



Effect of trehalose on post thaw semen quality profiles, sperm kinetic profiles and antioxidant and oxidative stress profiles in Mithun

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ABSTRACT

Present study was designed to assess the effect of trehalose on post thaw semen quality parameters (SQPs), sperm velocity and kinetic profiles, antioxidant and oxidative stress profiles and sperm cholesterol efflux in mithun. A total of 25 ejaculates were selected based on the biophysical parameters and each sample was split into four equal aliquots after dilution with the Tris-citrate-glycerol (TCG) extender such as Group I: control, Group II, III and IV: 50, 75 and 100 mM of trehalose, 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 integrity, plasma membrane integrity and nuclear integrity and sperm intra-cellular enzymatic leakage and biochemical (sperm cholesterol, antioxidants and malondialdehyde) profiles. Study revealed a significant enhancement in viability, sperm morphological and nuclear normalities, acrosome integrity, motility, sperm cholesterol content and reduction in leakage of intracellular enzymes in Group II. Moreover, intactness of acrosome and biochemical membranes were protected significantly in addition to significant improvement in kinetic and velocity profiles in extender containing 50 mM trehalose. These results clearly indicated that however the cryopreservation of mithun's spermatozoa in TCG was comparable with other species, inclusion of 50 mM trehalose holds a clear advantage over control or 75 or 100 mM trehalose. It can be concluded from the present study that trehalose 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, Mithun, Semen quality profiles, Trehalose

Mithun is a unique, magnificent domestic bovine species available in North Eastern Hilly region of India. Several reports revealed that mithun is affected with intensive inbreeding depression because of lack of suitable breeding bulls and lack of proper breeding management. Mithuns are reared under extensive free-range system with natural service with various limitations; therefore, loss of productive and reproductive performances occur and these limitations could be overcome by implementation of artificial breeding programmes. Preliminary research on trehalose effect on basic SQPs in liquid preservation revealed that 50 mM trehalose is suitable for liquid semen preservation in mithun (Perumal *et al.* 2015). Various stages of freezing process induce physical, osmotic and chemical stresses on the sperm membrane associated with an oxidative stress induced by free radicals (Chatterjee *et al.* 2001). All these deleterious stresses cause loss of motility, viability, intactness of acrosomal membrane, plasma membrane and nuclear integrity and large number of sperms

incapable to fertilize the ovum and ultimately infertility or sterility (Bernardini *et al.* 2011). Higher 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 the presence of oxygen free radicals (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-capability or cryo-resistance of the sperm. ROS effects on spermatozoa are irreparable loss of motility, sperm DNA disintegration and reduced fertilizing ability (Perumal *et al.* 2011). Therefore, addition or inclusion of exogenous additives or antioxidants in the semen preservation extender (Perumal *et al.* 2013) or feeding of flaxseed oil (Perumal *et al.* 2019) or antioxidants (Jayaganthan *et al.* 2013) or administration of slow release melatonin (Perumal *et al.* 2018) can minimise the adverse effects of oxidative and cryo stress during the semen cryopreservation process (Perumal *et al.* 2011). In recent years, studies have also been conducted on bovine semen extenders including additives/antioxidants such as taurine (Perumal *et al.* 2013), glutathione (Perumal *et al.*

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2013), catalase (Perumal *et al.* 2013), superoxide dismutase (Perumal 2014), melatonin (Perumal *et al.* 2015) and so on to improve the SQPs and *in vivo* or *in vitro* fertility.

Addition of additives such as trehalose to buffalo (Kumar and Atreja 2011), goat (Aboagla and Terada 2003) and boar (Hu *et al.* 2009) has been shown to protect the sperm against the harmful effects of ROS and improve the sperm motility and membrane integrity during sperm storage. Trehalose is a disaccharide, which acts as a non-permeating cryoprotective agent, which induces spermatozoa dehydration due to osmotically driven flow of water. Due to mild dehydration, spermatozoa have reduced intracellular water which in turn has reduced the formation of intra-cellular ice crystals. On the other hand, higher intra-cellular ice crystal formation results into sperm cell death leading to reduced fertility rate of the cryopreserved sperm. No information is available with regard to the effect of trehalose in Tris based semen extender cryopreservation on fertility of mithun. Therefore, it was hypothesized that application of trehalose in semen extender could be more beneficial on *in vitro* sperm functional parameters in mithun. With this, the objective of the present study was to assess the effect of different concentrations of trehalose in semen diluents on SQPs, kinetic and velocity profiles, oxidative stress profiles and leakage of intracellular enzymes of the cryopreserved sperm of mithun.

MATERIALS AND METHODS

Location of the study: The study was conducted at ICAR-National Research Centre on Mithun, Medziphema, Nagaland in summer season (May to July; THI: 76.06 ± 1.74 , sunshine hours: 6.55 ± 0.15). It is located between $25^{\circ}54'30''$ North latitude and $93^{\circ}44'15''$ East longitude and at an altitude range of 250–300 m above MSL.

Experimental animals: Ten apparently healthy (body condition score 5–6 of 10) mithun bulls of 4–6 years of age were selected. Mithun bulls were maintained under uniform feeding, lighting, housing and other managerial conditions as per farm schedule. Experimental mithuns 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 Tris (hydroxymethyl) aminomethane: 3.028 g, citric acid: 1.675 g; fructose: 1.250 g; glycerol (7%): 7 mL; streptomycin sulphate (μ mL): 1000; penicillin G sodium (IU/mL): 1000; and different concentrations of trehalose (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) contained no trehalose.

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, these ejaculates were kept in a water bath at 37°C and evaluated for preliminary SQPs. These ejaculates were evaluated for accepted for evaluation if the following criteria were met: concentration: > 500 million/mL, mass activity: $>3+$, individual motility: $>70\%$ and total morphological abnormalities $<10\%$ or below were processed further. 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 75 or 100 mM trehalose (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 $0.2\text{--}0.3^{\circ}\text{C}$ per min in a cold cabinet (IMV, L'Aigle, France) and maintained at 5°C for 2 h. Polyvinyl chloride (PVC) 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 30s. 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 $800 \times g$ 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 cryovials 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 $\mu\text{mol/dL}$. Similarly, the activity of the lactate dehydrogenase (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, K274; Bio Vision, CA, USA; mmol/mL) and superoxide dismutase (SOD; U/mL), glutathione (GSH; $\mu\text{mol/mL}$) and catalase (CAT; nmol/min mL) were estimated using commercially available ELISA kits (706002, 703002 and 707002, Cayman Chemical Co. USA, respectively) 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 peroxide level of spermatozoa was measured by determining the malondialdehyde (MDA) production at 535 nm, using thiobarbituric acid-trichloroacetic acid as per the method of Suleiman *et al.* (1996).

Sperm cholesterol content: The cholesterol content in spermatozoa was estimated as per the method of Bligh and Dyer (1959) with some modification. Hundred million washed spermatozoa 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 20s. 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 $\mu\text{g cholesterol}/10^8$ spermatozoa.

Statistical analysis: Means were analyzed by one way analysis of variance (ANOVA) followed by the Tukey's post-hoc test to determine the significant differences among the treatments and control groups on these sperm parameters using the SAS software (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001). Differences with values of $p < 0.05$ were considered to be statistically significant after arcsine transformation of percentage data. Associations between different experimental parameters were analysed for statistical significance using Pearson's correlation coefficient method. 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

Mithun semen samples ($n = 50$) are mostly creamy white to thick creamy in colour with an average semen volume of

2.35 ± 0.12 mL and an average sperm concentration of 865.14 ± 8.94 million per mL. SQPs, sperm cholesterol and antioxidant profiles were increased and leakage of intracellular enzymes, sperm morphological abnormalities and MDA were decreased significantly ($p < 0.05$) in 50 mM trehalose than those in 75 or 100 mM trehalose treated or untreated control group.

Semen quality parameters: Spermatozoa treated with trehalose 50 mM had significantly higher post thaw motility than those in control (7.42%), trehalose 75 mM (3.51%) and trehalose 100 mM (3.21%). Similarly viability was significantly higher in 50 mM trehalose than those in control (11.43%), 75 mM (3.12%) and 100 mM (4.56%). Acrosomal intactness of spermatozoa was significantly higher in 50 mM as compared to those in control (6.89%), 75 mM (3.56%) and 100 mM (4.12%); whereas the total sperm morphological abnormality was significantly ($p < 0.05$) reduced in 50 mM trehalose treated than that in control (10.23%), 75 mM (4.76%) and 100 mM (3.89%). Plasma membrane integrity was significantly ($p < 0.05$) affected with trehalose treatment such that 50 mM treated sperm had shown higher membrane intactness than those in untreated control (11.89%) and other treatment groups (75 mM: 4.34% and 100 mM: 6.89%). Nuclear integrity was also following same trend as HOST (50 mM > 75 mM or 100 mM or control: 4.12, 2.89 or 6.67%, respectively). Vanguard distance travelled by sperm in CMPT was significantly higher in 50 mM than those in 75 mM (6.43%) or 100 mM (3.56%) or control (6.67%) groups (Fig. 1).

Velocity and motility parameters by CASA: Forward progressive motility (FPM) of sperm was significantly ($p < 0.05$) higher in 50 mM than those in other groups (75 mM: 4.78%, 100 mM: 7.65% and control: 14.32%). Similarly total motility (TM) was significantly ($p < 0.05$) higher in 50 mM than those in other trehalose treated (4.35 to 5.67%) and untreated control (11.21%) groups. On the other hand, static motility (SM) was significantly ($p < 0.05$) reduced in trehalose treated than those in control groups (20.45% vs 26.23%). Velocity profiles (curvilinear motility: VCL, straight line velocity: VSL and average path velocity: VAP) were significantly ($p < 0.05$) higher in trehalose 50 mM than those in 75 mM (1.5–2.6%) or 100 mM (4.3–7.8%) or untreated control (2.2–8.4%) groups. Trehalose 50 mM had significantly ($p < 0.05$) higher amplitude of lateral head displacement (ALH) than those in control (14.56%), 75 (6.43%) and 100 mM (2.12%) and similar trend for beat cross frequency (BCF) (13.87, 6.43 and 11.67%). The straightness (STR) was 1.36 to 2.93% higher in 50 mM treated than other trehalose treated or control groups (Fig. 2).

Leakage of intracellular enzymes: Leakage of intracellular enzymes such as AST was significantly ($p < 0.05$) reduced in 50 mM treated than in untreated control (13.31%) or other trehalose treated (75 mM; 4.02% or 100 mM; 3.87%) groups. Similar observation was noted in ALT leakage (15.78, 4.34 or 2.89%, respectively). Similarly another enzyme, LDH also revealed that leakage was

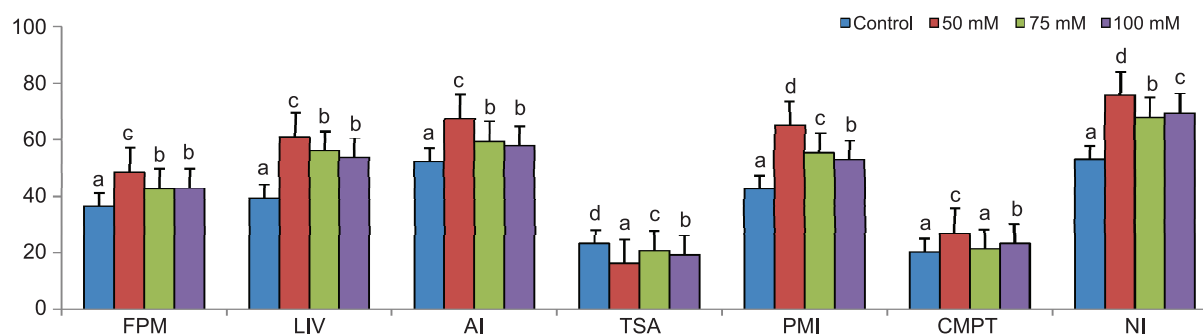


Fig. 1. Effect of Trehalose on post thaw semen quality profiles in mithun (mean $\pm$ SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); LIV, Livability (%); AI, Acrosomal Integrity (%); TSA, Total sperm abnormality (%); PMI, Plasma membrane integrity (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.

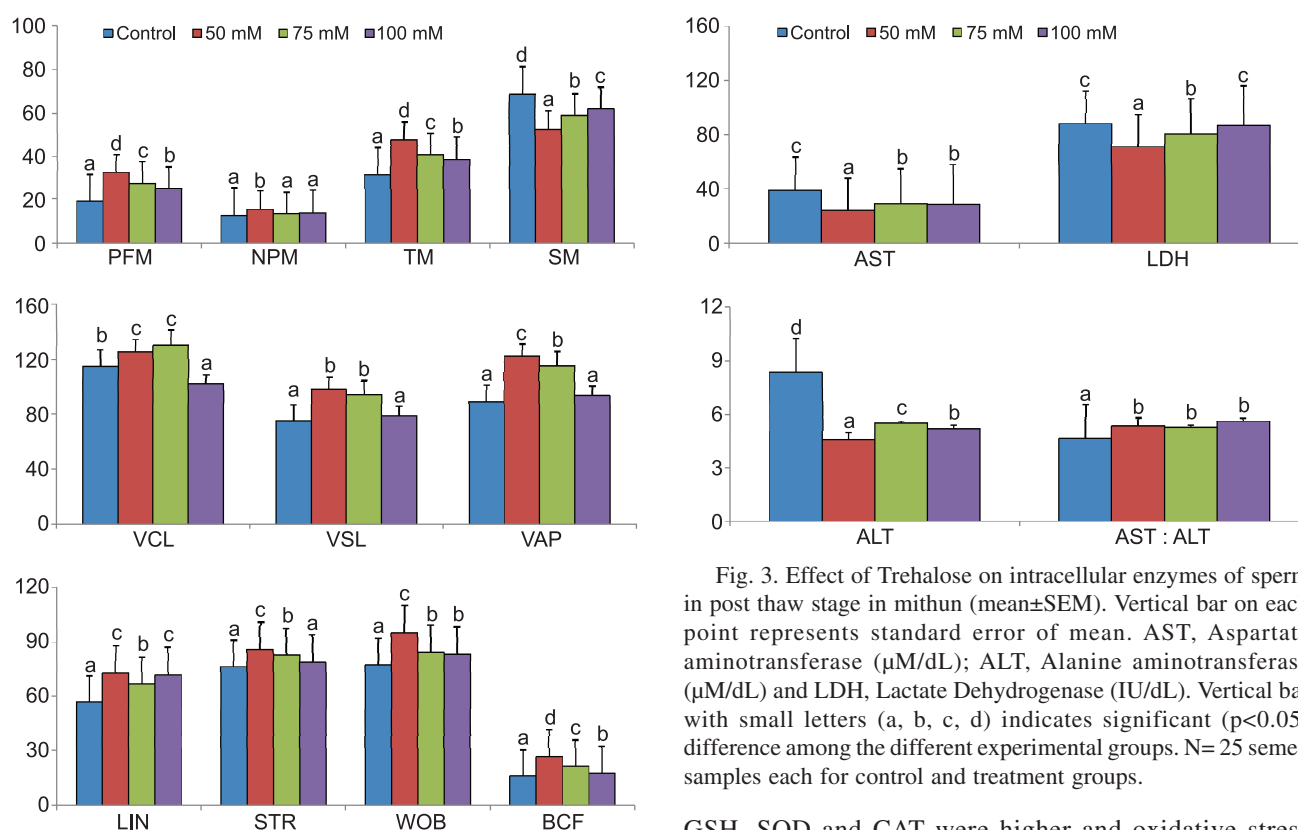


Fig. 2. Effect of Trehalose on post-thaw motility and velocity parameters by computer assisted sperm analyser (CASA) in mithun (mean $\pm$ SEM). Vertical bar on each point represents standard error of mean. FPM: Forward progressive motility (%), NPM: Non-progressive motility (%); TM, total motility; SM, Static sperm (%); VCL, curvilinear velocity (μ m/sec.); VSL, straight line velocity (μ m/sec.); VAP, average path velocity (μ m/sec.); 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.

significantly ($p < 0.05$) reduced in 50 mM than those in 75 mM (3.23%) or 100 mM (5.12%) or control (5.43%) groups (Fig. 3).

Antioxidant profiles: Antioxidant profiles such as TAC,

Fig. 3. Effect of Trehalose on intracellular enzymes of sperm in post thaw stage in mithun (mean $\pm$ SEM). Vertical bar on each point represents standard error of mean. AST, Aspartate aminotransferase (μ M/dL); ALT, Alanine aminotransferase (μ M/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.

GSH, SOD and CAT were higher and oxidative stress profiles such as MDA was lower significantly ($p < 0.05$) in 50 mM than those in 75 mM or 100 mM or untreated control groups. Trehalose 50 mM had significantly ($p < 0.05$) higher antioxidant profiles and lower MDA than in control (10.56–18.12% and 16.45%) or 75 mM (8.23–16.78% and 5.13%) or 100 mM (7.91–13.34% and 6.19%) in mithun bulls (Fig. 4).

Sperm cholesterol: Sperm cholesterol was higher significantly in 50 mM than those in 75 or 100 mM or untreated control groups. Trehalose 50 mM had significantly ($p < 0.05$) higher sperm cholesterol than in control (11.43%) or 75 mM (9.54%) or 100 mM (11.12%) in mithun bulls (Fig. 4).

Correlation study: Correlation analysis revealed that SQPs such as forward progressive motility, livability,

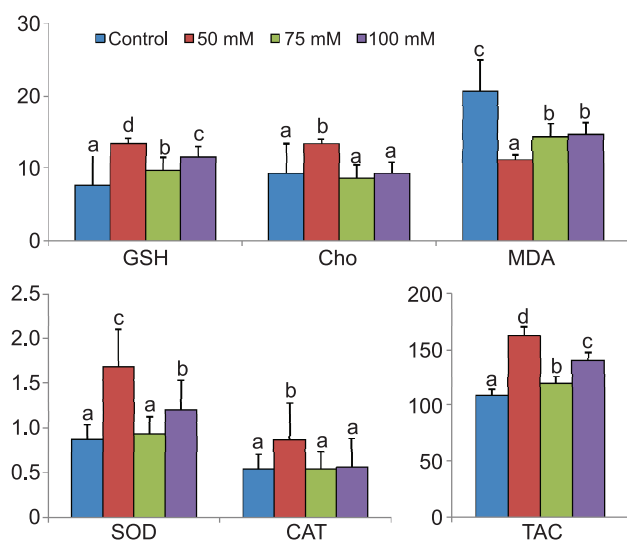


Fig. 4. Effect of Trehalose on antioxidant profiles in mithun (mean $\pm$ SEM). Vertical bar on each point represents standard error of mean. GSH, Glutathione (μ mol/mL); Cho, Cholesterol (μ g/10⁸ sperm); MDA, Malondialdehyde (nmol/10⁸ sperm); SOD, superoxide dismutase (U/ml of seminal plasma); CAT, Catalase (nmol/min/mL) and TAC, Total antioxidants (Trolox equivalents μ mol/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.

acrosomal integrity, plasma membrane integrity, cervical mucus penetration test and nuclear integrity, CASA parameters such as FPM, TM, VCL, VSL, VAP, LIN, STR, ALH and BCF, antioxidant parameters such as GSH, SOD, CAT and TAC and biochemical profile such as sperm cholesterol had significant ($p < 0.05$) positive correlation with each other whereas these profiles had significant ($p < 0.05$) negative correlation with TSA, SM, AST, ALT, LDH and MDA in trehalose treated sperm (Fig. 5).

Analysis of the present study revealed that inclusion of 50 mM trehalose in the semen extender improved the SQPs, level of antioxidants and total cholesterol of sperm whereas it reduced the leakage of intracellular enzymes, free radical formation and sperm morphological abnormalities in mithun. Thus trehalose protects efficiently the functions and structures of the spermatozoa. Moreover, the sperm treated with trehalose enhanced the SQPs by preserving efficiently during the procedure of artificial insemination. Perusal of available literature revealed no information on trehalose inclusion on these experimental parameters in mithun semen cryopreservation. Though several authors have reported trehalose has significant beneficial effects in SQPs and profiles of antioxidant and oxidative stress and biochemical profiles in different species like buffalo (Kumar and Atreja 2011), goat (Aboagla and Terada 2003) and boar (Hu *et al.* 2009), similar studies in mithun were lacking. The beneficial effect of trehalose in semen preservation is because it is a very potent membrane stabilizer (Chhillar *et al.* 2012).

Trehalose is a disaccharide which functions as a non-permeating cryoprotective agent which induces

spermatozoa dehydration due to the osmotically stimulated flow of water. Because of this mild dehydration, sperm cell had reduced intra-cellular water which in turn reduced the formation of intracellular ice crystal. This is significant beneficial for preservation of sperm as because intracellular ice crystal formation leads to sperm cell death and subsequently decreased the fertility rate of the cryopreserved sperm. This might be the reason that higher motility, viability, acrosomal and plasma membrane integrity of spermatozoa was observed in the semen extender containing trehalose.

Moreover, it maintains plasma membrane, mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. Trehalose also protects SOD and CAT level in the semen extender, which helps to maintain membrane transportation and fertility of the spermatozoa. The axosome and related dense fibres of sperm middle pieces are thickly packed by mitochondria that produce energy from the intra-cellular stores of ATP. These energy production systems of mitochondria and ATP are responsible for motility of spermatozoa (Garner and Hafez 1993). Based on the present observations, we can propose that inclusion of trehalose has displayed the protective effects on functions and structures of axosome as well as the mitochondria, thus sperm motility was improved in cryopreserved mithun sperm.

Mammalian sperm membrane has higher level of polyunsaturated fatty acids which makes the sperm very susceptible to lipid peroxidation; which occurs as a consequence of the membrane lipids oxidation due to the presence of the partially reduced oxygen molecules like superoxide, H₂O₂ and hydroxyl radicals. Lipid peroxidation of the sperm membranes induces the impairment of structure and function of sperm which is due to the attack by ROS, altered membrane integrity, sperm motility, sperm DNA damage and fertility rate through production of cytotoxic aldehydes and oxidative stress (Griveau *et al.* 1995). Endogenous antioxidant defence mechanism neutralises the harmful effect of these ROS (Jayaganthan *et al.* 2013). However, when antioxidant system exhausts, surplus free radicals contribute to oxidative stress process in spermatozoa and induces lipid peroxidation (Perumal *et al.* 2011b). In our study, the rate of lipid peroxidation was also found to be significantly ($p < 0.05$) higher in untreated than in trehalose treated group. Upon supplementation of trehalose to the extender, the rate of lipid peroxidation was significantly ($p < 0.05$) decreased. Our results are in close agreement with Hu *et al.* (2010) who showed that supplementation of 50 mM trehalose in egg yolk based extender improves sperm quality and oxidative stress parameters in liquid storage of mithun semen. Moreover, membrane stabilizer like disaccharide sugars of seminal plasma is compromised during semen processing and preservation (Alvarez and Storey 1992). Therefore, inclusion of sugars like trehalose exogenously may modulate the membrane structure which helps to preserve the mithun sperm effectively.

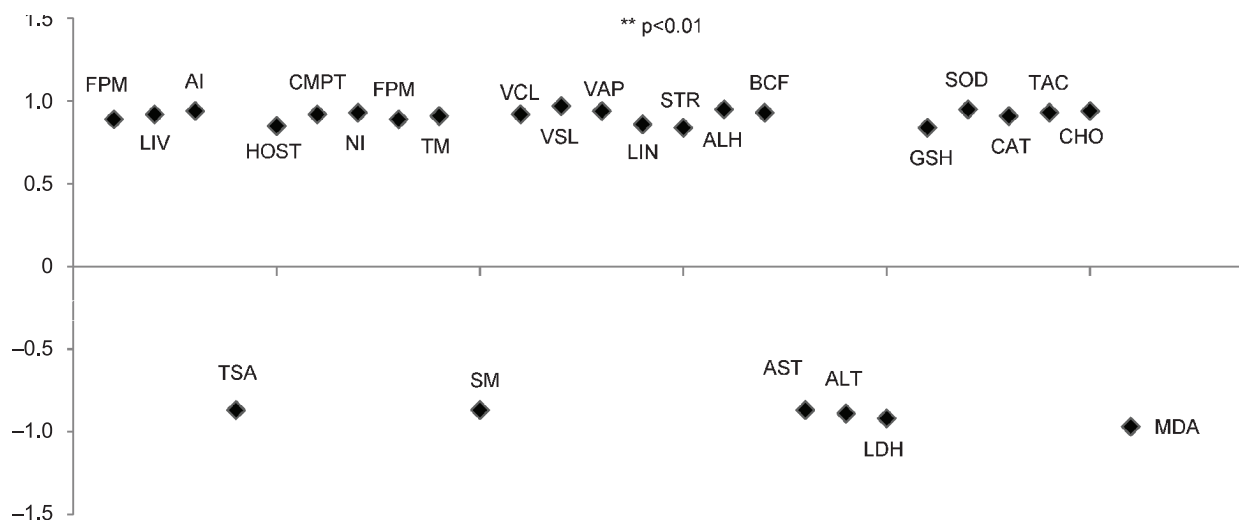


Fig. 5. Correlation coefficients among the semen quality parameters, kinetic parameters by computer assisted sperm analyser, biochemical profiles and antioxidant and oxidative profiles in mithun bulls. FPM, Forward progressive motility; LIV, livability; AI, acrosomal integrity; TSA, total sperm abnormality; 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, $p < 0.01$.

Level of the intra-cellular enzymatic profiles of seminal plasma is very important to measure the metabolism and functions of the sperm (Brooks 1990) which help to measure the degree of the sperm damage (Pesch *et al.* 2006). ALT and AST are the essential intra-cellular enzymes essential for metabolic processes which provide energy for sperm viability, motility and fertility and these transaminase activities in semen are good indicators of semen quality because they measure sperm membranes stability (Corteel 1980). Moreover, increase in AST and ALT activities of seminal plasma and semen during storage may be due to structural instability of the sperm (Buckland 1971). In our study, the level of ALT and AST was reduced in trehalose treated than in control group suggesting that trehalose maintained the integrity of acrosome membrane, plasma membrane, mitochondrial structure as well as flagella of the sperm.

In the present study, GSH was higher in the seminal plasma of trehalose added semen as they maintain the antioxidant system in mithun semen. SOD prevents premature capacitation and hyperactivation induced by superoxide radicals before ejaculating (de Lamirande and Gagnon 1995). In our study, concentration of SOD and CAT were higher in trehalose treated semen. However, the seminal plasma is a rich source of this potent antioxidant (Kobayashi *et al.* 1991). The high levels of readily peroxidizable polyunsaturated material expose spermatozoa to excessive oxidative stress and the SOD activity of sperm samples is a good predictor of their survival time. Trehalose, when included at a dose of 50 mM, significantly improved the sperm motility during the process of preservation and exhibited potent antioxidative characteristics, increased the

CAT level in association with SOD concentration. Further, trehalose is considered as a permeating cryoprotective agent and acts as a potent antioxidant and induces the membrane protein and lipid rearrangement, which enhanced membrane fluidity and higher dehydration at the lower temperatures of preservation; therefore the spermatozoa is able to survive under cryopreservation. This might be the reason that the spermatozoa treated with trehalose had significantly improved motility, viability and membrane integrity.

The results of the present study showed that addition of 50 mM trehalose improves the keeping quality of mithun semen preserved at -196°C . Beneficial effect on sperm parameters with reduced concentration of trehalose and adverse effect at higher trehalose concentration was reported in mithun as in porcine semen preservation (Hu *et al.* 2009). Sperm acrosomal membrane and plasma membrane integrity related with sperm viability and motility are reduced due to higher doses of trehalose in the semen extender. In the present study, highest protective effect of trehalose was observed at 50 mM and much reduced effect was at 100 mM. The latter concentration resulted in increased osmolarity of the extender which was in itself deleterious to the sperm cells (Hu *et al.* 2009). As demonstrated in previous studies, antioxidants exhibited cryoprotective activity on certain sperm variables in moderate doses, but increasing doses of antioxidant additives would result in a hypertonic property of extender and impair sperm functions (Bucak *et al.* 2007). The exact mechanism by which trehalose preserves the sperm membrane is not known, but it is theorized that these sugars probably play a key role in preventing deleterious alteration in the membrane structure and functions during reduced

water conditions (Aboagla and Terada 2003). Furthermore, Liu *et al.* (1998) and Aboagla and Terada (2003) proposed that trehalose easily penetrates into the spermatozoa plasma membrane and bind with the polar head groups of phospholipids to form hydrogen bonds. Thereby, they also create an osmotic pressure, inducing cell dehydration, increased membrane fluidity and a lower incidence of intracellular ice formation (Aisen *et al.* 2002). However, cryoprotective function of sugar like trehalose on spermatozoa may be depended upon their chemical structure and molecular weight (Molinia *et al.* 1994) and also the type of buffer used in the preservation (Abdelhakeam *et al.* 1991). Improvement of SQPs due to inclusion of exogenous trehalose recorded in the current study was reported earlier in the bovine semen in the form of motility, viability and intact acrosomal membrane (Chhillar *et al.* 2012). Further, inclusion of exogenous trehalose has significantly enhanced sperm viability and intact plasma membrane especially at the concentration of 50 mM trehalose. Significantly higher intact plasma membrane and acrosomal membrane was observed in our experiment in 50 mM trehalose treated sperm which might be the reason for higher motility and viability in these samples (Chhillar *et al.* 2012).

Trehalose prevents cholesterol efflux from the plasma membrane of sperm and free radicals production in the semen extender suggests that it prevents premature acrosomal reaction and capacitation as it functions as a potential membrane stabilizer. Cholesterol is required along with phospholipids for physical integrity of sperm cell and ensures the fluidity of the sperm cell membranes. Similarly cholesterol also plays a crucial role in sperm plasma membranes because its release initiates the important step in the capacitation and acrosome reaction that are crucial for fertilization process (Srivastava *et al.* 2013). Moreover, inclusion of cholesterol into the diluents before the defreezing process enhances the sperm resistance power to the stress induced by the freezing-defreezing procedures, maintaining the sperm motility as well as the fertilization potential of sperm (Moore *et al.* 2005). In our study, cholesterol efflux and malondialdehyde production were reduced in trehalose treated group than in the untreated control group. Therefore the semen samples treated with trehalose had higher cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that received 50 mM of trehalose were significantly higher than those of the other treatment and control groups. In this study, improvements observed in sperm quality may be attributed to prevention of excessive ice crystal generation and formation of protective coat over the sperm membrane by means of membrane stabilizer property of trehalose.

It was concluded that the possible protective effects of trehalose supplementation are that it maintains membrane structure of sperm, prevents efflux of cholesterol and phospholipids from cell membrane, prevents leakage of intracellular enzymes and MDA production. Thus it protects the spermatozoa during preservation and enhancing the

fertility in mithun species. Future studies by measuring the level of fertility rate in *in vitro* or *in vivo* fertility assay are warranted to confirm the present findings.

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