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Effect of addition of trehalose on the liquid storage (5°C) of mithun (*Bos frontalis*) semen

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ABSTRACT

The present study was undertaken to assess the effect of trehalose on sperm parameters, enzymatic profiles and biochemical profiles of seminal plasma of mithun. The semen ejaculates were collected from mithun and was split into four groups as group 1: semen without additives (control), group 2 to 4: semen with 50 mM, 75 mM and 100 mM of trehalose, respectively in EYTC extender. These parameters were assessed at 5°C for 0, 6, 12, 24 and 30 h of incubation. Inclusion of trehalose resulted in significant ($p < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities as compared with control group. Additionally, trehalose at 50 mM has significant improvement in quality of mithun semen in *in- vitro* storage. It was due to the protective effects of trehalose on sperm parameters as sperm membrane stabilizer and prevents efflux of cholesterol, lipid peroxide formation and biochemical enzymes from sperm cell during preservation.

Key words: Biochemical profiles, Enzymatic profiles, Mithun, Seminal parameters, Trehalose.

INTRODUCTION

Mithun (*Bos frontalis*) is a semi-wild free–range, rare bovine species present in the North-Eastern hill (NEH) region of India. The animal has an important place in the social, cultural, religious and economic life of the tribal population particularly in this region. Recent statistics indicates that the mithun population is decreasing gradually due to lack of suitable breeding bulls, increase in intensive inbreeding practices and lack of suitable breeding and feeding management. Since mithuns are semi wild animal, natural breeding is practiced in this species with accompanied limitations like cost and disease transmission. Thus, use of artificial insemination for improvement of its pedigree is utmost essential.

Cold storage of semen is used to reduce metabolism and to maintain sperm viability over an extended period of time. But the quality of semen is deteriorated during this extended storage period. Researches into extender development have focused on the subject of membrane stabilizing compounds, antioxidants and cryoprotectants. Cause of this decline is due to formation and action of the ice crystals and reactive oxygen species (ROS) during preservation leads to cryocapacitation and premature capacitation and acrosomal reaction (Perumal *et al*., 2011a, Perumal *et al*., 2013). Mithun semen normally contains anti-oxidants that can offset lipid peroxidation and sugars like fructose, glucose as membrane stabilizer. But the concentration of these antioxidants and sugars is reduced during dilution and storage that affect the semen quality during storage. Further, perusal of literatures revealed no information on the effect this membrane stabilizer trehalose, on the sperm viability during low temperature liquid storage of mithun semen. Hence, the objective of this study was to assess the effect of this additive on the seminal parameters, biochemical and enzymatic profiles of mithun semen to pursuit future sperm preservation protocols.

MATERIALS AND METHODS

Eight apparently healthy mithun bulls of approximately 4 to 6 years of age were selected from the herd derived from various hilly tracts of the north eastern region of India. The average body weight of the bulls was 501 kg at 4 - 6 years of age with good body condition 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 collection, the initial transparent secretions were discarded and semen drops were collected in a graduated test tube with the help of a funnel. During the study, all the experimental

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protocols was met for the Institute Animal Care and Use Committee regulations.

Total numbers of 40 ejaculates were collected from the mithun twice a week and semen pooled to eliminate individual differences. Immediately after collection, the samples were kept in a 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 then brought to the 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 >2.5+, individual motility: >70% and total abnormality: <10%.

Each pooled ejaculate was split into four equal aliquots and diluted with the TEYC extender with trehalose. Group 1: semen without additives (control), group 2 to group 4: semen with 50 mM, 75 mM and 100 mM of trehalose, 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, at a rate of 0.2–0.3°C/min in a cold cabinet and maintained at 5°C during liquid storage for up to a 30 hours 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 hours, respectively. The AST, ALT, ALP, ACP activity and cholesterol efflux of the seminal plasma was estimated by commercial available kit. Lipid peroxidation level of sperm and seminal plasma was measured by determining the malonaldehyde (MDA) production, using thiobarbituric acid (TBA) as per the method of Buege and Aust (1978) and modified by Suleiman *et al.* (1996).

RESULTS AND DISCUSSION

The effects of various doses of trehalose on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity at different hours of incubation in liquid storage (5°C) were presented in Figure 1, 2,3,4 and 5, respectively. Results also revealed that the inclusion of trehalose into diluent resulted in significant ($p < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities when semen samples were examined at different hours of storage periods compared with control group. Additionally, trehalose at 75 and 100 mM were inferior to trehalose 50 mM treatments as regards to these characteristics, and there were significant differences between trehalose at 75 and 100 mM in relation to these features. The enzymatic profiles revealed that lowest mean AST (Figure 8) and ALT (Figure 9) activity was recorded in trehalose treated semen than control group and were significantly ($p<0.05$) differed between groups. But the enzymatic profiles such as ALP and ACP were significantly higher in trehalose treated semen than untreated control group (Figure 6 and 7). Similarly cholesterol efflux (Figure 10) and MDA production (Figure 11) were significantly differed between the trehalose treated and control. It was obvious from the data of this experiment that the addition of trehalose

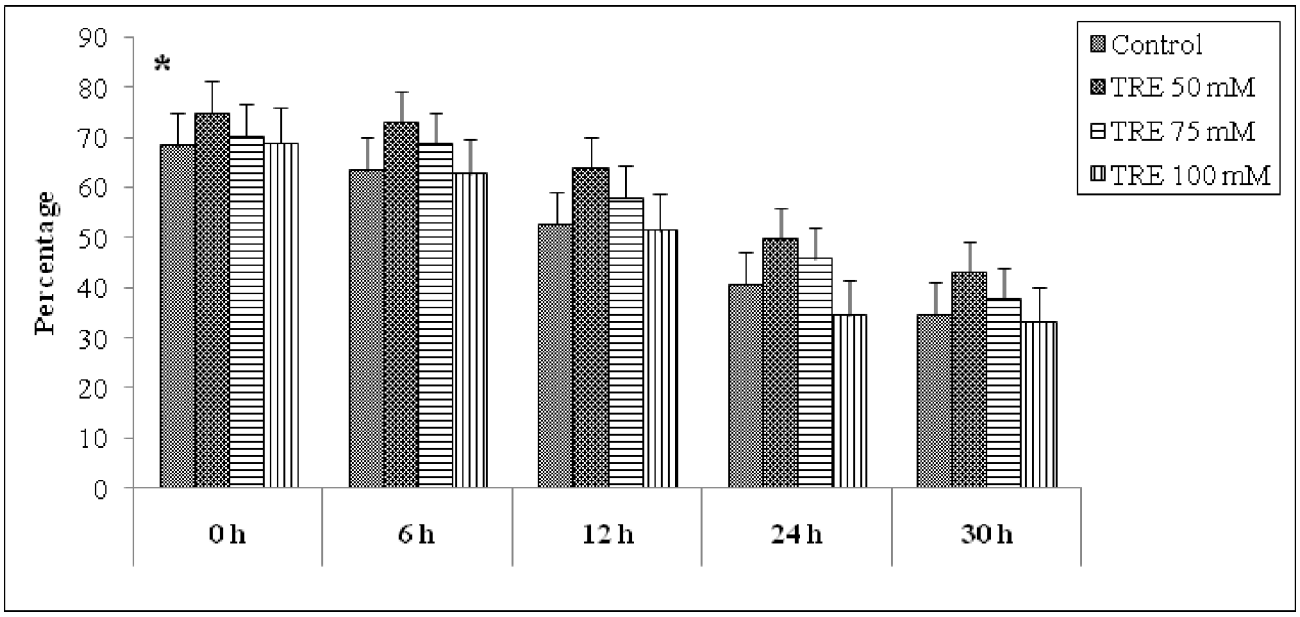

FIG 1: Effect of diluents supplementation with trehalose (TRE) on motility of spermatozoa of mithun semen (* indicates $p < 0.05$)

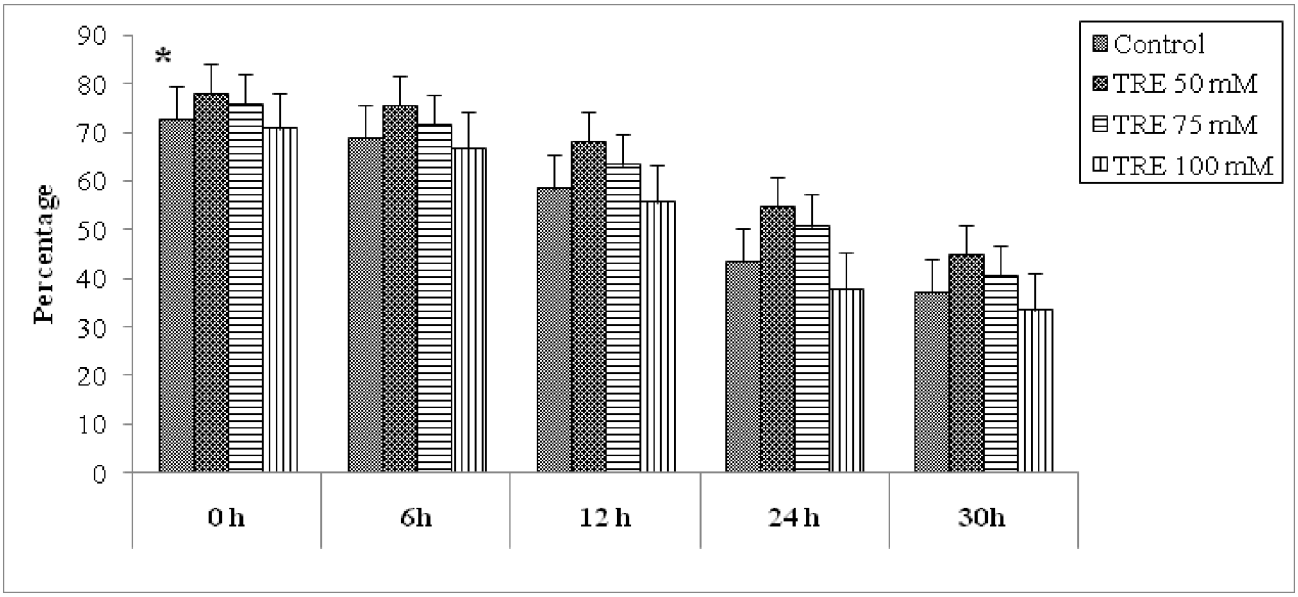

FIG 2: Effect of diluents supplementation with trehalose (TRE) on viability of spermatozoa of mithun semen (* indicates $p < 0.05$)

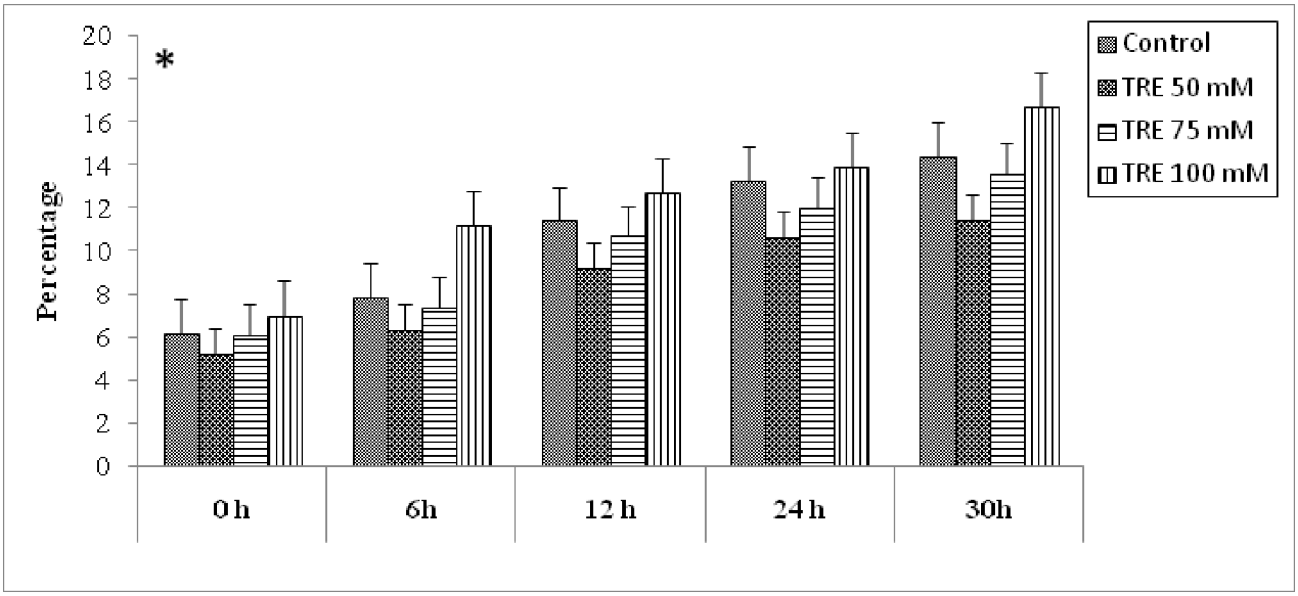

FIG 3: Effect of diluents supplementation with trehalose (TRE) on total sperm abnormality of spermatozoa of mithun semen (* indicates $p < 0.05$)

especially at the concentrations of 50 mM to the semen diluent resulted in significant improvement in quality and enzymatic activity and reduction of cholesterol efflux and MDA production of mithun semen in *in-vitro* stored for up to 30 h.

In the present study, the results revealed that addition of trehalose has improved the seminal parameters, enzymatic and biochemical profiles of mithun semen and thus it protects the 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 trehalose on seminal parameters in mithun and to the best of our knowledge this is the first report of the effect of trehalose on seminal parameters, enzymatic level and biochemical profiles in mithun semen. Analysis of various seminal parameters such as forward progressive motility, livability, acrosomal and plasma membrane integrity are important for extensive utilization of semen in artificial insemination. The beneficial effects of trehalose in semen preservation are due to it is a very potent membrane stabilizer (Chhillar *et al*., 2012).

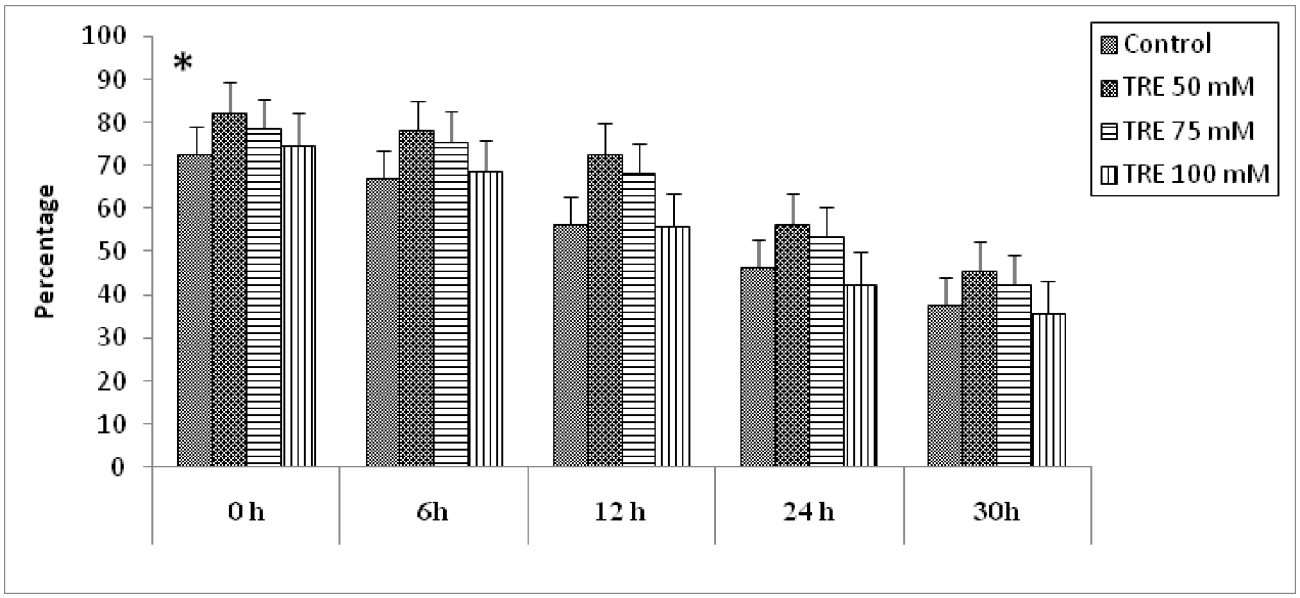

FIG 4: Effect of diluents supplementation with trehalose (TRE) on acrosomal integrity of spermatozoa of mithun semen (* indicates $p < 0.05$)

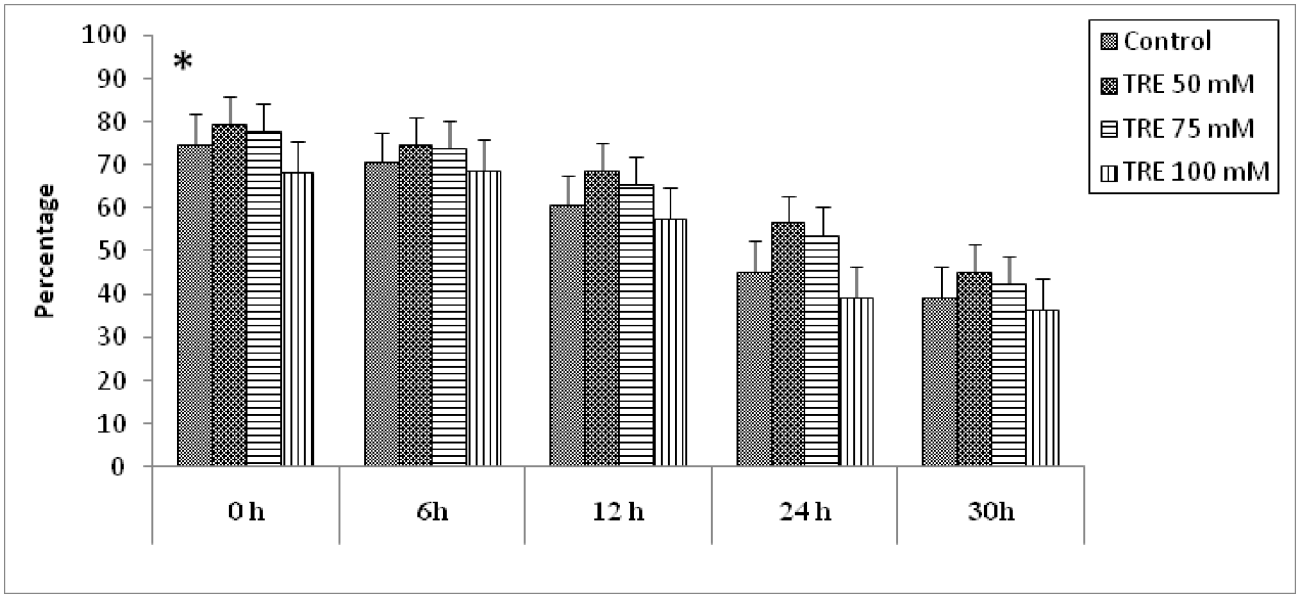

FIG 5: Effect of diluents supplementation with trehalose (TRE) on plasma membrane integrity (HOST) of spermatozoa of mithun semen (* indicates $p < 0.05$)

The present results show that supplementation of 50 mM trehalose to Tris–egg yolk extender prior to preservation led to significant ($P < 0.05$) increase in survival and quality parameters of Mithun spermatozoa. These results confirmed earlier reports of using this additive to freezing medium for improving quality parameters of buffalo (Reddy *et al*., 2010, Kumar and Atreja, 2011), goat (Aboagla and Terada, 2003) and boar (Hu *et al*., 2009) spermatozoa following cryopreservation. Trehalose, a disaccharide, acts as non-permeating cryoprotectant which causes dehydration of spermatozoa due to the osmotically driven flow of water. Due to this mild dehydration, spermatozoa have less intracellular water which results in reduced intracellular ice crystal formation. This is beneficial for sperm because intracellular ice crystal formation results in cell death and consequently reduced fertility of the cryopreserved semen. This could be one of the reasons for improved motility, viability, acrosomal and membrane integrity of spermatozoa, diluted in presence trehalose in the semen extender at different hours of incubation.

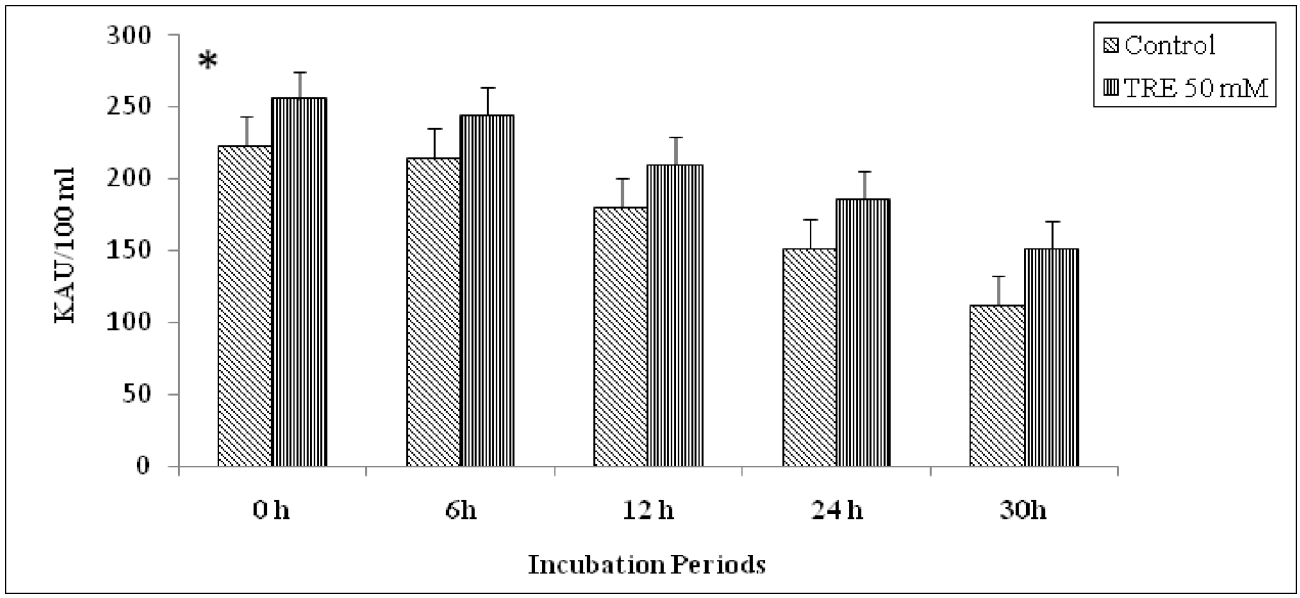

FIG 6: Effect of diluents supplementation with trehalose (TRE) on alkaline phosphatase (ALP) of mithun semen (* indicates $p < 0.05$)

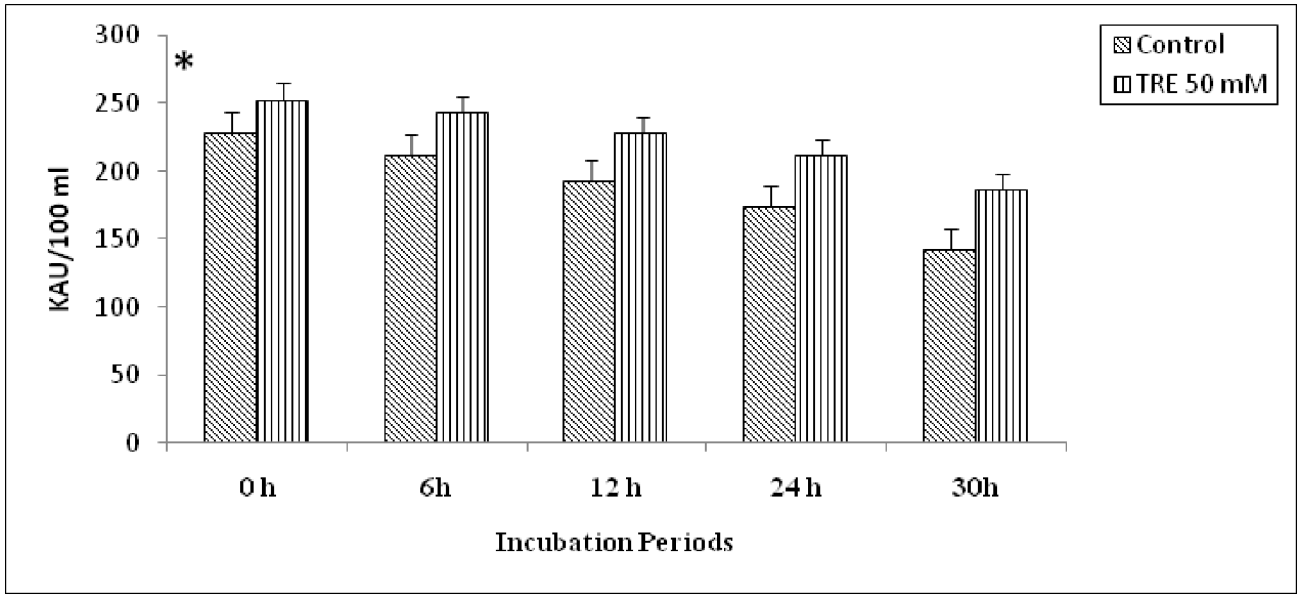

FIG 7: Effect of diluents supplementation with trehalose (TRE) on acid phosphatase (ACP) of mithun semen (* indicates $p < 0.05$)

Because of the mammalian sperm membrane has high polyunsatured fatty acids, 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. 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). Endogenous antioxidant defence mechanism neutralises harmful effect of these ROS (Jayaganthan *et al.*, 2013). However, when antioxidant mechanism exhaust, the surplus ROS contribute to oxidative stress in spermatozoa and causes lipid peroxidation (Aitken and Baker, 2004, McCarthy *et al.*, 2010, Perumal *et al.*, 2011b). In our study the rate of lipid peroxidation were also found to be significantly ($P < 0.05$) higher in untreated group than treated group. Upon supplementation of trehalose to the extender rate of lipid peroxidation were significantly ($P < 0.05$) decreased. Our results are in close agreement with Hu *et al*. (2010) where shown that supplementation of 50 mM

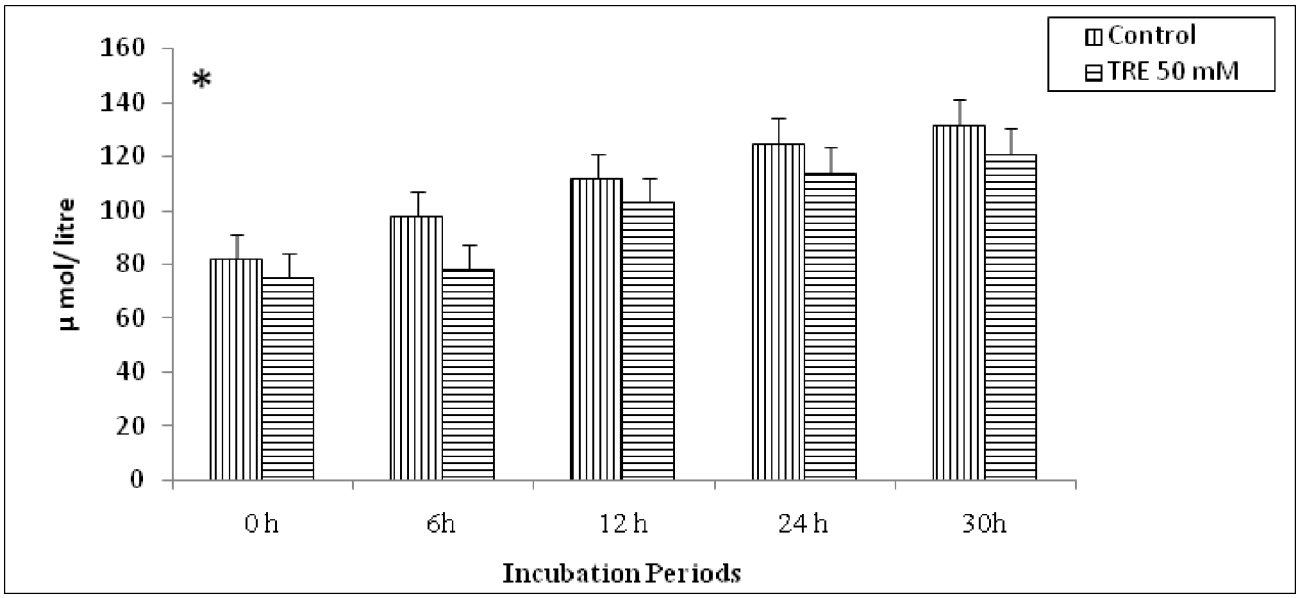

FIG 8: Effect of diluents supplementation with trehalose (TRE) on aspartate amino transaminase (AST) of mithun semen (* indicates $p < 0.05$)

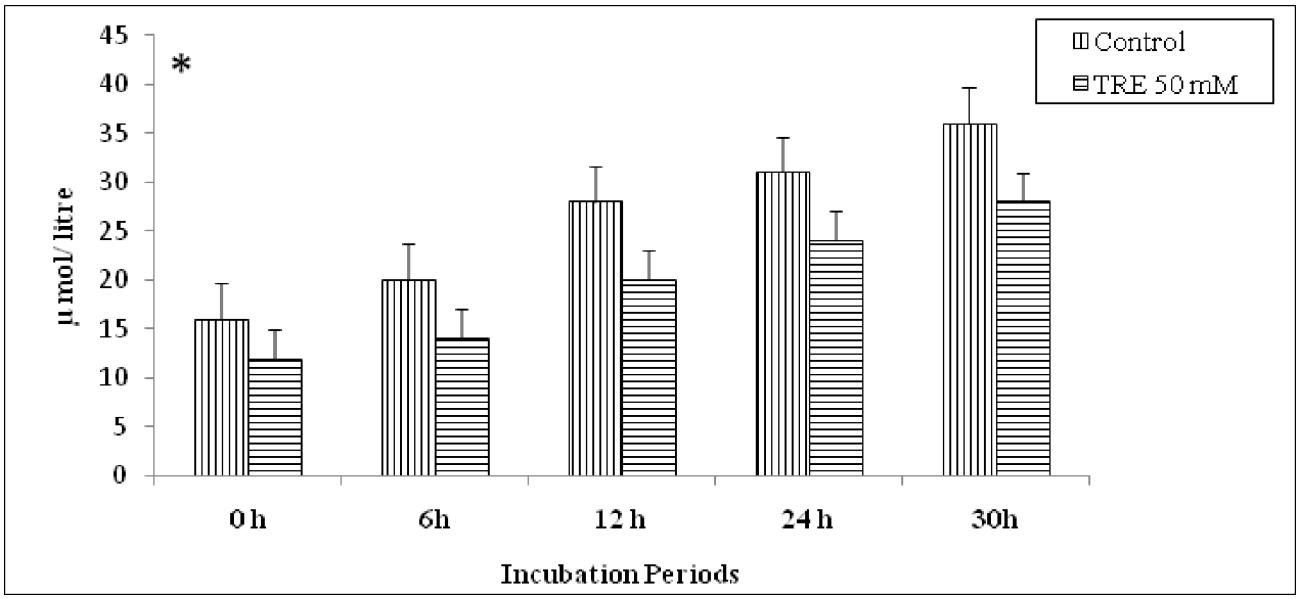

FIG 9: Effect of diluents supplementation with trehalose (TRE) on alanine amino transaminase (ALT) of mithun semen (* indicates $p < 0.05$)

trehalose in egg yolk based extender improves sperm quality and oxidative stress parameters in liquid storage of mithun semen. In addition, the membrane stabilizer system like disaccharide sugars of seminal plasma and spermatozoa is compromised during semen processing (Alvarez and Storey, 1992). Therefore, inclusion of sugars like trehalose exogenously may modulate the membrane structure help to preserve the mithun semen effectively.

The enzyme levels of seminal plasma are very important for sperm metabolism as well as sperm function (Brooks, 1990). Therefore, estimates of these enzymes have been recommended as markers for semen quality since they indicate sperm damage (Pesch *et al*., 2006). AKP in seminal plasma is primarily of testicular and epididymal origin and can be used as a clinical ejaculatory marker to differentiate azoospermia or oligospermia from ejaculatory failure (Turner and McDonnell, 2003). In the present study, the AKP were significantly higher in the semen of trehalose treated than control group because this sugar has maintained the membrane stability as this enzyme have high positive

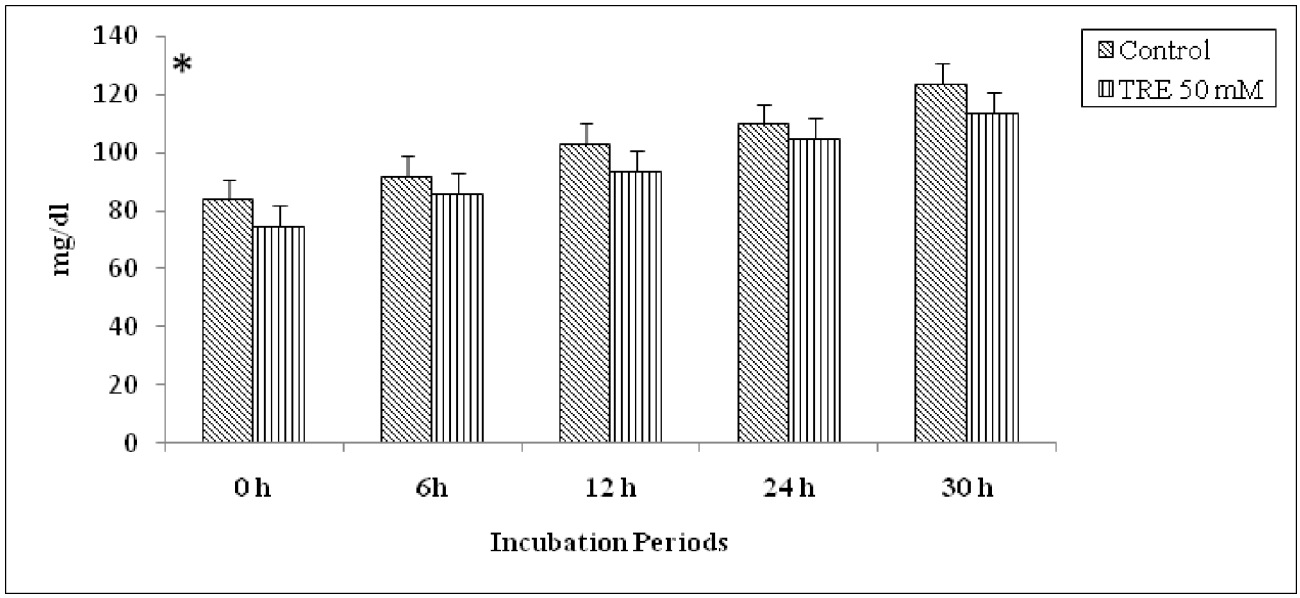

FIG 10: Effect of diluents supplementation with trehalose on cholesterol concentration of seminal plasma of mithun (* indicates $p < 0.05$)

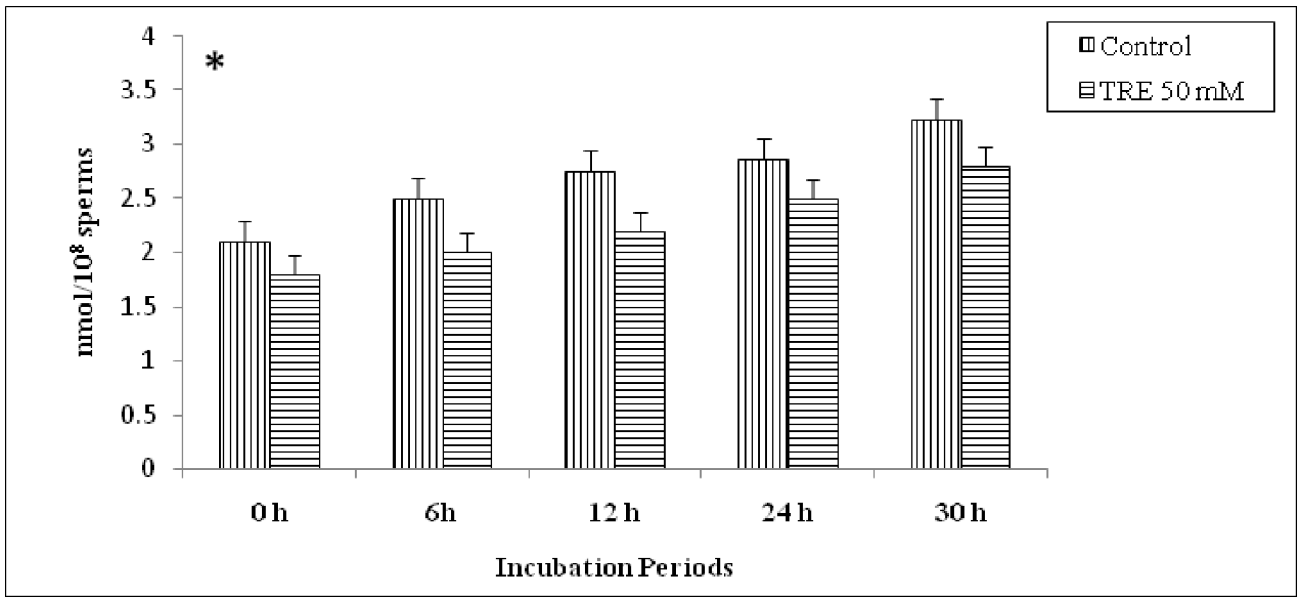

FIG 11. Effect of diluents supplementation with trehalose (TRE) on malonaldehyde (MDA) production of spermatozoa of mithun semen (* indicates $p < 0.05$)

correlation with semen quality, antioxidant content in the seminal plasma and negative correlation with ROS and free radical stress (Ciereszko *et al*., 1992). ACP is especially localized in corpus epididymidis, ductus epididymidis and vas deferens, but it is thought to be an indicator for the secretory function of prostate (López *et al*., 1989). The ACP concentration was higher in trehalose treated group than control because it has a positive correlation with semen concentration (Dogan *et al*., 2009).

Likewise, 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 sperm membrane stability (Corteel, 1980). Thus, increasing the percentage of abnormal spermatozoa in ejaculate causes high concentration of transaminase enzyme in the extra cellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa

(Gundogan, 2006). Moreover, increase in AST and ALT activities of seminal plasma and semen during storage may be due to structural instability of the sperm (Buckland, 1971). In the present study, AST and ALT levels were lowered as compared to control indicates that the trehalose maintained the membrane integrity of acrosome, plasma, mitochondria and flagella of the sperm.

The results of the present study showed that addition of 50 mM trehalose improve the keeping quality of mithun semen presented at 5°C. The sperm motility was declined by the time of storage and remained over 50% for up to 30 hours. In contrast, decline rate in the motility percentage was higher in semen samples treated with 100 mM trehalose or without trehalose. Favourable effect of the trehalose on sperm parameters at reduced concentration and deleterious effect at greater concentration was observed as in boar semen preservation (Hu *et al*., 2009). The functional integrity of sperm acrosomal membrane and plasma membrane associated with sperm motility can be expected to have been destroyed by large doses of trehalose. In the current study, the greatest protective effects of trehalose were at the concentration of 50 mM, and a much reduced extent at 100 mM. The latter concentration resulted in increased osmolarity of the extender was in itself deleterious to the sperm cells (Hu *et al*., 2009). When trehalose concentration was 100 mM, the percentages of linear motile sperm, intact acrosomal membrane, and intact-plasma membrane sperm of cold stored bovine semen were decreased. As demonstrated in previous studies, antioxidant additives 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 function *viz.* sperm motility, membrane integrity and fertility (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 to the membrane during reduced water states (Aboagla and Terada, 2003). Furthermore, Liu *et al*. (1998) and Aboagla and Terada (2003) hypothesized that trehalose penetrate into the plasma membrane of the spermatozoa and form hydrogen bonds with the polar head groups of phospholipids. Thereby, they also create an osmotic pressure, inducing cell dehydration, increased membrane fluidity and a lower incidence of intracellular ice formation (Molinia *et al*., 1994, Aisen *et al*., 2002). However, the cryoprotective ability of sugars on sperm may depend on their molecular weight (Molinia *et al*., 1994) and the type of buffer used (Abdelhakeam *et al*., 1991). It has been reported that the quality of chilled semen decreased with time and remained suitable for use up to 30 hours as judged by motility and morphology (Urata *et al*., 2001). The improvement of semen quality due to addition of exogenous trehalose recorded in the present study was previously reported in bull semen in the form of motility and intact acrosomal membrane (Chhillar *et al*., 2012). Moreover, the addition of exogenous trehalose was significantly improving the percentages of sperm viability and intact plasma membrane (swelling tails) especially at a level of 50 mM trehalose. The highest percentages of intact plasma and acrosomal membranes which were found in the present experiment due to 50 mM trehalose may be the reason for better motility in these samples (Chhillar *et al*., 2012).

It also prevents efflux of cholesterol from the sperm membrane and MDA production in diluents indicates it prevents premature capacitation and acrosomal reaction as act as membrane stabilizer. Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane. Cholesterol plays a special role in the sperm membrane because its release from the sperm membrane initiates the key step in the process of capacitation and acrosome reaction that is crucial for fertilization (Witte and Schafer-Somi, 2007, Srivastava *et al*., 2013). Moreover, adding cholesterol to diluents prior to defreezing increases sperm resistance to stress caused by the freezing-defreezing procedures, preserving sperm motility and fertilization potential (Moore *et al*., 2005). In the present study, the efflux of cholesterol and MDA production were decreased in treated group as compared to the control untreated group. So the semen samples treated with trehalose will have high cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that received at 50 mM of trehalose 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 ice crystal generation and formation of protective coat over the sperm membrane by means of their membrane stabilizer property of trehalose. It was concluded that the possible protective effects of trehalose supplementation are it maintains membrane structure of sperm and preventing efflux of cholesterol and phospholipids from cell membrane, intracellular enzymes and MDA production. Thus it may protect the spermatozoa during preservation and enhancing the fertility in mithun species. Future, sperm preservation/cryoprotective studies is warranted to confirm the present findings.

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