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Effect of reduced glutathione on the liquid storage (5°C) of mithun semen

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ABSTRACT

The present study was undertaken to assess effect of reduced glutathione (GSH) on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity, super oxide dismutase (SOD) and catalase, cholesterol efflux and malonaldehyde (MDA) production. Ejaculates (50) were collected twice a week from 8 mithun bulls and semen was split into 4 equal aliquots, diluted with the TEYC extender. Group 1: semen without additives (control), group 2 to group 4: semen was diluted with 5 mM, 10 mM and 15 mM of reduced glutathione (GSH), respectively. Inclusion of GSH into diluent resulted in significant decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal integrity at different hours of storage periods as compared with control group. Additionally, GSH at 5 and 15 mM were inferior to GSH 10 mM treatments as regards to these characteristics and GSH at 10 mM has significant improvement in quality of mithun semen in *in- vitro* stored for up to 30 h. It was concluded that the possible protective effects of GSH on sperm parameters are, it enhanced the function of antioxidant enzymes, prevented efflux of cholesterol from cell membrane, and reduced malonaldehyde (MDA) production during preservation.

Key words: Biochemical profiles, Enzymatic profiles, Mithun, Reduced glutathione (GSH), Seminal parameters

Mithun population is decreasing gradually due to lack of suitable breeding bulls, increase in intensive inbreeding practices, declining land area for grazing and lack of suitable breeding and feeding management in this region. Use of artificial insemination for improvement of its pedigree is utmost essential.

Cold storage of semen is used to maintain sperm viability over an extended period of time. During this extended storage period the quality of semen deteriorates due to action of reactive oxygen species (ROS) leading to irreversible loss in motility, damage to the sperm DNA and fertility (Maxwell and Watson 1996, Perumal *et al.* 2011a). Mithun semen normally contains anti-oxidants, including reduced glutathione (GSH), cysteine hydrochloride, catalase, superoxide dismutase (SOD) that can offset lipid peroxidation (Perumal *et al*. 2012 unpublished data). But the concentration of these antioxidants is reduced during dilution and storage that affect semen quality during storage in semen preservation. The addition of anti-oxidants such as GSH to equine sperm (Baumber *et al*. 2000), crossbred bull sperm (Perumal *et al*. 2011a, Perumal *et al*. 2011b), buffalo semen (El-Kon and Darwish 2011) protected sperm against harmful effects of ROS and improved sperm motility and membrane integrity during sperm liquid storage or in unfrozen state. Further, perusal of literature revealed no information on the effect of addition of anti-oxidant GSH, on maintenance of sperm viability during low temperature liquid storage of mithun semen. Hence, this study was conducted to assess the effect of additive GSH on the seminal parameters, biochemical and enzymatic profiles of mithun semen to pursuit future sperm preservation protocols.

MATERIALS AND METHODS

Apparently healthy mithun bulls (8), 4 to 6 year-old, weighing 501 kg (493 to 507 kg) with good body condition (score 5–6) were maintained under uniform feeding, housing and lighting conditions. Each experimental animal was fed in this experiment as per the farm schedule. Semen was collected from the animals through rectal massage method. During the study, all the experimental protocols met the Institute Animal Care and Use Committee regulations.

Ejaculates (50) were collected from mithuns twice a week and semen pooled to eliminate individual differences. Immediately after collection, the samples were kept in a

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water-bath at 37°C and evaluated for volume, colour, consistency, mass activity and pH. After the preliminary evaluations, samples were subjected to the initial dilution with pre-warmed (37°C) Tris egg yolk citrate extender (TEYC). The partially diluted samples were brought to laboratory in an insulated flask containing warm water (37°C) for further processing. The ejaculates were evaluated and accepted for evaluation if the following criteria were met: concentration: >500 million D ml; mass activity >3+, individual motility: >70% and total abnormality: <10%.

Each pooled ejaculate was split into 4 equal aliquots and diluted with the TEYC extender with GSH. Group 1: diluent without additives (control); group 2 to group 4: diluent with 5 mM, 10 mM and 15 mM of GSH, respectively. However, pH of diluents was adjusted to be 6.8 – 7.0 by using phosphate buffer solution. Diluted semen samples were kept in glass tubes and cooled from 37 to 5°C @ 0.2–0.3°C/min in a cold cabinet and maintained at 5°C during liquid storage for up to a 30 h period of the experiment. The percentage sperm motility, viability, total sperm abnormality, acrosomal integrity and the plasma membrane integrity by hypo-osmotic swelling test (HOST) were determined as per standard procedure in samples during storage of semen at 5°C for 0, 6, 12, 24 and 30 h, respectively. The SOD, catalase activity and cholesterol efflux of the seminal plasma were estimated by commercial kit. Lipid peroxidation level of preserved sperm cells was measured by determining the malonaldehyde (MDA) production, using thiobarbituric acid (TBA) as per Buege and Aust (1978) and modified by Suleiman *et al.* (1996).

The results were analysed statistically and expressed as the mean±SEM. Means were analyzed by analysis of variance, followed by the Tukey's post hoc test to determine significant differences between the 4 experimental groups i.e. with additives or no additive for 0, 6, 12, 24 and 30 h of storage on the sperm parameters using the SPSS/PC computer program. Differences with values of $P < 0.05$ were considered to be statistically significant after arcsine transformation of percentage data by using SPSS 15.

RESULTS AND DISCUSSION

The effects of various doses of GSH on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity at different hours of incubation in liquid storage (5°C) are presented in Figs 1– 5, respectively. Results revealed that the inclusion of GSH into diluent resulted in significant ($P < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal integrity when semen samples were examined at different hours of storage periods compared with control group. Additionally, GSH at 5 and 15 mM were inferior to GSH 10 mM treatments as regards to these characteristics, and there were significant differences between GSH at 5 and 15 mM in relation to these features. The antioxidant enzymatic profiles revealed that

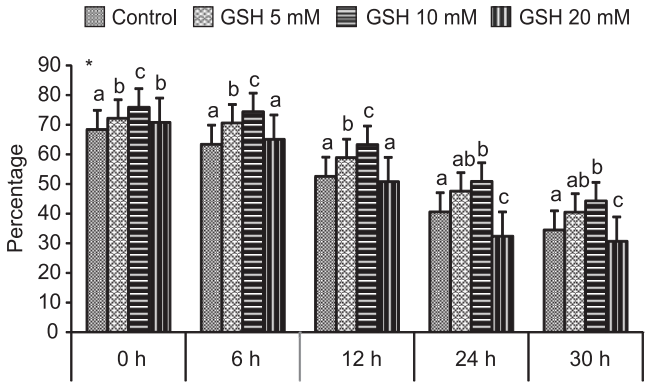

Fig. 1. Effect of diluents supplementation with reduced glutathione (GSH) on motility of spermatozoa of mithun semen (* indicates P< 0.05).

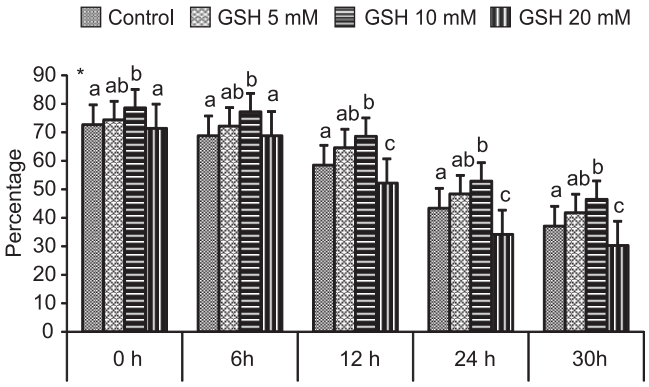

Fig. 2. Effect of diluents supplementation with reduced glutathione (GSH) on viability of spermatozoa of mithun semen (* indicates P< 0.05).

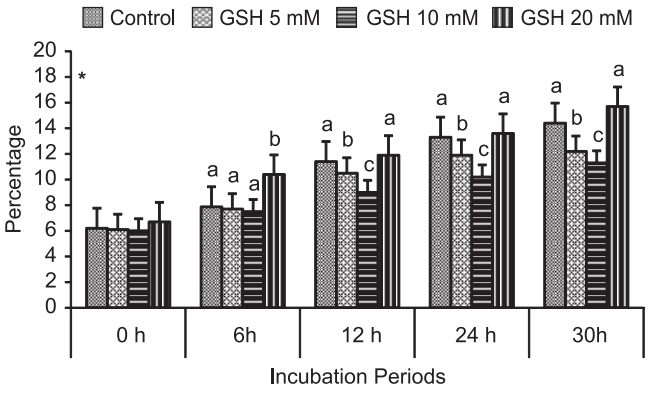

Fig. 3. Effect of diluents supplementation with reduced glutathione (GSH) on total sperm abnormality of spermatozoa of mithun semen (* indicates P< 0.05).

highest mean SOD (Fig. 6) and catalase (Fig. 7) activity were recorded in GSH treated semen than control group and significantly ($P<0.05$) differed between groups. Similarly cholesterol efflux (Fig. 8) and MDA production (Fig. 9) significantly differed between the GSH treated and control.

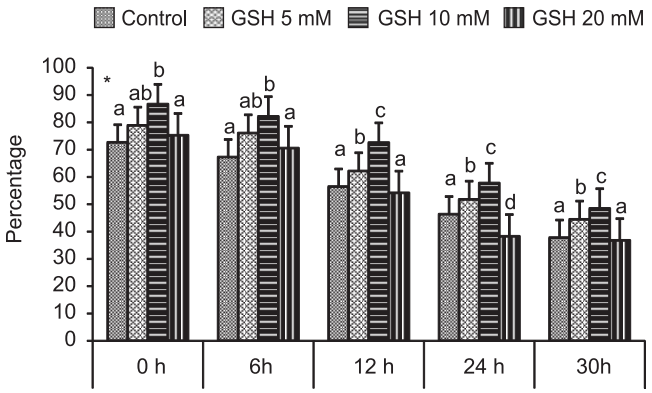

Fig. 4. Effect of diluents supplementation with reduced glutathione (GSH) on acrosomal integrity of spermatozoa of mithun semen (* indicates P< 0.05).

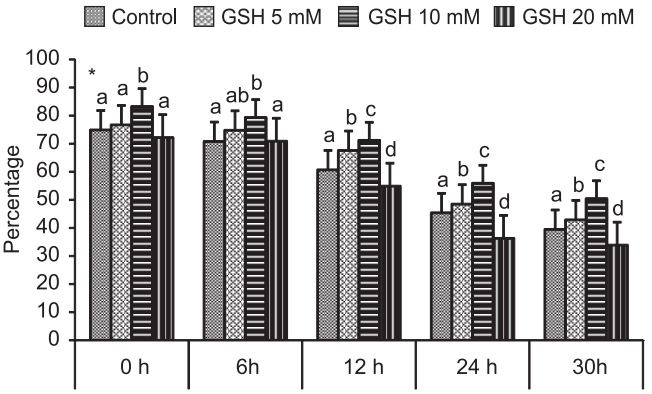

Fig. 5. Effect of diluents supplementation with reduced glutathione (GSH) on plasma membrane integrity (HOST) of spermatozoa of mithun semen (* indicates P< 0.05).

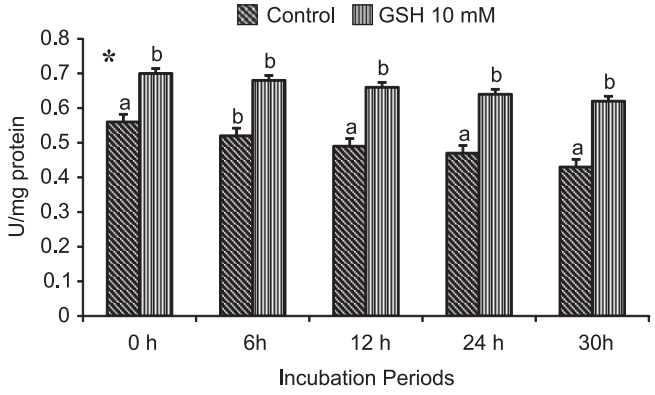

Fig. 6. Effect of diluents supplementation with reduced glutathione (GSH) on SOD activity of mithun semen (* indicates P< 0.05).

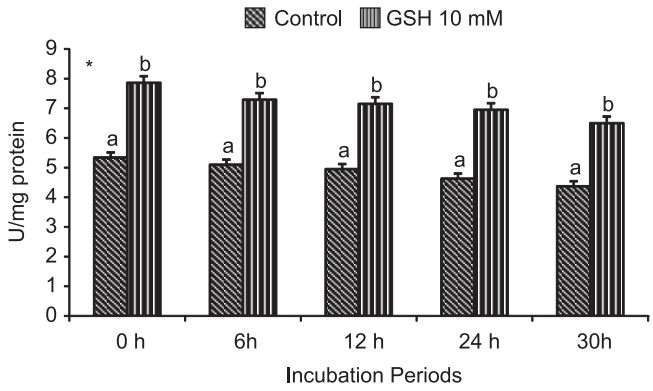

Fig. 7. Effect of diluents supplementation with reduced glutathione (GSH) on catalase activity of mithun semen (* indicates P< 0.05).

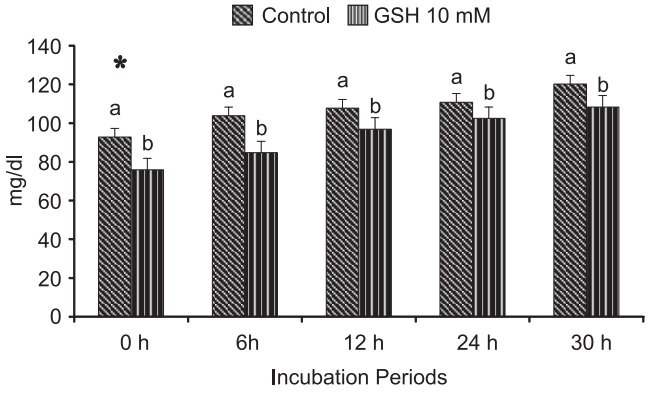

Fig. 8. Effect of diluents supplementation with reduced glutathione (GSH) on cholesterol efflux of spermatozoa of mithun semen (* indicates P< 0.05).

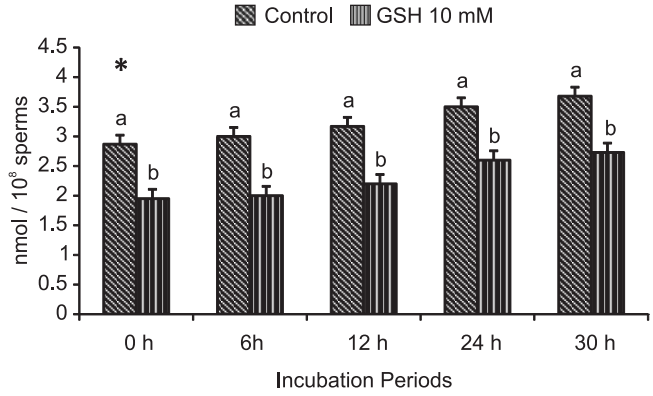

Fig. 9. Effect of diluents supplementation with reduced glutathione (GSH) on malonaldehyde (MDA) production in mithun semen (* indicates P< 0.05).

The data revealed that the addition of GSH especially at the concentrations of 10 mM to the semen diluent resulted in significant improvement in quality and antioxidant enzymatic activity and reduction of cholesterol efflux and MDA production of mithun semen in *in-vitro* stored for up to 30 h.

The results revealed that addition of GSH improved seminal parameters, enzymatic and biochemical profiles of mithun semen and thus it protects structures and functions

of spermatozoa efficiently. Thus, it may enhance the quality of semen by preserving efficiently during artificial insemination procedure.

There was no report on effect of addition of GSH on seminal parameters in mithun and to the best of our knowledge this is the first report of the effect of GSH on seminal parameters, antioxidative enzymatic level and biochemical profiles in mithun semen. Analysis of forward progressive motility, livability, acrosomal and plasma membrane integrity are important for extensive utilization of semen in artificial insemination. In the present study, GSH supplementation on these parameters revealed significant difference between the treatment groups. The beneficial effects of GSH in semen preservation are because of its being a very potent antioxidant (Perumal *et al.* 2011a).

Presence of high polyunsatured fatty acids in mammalian sperm membrane renders it very susceptible to LPO, which occurs due to oxidation of the membrane lipids by partially reduced oxygen molecules, such as superoxide, hydrogen peroxide, and hydroxyl radicals. Lipid peroxidation of sperm membrane ultimately leads to impairment of sperm function due to attacks by ROS, altered sperm motility and 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). Therefore, inclusion of exogenous antioxidants may modulate the antioxidant system of semen.

Our results showed that addition of 10 mM GSH improved the keeping quality of mithun semen preserved at 5°C. The sperm motility declined by the time of storage and remained over 50% for up to 30 h. In contrast, decline rate in the motility percentage was higher in semen samples treated with 20 mM GSH or without GSH. It was reported that the quality of chilled semen decreased with time and remained suitable for use up to 30 h as judged by motility and morphology (Urata *et al.* 2001). The improvement of semen quality due to addition of exogenous glutathione recorded in the present study was previously reported in bull semen in the form of motility and intact acrosomal membrane (Perumal *et al.* 2011a). Moreover, addition of exogenous GSH significantly improved percentages of sperm viability and intact plasma membrane (swelling tails) especially at a level of 10 mM GSH. The highest percentages of intact plasma and acrosomal membranes observed in the present experiment due to 10 mM glutathione may be the reason for better motility in these samples (Slaweta *et al.* 1987).

GSH helps maintaining integrity of normal acrosome (Sinha *et al*. 1996) and stabilize plasmalemma of spermatozoa and so increase motility. GSH, in sperm cells is able to react with many reactive oxygen species directly for protecting mammalian cells against oxidative stress, and hence maintaining sperm motility (Bilodeau *et al.* 2001). Therefore, as seen by this study, attempts to improve the motility and viability of the sperm cells by incorporating glutathione in liquid storage (Gupta and Tripathi 1984) and frozen semen form were investigated (Perumal *et al*. 2011a).

Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. GSH also protects SOD and catalase level in semen extender (Halvorsen *et al*. 2002), which helps to maintain membrane transportation (Alvarez and Storey 1992) and fertility of spermatozoa.

It also prevents efflux of cholesterol from the sperm membrane, and MDA production in diluents indicated that it prevented premature capacitation and acrosomal reaction as an antioxidant. Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane. Cholesterol plays a special role in sperm membrane because its release from sperm membrane initiates the key step in the process of capacitation and acrosome reaction that is crucial for fertilization (Witte and Schafer-Somi 2007). Moreover, adding cholesterol to diluents prior to defreezing increases sperm resistance to stress caused by the freezing-defreezing procedures, preserving sperm motility and fertilization potential (Moore *et al.* 2005). In the present study, the efflux of cholesterol and MDA production decreased in treated group as compared to the control untreated group. So semen samples treated with GSH will have high cryoresistance power than untreated control group. We observed that sperm parameters that received 10 mM of GSH were significantly higher than those of the other and control group.

In this study, improvements observed in sperm quality may be attributed to prevention of excessive generation of free radicals, produced by spermatozoa themselves, by means of their antioxidant property of GSH. It was concluded that the possible protective effects of GSH supplementation are—it enhances the antioxidant enzymes content, prevents efflux of cholesterol and phospholipids from cell membrane, and MDA production. Thus it may protect the spermatozoa during preservation and enhance the fertility in this species. Future, sperm preservation/cryoprotective studies are warranted to confirm the present findings.

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