a]	25.98**	70.96±0.30[a]	64.42±0.31[a]	11.01**
2 (5)	87.47±0.49[b]	77.25±0.36[b]	17.79**	67.50±0.48[b]	61.70±0.78[b]	9.56**
3 (4)	71.94±0.66[c]	60.95±0.55[c]	21.96**	62.16±0.56[c]	55.28±0.55[c]	82.29**
F value	519.13	731.43		109.02	73.35	

Means with different superscripts in the same column differ significantly. *, a b ($P < 0.05$); **, a c and b c ($P < 0.01$).

Table 4. Plasma membrane integrity, mitochondrial membrane potential and bovine cervical mucus penetration distance of frozen-thawed sperm cells of Holstein Friesian breeding bulls

Group (no. of bulls)	Plasma membrane integrity (%)	Mitochondrial membrane potential (%)			Sperm penetration distance (mm)
		High	Low	Lost	
1 (8)	67.35±0.45[a]	25.64±0.49[a]	49.04±0.42	21.82±0.44[a]	31.17±0.21[a]
2 (5)	61.07±0.20[b]	21.03±0.19[b]	48.61±0.24	30.52±0.28[b]	26.41±0.37[b]
3 (4)	54.56±0.41[c]	15.14±0.17[c]	46.77±0.41	38.10±0.44[c]	21.46±0.37[c]
F value	226.37	141.99	0.150	350.54	268.85

Means with different superscripts in the same column differ significantly. a b, $P < 0.05$); a c and b c, $P < 0.01$).

Table 5. The correlation between sperm total motility, progressive motility, sperm velocity parameters with plasma membrane integrity and mitochondrial membrane potential

	TM	PM	AVP	VSL	VCL	PMI	MMPHigh	MMPLow	MMPLost
PM	0.970**	1							
AVP	0.971**	0.981**	1						
VSL	0.947**	0.975**	0.960**	1					
VCL	0.975**	0.991**	0.990**	0.976**	1				
PMI	0.937**	0.949**	0.966**	0.909**	0.972**	1			
MMPHigh	0.929**	0.950**	0.965**	0.914**	0.967**	0.978**	1		
MMPLow	0.250	0.102	0.173	0.162	0.149	0.067	0.061	1	
MMPLost	-0.944**	-0.942**	-0.962**	-0.886**	-0.960**	-0.984**	-0.982**	-0.112	1
CMPT	0.956**	0.959**	0.973**	0.912**	0.971**	0.972**	0.962**	0.194	0.979**

**Correlation is significant at the 0.01 level (2-tailed). TM, Total motility; PM, progressive motility; AVP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; PMI, plasma membrane integrity; MMP, mitochondrial membrane potential; CMPT, cervical mucus penetration test.

may be due to the decline in mitochondrial membrane potential in bulls negative for HBPs and it is also a sign of apoptosis (Espinoza *et al*. 2009).

Velocity parameters of sperm cells: The velocity parameters of sperm in different group of bulls are presented in Fig. 1, which include average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL). Fresh ejaculates of bulls positive for HBPs had an average of higher ($P < 0.01$) VAP (103.73±0.68 µm/s), VSL (82.32±0.29 µm/s) and VCL (167.75±1.48 µm/s) in comparison to semen negative for HBPs (72.35±0.59, 62.10±0.32 and 93.65±0.88 µm/s). Similarly, the post-thaw sperm velocity parameters were significantly higher ($P < 0.01$) in frozen-thawed semen of bulls positive for HBPs. Further, velocity parameters showed a strong correlation ($P < 0.01$) with sperm motility, high MMP and the plasma membrane integrity (Table 5).

The amplitude of lateral head displacement (ALH) and

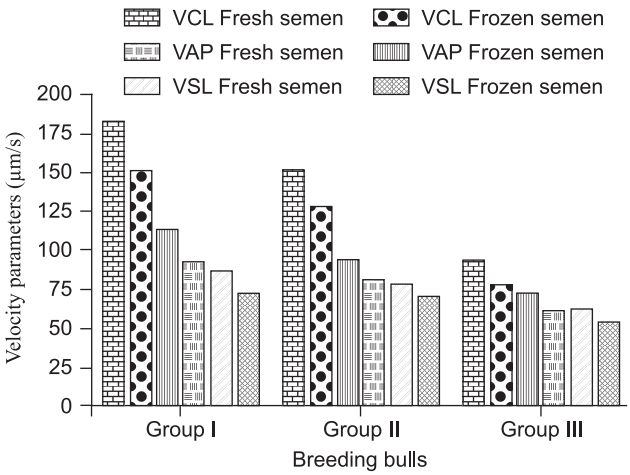

Fig. 1. Comparison of sperm average path velocity, straight line velocity and curvilinear velocity parameters (µm/s) in fresh and frozen-thawed semen of Holstein Friesian bulls.

beat frequency of sperm cells (BCF) in fresh and frozen-thawed semen are presented in Table 2. The ALH and BCF of sperm cells were higher ($P < 0.05$) in the semen positive for HBPs in comparison to semen lack of HBPs in fresh and frozen-thawed semen. Nevertheless, ALH and BCF of sperm cells were significantly higher ($P < 0.05$) in semen with 30 kDa than 24 kDa HBPs. The per cent of straightness (STR) and linearity (LIN) of sperm cells in the fresh and frozen-thawed semen of all the groups are presented in Table 3. The STR and LIN of sperm cells were significantly higher ($P < 0.01$) in the groups with HBPs than the negative group devoid of HBPs in the fresh and frozen-thawed semen. Among the bulls positive for HBPs, STR and LIN were significantly higher ($P < 0.05$) in the semen of bulls positive for 30 kDa than 24 kDa.

The results are in agreement with the findings of Mongielnicka-Brzozowska *et al*. (2014), Kumar *et al*. (2015) in buffalo and Sarsaifi *et al*. (2015). The semen with HBPs had significantly higher ($P< 0.05$) sperm velocity, which might be due to cholesterol efflux from the sperm membrane by HBPs thought to increase membrane fluidity and allow greater lateral movements in integral membrane proteins (Najjar *et al*. 2013). Kim *et al*. (2013) reported that the addition of HBPs had increased the sperm motility and showed a strong positive correlation with fertility. The semen of bulls devoid of HBPs had lower ($P < 0.05$) sperm velocity parameters as a result of more cryo-damage and oxidative stress. Karunakaran *et al*. (2012) also reported high content of lipid peroxidation in the frozen-semen without heparin binding proteins. Further, a decreased phosphorylation of axonemal proteins, inhibition of oxidative phosphorylation and glycolysis might have limited ATP generation by sperm cells. In addition, loss of motility due to destruction of structural integrity of plasma membrane is result of peroxidation of sperm membrane fatty acids (Najjar *et al*. 2013, Kumar *et al*. 2015). Besides peroxidation and toxic effects of glycerol, the phospholipids of damaged spermatozoa produce the reactive oxygen species, which may be toxic to the normal spermatozoa and in turn may contribute to reduction in sperm motion characteristics (Chaveiro *et al*. 2015).

Plasma membrane integrity: The statuses of post-thaw plasma membrane integrity of sperm cells in different groups and its relationship with sperm motility and mitochondrial membrane potential are presented in Tables 4, 5. The post-thaw sperm cells of bulls positive for 30 kDa HBP had 6.28% and 12.79% of higher ($P < 0.01$) intact plasma membrane integrity in comparison to semen positive for 24 kDa and lack of HBPs, respectively. Further, semen with 24 kDa HBP had 6.51% of more ($P < 0.01$) number of sperm with intact plasma membrane than semen devoid of HBPs. The plasma membrane integrity of sperm cells had highly ($P < 0.01$) positive correlation with motility, velocity parameters and high MMP. The results are in line with previous findings of Gohar *et al*. (2014), Singh *et al*. (2014) and Zodinsanga *et al*. (2015). The major BSP proteins (BSP-A1/A2, A3 and BSP-30 kDa) are known to safe guard and influence capacitation of acrosome by their ability to modulate membrane cholesterol (Moura *et al*. 2007). 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 protects the sperm membrane against cryo-injureis and oxidative stress. The presence of a 24 kDa band in the seminal plasma of buffalo had improved membrane integrity of frozen sperm in buffalo (Asadpour *et al*. 2007). The semen of bulls devoid of HBPs had higher ($P < 0.05$) percentage of impaired plasma membrane which might be due to the higher steroid content of sperm membrane and reduction in their membrane fluidity (Zodinsanga *et al*. 2015). In addition, the process of freezing and thawing of semen increased mechanical damage of sperm plasma membrane with increased permeability and subsequent loss of membrane integrity associated with osmotic and chemical changes (Ashrafi *et al*. 2013). Malik *et al*. (2015) also reported that cryopreservation reduces the functional and structural integrity of bovine spermatozoa with increased ROS production. The correlation was stronger ($P < 0.01$) between membrane integrity and total motility that could be attributed to the fact that motility is a function of intra-cellular ATP and plasma membrane integrity (Singh *et al*. 2014).

Mitochondrial membrane potential of sperm cells: Sperm cells with different levels of mitochondrial membrane potency in frozen-thawed semen of all the groups are presented in Table 4. The frozen-thawed semen of bulls positive for 30 kDa HBP had 4.61% and 10.50% of higher ($P < 0.01$) per cent of sperm with high MMP than semen with 24 kDa and devoid of HBPs. The semen with 24 kDa HBP had 5.89% of additional ($P < 0.01$) number of sperm with high MMP in comparison to negative semen. There were no significant ($P > 0.05$) differences in the per cent of sperm cells with low MMP among all the groups. However, lost MMP had highly ($P < .01$) negative correlation with sperm motility, velocity parameters and the plasma membrane integrity (Table 5). The MMP statuses of sperm cells in the present study are in accordance with previous reports of Kim *et al*. (2013), Reis *et al*. (2014) and Ajaoa *et al*. (2015). The HBPs protects mitochondrial membrane integrity by functional changes in the cell membrane of sperm and activates cyclic AMP production (Karunakaran *et al*. 2013, Kim *et al*. 2013). Spermatozoa that exhibit high MMP generally have intact acrosome function and high fertilizing capacity as well as normal motility and morphology (Grunewald *et al*. 2008). The semen devoid of HPBs had low MMP due to lack of protection against oxidative stress and mitochondrial impairment with the generation of reactive oxygen species (Aitken and Baker 2013). Likewise, low MMP sperm cells are inferior in quality and are associated with reduced fertility and elevated levels of reactive oxygen species (Espinoza *et al*. 2009). Recent reports showed a positive correlation between the percentage of sperm with high MMP and standard semen parameters like sperm concentration and motility (Kim *et*

al. 2013). High correlation of MMP with forward motility confirms the strong link between functional status of mitochondria, plasma membrane and sperm quality (Ajaoa *et al*. 2015).

Bovine cervical mucus penetration test: The results of bovine cervical mucus penetration distance of frozen-thawed sperm in different group of bulls are presented in Table 4. The frozen-thawed sperm cells of bulls positive for 30 and 24 kDa HBPs had travelled significantly an extra ($P < 0.01$) distance of 9.71 mm and 4.95 mm in bovine cervical mucus than semen lacking for HBPs, respectively. Further, sperm cells of semen positive for 30 kDa had travelled more ($P < 0.05$) distance than semen with 24 kDa HBP at a given time in the cervical mucus. The post-thaw mucus penetration distance showed a strong ($P < 0.01$) positive correlation with high MMP, plasma membrane integrity, sperm motility and velocity parameters (Table 5). The present results are in harmony with the findings of Srivastava and Kumar (2014), Kumar *et al*. (2015) in buffalo and Zodinsanga *et al*. (2015) in Holstein bulls. It could be explained by the fact that sperm of semen with HBP had a higher ($P < 0.01$) progressive motility, velocity parameters with high MMP (Kim e*t al*. 2013) and motility is associated with higher depth of penetration (Zodinsanga *et al*. 2015). Similarly, HBPs in semen changed the cholesterol concentrations in the plasma membrane over the mid piece and tail of sperm which might increase lateral mobility of the membrane components, supporting hyperactivated motility for penetrating an extra distance (Naseer *et al*. 2014). Sperm cells of the bulls lacking of HBPs had lower ($P < 0.01$) penetration distance, which might be due to lower per cent of progressive motility with lower per cent of MMP. Tas *et al*. (2007) reported that lower fertility bulls had lower number of spermatozoa at the determined penetration distance ranges in comparison with higher fertility bulls in the bovine cervical mucus.

The present study concluded that the semen positive for 30 and 24 kDa HBPs had higher ($P < 0.01$) per cent of total motile sperm with progressive motility in fresh and frozen-thawed semen than semen devoid of HBPs. Further, semen with HBPs was able to sustain and protect the sperm plasma membrane and high ($P < 0.01$) mitochondrial membrane potential against cryo-injuries and oxidative stress. Similarly, high MMP was depicted by higher sperm velocity parameters in the fresh and frozen-thawed semen of bulls positive for HBPs and it was confirmed by more cervical mucus penetration distance. Further, sperm motility and velocity parameters had a strong ($P < 0.01$) correlation with plasma membrane integrity, high mitochondrial membrane potential. Hence, the presence of HBPs in semen of breeding bulls may enhance the fertility rate by preserving and protecting sperm mitochondrial membrane potential and plasma membrane integrity against cryo-injuries and oxidative stress which may augment sperm motility.

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