Effect of superoxide dismutase on cryopreservation of Mithun semen

P PERUMAL[1⊠], A K DE[1], R VIKRAM[2], K KHATE[2], K VUPRU[2], M H KHAN[2], A K NAHAK[3] and P C MISHRA[3]

ICAR-National Research Centre on Mithun, Medziphema, Nagaland 797 106 India

ABSTRACT

The present study was designed to assess the effect of superoxide dismutase (SOD) on post thaw semen quality parameters (SQPs), kinetic profiles and seminal biochemical profiles in Mithun. Ejaculates (25) were split into four equal aliquots after dilution with Tris-citrate-glycerol (TCG) extender, viz. Group I (control), Group II, III and IV contained 50, 100 and 150 U/ml of SOD, respectively. Cryopreserved and thawed samples were analysed for their motility parameters (progressive forward and in bovine cervical mucus penetration test [BCMPT]), kinetic and velocity parameters by computer assisted sperm analyser (CASA), viability, sperm morphological and nuclear abnormalities, acrosomal, plasma membrane and nuclear integrities, sperm enzymatic leakage and biochemical (sperm cholesterol and antioxidant and oxidative stress) profiles. Study revealed a significant enhancement in viability, sperm morphological and nuclear normalities, acrosome integrity, motility (progressive and BCMPT), sperm cholesterol content and reduction in leakage of intracellular enzymes in Group III. Moreover, intactness of acrosome and biochemical membranes was protected significantly in addition to significant improvement in kinetic and velocity profiles in extender containing 100 U/ml SOD. These results clearly suggest that though the cryopreservation of Mithun's spermatozoa in TCG was comparable with other species but inclusion of 100 U/ml SOD holds a clear advantage over control or 50 or 150 U/ml SOD. It can be concluded that SOD supplementation in semen extender can be effectively utilized to reduce the oxidative stress and improve the antioxidant profiles with cascading beneficial effects on cryopreserved semen quality parameters in Mithun.

Keywords: Cryopreservation, Kinetic profiles, Mithun, Semen, Superoxide dismutase

Mithun is a unique magnificent domestic bovine species in NEH region of India. Mithuns are reared under extensive free-range system with natural service as the preferred breeding practice with various limitations which could be overcome easily by implementation of artificial breeding programmes. Preliminary research conducted on SOD effect on basic SQPs in liquid preservation indicated that 100 U/ml SOD is suitable for Mithun liquid semen preservation (Perumal 2014). Various stages of cryopreservation induce physical, osmotic and chemical stresses on the sperm membrane associated with an oxidative stress induced by free radical (Chatterjee *et al.* 2001). All these deleterious effects cause loss of motility, viability, intactness of acrosome, plasma membrane and nuclear integrities which renders large number of sperms incapable of fertilizing the ovum and ultimately infertility or sterility (Bernardini *et al.* 2011). High polyunsaturated fatty acids content in sperm membranes and lack of significant cytoplasmic component containing antioxidants makes the spermatozoa highly and easily susceptible to lipid peroxidation by

Present address: [1]ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands. [2]ICAR-National Research Centre on Mithun, Medziphema, Nagaland. [3]OUAT-College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha.⊠Corresponding author email: perumalponrajvet@gmail.com

the presence of oxygen free radicals and H_2O_2 (Sinha *et al.* 1996). Therefore, researchers have concentrated on extender preparation by inclusion of membrane stabilizing compounds, additives, antioxidants, cryoprotectants and anti-apoptotic agents to improve the cryo-resistance of the sperm. Therefore, supplementation of exogenous antioxidants in semen extender (Shoae and Zamiri 2008, Perumal *et al.* 2013) or feeding of antioxidants (Jayaganthan *et al.* 2013) or flaxseed oil (Perumal *et al.* 2019) or slow release melatonin implantation (Perumal *et al.* 2018) can reduce the deleterious effect of oxidative and cryo-stress during the cryopreservation process (Perumal *et al.* 2011). Addition of additives such as SOD to equine sperm (Cocchia *et al.* 2011), bovine sperm (Asadpour *et al.* 2012) and bubaline semen (El-Sisy *et al.* 2008) has been shown to protect the sperm against the deleterious effects of ROS and improve the SQPs during sperm storage. Superoxide dismutase is a potential antioxidant that catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. It scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. SOD spontaneously oxidizes dismutase (O_2^-) anion to form O_2 and H_2O_2. SOD also prevents premature hyper-activation and capacitation induced by superoxide radicals before ejaculating (de Lamirande and Gagnon 1995). No information is available with

regard to the effect of SOD in Tris based semen extender cryopreservation on fertility of Mithun. Therefore, it was hypothesized that application of SOD in semen extender could be more beneficial on *in vitro* sperm functional parameters in Mithun. With this backdrop, the present study was undertaken to assess the effect of different concentrations of SOD in semen diluents on SQPs, kinetic and velocity profiles, oxidative stress profiles and leakage of intracellular enzymes of the cryopreserved Mithun sperm.

MATERIALS AND METHODS

Location of the study: The study was conducted at ICAR-National Research Centre on Mithun, Medziphema, Nagaland. It is located between 25°54′30′ North latitude and 93°44′15′ East longitude and at an altitude range of 250-300 m above MSL. Temperature humidity index (THI) ranges from 54.41±1.09 in winter (November to January), 63.51±1.85 in spring (February to April), 74.00±1.77 in autumn and 76.06±1.74 in summer (May to July) season.

Experimental animals: Ten apparently healthy (body condition score 5-6 of 10, classified as good) Mithun bulls of 4-6 years of age were selected. Experimental animals were maintained under uniform feeding (farm schedule), lighting, housing and other managemental conditions. Experimental animals were offered *ad lib.* potable drinking water, 30 kg mixed jungle forages (18.40% and 10.20% dry matter and crude protein, respectively) and 4 kg concentrates (87.10% and 14.50% dry matter and crude protein, respectively) fortified with mineral mixture and salt.

Extender preparation: The extender used in this study contained 3.028 g Tris (hydroxymethyl) aminomethane, 1.675 g citric acid, 1.250 g fructose, 7 ml glycerol (7%), 1000 IU/ml streptomycin sulphate, 1000 IU/ml penicillin G sodium and different concentrations of SOD (25 or 50 or 100 mM, in Group II or III or IV, respectively) for 100 ml deionized water. The extender for the control (Group I) lacked SOD.

Semen collection and processing: Semen was collected not more than twice per week from any animal through trans-rectal massage method. Semen samples with mass activity of 3+ or above were selected for the experiment. At each collection day, a minimum of two good ejaculates per bull were obtained. Immediately after collection, the ejaculates were kept in a water bath at 37°C and evaluated for the preliminary SQPs. After discarding ejaculates with wide variation in *p*H (i.e. <6.7 and >7.2), colour or too low volume (< 0.5 ml), rest were evaluated microscopically. These ejaculates were evaluated and processed further if the following criteria were met: concentration> 500 million/ml, mass activity >3+, individual motility>70% and total morphological abnormalities <10% or below. Following the above-screening protocol, 50 ejaculates were selected. After the preliminary evaluations, two consecutive ejaculates of a same bull were pooled together (termed 'sample' hereafter, n = 25) and subjected to the two-fold initial dilution with pre-warmed (37°C) TCG extender.

Thus, from initial collections, 50 selected ejaculates were pooled to make 25 samples for the experiment. The partially diluted samples were brought to the laboratory in an insulated flask containing warm water (37°C) for further processing.

Each sample was split into four aliquots and diluted (to get final concentration of 60 million spermatozoa per ml) with the TCG extender containing either 0 or 50 or 100 or 150 U/ml SOD (Group I, II, III or IV, respectively). Diluted semen samples of each group were cooled simultaneously from 37°C to 5°C at a rate of 0.2–0.3°C per min in a cold cabinet (IMV, L'Aigle, France) and maintained at 5°C for 2 h. Polyvinyl chloride straws (0.5 ml) (IMV, L'Aigle, France) were filled and maintained in a cold cabinet at 5°C for 2.5 h. Subsequently, these straws were wipe-cleaned, dried and spread over the freezing rack. The rack containing straws was kept in biological programmable freezer for freezing (final temperature maintained at −124°C, 12 min) followed by plunging of straws into the liquid nitrogen (−196°C) and was stored therein.

Post thaw semen evaluation: At the time of evaluation, the stored semen straws were taken out of the cryocans and thawed in water at 37°C for 30 sec. SQPs such as post thaw sperm motility (Salisbury *et al.* 1985), kinetic, velocity and motility parameters by computer assisted sperm analyser (CASA; Hamilton Thorne Sperm Analyser, HTM-IVOS, version IVOS 11, Hamilton Thorne Research, USA; Perumal *et al.* 2014), viability and total sperm abnormality by Eosin–Nigrosin staining (Lasley and Bogart 1944), acrosomal integrity by Giemsa staining (Watson 1975), plasma membrane integrity by hypo-osmotic swelling test (Jeyendran *et al.* 1984), nuclear integrity by Feulgen's staining technique (Barth and Oko 1989) and vanguard distance travelled by sperm in the bovine cervical mucus penetration test (Prasad *et al.* 1999) were determined.

Biochemical assays: An aliquot of semen from each sample was centrifuged at 800 × g for 10 min; seminal plasma siphoned out and sperm pellets were separated and washed by resuspending in PBS and centrifuging (thrice). After final centrifugation, 1 ml of deionized water was added to the spermatozoa. The seminal plasma and sperm pellets were snap-frozen and stored in sterilized cryo-vials in deep freezer at −80°C until further analysis. At the time of estimation, concentration of spermatozoa was determined and then re-diluted to contain 100×10^6 cells/ml. Biochemical profiles such as AST, ALT, LDH, SOD, CAT, GSH and TAC in seminal plasma of frozen-thawed sample and MDA and cholesterol in frozen thawed sperm pellet were estimated.

Leakage of intracellular enzymes: The activities of intracellular enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated in the seminal plasma according to the method described by Reitman and Frankel (1957) and its activity was expressed as μmol/dL. Similarly, the activity of the lactate dehydrogenase activity (LDH) in the seminal plasma was determined as per the method described by Wotten (1964)

and its activity was expressed as IU/dL.

Antioxidant and oxidative stress profiles: Total antioxidant capacity (TAC, Bio Vision, CA, USA; mmol/ml) and superoxide dismutase (SOD; U/ml), glutathione (GSH; μmol/ml) and catalase (CAT; nmol/min/ml) were estimated using commercially available ELISA kits (Cayman Chemical Co., USA) at optical density (λ 570, 440-460, 405-424 and 540 nm, respectively). These antioxidants were estimated with use of microplate spectrophotometer (Thermo Scientific Multiskan GO Microplate Spectrophotometer, USA). Lipid peroxidation level of spermatozoa was measured by determining the malondialdehyde (MDA) production using thiobarbituric acid -trichlroacetic acid as per the method of Suleiman *et al.* (1996).

Sperm cholesterol content: The cholesterol (CHO) content in spermatozoa was estimated as per the method of Bligh and Dyer (1959) with some modification. Washed spermatozoa (100 million) were taken in a 10 ml vial. The sperm pellet was extracted with 20 volumes of chloroform: methanol (1:1 v/v) solution and vortexed for 20 sec. Thereafter, it was centrifuged at $800 \times g$ for 5 min. Spermatozoa were evaporated to dryness under liquid nitrogen gas and kept at −20°C. At the time of estimation, 0.5 ml of chloroform was added to each vial, cholesterol was estimated by cholesterol assay kit (Span Diagnostics Ltd., India) and results were expressed as μg cholesterol/10^8 spermatozoa.

Statistical analysis: Means were analyzed by one way ANOVA, followed by the Tukey's post hoc test to determine significant differences among the treatments and control groups (Statistical Analysis System for Windows, Version 9.3; SAS Institute, Inc., Cary, NC).Differences with values of $p<0.05$ were considered to be statistically significant. Associations between different experimental parameters were analysed for statistical significance using Pearson's correlation coefficient. If the r value is greater than 0.50, the correlation is considered as large, 0.50-0.30 is considered as moderate, 0.30-0.10 is considered as small.

RESULTS AND DISCUSSION

In the present study, Mithun semen samples (n=50) were creamy white to thick creamy in colour with an average semen volume of 2.35±0.12 ml with an average sperm concentration was 865.14±8.94 million per ml. Analysis of the present study revealed that inclusion of SOD (100 U/ml) in the semen extender improved the SQPs, level of antioxidants and total cholesterol of sperm whereas it reduced the leakage of intracellular enzymes, reactive oxygen species formation and sperm morphological abnormalities in Mithun. Thus it protects the structures and functions of spermatozoa efficiently. Moreover, the SOD treated sperm may enhance the quality of semen by preserving efficiently during artificial insemination procedure. Perusal of available literature revealed no information on SOD inclusion on *in vitro* SQPs, antioxidant and oxidative stress profiles and biochemical

profiles in Mithun semen cryopreservation and to best of our knowledge, this is the first report on effect of SOD in cryopreserved semen in Mithun. Though several authors have reported SOD has significant beneficial effects on SQPs and antioxidant and oxidative stress and biochemical profiles in different species like equine (Cocchia *et al.* 2011), bovine (Asadpour *et al.* 2012) and bubaline (El- Sisy *et al.* 2008), similar studies in Mithun are lacking. In the present study, SOD supplementation revealed significant difference between the treatment groups. The beneficial effects of SOD in semen preservation are due to its very potent antioxidant nature (Cocchia *et al.* 2011, Asadpour *et al.* 2012, El- Sisy *et al.* 2008).

As the mammalian sperm membrane has high polyunsatured fatty acids (PUFA), it renders the sperm very susceptible to LPO, which occurs as a result of the oxidation of the membrane lipids by partially reduced oxygen molecules such as superoxide, hydrogen peroxide, and hydroxyl radicals (Asadpour *et al.* 2012). Lipid peroxidation of the sperm membrane ultimately leads to the impairment of sperm function due to the attacks by ROS, altered sperm motility and membrane integrity and damage to sperm DNA and fertility through oxidative stress and the production of cytotoxic aldehydes (Griveau *et al.* 1995). In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing and cryopreservation (Alvarez and Storey 1992). The levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules (Kumar *et al.* 2011). Natural and synthetic antioxidant systems have been described as a defense functioning mechanism against lipid peroxidation in semen (Shoae and Zamiri 2008). Therefore, inclusion of exogenous antioxidants could reduce the impact of oxidative stress during the sperm cryo-storage process and thus improve the quality of chilled and cryopreserved semen (Asadpour *et al.* 2012).

In the present study, spermatozoa treated with SOD (100 U/ml) had significantly higher post thaw motility than those in control (8.59%), 50 U/ml SOD (3.67%) or 150 U/ml (4.45%). Similarly, viability was significantly higher in 100 U/ml SOD than those in control (11.24%), 50 U/ml (3.12%) and 150 U/ml (3.86%) treated spermatozoa. Acrosomal intactness of spermatozoa was significantly higher in 100 U/ml SOD as compared to those in control (7.17%), 50 U/ml (4.98%) and 150 U/ml (5.22%); whereas the total sperm morphological abnormality was significantly reduced in 100 U/ml SOD treated than that in control (10.48%), 50 U/ml (4.23%) and 150 U/ml (4.68%). Plasma membrane integrity was significantly affected with SOD treatment as 100 U/ml treated sperm showed higher membrane intactness than those in untreated control (11.27%) and other treatment groups (50 U/ml: 4.66% and 150 U/ml: 6.12%). Nuclear integrity also followed the same trend as HOST (100 U/ml > 50 or 150 U/ml or control: 4.14, 3.26 or 6.64%, respectively). Vanguard distance travelled by sperm in CMPT was significantly higher in 100 U/ml than
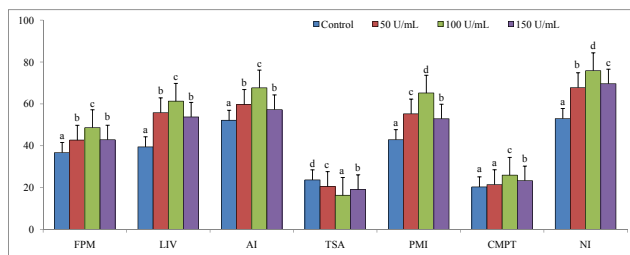
Fig. 1. Effect of superoxide dismutase on post thaw semen quality profiles in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); LIV, Livability (%); AI, Acrosomal integrity (%); TSA, Total sperm abnormality (%); PMI, Plasma membrane integrity (HOST; %); CMPT, Cervical mucus penetration test (vanguard distance travelled by sperm; mm/h) and NI, Nuclear integrity (%). Vertical bar with small letters (a, b, c, d) indicates significant (p<0.05) difference among the different experimental groups. N=25 semen samples each for control and treatment groups.

those in 50 U/ml (5.89%) or 150 U/ml (4.23%) or control (7.45%) groups (Fig. 1).

Sperm mobility and velocity parameters were estimated with use of computer assisted sperm analyser (CASA) in SOD treated semen samples. Forward progressive motility (FPM) of sperm was significantly higher in 100 U/ml than those in other groups (50 U/ml: 4.72%, 150 U/ml: 6.87% and control: 14.54%). Similarly, total motility (TM) was significantly higher in 100 U/ml than those in other SOD treated (4.67 to 5.45%) and untreated control (11.43%) groups. On the contrary, static motility (SM) was significantly reduced in SOD treated than those in control groups (20.87% vs 25.73%). Velocity profiles (curvilinear motility: VCL, straight line velocity: VSL and average path velocity: VAP) were significantly higher in SOD (100 U/ml) than those in 50 U/ml (1.4-2.6%) or 150 U/ml (4.7-7.9%) treated or untreated control (2.5-8.7%) groups. SOD (100 U/ml) had significantly higher amplitude of lateral head displacement (ALH) than those in control (14.86%), 50 U/ml (8.34%) and 150 U/ml (2.87%) and similar trend was observed for beat cross frequency (BCF) (12.67, 5.96 and 12.54%). The straightness (STR) was 2.36 to 3.89% higher in 100 U/ml treated than other SOD treated or control groups (Fig. 2).

The present results showed that addition of 100 U/ml of SOD improved the keeping quality of Mithun semen as compared to those semen samples treated with 50 or 150 U/ml SOD or without SOD. The different effects of the different levels of SOD might be explained by the fact that the excessive amount of antioxidants caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages (Asadpour *et al.* 2012, Shoae and Zamiri 2008). In addition, the concentration of antioxidants added to extender should be considered since high dosage of antioxidants may be harmful to spermatozoa due to the change in physiological condition of semen extender. In ram, survival of spermatozoa will
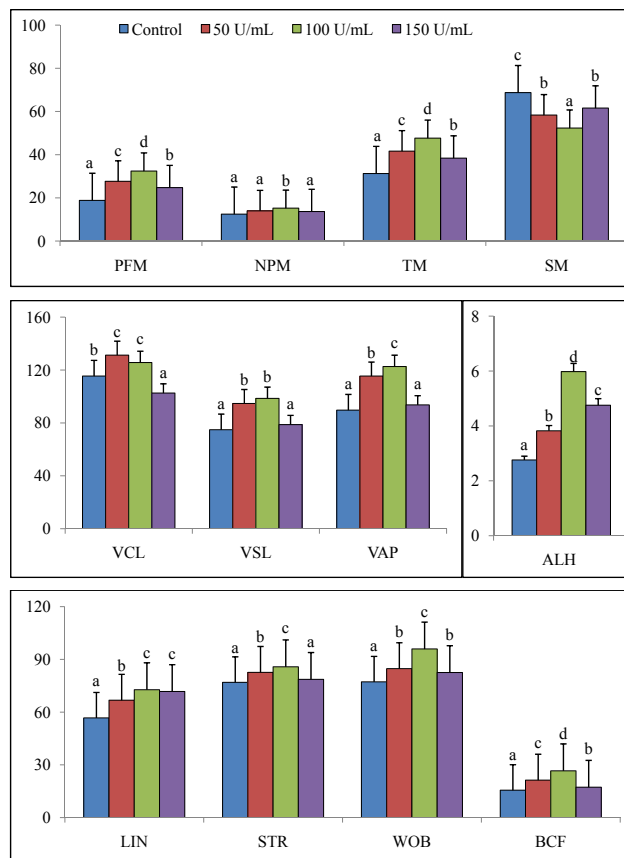

Fig. 2. Effect of superoxide dismutase on post thaw motility and velocity parameters by computer assisted sperm analyser (CASA) in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); NPM, Non-progressive motility (%); TM, Total motility; SM, Static sperm (%); VCL, Curvilinear velocity (µm/sec.); VSL, Straight line velocity (µm/sec.); VAP, Average path Velocity (µm /sec.); ALH, Amplitude of lateral head displacement (µm); LIN, Linearity (%); STR, Straightness (%); WOB, Wobble (%) and BCF, Beat/Cross Frequency (Hz). Vertical bar with small letters (a, b, c, d) indicates significant (p<0.05) difference among the different experimental groups. N= 25 semen samples each for control and treatment groups.

increase when the dosage of antioxidant added to extender increases. However, the antioxidant dosage higher than required amount was toxic to spermatozoa (Maxwell and Stojanov 1996). The over-expression of SOD may reflect a defect in the development or maturation of spermatozoa, as well as sperm cellular damage, resulting in decreased sperm fertilization potential (Gavella *et al.* 1996). Similarly, in the present study, increasing dosage of SOD, at 150 U/ml affected the seminal as well as biochemical parameters in Mithun semen with TEYC extender. At the same time, less dosage rate also affected the sperm parameters. Differences in preservation protocols and extender formulations among laboratories, the time of addition/exposure of sperm with antioxidant, concentration of antioxidants and between species may explain, at least in part, this variability. The improvement of semen quality

due to addition of exogenous SOD recorded in the present study was previously reported in the form of motility and intact acrosomal membrane in equine sperm (Cocchia *et al.* 2011), bovine sperm (Asadpour *et al.* 2012) and bubaline semen (El-Sisy *et al.* 2008). Moreover, the addition of exogenous SOD significantly improved the percentages of DNA morphology, sperm viability and intact plasma membrane (swelling tails) especially at a level of 100 U/ml of SOD. The highest percentages of intact plasma membrane and acrosomal membranes found in the present experiment in 100 U/ml SOD group may be the reason for better motility in these samples (Asadpour *et al.* 2012, El-Sisy *et al.* 2008).

SOD helps in maintaining the integrity of normal acrosome (Maxwell and Stojanov 1996) and stabilizes the plasmalemma of spermatozoa and hence increase motility. SOD, in sperm cells is able to react with many ROS directly for protecting mammalian cells against oxidative stress, and hence maintaining sperm motility (Bilodeau *et al.* 2001). Therefore, as seen by this study, attempts to improve the motility and viability of the sperm cells by incorporating SOD in liquid storage (Cocchia *et al.* 2011, Asadpour *et al.* 2012) and frozen semen form have been investigated (El- Sisy *et al.* 2008).

A recent report suggested that semen quality is deteriorated (Aitken *et al.* 2010) by which DNA damage is induced in the male gamete by oxidative stress and spermatozoa are particularly vulnerable to this because they generate ROS and are rich in targets for oxidative attack. The authors also draw attention to the fact that, because spermatozoa are transcriptionally inactive and have little cytoplasm, they are deficient in both antioxidants and DNA-repair systems (Aitken and Fisher 1994). Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in spermatozoa (Aitken and Fisher 1994). There are few studies on the effects of antioxidant addition to extenders during cooling and/or freezing mammalian spermatozoa (Kankofer *et al.* 2005). In Mithun semen, ROS are generated mainly by damaged and abnormal spermatozoa and by contaminating leukocytes. Reactive oxygen species damage cells by changes to lipids, proteins and DNA. Spermatozoa are potentially susceptible to peroxidative damage caused by ROS excess due to high amounts of polyunsaturated fatty acids in membrane phospholipids and to sparse cytoplasm. In the present study, addition of SOD reduced the DNA fragmentation especially at 100 U/ml in Mithun semen cryopreservation. Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. SOD also protects GSH, CAT and TAC level in the semen extender, which helps to maintain membrane transportation (Alvarez and Storey 1992) and fertility of the spermatozoa.

In the present study, leakage of intracellular enzyme such as AST was significantly reduced in 100 U/ml treated than in untreated control (11.76%) or SOD treated
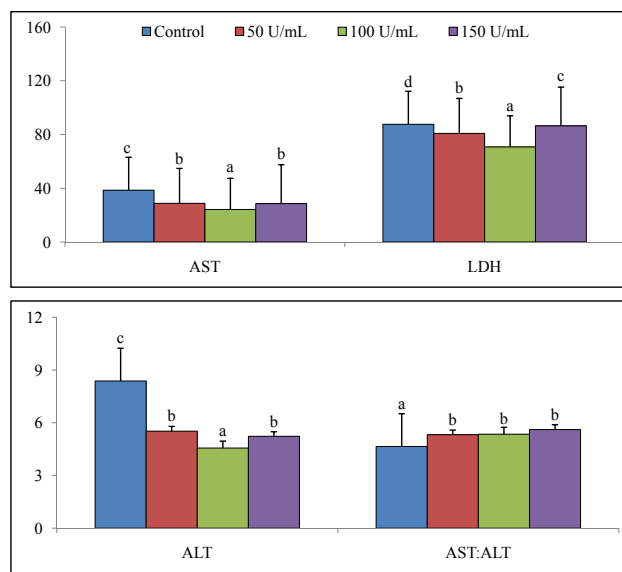

Fig. 3. Effect of superoxide dismutase on intracellular enzymes of sperm in post thaw stage in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. AST, Aspartate Aminotransferase (μM/dL); ALT, Alanine Aminotransferase (μM/dL) and LDH, Lactate Dehydrogenase (IU/dL). Vertical bar with small letters (a, b, c, d) indicates significant ($p < 0.05$) difference among the different experimental groups. N= 25 semen samples each for control and treatment groups.

(50 U/ml; 3.52% or 150 U/ml; 3.73%) groups. Similar observation was noted in ALT leakage (15.45, 4.34 or 2.46%, respectively). Similarly, another enzyme LDH also revealed that leakage was significantly reduced in 100 U/ml than those in 50 U/ml (3.05%) or 150 U/ml (4.97%) or control (5.65%) groups (Fig. 3). The enzyme such as AST and ALT levels in seminal plasma are very important for sperm metabolism as well as sperm function (Brooks 1990), provide energy for survival, motility and fertility of spermatozoa and these transaminase activities in semen are good indicators of semen quality because they measure sperm membrane stability (Corteel 1980). Thus, increasing the percentage of abnormal spermatozoa in the preservation causes high concentration of transaminase enzyme in the extra cellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa (Gundogan 2006). Moreover, increase in AST and ALT activities of seminal plasma and semen in cryopreservation may be due to structural instability of the sperm (Buckland 1971). In the present study, AST and ALT levels were lower in semen cryopreserved at 100 U/ml of SOD as it stabilises the membrane integrity of acrosome, plasma, mitochondria and flagella of the sperm.

Cholesterol was higher significantly in 100 U/ml SOD than those in 50 or 150 U/ml or untreated control groups. SOD @ 100 U/ml had significantly higher sperm cholesterol than in control (11.12%) or 50 U/ml (12.37%) or 150 U/ml (11.63%) in Mithun bulls (Fig. 4). SOD prevents efflux of cholesterol from the sperm membrane
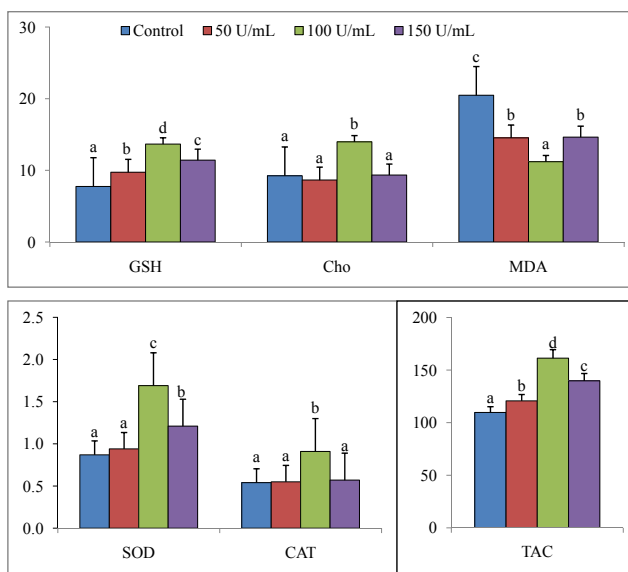
Fig. 4. Effect of superoxide dismutase on antioxidant profiles in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. GSH, Glutathione (μmol/ml); Cho, Cholesterol (μg/10^8 sperm); MDA, Malondialdehyde (nmol/10^8 sperm); SOD, Superoxide dismutase (U/ml of seminal plasma); CAT, Catalase (nmol/min/ml) and TAC, Total antioxidants (Trolox equivalents μmol/L). Vertical bar with small letters (a, b, c, d) indicates significant (p<0.05) difference among the different experimental groups. N=25 semen samples each for control and treatment groups.

and MDA production in diluents which indicates it prevents premature capacitation and acrosomal reaction that acts as an antioxidant (Asadpour *et al.* 2012). Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane. Cholesterol plays a special role in the sperm membrane because its release from the sperm membrane initiates the key step in the process of capacitation and acrosome reaction that is crucial for fertilization. Moreover, adding cholesterol to diluents prior to defreezing increases sperm resistance to stress caused by the freezing-defreezing procedures, preserving sperm motility and fertilization potential (Moore *et al.* 2005). In the present study, the efflux of cholesterol and MDA production were decreased in SOD treated group as compared to the control untreated group. Therefore, the semen samples treated with SOD had higher cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that received at 100 U/ml of SOD were significantly higher than those of the other treatment and control groups.

Antioxidant profiles such as TAC, GSH, SOD and CAT were higher and oxidative stress profile such as MDA was lower significantly in 100 U/ml than those in 50 U/ml or 150 U/ml or untreated control groups. SOD @ 100 U/ml had significantly higher antioxidant profiles and lower MDA than in control (11.43-18.89% and 17.74%) or 50 U/ml (8.28-16.76% and 5.42%) or 150 U/ml (7.98-13.37% and 6.46%) in Mithun bulls (Fig. 4). In the present study, GSH, CAT and TAC were higher in the seminal plasma of SOD

added semen as it maintains the antioxidant system in liquid storage of Mithun semen. But normally, seminal plasma is a potent source of this antioxidant, SOD (Kobayashi *et al.* 1991). The high levels of readily peroxidizable polyunsaturated material expose spermatozoa to excessive oxidative stress and the superoxide dismutase activity of sperm samples is a good predictor of their survival time. SOD, when applied at a dose of 100 U/ml, has improved sperm motility during preservation and displayed antioxidative properties, elevating the CAT level, in association with GSH and TAC concentration. Further, SOD, a permeating cryoprotectant acts as an antioxidant and cause membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures, and therefore increased ability of spermatozoa to survive during this preservation (Holt 2000).

In the present study, correlation analysis revealed that SQPs (forward progressive motility, livability, acrosomal integrity, plasma membrane integrity, vanguard distance in cervical mucus and nuclear integrity), CASA parameters (FPM, TM, VCL, VSL, VAP, LIN, STR, ALH and BC)F, antioxidants (GSH, SOD, CAT and TAC) and biochemical profile (sperm cholesterol) had significant positive correlation with each other whereas these profiles had significant negative correlation with TSA, SM, AST, ALT, LDH and MDA in SOD treated sperm (Fig. 5). This could be one of the reasons for improved motility, viability, plasma and acrosome membrane and DNA integrity of spermatozoa, diluted in presence of SOD in the semen extender.

It can be concluded that the possible protective effects of SOD supplementation are to enhance the antioxidant
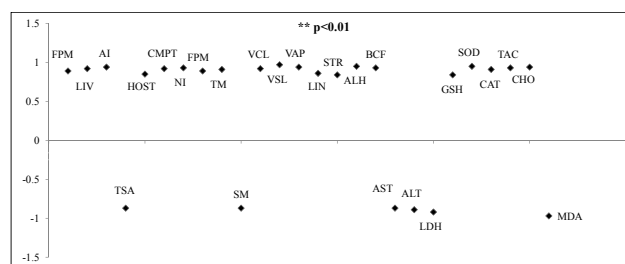

Fig. 5. Correlation coefficients among the semen quality parameters, kinetic parameters by computer assisted sperm analyser, biochemical profiles and antioxidant and oxidative profiles in mithun bulls. FPM, Forward progressive motility; LIV, Livability; AI, Acrosomal integrity; TSA, Total sperm abnormality; HOST/PMI, Hypo-osmotic swelling test/plasma membrane integrity; CMPT, Cervical mucus penetration test; NI, nuclear integrity; FPM, Forward progressive motility; TM, Total motility; SM, Static motility; VCL, Curvilinear velocity; VSL, Straight line velocity; VAP, Average path velocity; LIN, Linearity; STR, Straightness; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; LDH, Lactate dehydrogenase; GSH, Glutathione; SOD, Superoxide dismutase; CAT, Catalase; TAC, Total antioxidant capacity; CHO, Sperm cholesterol and MDA, Malondialdehyde. **Correlation coefficients were highly significant, p<0.01.

enzyme content and prevent efflux of cholesterol and phospholipids from cell membrane and lipid peroxide production. Thus, it protects the spermatozoa during cryopreservation and enhances the fertility in this species.

REFERENCES

Aitken J and Fisher H. 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioassays* **16**(4): 259–67.

Aitken R J, De Luliis G N, Finnie J M, Hedges A and McLachlan R. 2010. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. *Human Reproduction* **25**(10): 2415–26.

Alvarez J G and Storey B T. 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a model of sub lethal cryo damage to human sperm during cryopreservation. *Journal of Andrology* **13**(3): 232–41.

Asadpour R, Jafari R and Tayefi-Nasrabadi H. 2012. The effect of antioxidant supplementation in semen extenders on semen quality and lipid peroxidation of chilled bull spermatozoa. *Iranian Journal of Veterinary Research* **13**(3): 246–49.

Barth A D and Oko R J. 1989. Preparation of semen for morphological examination. *Abnormal Morphology of Bovine Spermatozoa.* Iowa State University Press, Ames, IA. p. 8–18.

Bernardini A, Hozbor F, Sanchez E, Fornés M, Alberio R and Cesari A. 2011. Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. *Theriogenology* **76**: 436–47.

Bilodeau J F, Blanchette S, Gagnon C and Sirard M A. 2001. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* **56**(2): 275–86.

Bligh E G and Dyer W J. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**: 911–17.

Brooks D E. 1990. Biochemistry of the male accessory glands. *Marshall's Physiology of Reproduction.* (Ed.) Lamming G E. 4th edn. Churchill Livingstone, Edinburgh. pp. 569–690.

Buckland R B. 1971. The activity of six enzymes of chicken seminal plasma and sperm. 1. Effect of *in vitro* storage and full sib families on enzyme activity and fertility. *Poultry Science* **50**(6): 1724–34.

Chatterjee S, de Lamirande E and Gagnon C. 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: Protection by oxidized glutathione. *Molecular Reproduction and Development* **60**: 498–506.

Cocchia N, Pasolini M P, Mancini R, Petrazzuolo O, Cristofaro I, Rosapane I, Sica A, Tortora G, Lorizio R, Paraggio G and Mancini A. 2011. Effect of SOD (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. *Theriogenology* **75**: 1201–10.

Corteel J M. 1980. Effects du plasma séminal sur la survie et la fertilité des spermatozoids conservés *in vitro*. *Reproduction Nutrition Development* **20**(4): 1111-23.

de Lamirande E and Gagnon C. 1995. Impact of reactive oxygen species on spermatozoa: A balancing act between beneficial and detrimental effects. *Human Reproduction* **10**(1): 15–21.

El-Sisy G A, El- Nattat W S and El- Sheshtawy R I. 2008. Effect of superoxide dismutase and catalase on viability of cryopreserved buffalo spermatozoa. *Global Veterinaria* **2**(2): 61–65.

Gavella M, Lipovac V, Vucic M and Rocic B. 1996. Relationship of sperm superoxide dismutase-like activity with other sperm specific enzymes and experimentally induced lipid peroxidation in infertile men. *Andrologia* **28**(4): 223–29.

Griveau J F, Dumont E, Renard P, Callegari J P and Le Lannou D. 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *Journal of Reproduction and Fertility* **103**(1): 17–26.

Gundogan M. 2006. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi Rams. *Turkish Journal of Veterinary and Animal Science* **30**(1): 95–100.

Holt W V. 2000. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology* **53**(1): 47–58.

Jayaganthan P, Perumal P, Balamurugan T C, Verma R P, Singh L P, Pattanaik A K and Meena K. 2013. Effects of *Tinospora cordifolia* supplementation on semen quality and hormonal profile of ram. *Animal Reproduction Science* **140**(1): 47–53.

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

Kankofer M, Kolm G, Aurich J and Aurich C. 2005. Activity of glutathione peroxidase, super oxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology* **63**(5): 1354–65.

Kobayashi M, Kakizono T and Nagai S. 1991. Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. *Journal of Fermentation and Bioengineering* **71**(5): 335–39.

Kumar R, Jagan Mohanarao G, Arvind R and Atreja S K. 2011. Freeze-thaw induced genotoxicity in buffalo (*Bubalus bubalis*) spermatozoa in relation to total antioxidant status. *Molecular Biology Report* **38**(3): 1499–1506.

Lasley J F and Bogart R. 1944. A comparative study of epididymal and ejaculated spermatozoa of boar. *Journal of Animal Science* **3**: 360–70.

Maxwell W M C and Stojanov T. 1996. Liquid storage of ram semen in the absence or presence of some antioxidants. *Reproduction, Fertility and Development* **8**(6): 1013–20.

Moore A I, Squires E L and Graham J K. 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. *Cryobiology* **51**(3): 241–49.

Perumal P, Chang S, Baruah K K and Srivastava N. 2018. Administration of slow release exogenous melatonin modulates, oxidative stress profiles and *in vitro* fertilizing ability of the cryopreserved Mithun spermatozoa. *Theriogenology* **120**: 79–90.

Perumal P, Chang S, Khate K, Vupru K and Bag S. 2019. Feeding supplementation of Flaxseed oil modulates semen production and its quality parameters, freezability, oxidative stress profiles, scrotal and testicular biometrics and endocrinological profiles in Mithun. *Theriogenology* **136**: 47–59.

Perumal P, Selvaraju S, Selvakumar S, Barik A K, Mohanty D N, Das R K, Das S and Mishra P C. 2011. Effect of pre-freeze addition of cysteine hydrochloride and reduced glutathione in semen of crossbred Jersey bulls on sperm parameters and conception rates. *Reproduction in Domestic Animal* **46**(4): 636–41.

Perumal P, Srivastava S K, Ghosh S K and Baruah K K. 2014. Computer-assisted sperm analysis of freezable and non-freezable Mithun (*Bos frontalis)* semen. *Journal of Animals* **2014**: 1–6.

Perumal P, Vupru K and Rajkhowa C. 2013. Effect of addition of reduced glutathione on the liquid storage (5°C) of Mithun (*Bos frontalis*) semen. *Indian Journal of Animal Sciences* **83**(10): 1024–28.

Perumal P. 2014. Effect of superoxide dismutase on the liquid storage (5°C) of Mithun (*Bos frontalis*) semen. *Journal of Animals* **2014**: 1–9.

Prasad J K, Kumar S, Mohan G, Agarwal S K and Shankar U. 1999. Simple modified method for cervical mucus penetration test for quality assessment of bull semen. *Indian Journal of Animal Sciences* **69**: 103–05.

Reitman S and Frankel S A. 1957. Colorimetric method for the determination of serum oxaloacetic and glutamic pyruvate transaminase. *American Journal of Clinical Pathology*
28: 56–63.

Salisbury G W, VanDemark N L and Lodge J R. 1985. *Physiology of Reproduction and Artificial Insemination of Cattle.* 2ⁿᵈ ed. W.H. Freeman and Company. p. 268–274.

Shoae A and Zamiri M J. 2008. Effect of butylated hydroxytoluene on bull sperm frozen in egg yolk-citrate extender. *Animal Reproduction Science* **104**(2): 414–18.

Sinha M P, Sinha A K, Sinka B K and Prasad P I. 1996. The effect of glutathione on motility, enzyme leakage and fertility of frozen goat semen. *Theriogenology* **41**: 237–43.

Suleiman S A, Ali M E, Zaki M S, Malik E M E A and Nast M A. 1996. Lipid peroxidation and human sperm motility: Protective role of vitamin E. *Journal of Andrology* **17**(5): 530–37.

Watson P F. 1975. Use of Giemsa stain to detect change in acrosome of frozen ram spermatozoa. *Veterinary Record* **97**: 12–15.

Wotten I D P. 1964. *Micro-analysis in Medical Biochemistry.* 4ᵗʰ edn. J and A Churchill Ltd, London.