Hindawi Publishing Corporation
Journal of Animals
Volume 2014, Article ID 821954, 9 pages
http://dx.doi.org/10.1155/2014/821954

Research Article

Effect of Superoxide Dismutase on Semen Parameters and Antioxidant Enzyme Activities of Liquid Stored (5°C) Mithun (*Bos frontalis*) Semen

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Received 15 November 2013; Revised 28 January 2014; Accepted 8 February 2014; Published 13 March 2014

Academic Editor: Mustafa N. Bucak

The present study was undertaken to assess the effect of superoxide dismutase (SOD) on sperm motility, and viability; total sperm abnormality; acrosomal and plasma membrane integrity; DNA abnormality; antioxidant profiles such as catalase (CAT), reduced glutathione (GSH), and total antioxidant capacity (TAC); enzymatic profiles such as aspartate amino transaminase (AST), and alanine amino transaminase (ALT); and biochemical profiles such as malondialdehyde (MDA) production and cholesterol efflux. Total numbers of 50 ejaculates were collected twice a week from eight mithun bulls and semen was split into four equal aliquots, diluted with the TEYC extender. Group 1: semen without additives (control), and group 2 to group 4: semen was diluted with 50 U/mL, 100 U/mL, and 150 U/mL of SOD, respectively. These seminal parameters, antioxidant, enzymatic, and biochemical profiles were assessed at 5°C for 1, 6, 12, 24, and 30 h of incubation. Inclusion of SOD into diluent resulted in significant ($P < 0.05$) decrease in percentages of dead spermatozoa, abnormal spermatozoa, and acrosomal abnormalities at different hours of storage periods as compared with control group. Additionally, SOD at 100 U/mL has significant improvement in quality of mithun semen than SOD at 50 or 150 U/mL stored in *in-vitro* for up to 30 h. It was concluded that the possible protective effects of SOD on sperm parameters are that it prevents MDA production and preserves the antioxidants and intracellular enzymes during preservation.

1. Introduction

Mithun (*Bos frontalis*) is a semiwild free-range, rare bovine species present in the North-Eastern Hill (NEH) region of India. It is believed to have originated more than 8000 years ago from wild Indian gaur (*Bos gaurus*) [1]. The animal has an important place in the social, cultural, religious, and economic life of the tribal population particularly in the states of Arunachal Pradesh, Nagaland, Manipur, and Mizoram. Recent statistics indicates that the 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 NEH region. Greater efforts are required from all quarters to preserve the mithun population to enhance the socioeconomic status of this region. Since mithuns are semiwild animals and not fully domesticated, natural breeding is practiced in this species with accompanied limitations like cost and disease transmission. Thus, use of AI 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. One cause of this decline is due to the action of the reactive oxygen species (ROS) generated by the cellular components of semen, abnormal spermatozoa, and by neutrophils, namely, a superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) [2, 3] as the sperm membrane has high polyunsaturated fatty acids (PUFA). It results in the inhibition of both sperm ATP production and sperm movement, particularly forward progression [4]. The effects of lipid peroxidation include irreversible a loss in motility, damage to the sperm DNA, and fertility [2, 5]. Glutathione,

glutathione peroxidase, reduced glutathione, catalase, superoxide dismutase, vitamin C, vitamin E, and melatonin are the major antioxidants naturally present in mammalian semen against ROS to protect the sperm from lipid peroxidation and to maintain its integrity [6–11]. The levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules [9, 12]. Natural and synthetic antioxidant systems have been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen [13, 14]. Thus, supplementation with natural antioxidants or synthetic antioxidants could reduce the impact of oxidative stress during the sperm storage process and thus improve the quality of chilled semen [15–17].

Superoxide dismutase (SOD) is an antioxidant that catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. It scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. SOD spontaneously dismutase (O_2^-) anion to form O_2 and H_2O_2. SOD also prevents premature hyperactivation and capacitation induced by superoxide radicals before ejaculating [18].

The addition of antioxidants such as SOD to equine sperm [19], bull sperm [20], and buffalo semen [21] has been shown to protect sperm against the harmful effects of ROS and improve sperm motility and membrane integrity during sperm liquid storage or in the unfrozen state.

Further, perusal of literatures revealed that no information and to the best of our knowledge this is the first report of the effect of SOD on seminal parameters, antioxidative enzymatic level, and biochemical profiles in mithun semen. Hence, the objective of this study was to assess the effect of this additive on the seminal parameters and biochemical and enzymatic profiles of mithun semen to pursue future sperm preservation protocols.

2. Material and Methods

2.1. Experimental Animals. Eight apparently healthy mithun bulls of approximately 4 to 6 yr of age were selected from the herd derived from various hilly tracts of the NEH region of India. The average body weight of the bulls was 501 kg (493 to 507 kg) at 4–6 yr of age with good body condition (score 5-6) maintained under uniform feeding, housing, and lighting conditions. Each experimental animal was fed in this experiment as per the farm schedule. Each experimental animal was daily offered ad libitum drinking water, 30 kg mixed jungle forages (18.4% dry matter and 10.2% crude protein), and 4 kg concentrates (87.1% dry matter and 14.5% crude protein) fortified with mineral mixture and salt. Semen was collected from the animals through rectal massage method [22]. During the study, all the experimental protocols met the Institutional Animal Care and Use Committee regulations.

2.2. Semen Collection and Processing. Total numbers of 58 ejaculates were collected from the eight mithun bulls twice a week with the success rate of 90.62%, of which 86.20% ejaculates (50 ejaculates) were used for semen processing and analysis. Semen samples were pooled to eliminate individual differences and individual ejaculate volume of semen was between 0.8 and 1.3 mL and was not sufficient to analyse the seminal parameters and measure the biochemical profiles. Moreover during collection, the initial and end transparent secretions were discarded and only good quality neat semen drops were collected in a graduated test tube with the help of a funnel. Immediately after collection, the samples were kept in a water bath at 37°C and routine preliminary evaluations were measured. After the preliminary evaluations, samples were subjected to the initial dilution with prewarmed (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/mL; mass activity >3+, individual motility >70%, and total abnormality <10%.

Each pooled ejaculate was split into four equal aliquots and diluted with the TEYC extender with SOD (SRL, Mumbai, India). Group 1: semen without additives (control) and group 2 to group 4: semen with 50 U/mL, 100 U/mL, and 150 U/mL of SOD, respectively [17]. 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 h period of the experiment. The percentage of sperm motility and viability, total sperm abnormality, acrosomal integrity, the plasma membrane integrity by hypoosmotic swelling test (HOST), and DNA integrity by Feulgen staining technique [23] was determined as per standard procedure in samples during storage of semen at 5°C for 1, 6, 12, 24, and 30 h, respectively.

Sperm motility was assessed by analyzing four to five fields of view of sample placed on a prewarmed slide (37°C) under prewarmed cover slip (37°C) using bright-field optics (Nikon, Eclipse 80i; magnification 400x). Before determination of progressive motility, the stored samples were warmed in a water bath at 37°C for 5 min [24].

The count of live spermatozoa was determined using eosin-nigrosin stain [5% (w/v) nigrosin water soluble, 0.6% (w/v) eosin yellow water soluble, and 3% sodium citrate dihydrate; filtered and pH adjusted to 7.0 by adding few drops of 0.1 M NaH_2PO_4 or 0.1 M Na_2HPO_4] according to a previously described method using bright-field optics (Nikon, Eclipse 80i; magnification 1000x) [24]. Spermatozoa (eosin-nigrosin stained; 200 per sample) were also evaluated under bright-field optics (Nikon, Eclipse 80i; magnification 1000x) for morphological abnormalities [24]. Acrosomal integrity was assessed by Giemsa staining as described by Watson [25].

The HOST was used as a complementary test to the viability assessment protocol to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to loss of permeability under stress condition of swelling in a hypoosmotic medium [26]. Sperm cells with resistant membranes exhibited swelling around the tail such that the flagella becomes curled and the membrane maintained a swollen bubble around the curled flagellum. The assay was performed by mixing 30 μL of semen with

a 300 μL 100 mOsm/kg hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) [27]. This mixture was incubated (37°C) for 1 h, and 0.2 mL of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated (Nikon, Eclipse 80i; 400x magnification) under a phase-contrast microscope. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

DNA abnormality of sperm was examined by Feulgen staining technique. Semen smears were made at the different hours of incubation and stained by the Feulgen technique [23]. Briefly, the smear was prepared and allowed it to air dry for at least 1 hour, fixed in 10 percent neutral buffered formal saline for 15 minutes and washed in running water for 10 minutes. The smear was hydrolyzed in 5 N HCL for 30 minutes, washed in running water for 5 minutes, and dipped in Schiff's reagent for 30 minutes. The slides were rinsed in sulfate water for 2 minutes and the same was repeated for three times. They were washed in running water for 10 minutes and dried in air and examined at ×1000 under phase-contrast microscope and the percentage of normal and abnormal staining spermatozoa was determined by counting at least 200 cells per sample. Sperm abnormalities found were classified into six categories: pyriform heads, giant-rolled-crested heads, pale staining cells, multiple vacuoles, single vacuoles, and clumped nuclear material [28]. Only spermatozoa with clumped nuclear material were classified as abnormally condensed for further comparisons.

2.3. Biochemical Assays

2.3.1. Lipid Peroxidation Assay. Lipid peroxidation level of sperm and seminal plasma was measured by determining the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) as per the method of Buege and Aust [29] and modified by Suleiman et al. [30]. The semen sample from the glass tube was taken and centrifuged at 3000 rpm for 15 min and the seminal plasma was removed. Then the sperm pellet was resuspended in 2 mL of PBS (pH 7.2) or a variable volume to obtain a sperm concentration of 20×10^6/mL. Lipid peroxide levels were measured in spermatozoa after the addition of 2 mL of TBA-TCA reagent (15% w/v TCA, 0.375% w/v TBA and 0.25 N HCL) to 1 mL of sperm suspension. The mixture was treated in a boiling water bath for 1 hour. After cooling, the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then separated, and absorbance was measured at 535 nm. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 μmol/cm^3).

Consider

$$\text{MDA produced}\left(\frac{\mu\text{mol}}{\text{mL}}\right) = \frac{\text{OD} \times 10^6 \times \text{Total volume (3 mL)}}{1.56 \times 10^5 \times \text{Test volume (1 mL)}} = \frac{\text{OD} \times 30}{1.56}. \quad (1)$$

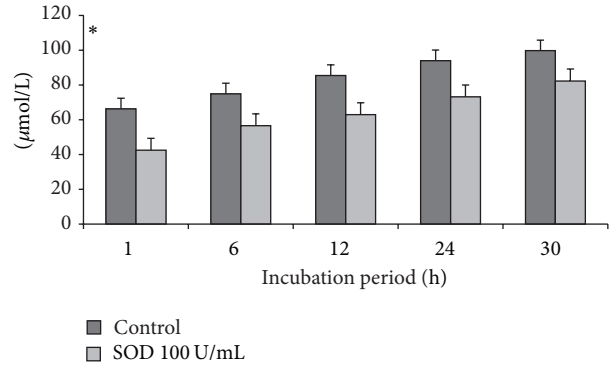

Figure 1: AST production in the extended mithun semen containing additive at different storage periods (*indicates $P < 0.05$).

2.3.2. Biochemical Profiles. The antioxidant profiles such as CAT, GSH, and TAC; intracellular enzymes such as AST and ALT activity; and cholesterol efflux of the seminal plasma were estimated by commercial available kit.

2.4. Statistical Analysis. The results were analysed statistically and expressed as the mean ± S.E.M. Means were analyzed by analysis of variance, followed by the Tukey's post hoc test to determine significant differences between the four experimental groups, that is, with additives or no additive for 1, 6, 12, 24, and 30 h of storage on the sperm parameters using the SPSS/PC computer program (version 15.0; SPSS, Chicago, IL). Differences with values of $P < 0.05$ were considered to be statistically significant after arcsine transformation of percentage data by using SPSS 15.

3. Results

The effects of various doses of SOD on sperm motility (Table 1) and viability (Table 2), total sperm abnormality (Table 3), acrosomal (Table 4), plasma membrane (Table 5) integrity, and DNA integrity (Table 6) at different hours of incubation in liquid storage (5°C) were presented. Results also revealed that the inclusion of SOD 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, SOD at 50 and 150 U/mL were inferior to SOD 100 U/mL treatments with regard to these characteristics, and there were significant differences between SOD at 50 and 150 U/mL in relation to these features. The enzymatic profiles revealed that lowest mean AST (Figure 1) and ALT (Figure 2) were recorded in SOD (100 U/mL) treated semen than control group and were significantly ($P < 0.05$) differed between groups. Similarly MDA production (Figure 3) and cholesterol efflux (Figure 4) were significantly differed between the SOD (100 U/mL) treated and control untreated group. Antioxidant profiles revealed significantly ($P < 0.05$) higher CAT (Figure 5), GSH (Figure 6), and TAC (Figure 7) in SOD (100 U/mL) treated semen than control group. It was

Table 1: Mean (±S.E.) motility percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage time				
	1 h	6 h	12 h	24 h	30 h
Control	68.27 ± 1.76^{aE}	64.00 ± 1.73^{aD}	54.16 ± 1.67^{aC}	41.36 ± 1.44^{bB}	34.72 ± 1.56^{bA}
SOD 50 U/mL	73.17 ± 2.24^{bcC}	68.27 ± 2.57^{abC}	59.23 ± 2.23^{bB}	45.89 ± 1.68^{cA}	40.18 ± 1.37^{cA}
SOD 100 U/mL	76.33 ± 1.57^{cE}	72.16 ± 1.74^{bD}	65.58 ± 1.83^{cC}	52.66 ± 1.52^{dB}	44.56 ± 1.42^{dA}
SOD 150 U/mL	71.29 ± 1.36^{abE}	65.88 ± 1.33^{aD}	50.92 ± 1.74^{aC}	33.28 ± 1.74^{aB}	29.50 ± 1.90^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

Table 2: Mean (±S.E.) viable sperm percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage time				
	1 h	6 h	12 h	24 h	30 h
Control	73.03 ± 1.69^{aE}	66.44 ± 1.57^{aD}	55.45 ± 2.00^{aC}	43.36 ± 2.13^{bB}	37.40 ± 1.91^{bA}
SOD 50 U/mL	74.35 ± 1.79^{aC}	71.25 ± 1.68^{bC}	63.94 ± 1.41^{bB}	44.11 ± 1.60^{bA}	40.87 ± 1.53^{cA}
SOD 100 U/mL	78.07 ± 1.44^{bD}	75.21 ± 1.40^{cD}	68.19 ± 1.61^{cC}	58.01 ± 1.87^{cB}	47.22 ± 1.60^{dA}
SOD 150 U/mL	72.70 ± 1.31^{aC}	67.88 ± 1.33^{aD}	56.99 ± 2.19^{aC}	36.80 ± 1.47^{aB}	32.21 ± 1.55^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

Table 3: Mean (±S.E.) total abnormal sperm percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage time				
	1 h	6 h	12 h	24 h	30 h
Control	6.16 ± 0.70^{abA}	7.75 ± 0.91^{bA}	11.41 ± 1.22^{cB}	12.92 ± 1.25^{cBC}	14.56 ± 1.30^{cC}
SOD 50 U/mL	5.99 ± 0.97^{aA}	6.87 ± 0.88^{aA}	9.52 ± 1.29^{bB}	10.94 ± 1.35^{bBC}	12.00 ± 1.28^{bC}
SOD 100 U/mL	5.59 ± 0.85^{aA}	6.76 ± 0.88^{aB}	7.78 ± 0.78^{aB}	9.06 ± 1.12^{aC}	10.13 ± 1.23^{aC}
SOD 150 U/mL	6.96 ± 0.70^{bA}	8.85 ± 0.96^{cB}	10.05 ± 0.89^{bcC}	11.70 ± 1.09^{bcD}	14.81 ± 0.99^{cE}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

Table 4: Mean (±S.E.) Acrosomal integrity (%) in semen of mithun for different storage times at 5°C.

Additives	Storage time				
	1 h	6 h	12 h	24 h	30 h
Control	73.62 ± 1.99^{aE}	66.31 ± 1.91^{aD}	57.08 ± 1.74^{aC}	43.83 ± 1.83^{bB}	36.03 ± 1.87^{aA}
SOD 50 U/mL	79.42 ± 1.57^{bE}	74.27 ± 1.70^{bD}	66.22 ± 1.61^{bC}	56.79 ± 1.96^{cB}	43.77 ± 1.43^{bA}
SOD 100 U/mL	83.71 ± 1.76^{cD}	80.80 ± 1.77^{cD}	74.65 ± 1.20^{cC}	57.31 ± 1.72^{cB}	47.17 ± 1.57^{cA}
SOD 150 U/mL	75.25 ± 1.72^{aD}	70.93 ± 1.80^{bC}	57.36 ± 1.74^{aB}	39.34 ± 1.54^{aA}	36.05 ± 1.46^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

Table 5: Plasma membrane integrity (HOST) (±S.E.) (%) for extended mithun semen containing additive at different storage times at 5°C.

Additives	Storage iime				
	1 h	6 h	12 h	24 h	30 h
Control	74.61 ± 1.41^{abE}	68.25 ± 1.51^{aD}	59.27 ± 1.84^{bC}	43.35 ± 2.33^{bB}	35.35 ± 2.19^{aA}
SOD 50 U/mL	77.54 ± 1.79^{bD}	74.17 ± 1.97^{bD}	65.13 ± 1.83^{cC}	45.90 ± 1.68^{bB}	41.55 ± 1.74^{bA}
SOD 100 U/mL	82.54 ± 1.80^{cE}	78.56 ± 1.57^{cD}	73.42 ± 1.29^{dC}	58.24 ± 1.56^{cB}	50.35 ± 1.47^{cA}
SOD 150 U/mL	72.93 ± 1.86^{aE}	68.25 ± 2.26^{aD}	55.89 ± 1.51^{aC}	36.26 ± 1.27^{aB}	32.31 ± 1.40^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

Table 6: DNA integrity (±S.E.) percentage for extended mithun semen containing additive at different storage times at 5°C.

Additives	Storage time				
	1 h	6 h	12 h	24 h	30 h
Control	75.07 ± 1.98^{aE}	67.89 ± 2.00^{aD}	58.66 ± 1.61^{aC}	44.99 ± 1.79^{bB}	37.27 ± 2.01^{aA}
SOD 50 U/mL	80.71 ± 1.72^{bE}	76.29 ± 1.45^{bD}	67.36 ± 1.58^{bC}	58.09 ± 1.97^{cB}	45.27 ± 1.35^{bA}
SOD 100 U/mL	86.14 ± 1.87^{cE}	82.49 ± 1.78^{cD}	77.44 ± 1.18^{cC}	59.76 ± 1.69^{cB}	49.27 ± 1.59^{cA}
SOD 150 U/mL	75.99 ± 1.55^{aE}	71.67 ± 1.91^{bD}	59.04 ± 1.95^{aC}	41.10 ± 1.71^{aB}	37.16 ± 1.38^{aA}

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).
Within rows means with different letters (A, B, C, D, E) differ significantly ($P < 0.05$).

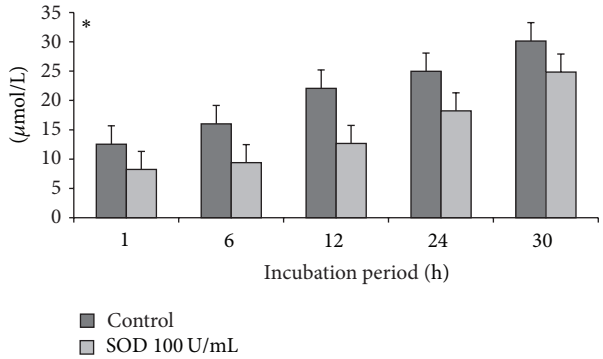

Figure 2: ALT production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

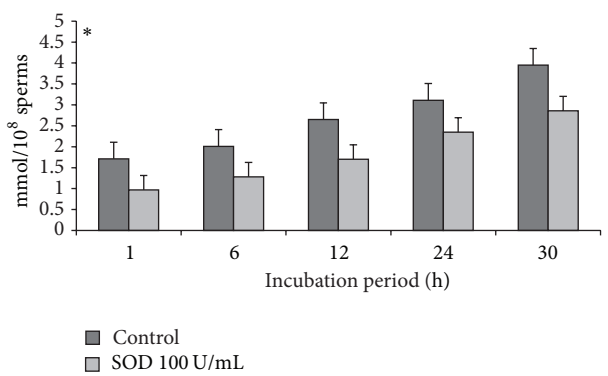

Figure 3: MDA production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

obvious from the data of this experiment that the addition of SOD especially at the concentrations of 100 U/mL to the semen diluent resulted in significant improvement in quality, reduction of MDA production, and protection of antioxidant profiles of mithun semen stored in *in vitro* for up to 30 h.

4. Discussion

In the present study, the results revealed that addition of GSH has improved the seminal parameters and enzymatic and biochemical profiles of mithun semen and thus it protects the structures and functions of spermatozoa efficiently. Thus,

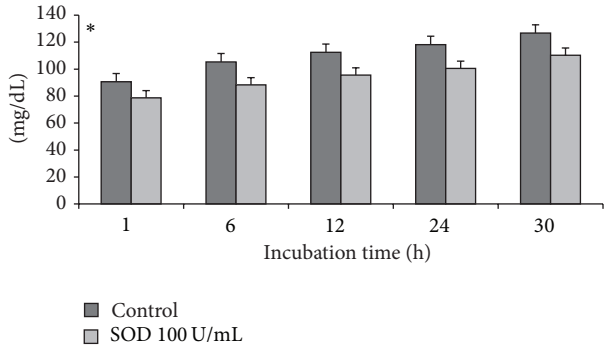

Figure 4: Cholesterol efflux for extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

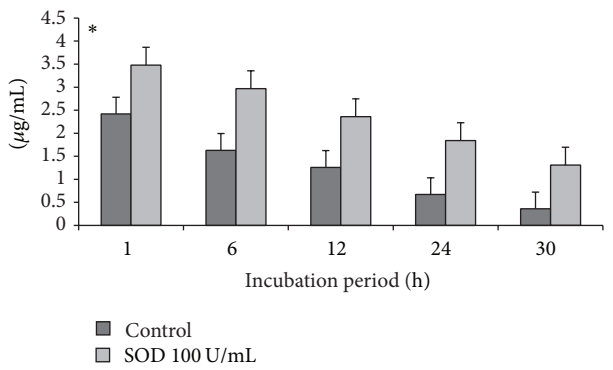

Figure 5: CAT production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

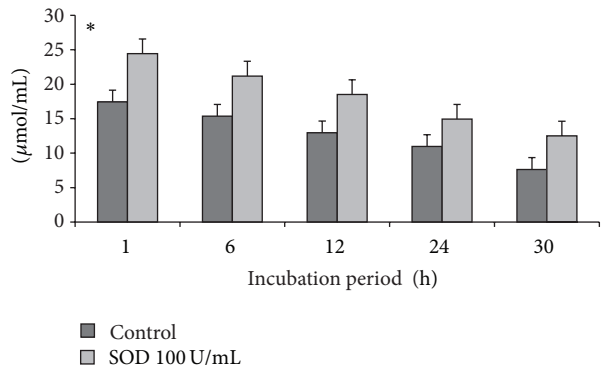

Figure 6: GSH production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

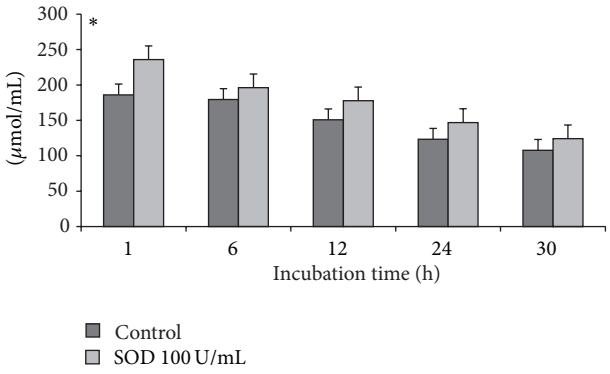

Figure 7: TAC production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

it may enhance the quality of semen by preserving efficiently during AI procedure.

Analysis of various seminal parameters such as forward progressive motility, livability, acrosomal, plasma membrane integrity, and DNA abnormality are important for extensive utilization of semen in AI. In the present study, SOD supplementation on these parameters revealed significant difference between the treatment groups. The beneficial effects of SOD in semen preservation are due to it being a very potent antioxidant [19–21].

Because the mammalian sperm membrane has high polyunsatured 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 [4, 20]. 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 [31]. In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing [32]. The levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules [9, 11]. Natural and synthetic antioxidant systems have been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen [13]. Therefore, inclusion of exogenous antioxidants with natural antioxidants could reduce the impact of oxidative stress during the sperm storage process, and thus improve the quality of chilled semen [4, 20].

The results of the present study showed that addition of 100 U/mL of SOD improved 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 150 U/mL SOD or without SOD. 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 [33]. The different effects of the different levels of SOD might be explained according to the report of Cocchia et al. [19] and Shoae and Zamiri [13] showed that the excessive amount of antioxidants caused high fluidity of plasma membrane above the desired point, making sperm more prone to acrosomal damages. 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 physiological condition of 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 [34]. The over expression of SOD may reflect a defect in the development or maturation of spermatozoa, as well as sperm cellular damage, resulting in decreased sperm fertilization potential [35, 36]. Similarly, in the present study, increasing dosage of SOD, at 150 U/mL affected the seminal as well as biochemical parameters in mithun semen TEYC 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 CAT recorded in the present study was previously reported in the form of motility and intact acrosomal membrane in equine sperm [19], bull sperm [20], and buffalo semen [21]. Moreover, the addition of exogenous SOD was significantly improving the percentages of DNA morphology, sperm viability, and intact plasma membrane (swelling tails) especially at a level of 100 U/mL of SOD. The highest percentages of intact plasma and acrosomal membranes which were found in the present experiment due to 100 U/mL SOD may be the reason for better motility in these samples [20, 21].

SOD helps maintaining the integrity of normal acrosome [33] and stabilizes the plasmalemma of spermatozoa and so increases motility. SOD, in sperm cells is able to react with many ROS directly for protecting mammalian cells against oxidative stress and hence maintaining sperm motility [6]. Therefore, as seen by this study, attempts to improve the motility and viability of the sperm cells by incorporating SOD in liquid storage [19, 20] and frozen semen form have been investigated [21].

A recent report suggested that semen quality is deteriorated [37] by which DNA damage is induced in the male gamete by oxidative stress and spermatozoa are particularly vulnerable to this because they generate ROS and are rich in targets for oxidative attack. The authors also draw attention to the fact that, because spermatozoa are transcriptionally inactive and have little cytoplasm, they are deficient in both antioxidants and DNA-repair systems [38]. Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in spermatozoa [38]. There are few studies on the effects of antioxidant addition to extenders during cooling and/or freezing mammalian spermatozoa [39]. In mithun semen, ROS are generated mainly by damaged and abnormal spermatozoa and by contaminating leukocytes. Reactive oxygen species damage cells by changes to lipids, proteins, and DNA. Spermatozoa are potentially susceptible to peroxidative

damage caused by ROS excess due to high amounts of polyunsaturated fatty acids in membrane phospholipids and to sparse cytoplasm. In the present study, addition of SOD has reduced the DNA fragmentation especially at 100 U/mL in mithun semen preservation at 5°C for 30 hrs.

Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. SOD also protects GSH, CAT, and TAC level in the semen extender, which helps to maintain membrane transportation [32] and fertility of the spermatozoa.

It also prevents efflux of cholesterol from the sperm membrane and MDA production in diluent, indicates it prevents premature capacitation and premature acrosomal reaction as acts as an antioxidant [20]. Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane [40]. 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 [41]. 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 [42]. In the present study, the efflux of cholesterol and MDA production were decreased in treated group as compared to the control untreated group [20]. So the semen samples treated with SOD will have high cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that were received at 100 U/mL of SOD were significantly higher than those of the other and control group.

The enzyme such as AST and ALT levels in seminal plasma is very important for sperm metabolism as well as sperm function [43], as it provides energy for survival and motility and fertility of spermatozoa and these transaminase activities in semen are good indicators of semen quality because they measure sperm membrane stability [44]. Thus, increasing the percentage of abnormal spermatozoa in the preservation causes high concentration of transaminase enzyme in the extra cellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa [45]. Moreover, increase in AST and ALT activities of seminal plasma and semen in liquid storage stage may be due to structural instability of the sperm [46]. In the present study, AST and ALT levels were lower in semen preserved at 100 U/mL of SOD at different storage period as it stabilises the membrane integrity of acrosome, plasma, mitochondria, and flagella of the sperm.

Glutathione (L-g-glutamyl-L-cysteinylglycine) is the most abundant nonprotein thiol in mammalian cells and is present mainly in reduced form (GSH) and only a small amount is in oxidized form (GSSG). Glutathione antioxidant system consists of reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GRX), glutathione peroxidase (GPX), and glutathione-s-transferase. GRX stimulates the reduction of GSSG to GSH. This ensures a steady supply of the reductive substrate (NADPH) to GPX. Glucose-6-phosphate dehydrogenase (G6PD) is required for the conversion of NADP to NADPH in GSH oxidization-reduction cycle in sperm and seminal plasma. In the present study GSH was higher in the seminal plasma of SOD added semen as it maintains the antioxidant system in liquid storage of mithun semen.

Similarly CAT is an antioxidant, a tetramer of four polypeptide chain antioxidants found in nearly all living organisms exposed to oxygen. It is derived from the epididymis and seminal vesicle and detoxifies both intracellular and extracellular hydrogen peroxide by reduces H_2O_2 to H_2O and O_2, by eliminating the potential ROS toxicity [47] and it can reduce the loss of motility caused by leukocyte generated ROS [48]. It scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. CAT also prevents premature hyperactivation and capacitation induced by superoxide radicals before ejaculating [16]. In the present study, the concentration of CAT was higher in SOD treated semen. But normally, seminal plasma is a potent source of this antioxidant, SOD [49]. The high levels of readily peroxidizable polyunsaturated material expose spermatozoa to excessive oxidative stress and the superoxide dismutase activity of sperm samples is a good predictor of their survival time. SOD, when applied at a dose of 100 U/mL, has improved sperm motility during preservation and displayed antioxidative properties, elevating the CAT level, in association with GSH and TAC concentration. Further, SOD, 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, and therefore increased ability of spermatozoa to survive during this preservation [50]. This could be one of the reasons for improved motility, viability and plasma and acrosome membrane and DNA integrity of spermatozoa, diluted in presence of SOD in the semen extender.

5. Conclusion

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 SOD. It was concluded that the possible protective effects of SOD supplementation are that it enhances the antioxidant enzymes content and prevents efflux of cholesterol and phospholipids from cell membrane and MDA production. Thus it may protect the spermatozoa during preservation and enhancing of the fertility in this species. Future, sperm preservation/cryoprotective studies are warranted to confirm the present findings.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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