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Effect of fertility associated proteins on lipid peroxidation production in Holstein Friesian semen

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ABSTRACT

Present study was designed to evaluate effects of fertility associated proteins on sperm motility, viability and level of lipid peroxidation (LPO) in semen. Holstein Friesian breeding bulls (17) were screened for fertility associated proteins (24, 28–30 and 55 kDa proteins), and categorized into 8 groups based on presence/absence in the seminal plasma and sperm membrane. Level of LPO compound malondialdehyde (MDA) was assessed in fresh and frozen-thawed semen using thiobarbituric acid method. Fresh semen of bulls positive for fertility associated proteins had lower MDA (0.433±0.03 µmol/ml) than bulls negative for fertility associated proteins (0.740±0.05 µmol/ml). Level of MDA increased in frozen-thawed sperm by 1.5 fold as that of fresh semen among bulls positive for fertility associated proteins and 2 fold in negative bulls. MDA level increased by 27% in all the frozen-thawed semen incubated at 37°C for 180 min, however it was lower in bulls with fertility associated proteins (2.380±0.14 µmol/ml) than negative bulls. Fresh semen with fertility associated proteins had higher number of motile sperm with progressive motility than negative bulls. Further, bulls with fertility associated proteins were able to sustain higher number of viable sperms with progressive motility than negative group at the end of 180 min of incubation. The results concluded that the fertility associated proteins were capable to sustain sperm viability and motility by limiting LPO production in fresh and frozen-thawed semen.

Key words: Bull, Fertility associated proteins, Lipid peroxidation, Semen, Sperm viability

Proteins present in seminal plasma and sperm membrane such as osteopontin (OPN), prostaglandin D synthase, bovine seminal plasma proteins (BSP A_1, A_2, A_3) and heparin binding proteins (24 and 28–30 kDa) are reported as markers of male fertility (Moura *et al.* 2008). OPN is expressed in the accessory sex gland fluid of high fertility bulls, which plays a role in sperm-binding and fertilization (Moura *et al*. 2006). Heparin binding proteins (HBP), present in seminal plasma and sperm membrane, are called fertility associated antigens (31 kDa). Tissue inhibitor of metalloproteinases-2 (24 kDa) contains factors that modulate fertilizing ability of sperm cells, preserve membrane integrity, minimize production of reactive oxygen species and lipid peroxidation in sperm membrane (Moura *et al*. 2006, Karunakaran *et al*. 2012).

LPO in biological membranes causes impairment of membrane function, decreased fluidity, inactivation of membrane-bound receptors and enzymes with increased non specific permeability to ions (Singh *et al.* 2014). The susceptibility of ruminant spermatozoa to oxidative stress is a consequence of the abundance of poly unsaturated fatty acids in sperm plasma membrane which are vulnerable to free radicals attack and the initiation of LPO cascade (Bansal and Bilaspuri 2011). The lipid peroxidation results in loss of membrane integrity, impaired cell function, along with impaired sperm motility and induction of sperm apoptosis. Reactive oxygen species (ROS) generated by spermatozoa play an important role in normal physiological processes but when its production exceeds the physiological limit results in oxidative stress to sperm (Asadpour *et al*. 2011). Further, dilution of semen with extender reduces the antioxidants level and the mechanism of freezing and thawing also causes death of certain per cent of sperm cells which generate ROS in excess (Parisi *et al*. 2014). Hence, present study was designed to evaluate the effects of presence or absence of fertility associated proteins on sperm motility, viability and the level of lipid peroxidation.

MATERIALS AND METHODS

Experimental animals: Adult Holstein Friesian breeding bulls (17) were selected from Central Frozen Semen

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Production and Training Institute, Hessarghatta, Bengaluru, India for the present study. All the bulls were in regular semen collection programme and maintained under standard managemental practices.

Semen collection and analysis of sperm motility: Semen samples were collected by artificial vagina method and with the quality control criteria of the institute. Motility analyses in fresh ejaculates were conducted using a computer-assisted semen analysis. Analysis was based on examination of 25 consecutive digitalized images per second using a 5 × negative phase contrast objective. Motility analysis was carried out in 5 µl of semen samples placed onto a pre-warmed (37ºC) microscopic slide covered with 22 mm × 22 mm cover slip. A minimum of 500 spermatozoa from at least 2 different drops were analyzed for each sample. The final analysis was done after removing the number of objects incorrectly identified as spermatozoa.

Isolation of fertility associated proteins: 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 and incubated at 4°C overnight. 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. The characterization of the fertility associated proteins in the seminal plasma and sperm membrane was carried out by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli 1970). Heparin binding proteins in the sperm membrane and seminal plasma were isolated using heparin-sepharose affinity chromatography (heparin-CL agarose pre-packed column) as per Manaskova *et al*. (2002) with slight modifications. The bulls were grouped based on the presence / absence of 24 and 28–30 heparin binding proteins, and 55 kDa proteins in seminal plasma as well as in sperm membrane. The bulls were categorized into 8 groups as follows, group 1 (positive for 24 kDa; n,10), group 2 (positive for 28–30 kDa, n,8), group 3 (positive for 24 and 28–30 kDa; n,11), group 4 (positive for 55 kDa; n,12), group 5 (positive for 24 and 55 kDa; n,13), group 6 (positive for 28–30 and 55 kDa; n,12), group 7 (positive for 24, 28–30 and 55 kDa; n,7) and group 8 (negative for 24, 28–30 and 55 kDa; n, 4).

Estimation of lipid peroxidation: Lipid peroxidation level of spermatozoa was estimated in semen samples by measuring the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) as per Suleiman *et al*. (1996) with slight modifications in sperm concentration and incubation time. The fresh and frozen-thawed semen was taken and washed twice in tris buffer by centrifugation (500 × *g* for 5 min). The sperm pellet was re-suspended in 1 ml of PBS (pH 7.2) or a variable volume of PBS to obtain a sperm concentration of 10 $\times 10^6$/ml. Lipid peroxide level was measured in spermatozoa after the addition of 2 ml of TBA-TCA reagent (15% w/v trichlor acetic acid and 0.375% w/v TBA in 0.25N HCl) to 1 ml of spermatozoa suspension. The mixture was kept in a boiling water bath for 45 min. After cooling, the suspension was centrifuged at 500 × *g* for 15 min. The supernatant was separated and the absorbance was measured at 535 nm under UV spectrophotometer. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5/mol/cm^3).

$$\text{MDA (µmol/ml)} = \frac{\text{OD} \times 10^6 \times \text{total volume (3ml)}}{1.56 \times 10^5 \times \text{test volume (1ml)}} = \frac{\text{OD} \times 30}{1.56}$$

Incubation test: The semen samples of each bull were incubated immediately after thawing at 37ºC for 180 min in the water bath. The incubated semen samples were monitored for their sperm viability, motility and progressive motility at 0, 30, 60, 120 and 180 min. The per cent of sperm motility and progressive motility were evaluated manually under phase contrast microscope. The per cent of sperm viability was assessed by eosin-nigrosin staining method. At least 500 spermatozoa were counted in different fields to arrive the per cent of viability. The lipid peroxidation level of spermatozoa was also estimated in the incubated frozen-thawed semen samples at the end of incubation period of 180 min.

Statistical analysis: All statistical analyses were carried out using the Statistical Package for Social Sciences programme (SPSS), version 16.00 software for windows. The comparison of lipid peroxidation level and motility pattern between groups based on the presence or absence of fertility associated 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 lipid peroxidation levels between fresh and frozen thawed semen was analyzed by paired 't' test. Results are expressed as mean±standard error of mean.

RESULTS AND DISCUSSION

Sperm motility pattern: The results of sperm motility and progressive motility assessed by computer assisted semen analysis in fresh semen of all the bulls are presented

Table 1. Total sperm motility and progressive motility (%) in fresh semen of Holstein Friesian breeding bulls

Animal group	No. of animals(n)	Total motility (%)	Progressive motility(%)
1	10	84.80±2.55^b	59.70±3.18^b
2	8	82.88±2.82^b	59.25±3.64^b
3	11	84.64±2.31^b	59.73±2.88^b
4	12	83.00±1.94^b	56.33±3.07^b
5	13	83.92±2.01^b	57.38±3.01^b
6	12	83.00±1.94^b	56.33±3.07^b
7	7	86.00±1.03^b	62.33±3.24^b
8	4	72.00±4.06^a	37.00±3.19^a
'F' value		1.703*	2.991**

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

Table 2. Malondialdehyde (MDA) level in fresh and frozen-thawed semen of Holstein Friesian breeding bulls

Animalgroup	No. of animals(n)	Malondialdehyde (µmol/ml)			
		Fresh semen	Frozen-thawed semen	't' value	MDA level at 180 min of incubation
1	10	0.415±0.03[a]	0.638±0.02[a]	8.260**	2.338±0.15[a]
2	8	0.411±0.03[a]	0.658±0.02[a]	9.558**	2.213±0.16[a]
3	11	0.416±0.03[a]	0.639±0.02[a]	9.154**	2.380±0.14[a]
4	12	0.459±0.04[a]	0.833±0.12[a]	3.505**	2.326±0.13[a]
5	13	0.448±0.04[a]	0.810±0.12[a]	3.675**	2.370±0.12[a]
6	12	0.459±0.04[a]	0.833±0.12[a]	3.505**	2.326±0.13[a]
7	7	0.382±0.03[a]	0.545±0.02[a]	8.365**	2.129±0.15[a]
8	4	0.740±0.05[b]	1.545±0.19[b]	3.131**	5.519±0.24[**a**]
'F' value		3.904**	3.578**		27.882**

Means with different superscripts in a column differ significantly (**P <0.01).

in Table 1. The fresh ejaculates of bulls positive for fertility associated proteins (groups 1 to 7) had significantly (P < 0.05) higher per cent of motile sperm with progressive motility in comparison with bulls (group 8) negative for fertility associated proteins. The present results are in conformity with the previous reports of Singh *et al*. (2014) and Sarsaifi *et al*. (2015). On ejaculation, the fresh semen get mixed with the BSP and fertility associated proteins, and the heparin binding proteins which leads to an initial cholesterol efflux along with release of phospholipids from the sperm membrane (Kutty *et al*. 2014). Further, the changes in sperm membrane phospholipid concentration in the mid piece and tail increase the intracellular Ca^{2+} and cAMP levels, which might augment the sperm motility. The bulls negative for fertility associated proteins had lower (P < 0.05) per cent of total sperm motility which could be the reason that lack of fertility associated proteins (Karunakaran *et al*. 2012).

The motility is a function of intracellular adenosine triphosphate (ATP) content which has highly correlated with progressive motility of spermatozoa in fresh and cryopreserved bull semen (Singh *et al*. 2014, Sarsaifi *et al*. 2015). While, Jobim *et al*. (2004) and Asadpour *et al*. (2007) reported that the presence of 24 kDa HBP in seminal plasma and sperm membrane had increased sperm progressive motility in fresh semen. However, the bulls (group 8) negative for fertility associated proteins had lower (P < 0.05) per cent of sperm progressive motility. This could be due to rapid leakage of intracellular ATP through the damaged sperm plasma membrane and oxidative stress (Asadpour *et al*. 2011).

Lipid peroxidation: The MDA levels of fresh and frozen-thawed sperm of all the bulls are presented in Table 2. The fresh semen of bulls positive for fertility associated proteins had significantly (P < 0.01) lower MDA (Average 0.433±0.03 µmol/ml) in comparison to bulls lacking for fertility associated proteins (group 8) which had 2 fold higher level of MDA (0.740±0.05 µmol/ml). There was no significant difference in the levels of MDA among the bulls positive for one or more fertility associated proteins. However, the fresh semen of bulls positive for all 3 fertility associated proteins had lowest level of MDA (0.382±0.03 µmol/ml) numerically, among all the bulls. The present results supported the earlier findings of Karunakaran *et al*. (2012) and Singh *et al*. (2014). This clearly indicated that bulls positive for fertility associated protein had better protection against oxidative stress and able to control the generation of lipid peroxides (Karunakaran *et al*. 2012).

The LPO production increased significantly (P < 0.01) in the frozen-thawed semen of all the bulls, irrespective of presence or absence of fertility associated proteins. The increase in level of MDA was around 1.5 fold as that of fresh sperm cells among the bulls positive for fertility associated proteins and it was 2 fold in bulls negative for fertility associated proteins. The process of cryopreservation is known to produce and accumulate more of ROS with reduced antioxidant defense system and initiation of lipid peroxidation in the sperm membrane (Asadpour *et al*. 2011). However, bulls positive for fertility associated proteins had significantly (P < 0.05) lower (0.708±0.06 µmol/ml) level of MDA compared to bulls negative for fertility associated proteins (1.445±0.19 µmol/ml) in the frozen-thawed semen. The fertility associated proteins might be counteracted the lipid peroxidation production and reduced cryopreservation effects on spermatozoa (Karunakaran *et al*. 2012, Bustamante-Filho *et al*. 2014).

Incubation test: The per cent of sperm viability and total motile sperm with progressive motility in the frozen-thawed semen of all the bulls incubated at 37°C for 180 min were assessed at different time intervals (Figs 1–3). The post-thaw sperm viability and motile sperm with progressive motility was significantly (P < 0.05) higher in bulls positive for fertility associated proteins than bulls negative for fertility associated proteins. The decrease in total sperm motility was (P < 0.01) lower (55.38±2.08 - 60.00±3.42%) in bulls positive for fertility associated proteins when compared with bulls negative for fertility associated proteins (38.75±3.15%) during 60 to 120 min of incubation. At the end of the incubation, semen with fertility associated proteins had (P < 0.01) higher per cent of total motile sperm (23.08±2.80 - 30.00±4.28%) with progressive motility (11.25±2.47 - 17.50±2.81%) while semen devoid of fertility

associated proteins had 4.00±2.04% of sperm with progressive motility. LPO production in terms of MDA was ($P < 0.01$) lower in the bulls positive for fertility associated proteins in comparison to bulls negative for fertility associated protein at the end of incubation period (Table 2). The present results are in accordance with previous reports of Karunakaran *et al*. (2012), Ashrafi *et al*. (2013) and Parisi *et al*. (2014). The presence of fertility associated proteins on the sperm membrane might have protected the spermatozoa from cryo-injuries and oxidative stress (Jobim *et al*. 2004). In addition, presence of 24 kDa heparin binding protein had increased sperm viability in frozen-thawed semen (Asadpour *et al*. 2007). Karunakaran *et al*. (2012) reported that addition of fertility associated proteins in particularly HBPs had facilitated in combating oxidative stress of sperm cells and reduced the lipid peroxidation. The fertility associated proteins had protected the membrane integrity and mitochondrial membrane potential by lowering the lipid peroxidation production (Karunakaran *et al*. 2012) and prolonged the maintenance of sperm motility (Kutty *et al*. 2014).

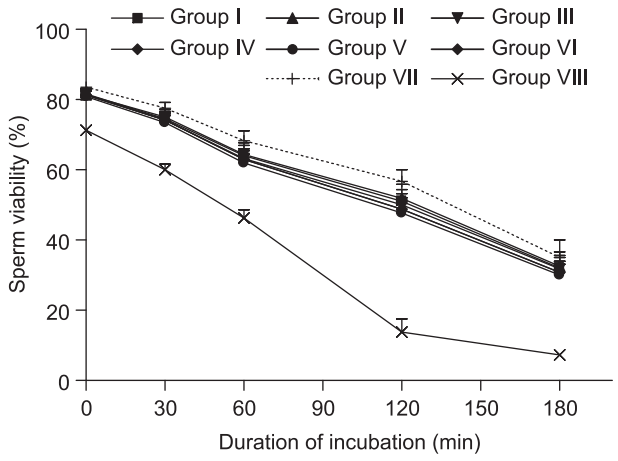

Fig. 1. The per cent of sperm viability in the frozen-thawed semen incubated at 37°C for 180 min.

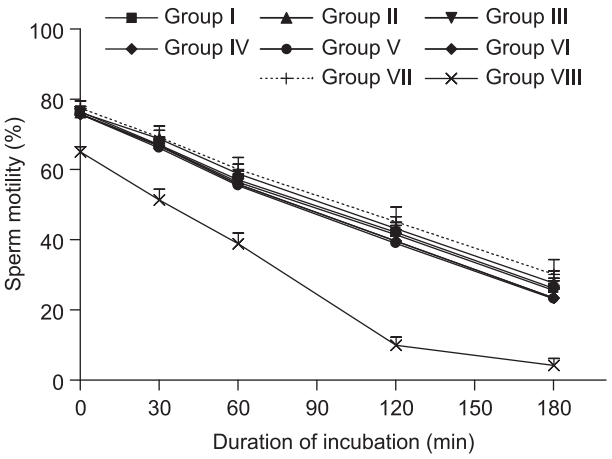

Fig. 2. The per cent of total sperm motility in the frozen-thawed semen incubated at 37°C for 180 min.

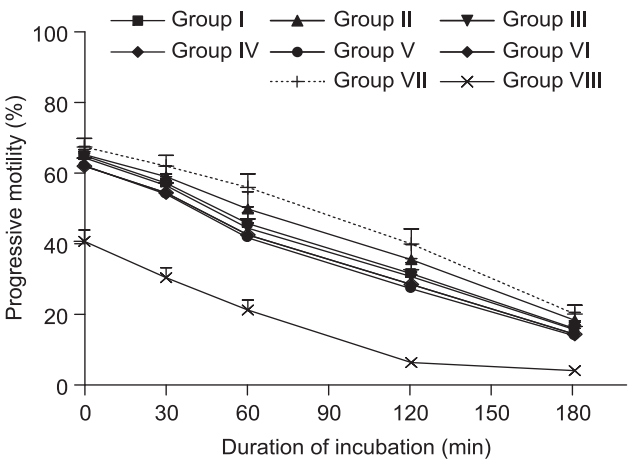

Fig. 3. The per cent of sperm progressive motility in the frozen-thawed semen incubated at 37°C for 180 min.

The semen without fertility associated proteins had higher ($P < 0.01$) level of MDA with lower number of motile sperm at 180 min of incubation. It could be due to the severe oxidative stress which resulted in lower viability of spermatozoa and higher level of ROS induced lipid peroxidation (Ashrafi *et al*. 2013). The decrease in motility during incubation might be due to a gradual decline in the ability of spermatozoa to generate ATP through mitochondrial respiration as a consequence of mitochondrial aging or the toxic effect of ROS during long storage in ambient temperature. It is well established that ROS can induce axonemal and mitochondrial damage which results in the immobilization of sperm cells (Bustamante-Filho *et al*. 2014, Parisi *et al*. 2014).

It may be concluded from the present results that fertility associated proteins are capable to sustain the sperm viability and motility by limiting lipid peroxidation production and protecting the sperm membrane against oxidative stress in the fresh and frozen-thawed semen. Further, the fertility associated proteins reduced the cryo-injuries and oxidative stress evidenced by higher per cent of motile sperm with progressive motility and lower level of lipid peroxidation in the frozen-thawed semen of bulls, which were positive for fertility associated proteins. Hence, it may be highly beneficial to include the criteria of fertility associated proteins in the bull selection programmes to achieve maximum fertility rate.

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