EFFECT OF FERTILITY ASSOCIATED PROTEIN ON OXIDATIVE STRESS OF BOVINE SPERM CELLS

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ABSTRACT

The present study addressed the effects of fertility associated protein on oxidative stress in frozen thawed bull spermatozoa. The frozen semen samples of 22 bulls were thawed at 37° C in a water bath and oxidative stress was measured indirectly by estimating malondialdehyde (MDA) level at immediate post thaw, 60, 120 and 180 min of incubation. To find whether addition of fertility associated protein would control the oxidative stress of the sperm cells, 25 µg of 28 – 30 kDa heparin binding protein of the sperm membrane was added to the thawed semen samples and MDA levels were estimated. MDA level was significantly lower ($P < 0.01$) in fertility associated protein treated semen sample than the untreated control semen at 60 min (1.95 ± 0.11 vs 2.85 ± 0.12), 120 min (2.40 ± 0.12 vs 3.20 ± 0.20) and 180 of incubation (2.99 ± 0.15 vs 4.31 ± 0.32). It was concluded that 28 – 30 kDa heparin binding protein of the sperm membrane could help in controlling the oxidative stress of the sperm cells during incubation.

Key words: Bull; Semen; Oxidative stress; Lipid peroxidation; Malondialdehyde; Fertility associated protein

INTRODUCTION

The imbalance between the production of reactive oxygen species (ROS) and the ability biological systems to detoxify the reactive intermediates or easily repair the resulting damage was known as oxidative stress (Agarwal *et al.,* 2003). Oxidative stress was believed to be the underlying cause for numerous cell dysfunctions. Mammalian spermatozoa represented a growing list of cell types that exhibited a capacity to generate ROS when incubated under aerobic conditions. Mature spermatozoa has little capacity for repairing oxidative damage because their cytoplasm contain low concentrations of scavenging enzymes (Alvarez and Storey, 1989). Seminal plasma endowed with low molecular non-enzymatic and enzymatic antioxidant capacity is capable of scavenging ROS to act as an additional protection of spermatozoa against oxidative stress. Two of the main factors contributing to ROS accumulation *in vitro* were the absence of endogenous defense mechanism and exposure of gametes to various manipulation techniques as well as environment that could lead to generation of oxidative stress (Agarwal *et al.,* 2005). Given that high levels of antioxidants in

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seminal plasma contributed to the preservation of spermatozoa, semen dilution or washing reduced the potential protective capacity provided by the plasma (Maxwell and Stojanov, 1996). During cryopreservation, spermatozoa are exposed to cold shock and atmospheric oxygen, which in turn increase the production of ROS and decrease the antioxidant level (Bucak *et al.*, 2010). The mechanism of ROS-induced damage to spermatozoa include an oxidative attack on the sperm membrane lipids leading to initiation of lipid peroxidation (LPO) cascade (Sharma and Agarwal, 1996). By measuring the level of malondialdehyde, a stable lipid peroxide compound, the level of oxidative stress in the sperm cells could be measured indirectly. It was reported that addition of seminal plasma proteins preserved not only the enzyme activity levels but also the distribution of antioxidant enzymes on the ram spermatozoa surface (Marti *et al.*, 2008) and controlled the oxidative stress. The present study was carried out with the objective to study the effect of fertility associated protein of sperm membrane in controlling the oxidative stress of sperm cells during incubation.

MATERIALS AND METHODS

Frozen semen samples (French mini straws) from 22 breeding bulls (10 Jersey, 10 Jersey crossbred and 2 Holstein Friesian bulls) maintained at Tamil Nadu Co-operative Milk Producer's Federation Limited, Nucleus Jersey and Stud Farm, Udhagamandalam and Semen Bank, Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai were procured. All the bulls were in regular semen collection programme for use in AI and maintained under standard management conditions. Frozen semen straws were thawed at 37° C for 60 sec. Thawed semen was emptied into an eppendorf tube, mixed thoroughly and maintained at thawing temperature in a water bath.

Two types of treatments viz., control (no treatment) and treatment with fertility associated protein were carried out. In the untreated control group lipid peroxidation status of the sperm cells was assessed at immediate post thaw, 60 min, 120 min and 180 min of post thaw incubation.

To assess the effect of fertility associated proteins on the lipid peroxidation status of sperm cells, 28- 30 kDa heparin binding proteins of the sperm membrane isolated from the bulls positive for protein were used. Sperm membrane proteins were extracted as per the method described by Nass *et al.* (1990) from the neat semen of the bulls and heparin binding proteins were isolated using heparin-sepharose affinity chromatography (Heparin- CL agarose prepacked column, Bangalore Genei, India) as per the method of Manaskova *et al.* (2002). Discontinuous sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS-PAGE) was performed according to Laemmli (1970) to characterize the proteins and check for the presence of fertility associated proteins. Frozen semen straws were thawed at 37° C for 60 s. Then the sperm cells were washed in phosphate buffered saline (PBS) by centrifugation (560 X g for 5 min). Heparin binding protein (25 µg) was added to the semen and incubated at 37° C and lipid peroxidation status were assessed at 60, 120 and 180 min of incubation.

Lipid peroxidation level of spermatozoa was estimated in semen samples by measuring the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) as per the method described by Suleiman *et al.* (1996) with slight modifications in sperm concentration and incubation time. The semen was thawed and washed twice in Tris buffer by centrifugation (500 X g for 5 min). Then 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 30×10^6/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 X g for 15 min. The supernatant was separated and the absorbance measured at 535 nm under UV spectrophotometer (Cecil CE 2021, 2000 series). The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5/molcm^{-3}).

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

Statistical analyses were carried out using the Statistical Package for Social Sciences programme (SPSS), version 15.00 software for windows (SPSS Inc. Chicago, IL, USA). Statistical analysis was performed after arcsine transforming the percentage values. Statistical significance was set at 0.05 probability level. If the effect was found significant, comparison of means was done by Duncan Multiple Range Test (DMRT). Results are expressed as Mean ± Standard Error of Mean. The effect of different duration of incubation on the lipid peroxidation status, the comparison between control and fertility associated protein treated group at each point of incubation were analyzed by one way ANOVA.

RESULTS AND DISCUSSION

MDA level (µ mol/ml) increased significantly ($P < 0.01$) from the immediate post thaw level of 1.67± 0.1 during different periods of incubation 60, 120 and 180 min post thaw both in control and treatment with fertility associated protein group. MDA level was significantly lower ($P < 0.01$)in fertility associated protein treated semen sample than control semen at 60 min (1.95 ± 0.11 vs 2.85 ± 0.12), at 120 min (2.40 ± 0.12 vs 3.20 ± 0.20) and at 180 min post thaw (2.99 ± 0.15 vs 4.31 ± 0.32) in 30 million sperm cells.

ROS generated by spermatozoa played an important role in normal physiological processes such as, sperm capacitation, acrosome reaction, maintenance of fertilizing ability and stabilization of the mitochondrial capsule in the mid-piece in bovine (Desai *et al.,* 2010). When in excess, ROS caused adverse effects on the sperm plasma membrane, DNA and physiological processes, thereby affecting the quality of spermatozoa. Sperm cells were sensitive to ROS attack which resulted in decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosome reaction (Bansal and Bilaspuri, 2007).

In the present study, in untreated control group the MDA level increased significantly ($P < 0.01$) during different periods of incubation from the immediate post thaw level. Elevation of MDA level during different periods of incubation suggests that the resumption of metabolic activity of the sperm cells after thawing leads to generation of excessive ROS (Agarwal *et al.,* 2005). Activation of an aromatic amino acid oxidase enzyme from dead sperm cells also might be an additional source of ROS in frozen thawed semen samples (Upretti *et al.,* 1999). The MDA values obtained for cattle bull sperms in the present study were in accordance with the reported values of Beorlegui *et al.* (1997) who reported values ranging between 0.34 ± 0.18 and 4.95 ± 0.31µM/ ml in frozen thawed bovine semen sample and lesser than the reported values of Selvaraju *et al.* (2008), who observed 8.00 ± 0.31 µM/ml at immediate post thaw and 9.36 ± 0.36 µM/ml at 120 min post thaw incubation in buffalo frozen semen. The elevated level of MDA concentration in buffalo sperm in comparison with bull sperm might be due to the fact that the sperm membrane of buffalo is rich in poly unsaturated fatty acids (Nair *et al.*, 2006).

The results indicated that fertility associated proteins helped in combating oxidative stress of sperm cells in bulls. The response to exogenous addition of protein in controlling MDA level may be because the seminal proteins might counteract the lipid peroxidation on dose dependent manner as reported by Schoneck *et al.* (1996). Marti *et al.* (2008) reported that seminal proteins added alone or with other compounds showed a protective effect and accounted for an increase in the sperm enzyme activity levels not only in the fresh sample but also after cooling and freezing/thawing. It was concluded from the study that oxidative stress to the sperm cells increased during incubation of frozen thawed semen samples and the addition of fertility associated proteins of sperm membrane helps in controlling the level of lipid peroxides in frozen thawed semen samples.

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