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Presence of Fertility-associated antigen on sperm membrane corresponds to greater freezability potential of Frieswal bull semen

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ABSTRACT

Several studies have shown relationship of semen freezability with Fertility-associated antigen (FAA) in purebreds. Objectives of present investigation were to determine presence of FAA in Frieswal bull semen and its relationship with freezability. Study involved separation of seminal plasma (SP, FAA – or +) and sperm membranes (SM, FAA – or +), electrophoresis, protein content and quality assessment (n=36).Five groups were: Group-I (19.4%, SP+ve/SM+ve); Group-II (22.2%, SP-ve/SM+ve); Group-III (25%, SP+ve/SM-ve); Group-IV (16.7%, SP-ve/SM-ve), and Group-V (16.7%, unprocessed, initial progressive motility ≤30%, negative control).At post-thaw stage, Group-I and -II showed significantly higher ($p< 0.001$) freezabilitythan FAA-SM-ve groups. Moreover, Group-III with FAA in seminal plasma but not on membrane showed better post-thaw motility than Group-IV, which totally lacked FAA. Our results have shown that 28–30 kDa protein is present in SP and SM of majority of Frieswal bulls and presence of FAA in SM confers appreciable improvement in freezability.

Key words: Frieswal bull, freezability, fertility associated antigen, seminal plasma/sperm membrane proteins

Freezing of spermatozoa is a rigorous process during which cells are subjected to variable range of temperature fluctuation that makes them liable to cryo-injuries. The sperm plasma membrane is the primary site of damage during freezing and thawing process (Januskauskas *et al.*2003). During the process of cryopreservation temperature fluctuations coupled with cell dehydration induce changes in lateral phase separation of lipids that leads to re-ordering of membrane components (Drobnis *et al.* 1993), efflux of poly-unsaturated fatty acids and cholesterol that induce cryo-capacitation and acrosome damage (Watson 1995). Several factors appear to play an important role in deciding about the fate of cryo-preserved spermatozoa *vis-à-vis*variable freezability effects.

Micro-environment surrounding the sperm cell at the time of ejaculation is believed to modulate the freezability of spermatozoa (Jobima *et al.* 2004). The seminal plasma proteins in this regard play important roles in the physiology and alterations of sperm cells (Boe-Hansen *et al.* 2015). Seminal proteins contribute to sperm maturation, metabolism, capacitation, defense, motility, modification of sperm membranes, acrosome reaction, interaction with the oviductal epithelium and fertilization (Rodriguez-Martinez *et al.* 2011, Caballero *et al.* 2012).Among several known seminal proteins,Binder of sperm proteins (BSP) are the major proteins of the bovine seminal plasma that bind to sperm as soon as theycome into contactwith cells (Manjunath*et al.* 2009). Homologs of bovine BSP proteins have been identified in several other mammalian species. In cattle, three BSP proteins, BSP1 (previously known as PDC-109), BSP3 (BSP-A3), and BSP5 (BSP-30 kDa), have been reported to play important role in fertilization (Manjunath*et al.* 2009). Of these BSP proteins, 28 to 30kDa protein (BSP5) is popularly known as Fertility associated antigen (FAA) (Bellin*et al.* 1996, 1998). It is considered as one of the genetic markers for male fertility and is shown to be a heritable character (Ax, 2008). It has also beenreported that bulls of beef breed positive for this protein on the sperm had 9–40% more conception than the negative bulls following natural mating (Ax, 2004). Karunakaranand Devanatha(2016) reported that sperm cells positive for FAA have better *in vitro*quality as well as better protection ability against oxidative stress. FAA, thus, is supposed to be an important constituent of spermatozoa milieu. However, relationship of presence of FAA either in seminal plasma or in sperm membrane or alternatively their cumulative effect has not been investigated in cross bred bulls. Available reports suggest that about 45 per cent of ejaculates from Frieswal bulls (Holstein Friesian and Sahiwal cross; having 5/8 Holstein Friesian inheritance)are non-freezable/ produce semen which is non-freezable (Tyagi*et al.* 2000). The varied

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freezability in semen samples of Frieswal bulls might be due to presence of FAA, a heritable character. Therefore the present study was envisaged with the objective to screen the presence of FAA in seminal plasma and sperm membrane of semen sample of breeding Frieswal bulls and its effecton freezabilityof spermatozoa.

MATERIALS AND METHODS

Semen collection, evaluation and processing

The present study was conducted at Semen Freezing Laboratory, ICAR-Central Institute for Research on Cattle, Meerut Cantt, India. Thirty six healthy Frieswal breeding bulls maintained at Bull rearing unit under uniform feeding and housing conditions were selectedfor the study. The collections were taken between 8:30 to 9:30 AM using artificial vagina as per the standard practice. A total of 36 semen samples one from each bull (first ejaculate of the day) was collected for the experiment. Immediately after collection, the tubes containing semen were placed in the water bath maintained at 35°C and samples were evaluated for semen quality parameters *viz.,* volume, pH value, motility and concentration. Semen volume was determined with a graduated glass tube and the pH value was measured using a pH-metrestrips. The concentration of spermatozoa (millions per mL) in the fresh semen was determined using a photometer (Accucell, IMV- France). Sperm motility was determined subjectively as the percentage of total motile sperm by microscopic examination. Semen samples with a volume of e"1 mL, motility of e" 70% and concentration of above 500 million/mL were only selected for further processing. Thereafter 1 mL of semen was kept for protein analysis and remaining portion of the sample was diluted in cryo-protective extender, composed of 3.028 g of Tris, 1.675 g of citric acid, 1.25 g of fructose, 7.0 mL of glycerol, 1 x 10^5 IU Penicillin G Sodium and 1 x 10^5 µg Streptomycin in 100 mL of deionized water. Subsequently viability, abnormality, acrosome integrity and membrane stability (HOS) tests were performed for fresh semen samples. The viability (per cent live spermatozoa) was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.* 1953)and the acrosome integrity (per cent normal acrosome) was assessed in Giemsa-stained smears (Watson, 1975).The plasma membrane integrity was evaluated using the hypo-osmotic swelling test (HOST) as described by Jeyendran*et al.*(1984).

The semen samples were diluted inegg yolk Trisglycerol extender in such a way to yield approximately 20 million motile sperm cells/ 0.25 mL straw. After ûlling and sealing, filled straws were then subjected to a combined cooling with an equilibration period of 4 h at 5°C. The rack along with the straws was transferred to Biological Cell Freezer (IMV- France) for automated freezing. Straws were then plunged into liquid nitrogen (–196°C) for storage until assayed. Following cryopreservation of semen samples for 48 h, three straws were randomly collected, thawed at 37°C for 30 s and tested for individual progressive motility. A variation of more than ±5% in frozen-thaw progressive motility of spermatozoa rendered whole batch of sample unsuitable for further use. For evaluation of post thaw semen quality variables, two straws were randomly selected from the bunch of cryopreserved semen straws, thawed and evaluated. In all assays, two replicates per sample were made for evaluation of quality parameters of spermatozoa.

Extraction of seminal plasma- and sperm membrane-proteins

Parallel to the assessment of semen quality parameters, the seminal plasma and sperm cells were separated post-collection by centrifugation (8000 x g for 10 min at 4°C). The supernatant i.e. seminal plasma of each sample was transferred into 1.5 mL micro-centrifuge tube and subsequently re-centrifuged twice to eliminate the remaining cells. Sperm cells pellet after first centrifugation was stored in 1.5 mL microfuge tubes at 4°C containing PBS for the duration of the dissecting period (generally not more than 2 h). Thereafter sperm cells were re-suspended with 1 mL ofTris Calcium chloride (TC) buffer (40 mMTris, 2 mM $CaCl_2$ and 0.01% sodium azide, pH 7.3) containing protease inhibitor (1 mM phenyl methyl sulfonyl fluoride) and washed thrice by centrifugation (8000 x g for 10 min at 4°C) to remove the left over seminal plasma. The sperm pellets were subsequently stored at –20° C until extraction of protein.

Proteins in the seminal plasma were precipitated by adding ice cold-ethanol nine times the volume of seminal plasma and incubating at refrigeration temperature overnight (Asadpour *et al.* 2007). Protein precipitates were separated by centrifugation (10,000 x g for 15 min at 4°C), air-dried and re-suspended in milli-Q water. Protein concentration was estimated using a spectrophotometer (Nanodrop, ND-1000, USA) at Protein A280 module and stored at –20°C.

Sperm membrane proteins were extracted as per the method described by Nass*et al.* (1990)with slight modifications. Washed pellets were re-suspended in 1 mL of TC buffer containing Triton X-100 (0.1% v/v) and incubated for 2 h at 5°C with vortexing at 15 min interval. After completion of 2 h incubation, the suspension was centrifuged (10,000 x g for 10 min at 4°C) to remove cellular debris. The supernatant containing sperm protein was recovered and the proteins were precipitated by adding ice-cold ethanol nine times the volume of supernatant and incubating at refrigeration temperature overnight with intermittent vortexing for initial 2 h. Protein precipitates were separated by centrifugation (10,000 x g for 15 min at 4°C), air-dried and re-suspended in milli-Q water. Protein concentration was estimated using a spectrophotometer (Nanodrop, ND-1000, USA) at Protein A280 module.

Characterization of seminal plasma and sperm proteins by electrophoresis

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Laemmli, 1970)to characterize the proteins. After electrophoresis, gels were stained with Coomassie Brilliant blue R–250 (0.15%)

including 50% methanol and 10% acetic acid for 10–12 h and de-stained in a mixture of methanol (25%) and acetic acid (10%) in distilled water until no background stain was detectable. Following determination of the apparent molecular mass using molecular weight markers and gel documentation and analysis system; the gels were stored in acetic acid (7%). Different protein bands were assigned molecular weight by comparing their relative mobilities to that of standards.

Grouping of bulls according to presence of FAA

Samples were identified and grouped according to the presence or absence of FAA in sperm membrane and seminal plasma as determined by SDS PAGE. The grouping is presented in Table 1.

Table 1. Grouping of semen samples based on presence of FAA in seminal plasma and sperm membranes

Fertility-associated antigen		On sperm membrane	
		Detected	Undetected
In seminal plasma	Detected	I	III
	Undetected	II	IV

Statistical analysis

The statistical analysis of the data was done as per standard procedures (Snedecor and Cochran, 1981). The data recorded in percent values were subjected to angular transformation before the analysis. Analysis of variance (ANOVA) was performed using a generalized liner model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001). Statistical significance was set at 0.05 probability level. If the effect was found significant, comparison of means was done by Duncan's multiple range test. To establish the correlation between parameters Pearson's correlation coefficient was performed. Results are expressed as mean±standard error of mean.

RESULTS AND DISCUSSION

Grouping of semen samplesand electrophoretic profile of semen of Frieswal bulls

This study involved application of one-dimensional SDS-PAGE to investigate the presence of FAA in seminal plasma and sperm membrane; and establish its relationship with freezability, if any, in Frieswal bull semen.Study revealed protein bands in the molecular weights ranging from 12 to 220 kDa in the SDS-PAGE. Five groups were made based on presence of FAA in 36semen samples. Seven samples (19.4%, n=8) had detectable-FAA in both seminal plasma as well as sperm membrane (Group I). Group II contained samples (22.2%, n= 8) with FAA detected only in sperm membrane. Samples with detectable-FAA in seminal plasma and not on sperm membrane were assigned to Group III (25%, n=9). Samples with undetectable FAA in both seminal plasma as well as sperm membrane were placed in Group IV (16.7%, n=6) whereas Group V (16.7%, n=6) comprised of unprocessed samples which had initial progressive motility ≤ 30%.

In the present study, protein bands in the molecular weights ranging from 12 to 220 kDawere observed in the semen samples of Frieswal bulls. Previous studies using one-dimensional SDS-PAGE have detected the molecular weights ranging from 15 to 250 kDa (Fernandes*et al.* 2008) and 14/15 to 205 kDa (Karunakaran and Devanatha, 2016) in other crossbred (*Bos Taurus indicus*)bulls. The bovine seminal plasma/sperm membrane proteome is known to be composed of many different proteins. Although the functions of the majority remain unknown, many of them are believed to be involved in certain steps of fertilization (Rodriguez-Martinez *et al.* 2014). Hence, the absence or presence of specific proteins could alter sperm functions, jeopardizing its fertilizing abilities, affecting the bull fertility.

In our study, 24 out of 36 bulls (66.7%) tested positive for 28–30 kDa protein irrespective of its existence in seminal plasma or sperm membrane. Earlier reports on exotic bulls have shown that almost 60% of the bulls fall under this category (Bellin*et al.* 1996). Karunakaran and Devanatha (2016) reported that 50% of the breeding bulls are positive for the said protein, whereas,Bellin*et al.* (1998) reported that the percentage of bulls that were FAA negative ranged from 0% to 50% (average, 12%; n=2191 bulls). The differences in the relative abundance, structure and patterns of expression of these seminal proteins seems to determine

Table 2.Detectable-FAA vis-à-vis fresh semen quality parameters (Means± SEM)

Semen Attributes	I	II	III	IV	V
N	7	8	9	6	6
Volume (ml)	5.94±0.20	5.48±0.17	5.53±0.14	5.18±0.47	5.00±0.12
pH	6.71 ± 0.06 [a]	6.61 ± 0.08 [a]	6.51 ± 0.09 [a]	6.43±0.08 [a]	7.20±0.19[b]
IPM (%)	75.71 ±2.02[b]	75.00±1.89[b]	73.33±1.67[b]	70.00±0.00[b]	20.00±3.65[a]
Concentration (10^6/ml)	987.57±45.8	959.12±44.0	909.44±59.4	836.33±77.4	717.16±166.5
Viability (%)	77.71±2.07 [a]	78.50±1.84 [a]	78.33±1.80 [a]	75.67±0.42 [a]	27.83±0.39 [b]
HOST (%)	73.86±2.43 [a]	73.88±2.42 [a]	72.33±2.40 [a]	68.83±0.48 [a]	21.67±4.94 [b]
Acr-Int (%)	74.28±2.25 [a]	72.88±2.55 [a]	73.22±2.13 [a]	72.17±0.87 [a]	22.67±4.98 [b]
Abnormality (%)	6.28±0.92 [a]	7.87±0.59 [a]	12.11±1.20 [b]	15.67±0.33 [c]	21.17±1.89 [d]

N, number of samples; IPM, initial progressive motility; HOST, hypo-osmotic swelling test; Acr-Int, acrosome integrity; [abcd]Values bearing different superscripts in a row differ significantly ($p<0.05$).

the species-specific effects of homologous proteins (Calvete and Sanz, 2007).

Semen quality parameters of fresh semen

The fresh semen attributes in Group I, II, III, IV and V are presented in Table 2. There was no significant difference in volume, pH, initial progressive motility, viability, membrane integrity and acrosome integrity of fresh semen samples between groups I, II, III and IV. However, in the Group V (poor quality, unprocessed) all these values were significantly ($p<0.001$) lower than other four groups. Per cent abnormality was lowest in Group I and highest in Group IV. There was no significant difference between the spermatozoa concentration among different groups.

Semen quality parameters at post-thaw

The post-thaw motility, viability, membrane integrity, acrosome integrity and abnormality per cent of frozen-thawed semen sample in all the four groups is presented in Table 3.

in I and II that were positive for FAA in sperm membranes were able to sustain higher ($p< 0.001$) per cent of post-thaw motile spermatozoa than semen lacking FAA in their sperm membrane. Even Group III (FAA in seminal plasma but not on membrane)had significantly ($p<0.05$) better post-thaw motility than Group IV which totally lacked FAA.

Although values of semen quality parameters in Group I having detectable-FAA in both plasma and membrane were higher, they were comparable with those from group showing detectable-FAA in sperm membrane only. This important observation suggests that presence of FAA in sperm membrane is more crucial for better quality post-thaw. In agreement, several researchers (Souza *et al.* 2008, Rodríguez-Villamil*et al.* 2016) have reported that FAA, along with other proteins of its group binds with spermatozoa at ejaculation; participate in cholesterol efflux from sperm membrane (first cholesterol efflux) and render membrane more stable than before. Thus, improved post-thaw sperm quality might be due to the presence of FAA on sperm membrane, stabilizing it to withstand rigors of cryo-

Table 3.Effect of presence of FAA on semen quality parameters of Frieswal bulls post-thaw (Means ± SEM)

Groups	n	Motility (%)	Viability (%)	HOST	Acr-Int (%)	Abnormality
I	7	55.71±2.02^{c}	60.14±2.32^{c}	55.14±2.74^{c}	49.86±2.35^{c}	11.14±1.26^{a}
II	8	53.75±1.82^{c}	57.50±1.86^{c}	52.87±1.91^{c}	48.12±1.99^{c}	12.25±0.41^{a}
III	9	34.44±1.76^{b}	38.78±1.86^{b}	34.89±1.84^{b}	36.00±4.30^{b}	20.78±0.98^{b}
IV	6	20.00±0.00^{a}	25.67±0.42^{a}	22.33±0.33^{a}	23.50±0.71^{a}	21.67±0.61^{b}

HOST, hypo-osmotic swelling test; Acr-Int, acrosome integrity; ^{abc}Values bearing different superscripts in a column differ significantly ($p<0.05$).

Table 3 shows descending order of total motile sperm post-thaw from Group I to IV.A similar trend was observed when per cent viability, HOS value, acrosome integrity and sperm abnormality amongst different groups were compared.

The per cent progressive motility had decreased in all the frozen-thawed semen samples when compared with fresh semen irrespective of presence or absence of FAA (Fig. 1). However, frozen-thawed semen samples grouped

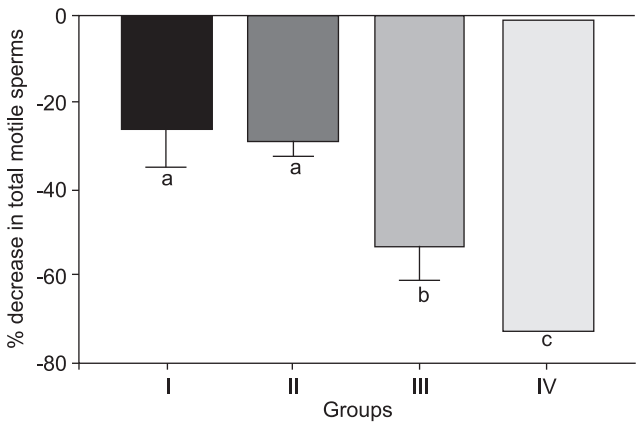

Fig. 1. The per cent decrease in progressive motility in frozen-thawed semen samples as compared to fresh semen amongst different groups.

preservation protocol. Moreover, the presence of 28–30 kDa on sperm membrane is reported to result in better conception rates (Bellin*et al.* 1996, Karunakaran*et al.* 2012, Karunakaran and Devnatha, 2016). As post-thaw sperm motility has been and will likely continue to be as one of the most important predictors of fertility (Matthew, 2016), we can safely conclude that FAA has a major role to play in fertility of Frieswal bulls.

Cryopreservation of semen samples have been shown to alter the coating of bull sperm by binder of sperm proteins (Ardon and Suarez, 2013) of which FAA is important. Therefore, the presence of FAA in seminal fluid might have resulted in some cladding over sperm surface of the said protein, resulting in better membrane stability and post thaw motility, albeit value was lower than Group I and II. Karunakaran*et al.*(2012) also reported high content of lipid peroxidation in the frozen-semen without heparin binding proteins.

The similar trends were observed when viability, acrosome integrity and plasma membrane integrity between fresh and frozen-thawed samples were compared. Krishnan *et al.*(2016) and Karunakaran and Devanatha(2016) have also reported that bulls positive for 30 kDa protein have more number of undamaged plasma membrane and acrosome intact sperms. The findings suggest that FAA may have some protective and stabilizing effects on the

spermatozoa membrane. Likewise, major binders of sperm proteins are known by their ability to modulate membrane cholesterol efflux from sperm membrane (Manjunath*et al.* 2007) and potential interaction with phospholipase A2 (Moura *et al.* 2006). This interaction confers protective effect on spermatozoa against cryo-damages and influence capacitation of spermatozoa by their ability to modulate membrane cholesterol efflux (Krishnan *et al.* 2016). Furthermore, it has been shown that bulls positive for FAA have low ROS-induced damage thus better protection against oxidative damage (Krishnan *et al.* 2016). This again justifies better semen attributes in groups positive for the said protein.

Total seminal plasma proteins, FAA status and freezability

The total seminal plasma proteins (TSPP) in fresh semen samples of Frieswal bulls in Group I, II, III, IV and V were 91.23±2.28, 92.13±1.93, 99.91±0.89, 104.67±1.33 and 104.43±4.05 mg/ mL, respectively. Though the Group I and II showed no significant difference amongst themselves,they hadsignificantly ($p < 0.01$) lesser TSPP than Group III, IV and V. Group III also had significantly ($p < 0.01$) lesser TSPP than Group IV and V. There was no significant difference observed in TSPP in Group IV and V.

The total seminal plasma proteins in fresh semen samples of Frieswal bulls had negative significant correlation with post-thaw motility ($r = –0.810$, $p< 0.01$), viability ($r = –0.814$, $p< 0.01$), plasma membrane integrity ($r = –0.823$, $p< 0.01$), acrosome integrity ($r = –0.863$, $p< 0.01$) and abnormality ($r = –0.758$, $p< 0.01$) of semen samples following frozen-thawing. The linear regression analysis between TSPP of fresh semen samples and post-thaw motility with regression equation is presented in Fig. 2.

Seminal plasma contains a variety of proteins, which are involved in several essential steps preceding fertilization. Some proteins are higher in the semen of fertile bulls, whereas others are more abundant in the semen of bulls of lower fertility (Gerena*et al.* 1998; McCauley *et al.* 2001). The composition, volume and character of seminal plasma may even differ within individuals (Yanagimachi, 1994). The total seminal plasma proteins in fresh semen samples of Frieswal bulls showed negative correlation with motility, viability, plasma membrane integrity, acrosome integrity and abnormality at their corresponding frozen-thawed stage. BSP proteins are known to exert both beneficial and detrimental effects on spermatozoa to maintain it in an appropriate state at the time of fertilization (Juyena and Stelleta, 2012). In our findings the negative correlation was probably due to the reason that more quantity of detrimental proteins may have resulted in continuous exposure of sperm to the same and hence greater efflux of cholesterol and phospholipids from the plasma membrane, rendering it destabilized. In agreement, Srivastava *et al.* (2013) have shown detrimental effect of BSP proteins in concentration and time dependent manner.

Total sperm membrane proteins, FAA status and freezability

The total sperm membrane proteins (TSMP) in Group I, II, III, IV and V were 4.71±0.44, 3.61±0.27, 2.16±0.23, 2.83±0.20 and 1.08±0.25 mg/10^9sperms, respectively. All the five groups had significant ($p<0.05$) difference in their TSMB, with highest value in Group I lowest in the Group V. A significant ($p<0.01$) positive correlation ($r = –0.66$) was observed between post thaw motility and total sperm membrane proteins.

The total sperm membrane proteins in fresh semen samples had positive correlation with post-thaw motility ($r = 0.666$, $p< 0.01$), viability ($r = 0.692$, $p< 0.01$), plasma membrane integrity ($r = 0.700$, $p< 0.01$), acrosome integrity ($r = 0.680$, $p< 0.01$) and abnormality ($r = 0.653$, $p< 0.01$) of semen samples following freezing-thawing. The linear regression analysis between total sperm membrane proteins of fresh semen samples and post-thaw motility with regression equation is presented in Fig. 3.

In the present investigation, the total sperm membrane proteins in fresh semen samples of Frieswal bulls had positive correlation with post-thaw motility, viability, plasma membrane integrity, acrosome integrity and

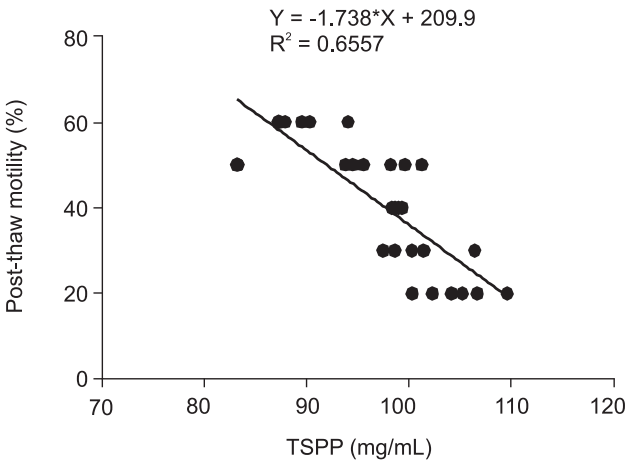

Fig. 2. Linear regression analysis between total seminal plasma proteins (TSPP) of fresh semen samples and post-thaw motility

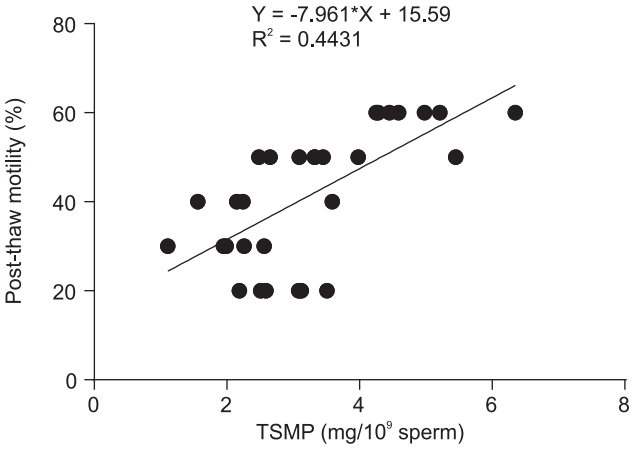

Fig. 3. Linear regression analysis between total sperm membrane proteins (TSMP) of fresh semen samples and post-thaw motility

abnormality of frozen-thawed semen samples. Increase in the concentration of total sperm membrane protein could have resulted as a consequence of increase in the binding of BSP, including FAA to sperm membrane. Improvement in freezability and bull fertility due to greater concentration of FAA in sperm membrane was reported by Bellin *et al*. (1996). This is in contrast to earlier observation of negative co-relation of TSPP with semen quality parameters within this study. This implies that beneficial proteins, as compared to detrimental ones, have greater affinity to bind with sperm membrane resulting in improved quality parameters. The reasons have already been discussed in this treatise for the stability of sperm membrane arising from binding of BSP, including FAA, to sperm membrane.In agreement, differential concentrations of sperm membrane proteins were shown to be relating to freezability of buffalo spermatozoa (Dhanju*et al.* 2001).

Our results have shown that 28–30 kDa protein is present in seminal plasma and sperm membranes of majority of Frieswal bulls. The samples with detectable-FAA on sperm membranes show better semen quality parameters than bulls with undetectable FAA in their sperm membranes. The total seminal plasma proteins in fresh semen samples of Frieswal bulls show negative correlation with semen quality parameters, the trend, contrariwise in sperm membrane proteins. This implies that the relative content of seminal plasma proteins and sperm membrane proteins could be an essential index to evaluate Frieswal bull semen quality.Eventhough the study is not conclusive, it anyway provides a guiding pathway to undertake a larger investigation and inclusion of FAA screening for discrimination of semen samples and bulls for their freezability and fertility attributes.

REFERENCES

Ardon F and Suarez S S. 2013. Cryopreservation increases coating of bull sperm by seminal plasma binder of sperm proteins BSP1, BSP3 and BSP5. *Reproduction* **146**(2): 111–17.

Asadpour R, Alavi-Shoushtari S, Rezaii A and Ansari A H K. 2007. SDS-polyacrylamide gel electrophoresis of buffalo bulls seminal plasma proteins and their relation with semen freezability. *Animal Reproduction Science* **102**: 308–13.

Ax R L. 2004. A new test screens bulls for a protein that enables them to settle more cows earlier. *Western Cowman* **7**: 28–32.

Bellin M E, Hawkins H E, Oyarzo J N, Vanderboom R J and Ax R L. 1996. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *Journal of Animal Science* **74**: 173–82.

Bellin M E, Oyarzo J N, Hawkins H E, Zhang H, Smith R G, Forrest D W, Sprott L R and Ax R L. 1998. Fertility-associated antigen (FAA) on bull sperm indicates fertility potential. *Journal of Animal Science* **76**: 2032–39.

Boe-Hansen G B, Rego J P A, Crisp J M, Moura A A, Nouwens A S, Li Y, Venus B, Burns B M and McGowan M R. 2015. Seminal plasma proteins and their relationship with percentage of morphologically normal sperm in 2–year-old Brahman (*Bos indicus*) bulls. *Animal Reproduction Science* **162**: 20–30.

Caballero I, Parrilla I, Alminana C, delOlmo D, Roca J, Martinez E A and Vázquez J M. 2012. Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. *Reproduction in Domestic Animals* **47**(3): 12–21.

Calvete J J and Sanz L. 2007. Insights into structure-function correlations of ungulate seminal plasma proteins. *Proceedings of the 10th International Symposium on Spermatology,* Spain, pp 201–215.

Campbell R G, Hancock J L and Rothschild L. 1953. Counting live and dead bull spermatozoa. *Journal of Experimental Biology* **130**: 44–45.

Dhanju C K, Cheema R S and Kaur S P. 2001. Effects of freezing on proteins and protein profiles of sperm membrane extracts and seminal plasma of buffalo bulls. *Asian Australasian Journal of Animal Science* **14**: 1678–1682.

Drobnis E Z, Crowe L M, Berger T, Anchordoguy T J, Overstreet W and Crowe J H. 1993. Cold shock damage is due to lipid phase transitions in cell membranes, a demonstration using sperm as a model. *Journal of Experimental Zoology* **265**: 432–37.

Fernandes C E, de Souza F F, Souza-Neto J A and Ribola P E M. 2008. Heparin-binding proteins of seminal plasma in Nellore bulls. *Ciencia Rural* **39**: 20–26.

Gerena R L, Irikura D, Urade Y, Eguchi N, Chapman D A and Killian G J. 1998. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biology of Reproduction* **58**: 826–33.

Januskauskas A, Johannisson A and Rodriguez-Martinez H. 2003. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology* **60**: 743–58.

Jeyendran R S, Vander Ven H H, Perz-Pelaez M, Crabo B G and Zaneveld L J D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other sperm characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.

Jobim M I M, Oberst E R, Salbego C G, Souza D O, Wald V B, Tramontina F and Mattos R C. 2004. Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. *Theriogenology* **61**: 255–66.

Juyena N S and Stelletta C. 2012. Seminal plasma: An essential attribute to spermatozoa. *Journal of Andrology* **33**: 536–51.

Karunakaran M and Devanatha T G. 2016. Evaluation of bull semen for fertility-associated protein, *in vitro* characters and fertility. *Journal of Applied Animal Research* **45**(1): 136–44.

Karunakaran M, Devanathan T G, Jawahar T P, Manimaran K, Chitra A, Dhali A and Selvaraju S. 2012. Electrophoretic profile of bull sperm membrane proteins as a tool for selection of breeding bull. *Indian Journal of Animal Sciences* **82**(11): 1303–05.

Krishnan G, Thangvel A, Loganathasamy K, Veerapandian C, Kumarasamy P and Karunakaran M. 2016. The presence of heparin binding proteins and their impact on semen quality of Holstein Friesian bulls. *Indian Journal of Animal Sciences* **86**(4): 392–96.

Laemmli V K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–85.

Manjunath P, Bergeron A, Lefebvre J and Fan J. 2007. Seminal plasma proteins: functions and interaction with protective agents during semen preservation. *Society of Reproduction and Fertility Supplement* **65**: 217–28.

Manjunath P, Lefebvre J, Jois P S, Fan J and Wright M W. 2009.

New nomenclature for mammalian BSP genes. *Biology of Reproduction* **80**: 394–97.

Matthew D U. 2016. Prediction of bull fertility. *Animal Reproduction Science* **169**: 37–44.

McCauley T C, Zhang H M, Bellin M E and Ax R X. 2001. Identification of a heparin- binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases–2. *Molecular Reproduction and Development* **58**: 336–41.

Moura A A, Koc H, Chapman D A and Killian G J. 2006. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *Journal of Andrology* **27**: 201–11.

Nass S J, Miller D J, Winner M A and Ax R L. 1990. Male accessory sex glands produce heparin-binding proteins that bind to caudaepididymal spermatozoa and are testosterone dependent. *Molecular Reproduction and Development* **25**: 237–46.

Rodriguez-Martinez H, Kvist U, Ernerudh J, Sanz L and Calvete J J. 2011. Seminal plasma proteins: what role do they play? *American Journal of Reproductive Immunology* **66** (1): 11–22.

Rodriguez-Martinez H. 2014. Semen evaluation and handling. Animal andrology theories and applications. (Eds) Chenoweth P J and Lorton S P. London, UK, pp 526–528.

Rodríguez-Villamil P, Hoyos-Marulanda V, Martins J A M, Oliveira A N, Aguiar L H, Moreno F B, Velho A L M C S, Monteiro-Moreira M C, Moreira R A, Vasconcelos I M, Bertolini M and Moura A A. 2016. Purification of binder of sperm protein 1 (BSP1) and its effects on bovine *in vitro* embryo development after fertilization with ejaculated and epididymal sperm. *Theriogenology* **85**(3): 540–54.

Snedecor G W and Cochran W G. Statistical methods, 7th ed. The Iowa State University Press, Ames, Iowa, USA, pp 115.

Souza C E, Moura A A, Monaco E and Killian G J. 2008. Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. *Animal Reproduction Science* **105**: 72–89.

Srivastava N, Jerome A, Srivastava S K, Ghosh S K and Amit K. 2013. Bovine seminal PDC–109 protein: an overview of biochemical and functional properties. *Animal Reproduction Science* **138**: 1–13.

Tyagi S, Mathur A K and Agarwal S C. 2000. Semen production performance of Frieswal bulls. *Indian Journal of Animal Reproduction* **70**: 1032–34.

Watson P F. 1975. Use of Giemsa stain to detect changes in acrosome of frozen ram spermatozoa. *Veterinary Record* **79**: 12–15.

Watson P F. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reproduction Fertility and Development* **7**: 871–91.

Yanagimachi R. 1994. Fertility of mammalian spermatozoa: its development and relativity. *Zygote* **3**: 371–72.