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Inclusion of catalase in semen extender modulates the post thaw semen quality and oxidative stress profiles in mithun spermatozoa

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ABSTRACT

Present study was designed to assess the effect of catalase on post-thaw semen quality parameters (SQPs), sperm kinematics, antioxidant and oxidative stress profiles and sperm cholesterol efflux in mithun (*Bos frontalis*). A total of 25 ejaculates were selected based on biophysical parameters and each sample was split into four equal aliquots and diluted (to get final concentration of 60 million spermatozoa per mL) with the TCG extender containing either 0 U/mL, 50 U/mL, 100 U/mL, and 150 U/mL catalase in the Group I, II, III and IV, respectively. Frozen-thawed samples were analysed for motility parameters (progressive forward and in bovine cervical mucus [BCMPT]), kinetic and velocity parameters by computer-assisted sperm analyser (CASA), viability, sperm morphological and nuclear abnormalities, acrosomal integrity, plasma membrane and nuclear integrities, sperm intra-cellular enzymatic leakage and seminal plasma biochemical (sperm cholesterol and oxidative stress markers) profiles. Study revealed an enhancement in viability, acrosomal integrity, plasma membrane integrity, motility (progressive and in cervical mucus), sperm cholesterol content and reduction in sperm morphological and nuclear abnormalities, leakage of intracellular enzymes in Group III. Moreover, intactness of acrosome and biochemical membranes were protected significantly in addition to significant improvement in kinetic and velocity profiles in extender containing 100 U/ml catalase. Correlation analysis revealed that sperm kinetic parameters, SQPs and antioxidant parameters had significant positive correlation with each other whereas these profiles were negatively correlated with sperm morphological abnormalities, enzymatic leakage and lipid peroxidation in catalase treated sperm. The results clearly indicated that inclusion of 100 U/ml catalase holds a clear advantage over control or 50 U/ml or 150 U/ml catalase in cryopreservation of mithun semen. It can be concluded from the present study that catalase supplementation in semen extender can be effectively utilized to reduce the oxidative stress and to improve the post-thaw semen quality in mithun.

Keywords: Antioxidants, Catalase, Cryopreservation, Kinematic profiles, Mithun, Oxidative stress, Spermatozoa

Mithun is a unique, magnificent domestic bovine species in North-Eastern Hilly (NEH) region of India. Several reports revealed mithun is affected with intensive inbreeding depression because of repeated breeding of dominant bulls, lack of suitable breeding bulls and lack of suitable breeding management. Mithun is reared under extensive free-range system with natural service as the preferred breeding practice with various limitations; therefore, loss of productive and reproductive performances would be overcome by implementation of artificial breeding programmes. Preliminary research on catalase effect on basic SQPs revealed that 100 U/mL catalase is suitable for liquid semen preservation in mithun (Perumal *et al.* 2013). Artificial insemination contributes in genetic improvement; in which a single ejaculate from a male is used to impregnate many females. Various stages of freezing process induce physical, osmotic and chemical stresses on the sperm membranes associated with an oxidative stress induced by free radicals (Chatterjee *et al.* 2001). All these deleterious effects resulting in to loss of motility, viability, intactness of acrosomal membrane, plasma membrane and nuclear integrity, and large number of sperms are incapable to fertilize the ovum leads to fertilization failure (Bernardini *et al.* 2011). High polyunsaturated fatty acids content in sperm membranes and lack of significant cytoplasmic component containing antioxidants make the spermatozoa highly susceptible to lipid peroxidation (Sinha *et al.* 1996). Reactive oxygen species (ROS) effects on spermatozoa are irreparable loss of motility, sperm DNA disintegration and reduced fertilizing ability (Perumal *et al.* 2011). Therefore, supplementation of exogenous antioxidants in the semen extender (Perumal *et al.* 2013) or feeding of antioxidants (Jayaganthan *et al.* 2013) or flaxseed oil (Perumal *et al.* 2019) or slow release implantation of melatonin (Perumal

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et al. 2018) can reduce the deleterious effect of oxidative and cryo-stress during semen cryopreservation (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.* 2013), superoxide dismutase (Perumal 2014), trehalose (Perumal *et al.* 2015), melatonin (Perumal *et al.* 2015), etc. to improve the SQPs and *in vivo* or *in vitro* fertility.

Addition of catalase in the extender of bull (Asadpour *et al.* 2011), buffalo (El-Sisy *et al.* 2008), ram (Maxwell and Stojanov 1996) and boar sperm (Roca *et al.* 2005) protected the sperm against the harmful effects of ROS and improved the semen quality during sperm preservation. Catalase is a tetramer with four polypeptide chain antioxidant, found in nearly all living organisms exposed to oxygen. It is derived from the epididymis, and seminal vesicle. It detoxifies both intracellular and extracellular hydrogen peroxide by reducing H_2O_2 to H_2O and O_2, and eliminates the potential ROS toxicity (Aitken 1995). It reduces the loss of spermatozoa motility caused by leukocytes generated ROS in the semen (de Lamirande *et al.* 1997). It scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. Catalase also prevents premature hyper activation and capacitation induced by superoxide radicals before ejaculating (de Lamirande and Gagnon 1995).

No information is available on effect of catalase in Tris based semen extender cryopreservation in mithun. Therefore, it was hypothesized that supplementation of catalase in semen extender would improve the post-thaw quality of mithun spermatozoa. With this, the objective of the present study was to assess the effect of different concentrations of catalase 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

Experimental animals: The proposed study was conducted at the mithun breeding farm, ICAR-National Research Centre on Mithun, Medziphema, Nagaland, India. 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 mean sea level. Ten healthy (body condition score 5-6) mithun bulls of 4-6 years of age were selected. Experimental animals were maintained under uniform feeding, lighting, housing and other managemental conditions as per farm schedule.

Extender preparation: The extender used in this study contained 3.028 g Tris (hydroxymethyl) amino methane, 1.675 g citric acid, fructose 1.250 g, 7 mL glycerol (7%), 1000 µ/mL streptomycin sulphate, 1000 IU/mL penicillin G sodium, and 20 mL egg yolk (20%), and different concentrations of catalase (50 U/ml, 100 U/ml and 150 U/ml, in Group II or III or IV, respectively) for 100 mL deionized water. The extender for the control (Group I) contained no catalase. The final *p*H of extender used in all three groups was adjusted to 6.8-7.0.

Semen collection and processing: Semen was collected not more than twice per week from any animal through standardised trans-rectal massage method during summer season (May to July; THI: 76.06±0.45; sunshine hours: 6.55±0.15). Semen samples with mass activity of 3+ or above were selected for the experiment. Immediately after collection, the ejaculates were kept in a water bath at 35°C and evaluated the preliminary semen quality parameters. After discarding the ejaculates with wide variation in *p*H (i.e. <6.7 and >7.2), colour or too low volume (< 0.50 mL), rest were evaluated microscopically. The ejaculates having 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 of 102 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 (35°C) TCG extender. Thus, from 102 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 (35°C) for further processing.

Each sample was split into four equal aliquots and diluted (to get final concentration of 60 million spermatozoa per mL) with the TCG extender containing either 0 U/mL, 50 U/mL, 100 U/mL, and 150 U/mL catalase in the Group I, II, III and IV, respectively. Diluted semen samples of each group were filled in polyvinyl chloride straws (0.5 mL; IMV, L'Aigle, France) by manual sucking and sealed with polyvinyl alcohol powder (IMV, L'Aigle, France). These straws were cooled simultaneously from 35°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 4 hrs. 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 were stored therein for at least 24 h before starting the next phase of experiment.

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 s. Semen quality parameters (SQPs), viz. 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 morphological abnormalities 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 (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 phosphate buffer saline and centrifugation (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: Intracellular enzymes such as aspartate aminotransferase (AST; µmol/dL), alanine aminotransferase (ALT; µmol/dL) and lactate dehydrogenase (LDH; IU/dL) were estimated in the seminal plasma by commercially available assay kits (Span Diagnostics Ltd., India).

Antioxidant and oxidative stress profiles: Total antioxidant capacity (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 of 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 at 535 nm, using thiobarbituric acid (TBA)-trichlroacetic acid (TCA) 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. 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 20 s. Thereafter, it was centrifuged at 800 × 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 analysed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test to determine the significant differences among the experimental groups on these sperm parameters using the SAS software (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001). Figures present the non-transformed data. Differences with values of $P<0.05$ were considered to be statistically significant after arcsine transformation of percentage data. Associations between different SQPs were analysed for statistical significance using Pearson's correlation coefficient using SAS 9.3.1 Software. 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) were mostly creamy white to thick creamy in colour with an average semen volume of 2.35 ± 0.12 mL and average sperm concentration was 865.14 ± 8.94 million per mL. Statistical analysis

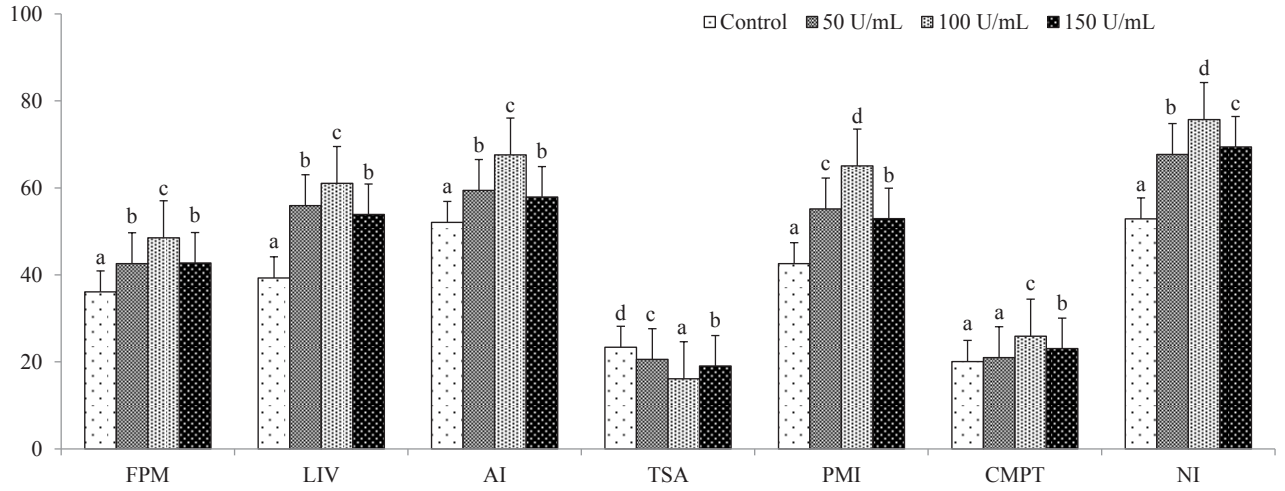

Fig. 1. Effect of catalase on post thaw semen quality profiles in mithun (mean ± SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); LIV, Liveability (%); AI, Acrosomal Integrity (%); TSA, Total sperm abnormality (%); PMI, Plasma membrane integrity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

revealed a significant (P<0.05) enhancement in quality parameters in ejaculates diluted with extender containing 100 U/ml catalase. Enzyme leakage was reduced significantly (P<0.05) in group supplemented with 100 U/ml catalase as compared to other treatment and control groups. Sperm cholesterol and seminal plasma antioxidant profiles revealed significant (P<0.05) improvement with simultaneous reduction of lipid peroxide (MDA) content of spermatozoa. Spermatozoa treated with 100 U/ml catalase had significantly (P<0.05) higher post thaw motility, viability, acrosomal intactness, plasma membrane integrity, nuclear integrity and vanguard distance travelled by sperm in cervical mucus compared to those in control and catalase 50 U/ml or 150 U/ml treated groups. On the other hand, the total sperm morphological abnormality was reduced (P<0.05) in 100 U/ml CAT compared to those in control and other CAT treated groups (Fig. 1).

Forward progressive motility (FPM) and total motility (TM) of sperm measured by CASA were significantly (P<0.05) higher in 100 U/ml catalase compared to those in control and other catalase treated groups. On the other hand, static motility (SM) was reduced significantly (P<0.05) in 100 U/ml catalase treated compared to those in control or other catalase treated groups. Velocity profiles (curvilinear motility: VCL, straight line velocity: VSL and average path velocity: VAP) were significantly (P<0.05) higher in 100 U/ml catalase compared to those in 50 U/ml or 150 U/ml treated or untreated control groups. Similarly, amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and straightness (STR) were significantly higher (P<0.05) in 100 U/ml catalase treated compared to those in control or other catalase treated groups (Fig. 2).

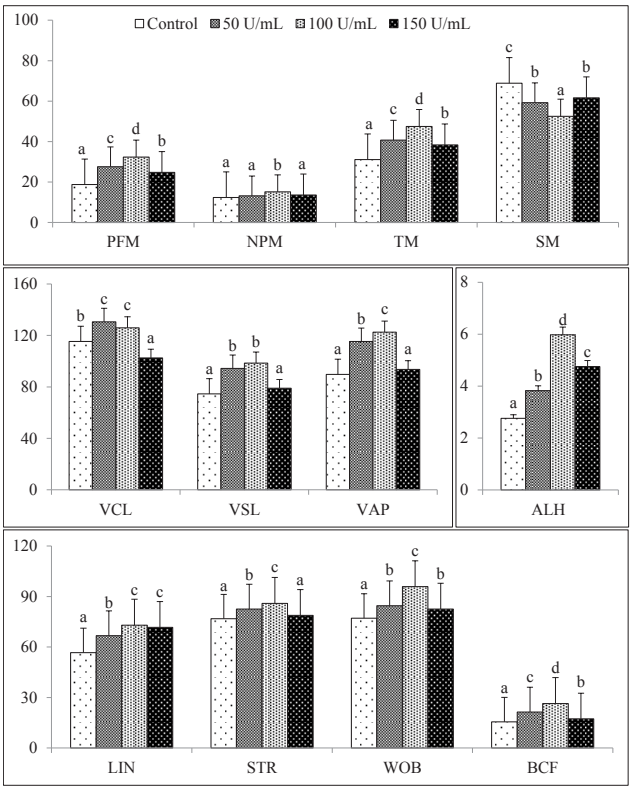

Fig. 2. Effect of catalase on post thaw motility and velocity parameters by computer assisted sperm analyser (CASA) in mithun (mean ± SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); NPM, Non-progressive motility (%); TM, Total motility; SM, Static 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.

Leakage of intracellular enzymes (AST, ALT and LDH) was significantly *(P < 0.05)* reduced in 100 U/ml catalase treated compared to those in untreated control or other catalase treated groups (Fig. 3). Antioxidants such as TAC, GSH, SOD and CAT were higher and oxidative stress marker such as MDA was significantly (P<0.05) lower in 100 U/ml catalase treated compared to those in other catalase treated or untreated control groups. Cholesterol was significantly (P<0.05) higher in 100 U/ml catalase treated compared to those in other catalase (50 U/ml or 150 U/ml) treated or untreated control groups. Similarly, sperm cholesterol was significantly (P<0.05) higher in catalase treated spermatozoa as compared to the control groups (Fig. 4).

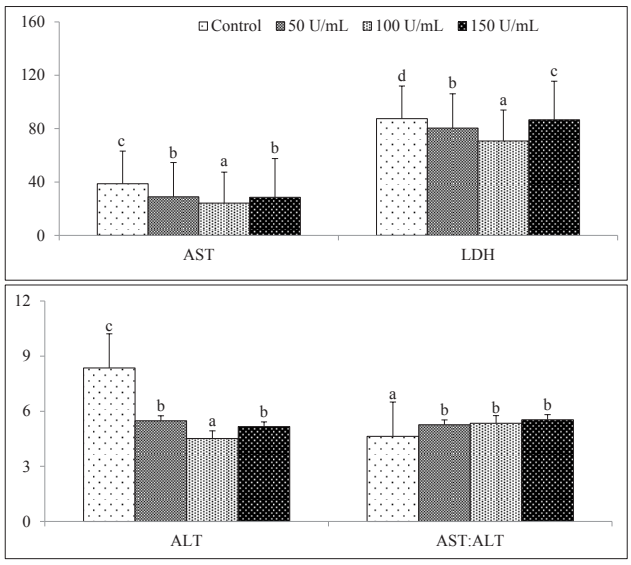

Fig. 3. Effect of catalase on intracellular enzymes of sperm in post thaw stage in mithun (mean ± SEM). Vertical bar on each point represents standard error of mean. AST, Aspartate Aminotransferase (µM/dL); ALT, Alanine Aminotransferase (µM/dL) and LDH, Lactate Dehydrogenase (IU/dL). Vertical bar with 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.

Correlation analysis revealed that sperm kinetic parameters, SQPs and antioxidant parameters had positive (P<0.05) correlation with each other whereas, these profiles were negatively correlated (P<0.05) with sperm morphological abnormalities, enzymatic leakage and lipid

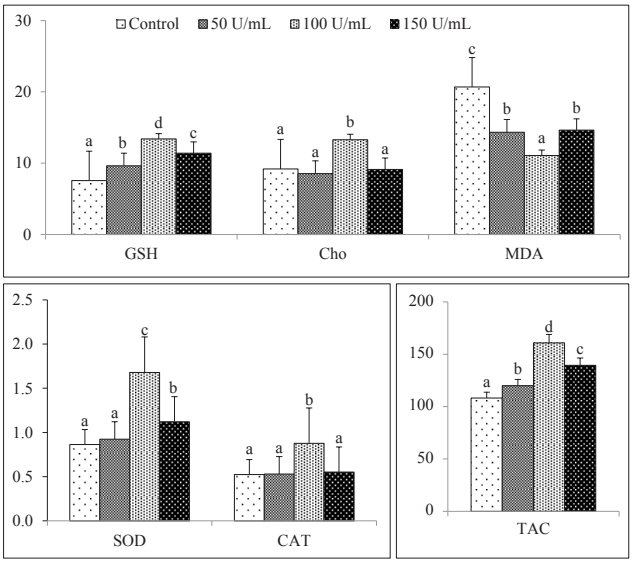

Fig. 4. Effect of catalase on antioxidant profiles in mithun (mean ± SEM). Vertical bar on each point represents standard error of mean. GSH, Glutathione (µmol/mL); Cho, Cholesterol (µg/10^8sperm); MDA, Malondialdehyde (nmol/10^8 sperm); SOD, Superoxide dismutase (U/ml of seminal plasma); CAT, Catalase (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.

peroxidation in catalase treated sperm (Fig. 5).

Inclusion of catalase in semen extender improved the SQPs, level of antioxidants, and total cholesterol of sperm and reduced the leakage of intracellular enzymes, free radical formation and sperm morphological abnormalities in mithun. Thus, it protected the structures and functions of spermatozoa efficiently. Though several authors had reported that catalase has significant beneficial effects on SQPs and oxidative stress markers in different species like bull (Asadpour *et al.* 2011), buffalo (El-Sisy *et al.* 2008), ram (Maxwell and Stojanov 1996) and boar sperm (Roca *et al.* 2005), similar studies in mithun were lacking. The beneficial effects of catalase in semen preservation are due to its potent antioxidant properties (Roca *et al.* 2005, El-Sisy *et al.* 2008, Asadpour *et al.* 2011).

Because the mammalian sperm membrane has high polyunsatured fatty acids, it renders the sperm highly susceptible to lipid peroxidation. It occurs due to the oxidation of the membrane lipids by partially reduced oxygen molecules (Dandekar *et al.* 2002, Asadpour *et al.* 2011). Lipid peroxides impair the sperm function through altered sperm motility, membrane integrity and damage to sperm DNA and fertility through oxidative stress and production of cytotoxic aldehydes (Griveau *et al.* 1995). In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing (Alvarez and Storey 1992). These results are in agreement with works of Maxwell and Stojanov (1996) that addition of catalase to the extender improved cryosurvival of ram spermatozoa. Therefore, inclusion of exogenous antioxidants may modulate the antioxidant system of semen (Dandekar *et al.* 2002, Asadpour *et al.* 2011).

In the present study, 100 U/ml CAT improved the post-thaw/cryopreservation quality of mithun semen. Different effects of three levels of catalase might be explained according to the report of Shoae and Zamiri (2008) who shown that excessive amount of antioxidants caused high fluidity of plasma membrane above the desired point, making the sperm more prone to acrosomal damages.

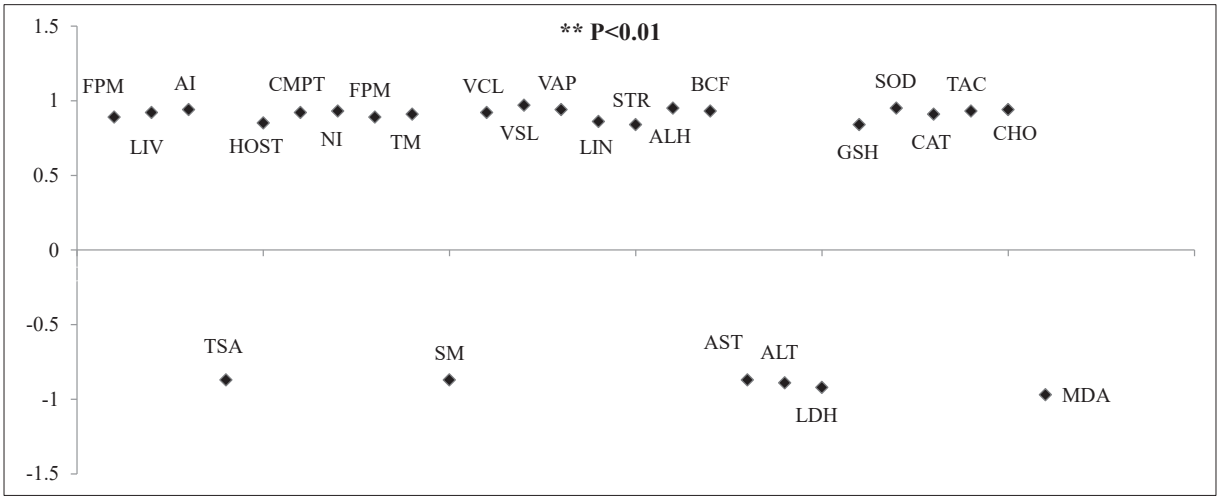

Fig 5. Correlation coefficients among the semen quality parameters, kinetic parameters by computer assisted sperm analyser, biochemical profiles and antioxidant and oxidative profiles in mithun bulls. FPM, Forward progressive motility; LIV, liveability; AI, acrosomal integrity; TSA, total sperm abnormality;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

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 the physiological state of semen extender. Also, this result showed that high concentration of catalase was toxic for sperm quality parameters (Maxwell and Stojanov 1996). In ram, survival of spermatozoa was increased as the dosage of catalase in extender increased. However, catalase at dosage higher than 200 U/ml was toxic to ram spermatozoa (Maxwell and Stojanov 1996). Similarly, in the present study, increased dosage of catalase at 150 U/ml affected the seminal as well as biochemical parameters in mithun semen 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 catalase recorded in the present study had also been reported previously in the form of motility and acrosomal membrane integrity in bull (Asadpour *et al.* 2011) and boar sperm (Roca *et al.* 2005). Moreover, the addition of exogenous catalase significantly improved the sperm viability and plasma membrane integrity, especially at the level of 100 U/ml (Asadpour *et al.* 2011).

Semen extender containing 100 U/ml of catalase enhanced motility, velocity and kinematic parameters of the sperm in mithun. This improvement in post□thaw CASA parameters might be due to the presence of higher level of antioxidants in 100 U/ml of catalase which in-turn protected the sperm from lipid peroxidation. Thus, the motility and velocity parameters were increased and cryopreservability was improved. A higher VCL and ALH of the spermatozoa indicate major bending of the mid piece and large amplitude of lateral head displacement. These signify the hyper activation of the spermatozoa and mitochondrial energy production efficiency of the sperm. Hyper activation implies high energy state of the spermatozoa, which is essential for sperm penetration through cervical mucus to fuse with the oocytes for successful fertilization. Similar result was obtained in the present study in catalase treated sperm.

Catalase maintains the integrity of normal acrosome (Maxwell and Stojanov 1996) and stabilizes the plasmalemma of spermatozoa; therefore, increased motility. Catalase is able to react with many ROS directly for protecting the 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 catalase in liquid storage (Maxwell and Stojanov 1996, Roca *et al.* 2005) and frozen semen form have been investigated (El-Sisy *et al.* 2008, Asadpour *et al.* 2011). Moreover, it maintains plasma membrane and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. Catalase also protects SOD, GSH and TAC level in the semen extender (Halvorsen *et al.* 2002), which in turn helps to maintain membrane transportation (Perumal *et al.* 2011a, b, Alvarez and Storey 1992) and fertility of the spermatozoa.

Catalase prevented cholesterol efflux from the sperm membrane and MDA production in diluents indicated that it prevents premature capacitation and acrosomal reaction as act as antioxidant. Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane. Cholesterol plays a crucial 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 are crucial for fertilization (Witte and Schäfer-Somi2007). In the present study, cholesterol efflux and MDA production were decreased in catalase treated group as compared to the control group. Therefore, the semen samples treated with catalase had high cryoresistance compared to control group.

AST and ALT are essential for metabolic processes which provide energy for survival, motility and fertility of spermatozoa and these transaminase activities in semen are good indicators of semen quality because they measure the sperm membrane stability (Corteel 1980). Thus, increasing the abnormal spermatozoa in storage 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, decrease of AST and ALT in the seminal plasma of catalase treated semen may be due to it maintains the structural stability of the sperm (Buckland 1971). Similar result was obtained in the present study that catalase treated semen had lower level of AST and ALT as it stabilizes the plasma membrane of acrosome, plasma membrane, mitochondria and flagella of the sperm.

Glutathione is the most abundant non-protein thiol in mammalian cells and is present mainly in reduced form (GSH) and only a small amount is in oxidized form (GSSG). In the present study, GSH was higher in the seminal plasma of catalase added semen as it maintains the antioxidant system in storage of mithun semen. Similarly, superoxide dismutase scavenges both extra-cellular and intra-cellular superoxide anion and prevents lipid peroxidation of the plasma membrane. In the present study, the concentration of SOD was higher in catalase treated semen. Catalase at a dose of 100 U/ml improved the sperm motility in cryopreserved semen and displayed antioxidative properties and elevated the SOD level in association with higher GSH concentration. Further, catalase is a permeating cryoprotectant, acts as an antioxidant and causes membrane lipid and protein rearrangement, which results in increased membrane fluidity, greater dehydration at lower temperatures; therefore, increased ability of spermatozoa to survive during the cryopreservation process (Holt 2005). This could be one of the reasons for improved motility, viability and membrane integrity of spermatozoa

in catalase treated semen extender.

In this study, improved SQPs in catalase treated extender may be because it prevents excessive generation of free radicals by means of its antioxidant property. The study concluded that catalase supplementation @100 U/mL enhanced the antioxidant properties of semen and prevented efflux of enzymes, cholesterol, and phospholipids from the cell membrane, and MDA production. Thus, it protected the spermatozoa during cryopreservation and enhanced the *in vitro* sperm functional properties in this 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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