



Glutathione in semen extender modulates post thaw semen quality profiles, and antioxidant and oxidative stress profiles in mithun

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ABSTRACT

Present study was designed to assess the effect of glutathione (GSH) on post thaw semen quality parameters (SQPs), sperm kinetic profiles, antioxidant and oxidative stress profiles and sperm cholesterol efflux in mithun. Ejaculates (25) were selected based on biophysical parameters for the present experiment. Each sample was split into four equal aliquots after dilution with the Tris-citrate-glycerol (TCG) extender, viz. Group I (control), Group II, III and IV contained 5, 10 and 15 mM of GSH, 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 and nuclear abnormalities, acrosomal integrity, 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 and nuclear normalities, acrosomal integrity, motility (progressive and in cervical mucus), sperm cholesterol content and reduction in 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 10 mM GSH. These results clearly indicate that though the cryopreservation of mithun's spermatozoa in TCG was comparable with other species, inclusion of 10 mM GSH holds a clear advantage over control and 5 or 15 mM GSH. The study concludes that GSH 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 bull.

Keywords: Antioxidants, Cryopreservation, Glutathione, Kinetic profiles, Mithun, Oxidative stress, Semen quality

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 lack of suitable breeding bulls and breeding management. Mithuns are reared under extensive free-range system with natural service as the preferred breeding practice with various limitations; therefore, loss of productive as well as reproductive performances and these limitations could be overcome easily by implementation of artificial breeding programmes. Preliminary research conducted on the effect of GSH on semen quality profiles in liquid preservation revealed that 10 mM GSH is suitable for mithun liquid semen preservation (Perumal *et al.* 2013). Artificial insemination contributes significantly 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 membrane associated with an oxidative stress induced by free radical

(Chatterjee *et al.* 2001). All these deleterious effects causes loss of motility, viability, intactness of acrosome, plasma membrane and nuclear integrity, large number of sperms incapable to fertilize the ovum and ultimately infertility or sterility (Bernardini *et al.* 2011). High unsaturated 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 and H₂O₂ (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.* 2011a,b). Therefore, supplementation/inclusion/addition of exogenous antioxidants in the semen extender (Perumal *et al.* 2013) or feeding of the natural/synthetic antioxidants (Jayaganthan *et al.* 2013) or flaxseed oil (Perumal *et al.* 2019) or slow release implantation of antioxidants (Perumal *et al.* 2018) can reduce the deleterious effect of oxidative as well as cryo stress during the process of semen

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cryopreservation (Perumal *et al.* 2011a,b). Studies have also been conducted on bovine semen extenders including additives/antioxidants such as taurine (Perumal *et al.* 2013), catalase (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.

The addition of additives such as GSH to equine sperm (Baumber *et al.* 2000), crossbred bull sperm (Perumal *et al.* 2011a,b), buffalo semen (El-Kon and Darwish 2011) has been shown to protect the sperm against the deleterious or harmful effects of ROS and improve the sperm motility and membrane integrity during sperm storage. 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. Glutathione system contains reduced glutathione, oxidized glutathione, glutathione reductase (GRD) and glutathione-s-transferase and glutathione peroxidase. No information is available with regard to the effect of GSH in Tris based semen extender cryopreservation on fertility of mithun bulls. Therefore, it was hypothesized that application of GSH in semen extender could be more beneficial on *in vitro* sperm functional parameters in mithun. Hence, the present study was undertaken to assess the effect of different concentrations of GSH 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 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. The 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 out of 10, classified as good) mithun bulls of 4–6 years of age were selected. Experimental animals were maintained under uniform feeding, lighting, housing and other managerial conditions as per farm schedule. 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 glutathione (5 or 10 or 15 mM, in Group II or III or IV, respectively) for 100 mL deionized water. The extender for the control (Group I) contained no glutathione.

Semen collection and processing: Semen was collected not more than twice per week from any animal through trans-rectal massage method. After discarding the initial transparent secretions, neat semen drops were collected in a graduated test tube with the help of a funnel. 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 volume, colour, consistency, pH, concentration and mass activity. After discarding ejaculates with wide variation in pH (i.e. <6.7 and >7.2), colour or too low volume (< 0.5 mL), rest were evaluated microscopically. These ejaculates were evaluated and 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 twofold initial dilution with pre-warmed (37°C) Tris-citrate-glycerol (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 5 or 10 or 15 mM GSH (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 (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 30 sec. 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 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 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 (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 (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 peroxidation level of spermatozoa was measured by determining the malondialdehyde (MDA) production at 535 nm, using thiobarbituric acid (TBA)-trichloroacetic 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 Blich and Dyer (1959) with some modification with use of cholesterol assay kit (Span Diagnostics Ltd, India) and results were expressed as μg cholesterol/ 10^8 spermatozoa.

Statistical analysis: Analysis of variance (ANOVA) was performed using a generalized liner model (Statistical Analysis System for Windows, SAS Version 9.3; SAS Institute, Inc., Cary, NC, 2001). Means were analyzed by one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences among the treatments and control groups. Differences with values of $p < 0.05$ were considered to be statistically significant. Associations between different parameters were analysed 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

Mithun semen samples ($n=50$) were creamy white to thick creamy with an average semen volume of 2.35 ± 0.12 mL with an average sperm concentration was 865.14 ± 8.94 million per mL. Result revealed a significant ($p < 0.05$) enhancement in SQPs in ejaculates diluted with 10 mM GSH. Intracellular enzymes showed a significant ($p < 0.05$) reduction and AST, ALT and LDH and were as reduced in 10 mM GSH as in other treatment and control groups. Sperm cholesterol and seminal plasma antioxidant profiles showed significant ($p < 0.05$) improvement with simultaneous reduction of lipid peroxide (MDA) content of spermatozoa.

Semen quality parameters: Spermatozoa treated with 10 mM GSH had significantly higher post thaw motility than those in control (8.61%), 5 mM GSH (3.69%) and 15 mM GSH (4.52%). Similarly, viability was significantly higher in 10 mM GSH than those in control (11.35%), 5 mM (3.22%) and 15 mM (3.78%). Acrosomal intactness of spermatozoa was significantly higher in 10 mM as compared to those in control (7.22%), 5 mM (4.87%) and 15 mM (5.24%); whereas the total sperm morphological abnormality was significantly ($p < 0.05$) reduced in 10 mM GSH treated than that in control (10.42%), 5 mM (4.26%) and 15 mM (4.63%). Plasma membrane integrity was significantly ($p < 0.05$) affected with GSH treatment that 10 mM treated sperm showed higher membrane intactness than those in untreated control (11.30%) and other treatment groups (5 mM: 4.62% and 15 mM: 6.23%). Nuclear integrity also followed the same trend as HOST (10 mM > 5 or 15 mM or control: 4.32, 3.16 or 6.68%, respectively). Vanguard distance travelled by sperm in CMPT was significantly higher in 10 mM than those in 5 mM (5.92%) or 15 mM (4.33%) or control (7.37%) groups (Fig. 1).

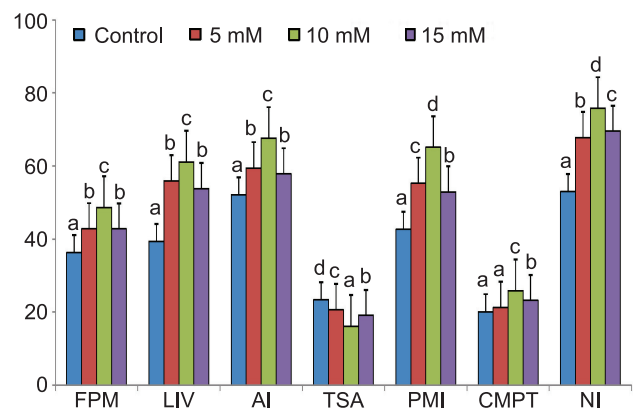


Fig. 1. Effect of glutathione on post thaw semen quality profiles in mithun (mean \pm SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); LIV, Livability (%); AI, Acrosomal integrity (%); TSA, Total sperm abnormality (%); PMI, Plasma membrane integrity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

Velocity and motility parameters by CASA: Forward progressive motility (FPM) of sperm was significantly ($p < 0.05$) higher in 10 mM than those in other groups (5 mM:

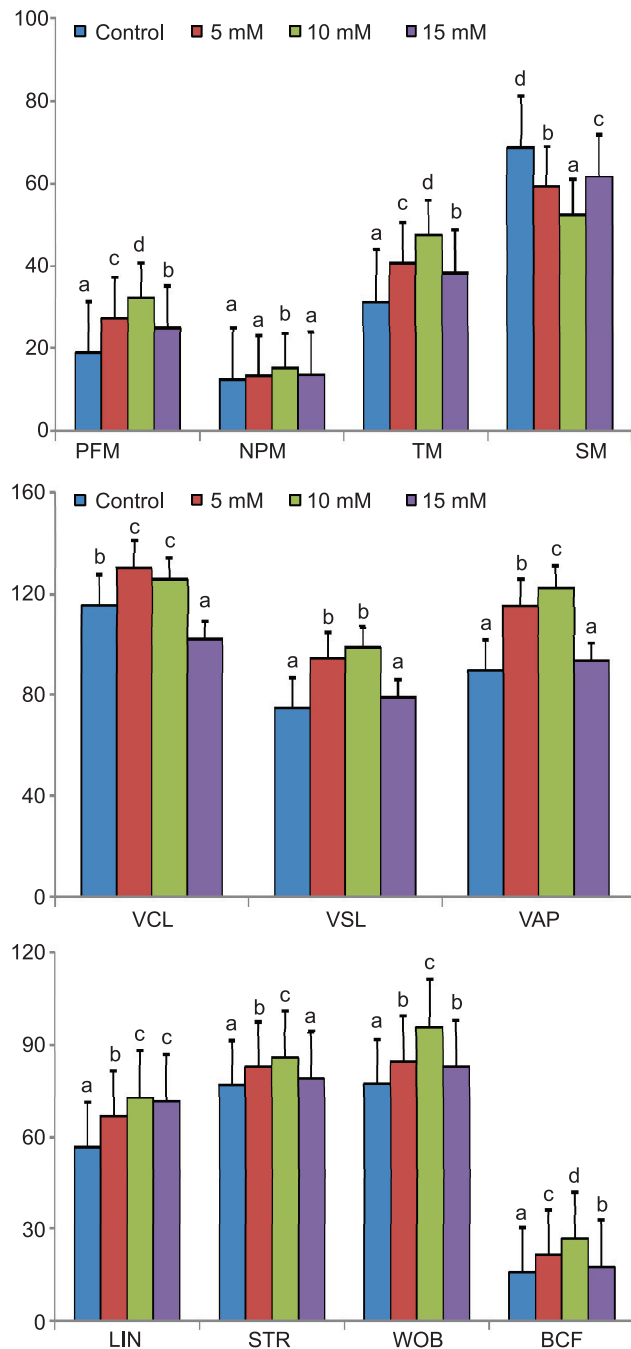


Fig. 2. Effect of glutathione on post thaw motility and velocity parameters by computer assisted sperm analyser (CASA) in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. FPM, Forward progressive motility (%); NPM, Non-progressive motility (%); TM, Total motility; SM, Static sperm (%); VCL, Curvilinear velocity (µm/sec.); VSL, Straight line velocity (µm/sec.); VAP, Average path velocity (µm/sec.); 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.

4.74%, 15 mM: 6.85% and control: 14.42%). Similarly, total motility (TM) was significantly ($p < 0.05$) higher in 10 mM than those in other GSH treated (4.64 to 5.47%) and untreated control (11.61%) groups. On the other hand, static motility (SM) was significantly ($p < 0.05$) reduced in GSH treated than those in control group (20.32% vs 25.64%). Velocity profiles (curvilinear motility: VCL, straight line velocity: VSL and average path velocity: VAP) were significantly ($p < 0.05$) higher in 10 mM GSH than those in 5 mM (1.5–2.9%) or 15 mM (4.8–7.2%) or untreated control (2.8–8.9%) groups. GSH (10 mM) had significantly ($p < 0.05$) higher amplitude of lateral head displacement (ALH) than those in control (14.92%), 5 mM (8.46%) and 15 mM (2.91%) and similar trend was observed for beat cross frequency (BCF) (12.72, 6.16 and 12.65%). The straightness (STR) was 2.42 to 3.76% higher in 10 mM treated than other GSH treated or control groups (Fig. 2).

Leakage of intracellular enzymes: Leakage of intracellular enzyme such as AST was significantly ($p < 0.05$) reduced in 10 mM treated than in untreated control (11.87%) or GSH treated (5 mM; 3.67% or 15 mM; 3.86%) groups. Similar observation was noted in ALT leakage (15.45, 4.64 or 2.32%, respectively). Enzyme LDH also revealed that leakage was significantly ($p < 0.05$) reduced in 10 mM than those in 5 mM (3.23%) or 15 mM (4.83%) or control (5.67%) groups (Fig. 3).

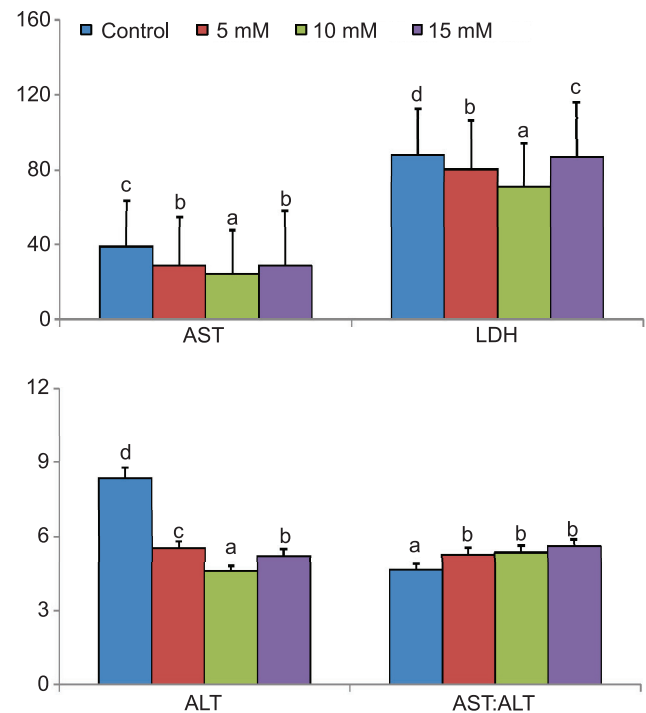


Fig. 3. Effect of glutathione on intracellular enzymes of sperm in post thaw stage in mithun (mean±SEM). Vertical bar on each point represents standard error of mean. AST, Aspartate aminotransferase (µM/dL); ALT, alanine aminotransferase (µM/dL); and LDH, Lactate dehydrogenase (IU/dL). Vertical bar with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

Antioxidant enzymes: Antioxidant profiles such as TAC, GSH, SOD and CAT were higher and oxidative stress profile such as MDA was lower significantly ($p < 0.05$) in 10 mM than those in 50 mM or 15 mM or untreated control groups. GSH (10 mM) had significantly ($p < 0.05$) higher antioxidant profiles and lower MDA than in control (11.51–18.93% and 17.86%) or 5 mM (8.32–16.71% and 5.55%) or 15 mM (7.87–13.41% and 6.53%) in mithun bulls (Fig. 4).

Sperm cholesterol: Cholesterol was higher significantly in 10 mM than those in 5 mM or 15 mM or untreated control groups. GSH (10 mM) had significantly ($p < 0.05$) higher sperm cholesterol than in control (11.23%) or 5 mM (12.56%) or 15 mM (11.74%) in mithun bulls (Fig. 4).

Correlation study: Correlation analysis revealed that SQPs such as forward progressive motility, livability, 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, antioxidants 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 glutathione treated sperm (Fig. 5).

Analysis of the present study revealed that inclusion of GSH 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 it protects the structures and functions of spermatozoa efficiently. Moreover, the GSH treated sperm may enhance the quality of semen by preserving efficiently during artificial insemination procedure. Perusal of available literature revealed no information on GSH 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 GSH in cryopreserved semen in mithun. Though several authors have reported GSH has significant beneficial effects in SQPs and profiles of antioxidant and oxidative stress and biochemical profiles in different species like equine (Baumber *et al.* 2000), crossbred bull (Perumal *et al.* 2011a,b), and buffalo (El-Kon and Darwish 2011), similar studies in mithun were lacking. In the present study, GSH supplementation on these parameters revealed significant difference between the treatment groups. The beneficial effects of SOD in semen preservation are due to its antioxidant nature (Perumal *et al.* 2011a,b).

Because the mammalian sperm membrane has high polyunsaturated 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

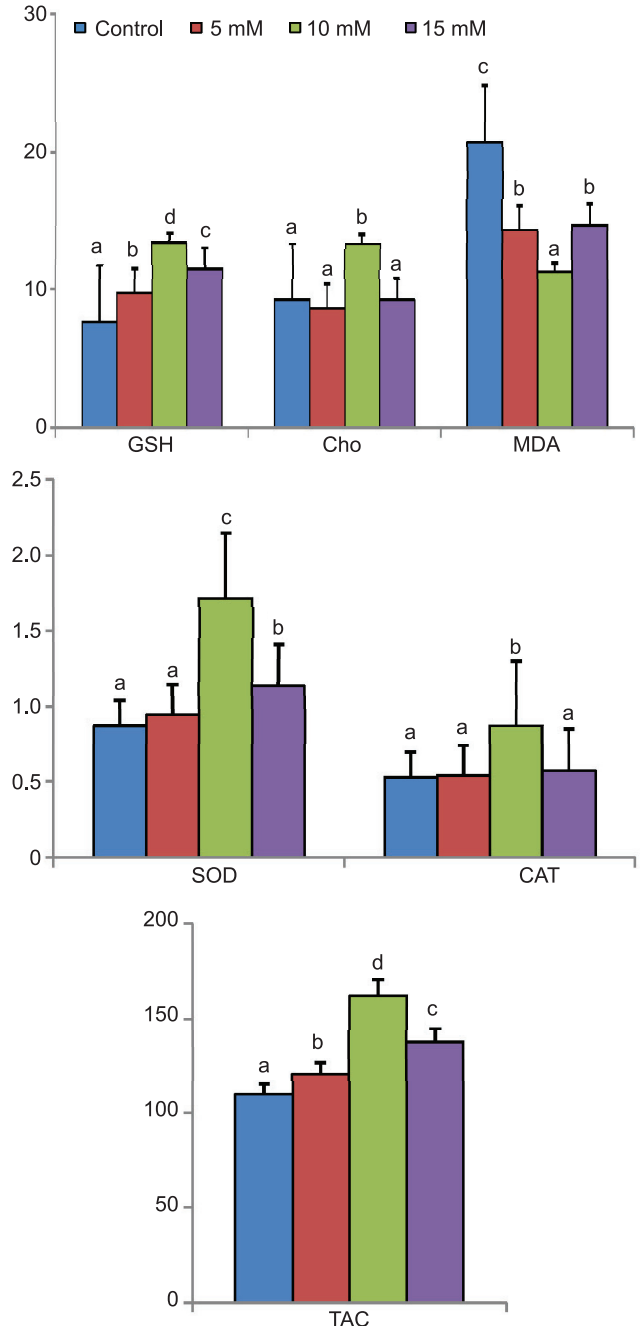


Fig. 4. Effect of glutathione on antioxidant profiles in mithun (mean \pm SEM). Vertical bar on each point represents standard error of mean. GSH, Glutathione ($\mu\text{mol/mL}$); Cho, Cholesterol ($\mu\text{g}/10^8$ sperm); MDA, Malondialdehyde ($\text{nmol}/10^8$ sperm); SOD, Superoxide dismutase (U/ml of seminal plasma); CAT, Catalase ($\text{nmol}/\text{min}/\text{mL}$); and TAC, Total antioxidants (Trolox equivalents $\mu\text{mol/L}$). 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.

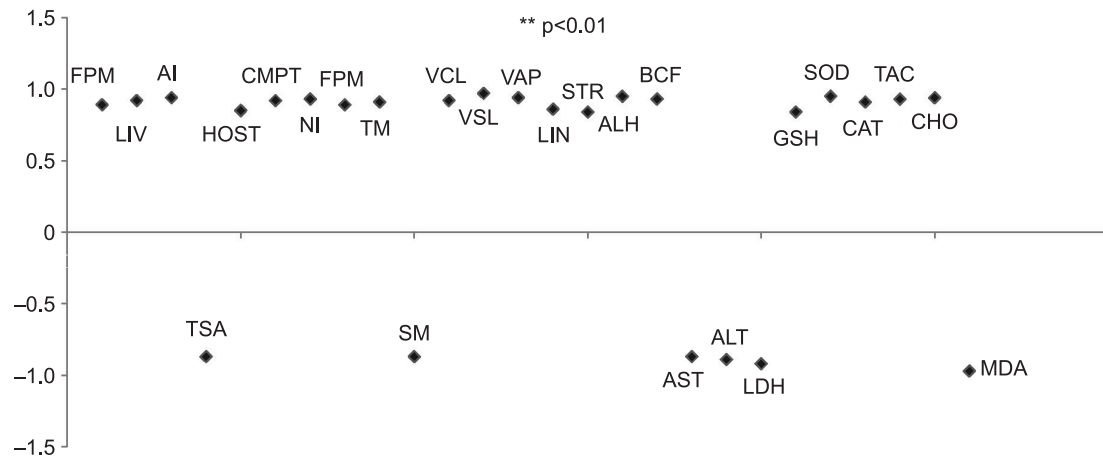


Fig. 5. Correlation coefficients among the semen quality parameters, kinetic parameters by computer assisted sperm analyser, biochemical profiles, and antioxidant and oxidative profiles in mithun bulls. FPM, Forward progressive motility; LIV, Livability; AI, Acrosomal integrity; TSA, Total sperm abnormality;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

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 (LPO) in semen (Shoae and Zamiri 2008). Therefore, addition of antioxidants could decrease the effect of oxidative or lipid peroxidation stress on sperm during the cryo preservation process and thus we can enhance the quality of liquid and cryopreserved semen (Asadpour *et al.* 2012).

The results of the present study showed that addition of 10 mM GSH improve the keeping quality of mithun semen as compared to those semen samples treated with 5 or 15 mM GSH or without GSH. The different effects of the different levels of GSH might be explained according to the report of Perumal *et al.* (2011a). Earlier reports (Perumal *et al.* 2011b) suggested that the excessive concentration of antioxidants induced high fluidity in the sperm plasma membrane above the desired or optimum point, making sperm more suffer to the acrosomal damages. Moreover, concentration of antioxidants included in the extender need to be monitored with utmost care as high concentration of antioxidants can be harmful to spermatozoa as higher dosage may change the physiological condition as well as osmolarity of the semen extender. In ram, survival of spermatozoa will 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 GSH 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 GSH, at 15 mM affected the seminal

as well as biochemical parameters in mithun semen TEYC extender. On the other hand, less concentration of antioxidant also significantly affected the *in vitro* sperm quality parameters. Moreover, the addition of exogenous GSH significantly improved the percentages of DNA morphology, sperm viability and intact plasma membrane especially at 10 mM GSH. The highest percentages of intact plasma and acrosomal membranes were found in 10 mM GSH which may be the reason for better motility in these samples (Perumal *et al.* 2011a,b).

GSH helps maintaining the integrity of normal acrosome (Sinha *et al.* 1996) and stabilize the plasmalemma of spermatozoa and so increases motility. GSH is capable to react with different ROS in the sperm cells directly to protect the mammalian cells against lipid peroxidation 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 glutathione in liquid storage (Gupta and Tripathi 1984) and frozen semen form have been investigated (Perumal *et al.* 2011a). Moreover, it maintains plasma membrane and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. GSH has also protects SOD and catalase level in the semen extender (Halvorsen *et al.* 2002), which helps to maintain membrane transportation (Alvarez and Storey 1992) and fertility of the spermatozoa.

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 vulnerable particularly to this oxidative stress as they generate lipid peroxide and are also rich targets for oxidative radicals attack. The sperm cells are transcriptionally inactive and have small quantity cytoplasm

and deficient in antioxidant level and DNA-repair systems (Aitken and Fisher 1994). Oxidative stress is a causative agent for male infertility and induces the DNA fragmentation in spermatozoa (Aitken and Fisher 1994). Further some investigations were conducted on effects of different antioxidant addition into extenders while liquid or cryopreservation procedure in mammalian spermatozoa (Kankofer *et al.* 2005). Lipid peroxides are generated in semen by damaged as well as the abnormal spermatozoa and also contaminated seminal leukocytes. ROS damages the sperm cells by alteration of lipids, proteins and DNA molecules. Spermatozoa are easily susceptible to lipid peroxidative effect which is caused by higher level of reactive oxygen species as because higher concentration of polyunsaturated fatty acids in plasma membrane phospholipids and little in the cytoplasm. In our study, inclusion of GSH had decreased the DNA fragmentation mainly at the dose of 10 mM in semen cryopreservation of mithun. Moreover, GSH also protects the plasma membrane as well as the mitochondrial membrane integrity and also cytoskeleton structure of the sperm flagella as cell protecting effects. Glutathione had also protected CAT, SOD and TAC in semen extender, which in turn helps to maintain the function of the membrane transportation (Alvarez and Storey 1992) and also fertility of the spermatozoa.

Glutathione prevents cholesterol efflux from the sperm plasma membrane and malondialdehyde production in semen extenders suggests it prevents premature acrosomal reaction and capacitation as acts as potential antioxidant (Asadpour *et al.* 2012). Along with phospholipids, cholesterol is important for sperm cell physical integrity and assures the fluidity of the cell membrane. Cholesterol is key component in sperm plasma membrane as its release from sperm plasma membrane initiates the important step in the capacitation process and acrosome reaction that are key role in the fertilization process (Witte and Schäfer-Somi 2007). Further addition of cholesterol into the diluents before defreezing process increases the capability of sperm to resist the stress caused by the freezing-defreezing process, which preserve the motility and fertilization potential of the spermatozoa (Moore *et al.* 2005). In our study, cholesterol efflux and LPO production were reduced in GSH treated than in untreated control group (Asadpour *et al.* 2012). Therefore GSH treated semen samples have higher cryoresistance power than the sperm of untreated control group. In our study, it was also observed that 10 mM of GSH treated sperm had significantly higher SQPs than those of the other treatment and control groups.

Intracellular enzymes such as AST and ALT in seminal plasma are required for sperm metabolism and function (Brooks 1990), provide energy for sperm survival, motility and fertility and these transaminase activities in semen are good indicators of semen quality as because they estimate the sperm membrane stability (Cortee 1980). Thus, higher percentage of abnormal spermatozoa in the cryopreservation causes high amount of transaminase enzyme in the extracellular fluid due to damage of sperm membrane and ease

of leakage of intracellular enzymes from spermatozoa (Gundogan 2006). Further, higher AST and ALT activities in seminal plasma and semen during the cryopreservation process are due to the sperm structural instability (Buckland 1971). In our study, concentration of intracellular enzyme was lower in cryopreserved semen with 10 mM GSH as it maintains the membrane integrity of acrosome, plasma membrane, mitochondria and flagella of the sperm. In our study, cholesterol efflux and malondialdehyde production were reduced in GSH treated group than those in untreated control group. Therefore the semen ejaculates treated with GSH had higher cryoresistance power than those in untreated control group.

In our study, concentration of catalase was significantly higher in glutathione treated semen. Generally, seminal plasma is a potent source of SOD (Kobayashi *et al.* 1991). Higher concentration of readily peroxidizable polyunsaturated material exposes the spermatozoa to excessive oxidative stress and the superoxide dismutase activity of sperm samples is a good predictor of their survival time. GSH @10 mM significantly enhanced the sperm motility during cryopreservation and exhibited the antioxidative characters, elevating the CAT level in association with GSH and TAC concentration.

The study concluded that GSH protects the sperm as it enhances the antioxidants level, prevents efflux of cholesterol and phospholipids from sperm cell membrane and lipid peroxide production. Thus it protected the spermatozoa during cryopreservation and enhanced the fertility in this 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.

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. pp. 8–18.
- Baumber J, Ball B A, Gravance C G, Medina V and Davies-Morel M C G. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *Journal of Andrology* **21**: 895–902.

- 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 H₂O₂-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.) G E. Lamming. 4th edn, Edinburgh, Churchill Livingstone, 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.
- Corteel J M. 1980. Effets du plasma séminal sur la survie et la fertilité des spermatozoïdes conservés *in vitro*. *Reproduction Nutrition Development* **20**(4): 1111–23.
- El-Kon I I and Darwish S A. 2011. Effect of glutathione (GSH) on microscopic parameters and DNA integrity in Egyptian buffalo semen during liquid and frozen storage. *Journal of Reproduction and Fertility* **2**(3): 32–40.
- 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.
- Gupta H P and Tripathi S S. 1984. Preservation of semen from Red Dane bull with different room temperature dilutors. *Indian Journal of Animal Sciences* **54**: 494–97.
- Halvorsen B, Holte K, Myhrstad M C W, Barikmo I, Havattum E, Remberg S F, Wold A B, Haffner K, Baugerod H, Andersen L F, Moskaug O, Jacobs D R and Blomhoff R. 2002. A systematic screening of total antioxidants in dietary plants. *American Society for Nutritional Sciences Journal of Nutrition* **132**: 461–471.
- 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, Perez-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, Chamuah J K, Nahak A K and Rajkhowa C. 2015. Effect of melatonin on the liquid storage (5°C) of semen with retrospective study of calving rate at different season in mithun (*Bos frontalis*). *Asian Pacific Journal of Reproduction* **4**(1): 1–12.
- Perumal P, Chamuah J K and Rajkhowa C. 2013. Effect of catalase on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Asian Pacific Journal of Reproduction* **2**(3): 209–14.
- Perumal P, Selvaraju S, Selvakumar S, Barik A K, Mohanty D N, Das R K, Das S and Mishra P C. 2011a. 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 Animals* **46**(4): 636–41.
- 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, Vupru K and Rajkhowa C. 2013. Effect of addition of taurine on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Veterinary Medicine International* **2013**: 1–7.
- Perumal P, Vupru K and Rajkhowa C. 2015. Effect of addition of trehalose on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Indian Journal of Animal Research* **49**(6): 837–46.
- Perumal P. 2014. Effect of superoxide dismutase on the liquid storage (5°C) of mithun (*Bos frontalis*) semen. *Journal of Animals* **2014**: 1–9.
- 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, 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, 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, Selvaraju S, Barik A K, Mohanty D N, Das S and Mishra P C. 2011b. Role of reduced glutathione in improving post-thawed frozen seminal characters of poor freezable Jersey crossbred bull semen. *Indian Journal of Animal Sciences* **81**(8): 807–10.
- 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, pp. 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.
- Witte T S and Schäfer-Somi S. 2007. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Animal Reproduction Science* **102**(3–4): 181–93.
- Wotten I D P. 1964. *Micro-analysis in Medical Biochemistry*. Fourth ed. J and A Churchill Ltd, London.