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Effect of heparin binding protein and hydrogen peroxide on lipid peroxidation status of bovine sperm cells

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ABSTRACT

Level of lipid peroxides in frozen thawed bull semen samples was studied in frozen semen straws procured from 22 bulls maintained at bull stations. The semen samples were thawed at 37°C in a water-bath and the level of lipid peroxide was measured by estimating malondialdehyde (MDA) concentration in the semen samples at 0, 60, 120 and 180 min of incubation by thiobarbituric acid assay. 28–30 kDa heparin binding protein (HBP) of sperm membrane @ 25 µg was added to the frozen thawed semen samples and the level of MDA was estimated to check whether addition of HBP of sperm membrane would control the production of lipid peroxides. MDA level (µmol/ml) increased significantly during different periods of incubation-60 min, 120 min and 180 min post thaw in both control and treatment groups. MDA level was significantly lower ($P < 0.01$) in HBP treated semen sample than control semen at each period of incubation 1.95 vs 2.85; 2.40 vs 3.20; 2.99 vs 4.31 at 60, 120 and 180 min, respectively post thaw. MDA level in H_2O_2 treated samples increased significantly from 0 min level 1.67±0.1 to 1.85±0.1 at 10 min, 3.05±0.11 at 30 min and to 3.28±0.11 at 60 min incubation. The MDA level in H_2O_2 treated group (3.28±0.11) was significantly higher than the control (2.85±0.12) and HBP treated group (1.95±0.11) at 60 min incubation period. It was concluded that excessive H_2O_2 causes significant increases in lipid peroxides level and 28 – 30 kDa HBP of the sperm membrane could help in controlling the oxidative stress of the sperm cells.

Key words: Bull sperm, Heparin binding protein, Hydrogen peroxide, Lipid peroxidation, Melondialdehyde

Reactive oxygen species (ROS) generated by spermatozoa play an important role in normal physiological processes (Desai *et al.* 2010). Excess ROS causes oxidative stress to sperm cells (Bansal and Bilaspuri 2007). Mature spermatozoa have little capacity for repairing oxidative damage (Alvarez and Storey 1989). Seminal plasma is endowed with enzymatic and non-enzymatic antioxidant capacity (Jimenez *et al*. 1990) and should be capable of scavenging ROS and protect spermatozoa against oxidative stress. Semen dilution with extenders reduces the potential protective capacity of plasma (Maxwell and Stojanov 1996). During cryopreservation, sperm cells are exposed to cold shock and atmospheric oxygen, which increases production of ROS and decreases antioxidant level (Bucak *et al*. 2010). The freezing and thawing cause damages and/or death to certain proportion of sperm cells, which generate ROS in excess (Sariozkan *et al*. 2009). The ROS-induced damage to spermatozoa leads to initiation of lipid peroxidation (LPO) cascade (Sharma and Agarwal 1996). This results in a subsequent loss in membrane and morphological integrity, impaired cell functions, along with impaired sperm motility and induction of sperm apoptosis (Bucak *et al*. 2010). A simple tool to evaluate the level of lipid peroxidation in the spermatozoan is the assay of sperm malondialdehyde (MDA; Kasimanickam *et al*. 2006). The present study was carried out to study level of lipid peroxides in frozen thawed semen samples during incubation and to study effect of heparin binding protein of sperm membrane and hydrogen peroxide on the lipid peroxidation status of sperm cells.

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MATERIALS AND METHODS

Frozen semen samples in French mini straws of 22 adult

bulls (10 Jersey, 10 Jersey crossbred and 2 Holstein Friesian bulls) were procured from a farm and semen bank at Madras Veterinary College, Chennai. All the bulls were in regular semen collection for breeding purpose and maintained under standard management conditions. Frozen semen straws were thawed at 37° C for 60 sec. The contents were emptied into an eppendorf tube, mixed thoroughly and maintained at thawing temperature in a water bath.

Treatments, viz. control, treatment with HBP and treatment with H_2O_2 were carried out. In the control group lipid peroxidation status of the sperm cells was assessed at 0, 60, 120 and 180 min of post thaw incubation (Bollwein *et al*. 2008). To assess the effect of HBP on the lipid peroxidation status of sperm cells, 28– 30 kDa HBP of the sperm membrane isolated from the bulls positive for protein were used. Frozen semen straws were thawed at 37° C for 60 sec. The sperm cells were washed in phosphate buffered saline (PBS) by centrifugation (560g for 5 min). 25 µg of heparin binding protein was added to the semen and incubated at 37° C (Schoneck *et al.* 1996) and lipid peroxidation status was assessed at 60, 120 and 180 min of incubation. To study the effect of H_2O_2 on the induction of lipid peroxidation, 0.1 mM of H_2O_2 was added, and MDA level was estimated at 10, 30 and 60 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 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*g* for 5 min). Then the sperm pellet was re-suspended in PBS (pH 7.2) 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 (0.375% w/v TBA in 0.25N HCl and 15% w/v Trichlor acetic acid) 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 500g 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/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 SPSS, version 15.00 software. 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's multiple range test (DMRT). The effect of different duration of incubation on the lipid peroxidation status, the comparison between control and HBP treated group at each period of incubation were analyzed by one way ANOVA.

RESULTS AND DISCUSSION

MDA level increased significantly (P<0.01) during different periods of incubation (Table 1) in control and treatment groups. MDA level was significantly lower (P<0.01) in HBP treated semen sample than control semen at each period of incubation MDA level increased significantly (P < 0.01) from 0 min (immediate post thaw) to 30 min incubation with H_2O_2. Though MDA level increased to 3.28±0.11 at 60 min incubation, it did not differ significantly from the 30 min incubation level. But the MDA level in H_2O_2 treated group was significantly higher than control and HBP treated group at 60 min incubation period.

The imbalance between the production of reactive oxygen species (ROS) and a biological systems ability to detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress (Agarwal *et al.* 2003). Oxidative stress was believed to be the underlying cause for numerous cell dysfunctions. Mature spermatozoa had little capacity for repairing oxidative damage because their cytoplasm contained low concentrations of scavenging enzymes (Alvarez and Storey 1989). In the present study, in untreated control the MDA level increased significantly (P < 0.01) during different periods of incubation than at 0 min. 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 (Upreti *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 0 min 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).

In HBP treated semen samples, though the MDA level showed an increase during incubation, it was significantly (P < 0.01) lower than the untreated control samples at different

Table 1. Lipid peroxidation levels (MDA µ mol/ml) in frozen thawed semen samples treated with heparin binding protein during different period of incubation

Group	0 min	60 min	120 min	180 min
Control	1.67±0.1^{a}	2.85±0.12 ^{b1}	3.20±0.20 ^{b1}	4.31±0.32^{c1}
HBP treatment	1.67±0.1^{a}	1.95±0.11^{b2}	2.40±0.12^{c2}	2.99±0.15^{d2}

Means with different superscripts (a, b, c, d) in a row and (1, 2) in a column differ significantly (P< 0.01).

periods of incubation. The results indicated that HBP helped in combating oxidative stress of sperm cells in bulls. Comparatively lower level of MDA in HBP treated semen samples might be due to its counter action on lipid peroxidation (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 semen sample but also after cooling and freezing/thawing.

Treatment of semen samples with 0.1 mM H_2O_2 resulted in significant ($P < 0.01$) increase in MDA levels from 0 min to different incubation period of thawing in the present study. The MDA level in H_2O_2 treated group was significantly higher than their controls at 60 min of incubation. Garg *et al*. (2009) reported a 2-fold increase in MDA level when the buffalo semen samples were treated with 50 µM H_2O_2 for 1h. They further observed that H_2O_2 induced lipid peroxidation of sperm lipids in a dose and time dependent manner and cryopreserved semen samples were more susceptible to lipid peroxidation than fresh semen. H_2O_2 reacts with transition metals (Fe, Cu) to form more reactive radicals like hydroxyl radicals that were believed to damage proteins and lipids (Halliwell and Gutterridge 1984) and start lipid peroxidation.

It was concluded from the study that lipid peroxidation level to the sperm cells increased during incubation of frozen thawed semen samples and the exogenous addition of hydrogen peroxide abruptly elevates the lipid peroxides. The addition of HBP of sperm membrane helps in controlling the level of lipid peroxides in frozen thawed semen samples.

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