Research Article

Effect of Addition of Melatonin on the Liquid Storage (5°C) of Mithun (*Bos frontalis*) Semen

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The present study was undertaken to assess the effect of melatonin (MT) on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity, DNA abnormality, antioxidant profiles such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and total antioxidant capacity (TAC), enzymatic profiles such as aspartate amino transaminase (AST), alanine amino transaminase (ALT), and biochemical profiles such as malonaldehyde (MDA) production and cholesterol efflux. Total numbers of 30 ejaculates were collected twice a week from eight mithun bulls and semen was split into five equal aliquots, diluted with the TEYC extender. Group 1 has semen without additives (control) and group 2 to group 5 have semen that was diluted with 1 mM, 2 mM, 3 mM, and 4 mM of melatonin, respectively. These seminal parameters, antioxidant, enzymatic, and biochemical profiles were assessed at 5°C for 0, 6, 12, 24, and 30 h of incubation. Inclusion of melatonin 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, melatonin at 3 mM has significant improvement in quality of mithun semen than melatonin at 1 mM, 2 mM or 4 mM stored *in vitro* for up to 30 h. It was concluded that the possible protective effects of melatonin on sperm parameters are it prevents MDA production and preserve 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 animal and not fully domesticated, 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. 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 poly unsaturated 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 [5, 6]. Glutathione,

glutathione peroxidase, reduced glutathione, catalase, superoxide dismutase, and vitamin C and E are the major antioxidants naturally present in mammalian semen against ROS to protect the sperm from lipid peroxidation and to maintain its integrity [7–11]. The levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules [10, 12]. Natural and synthetic antioxidant systems have been described as a defense functioning mechanism against lipid peroxidation (LPO) in semen [13]. Thus, the supplementation with natural antioxidants or synthetic antioxidants [2, 3, 5] or feeding of the antioxidants [14] could reduce the impact of oxidative stress during the sperm storage process, and thus improve the quality of chilled semen [3].

Melatonin (N-acetyl-5-methoxy tryptamine; MW = 232) is an indole derivative endogenous compound secreted rhythmically by the pineal gland in the brain [15] and plays a major role in regulating the circadian clock and seasonal reproduction in mammals [16]. Melatonin was discovered about 40 years ago, as a ubiquitously acting molecule related to neuroendocrine physiology, especially reproductive physiology [17, 18]. More recent studies have demonstrated that, besides its multiple actions on different physiological processes, melatonin as well as its metabolites is indirect antioxidants and powerful direct scavengers of free radicals [19]. In contrast to the majority of other known radical scavengers, melatonin is multifunctional and universal [20, 21]. It is soluble both in water and in lipids and hence acts as a hydrophilic and hydrophobic antioxidant. Melatonin also stimulates the activities of enzymes involved in metabolising ROS and preserves cell membrane fluidity. The discovery that melatonin is effective in antioxidative defense is related to the finding that under both *in vitro* and *in vivo* conditions, this molecule directly scavenges the highly toxic $\text{OH}^\cdot$ to form cyclic 3-hydroxymelatonin (3-OHM), a stable metabolite of melatonin [22, 23]. Indeed, Melatonin was shown to be twice as potent as vitamin E in removing peroxyl radicals [24] and it is more effective in scavenging hydroxyl radicals than glutathione and mannitol [25]. However, it has been reported recently that melatonin prevents *in vitro* sperm capacitation and apoptotic like changes [26], which can be explained by a direct action of this hormone on spermatozoa. The effect of melatonin in preventing apoptotic like changes may be related to its antioxidant and free radical scavenging activities.

The addition of antioxidant such as melatonin to ram sperm [27, 28], boar sperm [29] and bull sperm [30] 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 no information on the effect of addition of this antioxidant melatonin, on the maintenance of 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, antioxidant, biochemical, and enzymatic profiles of mithun semen to pursuit future sperm preservation protocols.

2. Material and Methods

2.1. Experimental Animals. 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 NEH region of India. The average body weight of the bulls was 501 kg (493 to 507 kg) at 4–6 years 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 (18.40% dry matter and 10.20% crude protein) and 4 kg concentrates (87.10% dry matter and 14.50% crude protein) fortified with mineral mixture and salt. Semen was collected from the animals through rectal massage method. Oxytocin (5 IU, intramuscular) was injected just prior to rectal palpation. Briefly, seminal vesicles were massaged centrally and backwardly for 5 min followed by the gentle milking of ampullae one by one for 3–5 min, which resulted into erection and ejaculation. During collection, the initial transparent secretions were discarded and neat semen drops were collected in a graduated test tube with the help of a funnel. 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 30 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 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 five equal aliquots and diluted with the TEYC extender with melatonin. Group 1 semen without additives (control), group 2 to group 5 semen with 1 mM, 2 mM, 3 mM, and 4 mM of melatonin, 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 h period of the experiment. The percentage of sperm motility, viability, total sperm abnormality, acrosomal integrity, the plasma membrane integrity by hypoosmotic swelling test (HOST), and DNA integrity by Feulgen staining technique [31] were determined as per standard procedure in samples during storage of semen at 5°C for 30 h.

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 the determination of progressive motility, the stored samples were warmed in a water bath at 37°C for 5 min [32].

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 NaH₂PO₄ or 0.1 M Na₂HPO₄) according to a previously described method using bright-field optics (Nikon, Eclipse 80i; magnification 1000x) [32]. Spermatozoa (eosin-nigrosin stained; 200 per sample) were also evaluated under bright-field optics (Nikon, Eclipse 80i; magnification 1000x) for morphological abnormalities [32]. Acrosomal integrity was assessed by Giemsa staining as described by Watson [33].

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 [34]. Sperm cells with resistant membranes exhibited swelling around the tail such that the flagella become 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 hypo osmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) [35]. 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 [31]. 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 slide were rinsed in sulfate water for 2 minutes and 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 [36]. Only spermatozoa with clumped nuclear material were classified as abnormally condensed for further comparisons.

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 [37] and modified by Suleiman et al. [38]. The semen sample was 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 ($1.56 \times 10^5 \mu\text{mol}/\text{cm}^3$) as follows:

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

2.3. Biochemical Assays. An aliquot of semen from each sample was centrifuged at 800 ×g for 10 min; sperm pellets were separated and washed by resuspending in PBS and recentrifuging (thrice). After the final centrifugation, 1 mL of deionised water was added to the spermatozoa and the spermatozoa and seminal plasma were snap-frozen and stored at -70°C until further analysis [39]. The antioxidant profiles such as CAT, SOD, GSH, and TAC, intracellular enzymes such as AST, 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 one way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences between the five experimental groups that is with additives or no additive at 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 melatonin on sperm motility (Table 1), viability (Table 2), total sperm abnormality (Table 3), acrosomal (Table 4) plasma membrane (Table 5), and DNA integrity (Table 6) in liquid storage (5°C) for 30 hrs were presented in tables. Results revealed that inclusion of melatonin into diluent resulted in decreases ($P < 0.05$) in percentages of dead spermatozoa, abnormal spermatozoa and acrosomal abnormalities when semen samples were examined at different hours of storage periods compared with the control group. Averaged over time, mean total sperm abnormalities were 14.73 ± 1.15 , 13.36 ± 1.28 , 12.24 ± 1.15 , 10.15 ± 1.03 and 13.52 ± 0.90 , respectively for control, 1 mM, 2 mM, 3 mM, and 4 mM of melatonin treated mithun semen at 30 h of storage. Additionally, melatonin at 1, 2, and 4 mM

TABLE 1: Mean ($\pm$ S.E.) motility percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage period				
	0 h	6 h	12 h	24 h	30 h
Control	70.28 $\pm$ 1.90 ^a	65.63 $\pm$ 1.64 ^{ab}	47.49 $\pm$ 1.86 ^a	35.82 $\pm$ 1.58 ^a	31.98 $\pm$ 1.74 ^a
MT 1 mM	72.92 $\pm$ 1.42 ^{ab}	68.59 $\pm$ 1.73 ^{bc}	50.22 $\pm$ 1.91 ^{ab}	40.99 $\pm$ 1.50 ^b	36.16 $\pm$ 1.52 ^b
MT 2 mM	74.77 $\pm$ 2.31 ^{bc}	69.29 $\pm$ 2.60 ^{bc}	53.37 $\pm$ 1.77 ^{bc}	43.82 $\pm$ 1.33 ^b	39.66 $\pm$ 1.47 ^c
MT 3 mM	77.37 $\pm$ 1.60 ^c	73.67 $\pm$ 1.68 ^c	56.48 $\pm$ 1.93 ^c	51.45 $\pm$ 1.22 ^c	45.77 $\pm$ 1.62 ^d
MT 4 mM	72.92 $\pm$ 1.42 ^{ab}	61.28 $\pm$ 2.44 ^a	46.87 $\pm$ 1.66 ^a	34.90 $\pm$ 1.69 ^a	31.51 $\pm$ 1.81 ^a

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).

TABLE 2: Mean ($\pm$ S.E.) viability percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage period				
	0 h	6 h	12 h	24 h	30 h
Control	74.10 $\pm$ 1.70 ^a	66.77 $\pm$ 1.49 ^a	50.69 $\pm$ 1.53 ^{ab}	41.04 $\pm$ 1.68 ^a	35.90 $\pm$ 1.57 ^a
MT 1 mM	74.70 $\pm$ 1.51 ^a	70.76 $\pm$ 1.50 ^b	54.34 $\pm$ 1.34 ^{bc}	44.41 $\pm$ 1.21 ^b	39.94 $\pm$ 1.53 ^b
MT 2 mM	76.06 $\pm$ 1.54 ^a	71.41 $\pm$ 1.28 ^b	56.69 $\pm$ 1.79 ^c	46.49 $\pm$ 1.23 ^{bc}	41.10 $\pm$ 1.22 ^b
MT 3 mM	79.14 $\pm$ 1.47 ^b	76.19 $\pm$ 1.70 ^c	61.28 $\pm$ 1.84 ^d	48.93 $\pm$ 1.97 ^c	45.72 $\pm$ 1.35 ^c
MT 4 mM	73.84 $\pm$ 1.31 ^a	68.88 $\pm$ 1.51 ^{ab}	48.43 $\pm$ 2.04 ^a	38.27 $\pm$ 1.48 ^a	33.91 $\pm$ 1.40 ^a

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).

TABLE 3: Mean ($\pm$ S.E.) total abnormal sperm percentage for mithun semen following storage at 5°C for different storage times.

Additives	Storage period				
	0 h	6 h	12 h	24 h	30 h
Control	6.18 $\pm$ 0.67 ^b	7.91 $\pm$ 0.91 ^{cd}	10.76 $\pm$ 1.22 ^c	12.91 $\pm$ 1.13 ^c	14.73 $\pm$ 1.15 ^c
MT 1 mM	5.82 $\pm$ 0.81 ^{ab}	7.29 $\pm$ 0.63 ^{bc}	9.81 $\pm$ 1.16 ^{bc}	11.85 $\pm$ 1.01 ^{bc}	13.36 $\pm$ 1.28 ^{bc}
MT 2 mM	5.37 $\pm$ 0.76 ^a	6.60 $\pm$ 0.79 ^{ab}	9.16 $\pm$ 1.21 ^b	10.91 $\pm$ 1.12 ^b	12.24 $\pm$ 1.15 ^b
MT 3 mM	5.39 $\pm$ 0.82 ^a	6.33 $\pm$ 0.91 ^a	7.34 $\pm$ 0.64 ^a	9.05 $\pm$ 0.93 ^a	10.15 $\pm$ 1.03 ^a
MT 4 mM	6.96 $\pm$ 0.64 ^c	8.67 $\pm$ 0.57 ^d	10.36 $\pm$ 0.84 ^{bc}	11.64 $\pm$ 0.85 ^{bc}	13.52 $\pm$ 0.90 ^{bc}

Within columns means with different letters (a, b, c) differ significantly ($P < 0.05$).

TABLE 4: Mean ($\pm$ S.E.) Acrosomal Integrity (%) in semen of mithun for different storage times at 5°C.

Additives	Storage Period				
	0 h	6 h	12 h	24 h	30 h
Control	74.69 $\pm$ 1.60 ^a	67.43 $\pm$ 1.90 ^a	55.91 $\pm$ 1.52 ^a	44.49 $\pm$ 1.67 ^b	36.73 $\pm$ 1.92 ^a
MT 1 mM	76.16 $\pm$ 1.53 ^a	69.82 $\pm$ 1.91 ^{ab}	59.02 $\pm$ 1.52 ^a	47.27 $\pm$ 1.73 ^b	40.19 $\pm$ 1.18 ^b
MT 2 mM	79.18 $\pm$ 1.30 ^b	75.95 $\pm$ 1.64 ^c	67.55 $\pm$ 1.50 ^b	57.99 $\pm$ 1.85 ^c	44.82 $\pm$ 1.33 ^c
MT 3 mM	81.26 $\pm$ 1.25 ^b	78.22 $\pm$ 1.32 ^c	75.52 $\pm$ 1.00 ^c	58.50 $\pm$ 1.70 ^c	48.12 $\pm$ 1.66 ^d
MT 4 mM	75.65 $\pm$ 1.76 ^a	71.45 $\pm$ 1.65 ^b	57.96 $\pm$ 1.88 ^a	39.97 $\pm$ 1.61 ^a	36.70 $\pm$ 1.48 ^a

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).

TABLE 5: HOST ($\pm$ S.E.) percentage for extended mithun semen containing additive at different storage times.

Additives	Storage Period				
	0 h	6 h	12 h	24 h	30 h
Control	74.23 $\pm$ 1.70 ^{ab}	69.78 $\pm$ 1.55 ^{ab}	56.58 $\pm$ 1.92 ^a	44.85 $\pm$ 2.01 ^b	36.67 $\pm$ 1.95 ^b
MT 1 mM	76.92 $\pm$ 1.80 ^{bc}	73.72 $\pm$ 2.07 ^{bc}	57.48 $\pm$ 1.93 ^a	45.43 $\pm$ 1.73 ^{bc}	41.72 $\pm$ 1.27 ^c
MT 2 mM	78.70 $\pm$ 1.69 ^{cd}	74.45 $\pm$ 2.30 ^c	62.07 $\pm$ 1.65 ^b	49.25 $\pm$ 1.48 ^c	42.88 $\pm$ 1.73 ^{cd}
MT 3 mM	82.26 $\pm$ 1.82 ^d	77.24 $\pm$ 1.78 ^c	66.07 $\pm$ 1.29 ^c	54.55 $\pm$ 1.91 ^d	46.25 $\pm$ 1.46 ^d
MT 4 mM	72.07 $\pm$ 1.90 ^a	66.25 $\pm$ 1.89 ^a	55.72 $\pm$ 1.30 ^a	36.26 $\pm$ 1.34 ^a	32.84 $\pm$ 1.51 ^a

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).

TABLE 6: DNA integrity (normality) ($\pm$ S.E.) percentage for extended mithun semen containing additive at different storage times.

Additives	Storage period				
	0 h	6 h	12 h	24 h	30 h
Control	75.01 $\pm$ 1.64 ^{ab}	68.32 $\pm$ 1.93 ^a	60.18 $\pm$ 1.51 ^b	45.82 $\pm$ 1.96 ^b	38.09 $\pm$ 2.06 ^a
MT 1 mM	77.86 $\pm$ 1.50 ^{bc}	72.52 $\pm$ 1.44 ^b	63.84 $\pm$ 1.70 ^{bc}	50.89 $\pm$ 1.93 ^c	42.71 $\pm$ 1.72 ^b
MT 2 mM	78.93 $\pm$ 1.45 ^c	74.19 $\pm$ 1.46 ^b	65.29 $\pm$ 1.95 ^c	53.22 $\pm$ 1.69 ^c	44.06 $\pm$ 1.55 ^b
MT 3 mM	83.93 $\pm$ 1.31 ^d	80.24 $\pm$ 1.39 ^c	71.80 $\pm$ 1.97 ^d	60.18 $\pm$ 1.41 ^d	48.73 $\pm$ 2.00 ^c
MT 4 mM	73.59 $\pm$ 1.60 ^a	68.56 $\pm$ 1.62 ^a	54.87 $\pm$ 1.96 ^a	38.88 $\pm$ 1.32 ^a	35.67 $\pm$ 1.28 ^a

Within columns means with different letters (a, b, c, d) differ significantly ($P < 0.05$).

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

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

were inferior to melatonin 3 mM treatments for these characteristics, and there was a significant ($P < 0.05$) difference between melatonin at 1, 2, 4, and 3 mM for these response. The antioxidant enzymatic profiles revealed that highest mean SOD (Figure 1) and CAT (Figure 2), GSH (Figure 3) and TAC (Figure 4) were recorded in melatonin treated semen than control group, and differed ($P < 0.05$) between groups. Intracellular enzymes such as AST (Figure 5), ALT (Figure 6) decreased ($P < 0.05$) in melatonin treated semen. Similarly cholesterol efflux (Figure 7) and MDA production (Figure 8) differed significantly between the melatonin treated and control and were lower in the melatonin treated group. It was obvious from the data of this experiment that addition of melatonin, especially at 3 mM, to the semen diluent resulted in significant improvement in quality, seminal parameters, antioxidant, enzymatic activity and reduction of cholesterol efflux and MDA production of mithun semen stored in *in-vitro* at 5°C.

4. Discussion

In the present study, the results revealed that addition of melatonin 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 melatonin on seminal parameters in mithun and to the best of our

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

FIGURE 4: TAC production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

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

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

FIGURE 7: Cholesterol efflux for extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

FIGURE 8: MDA production in the extended mithun semen containing additive at different storage periods (* indicates $P < 0.05$).

knowledge this is the first report of the effect of melatonin on routine seminal, antioxidative, enzymatic level and biochemical profiles in mithun semen. But many authors reported that melatonin has beneficial effects on preservation of mammalian sperm and improves the functional parameters of spermatozoa [27–30, 40–42]. 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 artificial insemination. In the present study, melatonin supplementation on these parameters revealed significant difference between the treatment groups. The beneficial effects of melatonin in semen preservation are due to it is a very potent antioxidant [27–30].

Because of the mammalian sperm membrane has high polyunsaturated fatty acids, 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, 43]. 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 [44]. In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing [45]. The levels of antioxidant decreased during the preservation process by dilution of semen with extender and excessive generation of ROS molecules [10, 12]. 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 [3, 28, 46].

The results of the present study showed that addition of 3 mM of melatonin 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 4 mM melatonin or without melatonin. But inclusion of 3 mM melatonin, the motility and viability parameters were increased as compared to control group [42, 47]. 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 [48]. The different effects of the different levels of melatonin might be explained according to the report of Ashrafi et al. [28] and Shoaie 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 mithun, 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 [49]. The over expression of melatonin

may reflect a defect in the development or maturation of spermatozoa, as well as sperm cellular damage, resulting in decreased sperm fertilization potential [30]. Similarly, in the present study, increasing dosage of melatonin, at 4 mM 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. But as per the dosage, the parameters were increased upto 3 mM then decreased to 4 mM. 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 melatonin recorded in the present study was previously reported in the form of motility and intact acrosomal membrane in ram sperm [27, 28], bull sperm [30] and boar sperm [29]. Moreover, the addition of exogenous melatonin was significantly improving the percentages of DNA morphology, sperm viability and intact plasma membrane (swelling tails) especially at a level of 3 mM of melatonin [42, 47]. The highest percentages of intact plasma and acrosomal membranes which were found in the present experiment due to 3 mM melatonin may be the reason for better motility in these samples [30]. Mitochondria in sperm cells encase the axosome, connect with dense fibers in the middle pieces and produce adenosine triphosphate (ATP). It has been reported [50] that the axoneme and mitochondria in sperms may be damaged by a high level of ROS. The studies have shown that melatonin can stabilise and protect mitochondria via several mechanisms [51–54].

Melatonin helps maintaining the integrity of normal acrosome and stabilizes the plasmalemma of spermatozoa and so increase motility [40–42, 55–57]. Melatonin, in sperm cells is able to react with many ROS directly for protecting mammalian cells against oxidative stress, and hence maintaining sperm motility [30]. Therefore, as seen by this study, attempts to improve the motility and viability of the sperm cells by incorporating melatonin in liquid storage [28] and frozen semen form have been investigated [27, 30].

A recent report suggested that semen quality is deteriorated [58] 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 [59]. Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in spermatozoa [59]. There are few studies on the effects of antioxidant addition to extenders during cooling and/or freezing mammalian spermatozoa [30, 60]. In mithun semen, ROS are generated mainly by damaged and abnormal spermatozoa and by contaminating leukocytes. ROS 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 melatonin has reduced the DNA

fragmentation especially at 3 mM in mithun semen preservation at 5°C for 30 hrs. It similar to reports of Succu et al. [47] that the addition of melatonin preserved DNA integrity in cryopreserved ram spermatozoa.

Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects [51–54]. Melatonin has also protects and stimulates the activities of antioxidant enzymes such as SOD, GSH, and CAT [61], which helps to maintain membrane transportation [45] and fertility of the spermatozoa. It results indirectly reduces the number of free radicals, ROS, and also may increase the production of molecules protecting sperm cells against oxidative stress. Indeed, Melatonin was shown to be twice as potent as vitamin E in removing peroxy radicals [24, 62] and it is more effective in scavenging hydroxyl radicals than glutathione and mannitol [25].

SOD, CAT, and GPX are important parts of antioxidant enzyme defence systems in sperm that convert superoxide ($O_2^{\bullet-}$) and peroxide (H_2O_2) radicals into O_2 and H_2O . GPX excludes peroxy radicals from various peroxides [63]. CAT and SOD also eliminate $O_2^{\bullet-}$ produced by nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase [64]. In our study, the activity of CAT, SOD, GSH increased by inclusion of various concentrations of melatonin in the semen extender [30].

The enzyme such as AST and ALT levels in seminal plasma are very important for sperm metabolism as well as sperm function [65], 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 [66]. 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 [67]. 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 [68]. In the present study, AST and ALT levels were lower in semen preserved at 3 mM of melatonin at different storage period as it stabilises the membrane integrity of acrosome, plasma, mitochondria and flagella of the sperm [51–54].

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 antioxidant [30, 69]. Along with phospholipids, cholesterol is necessary for cell physical integrity and ensures fluidity of the cell membrane [70]. 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 [71]. 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 [72]. In the present study, the efflux of cholesterol and MDA production were decreased in melatonin treated group as compared to the control untreated group [30]. However, it has been reported recently that melatonin prevents *in vitro* sperm capacitation and

apoptotic like changes [26], which can be explained by a direct action of this hormone on spermatozoa. The effect of melatonin in preventing apoptotic like changes may be related to its antioxidant and free radical scavenging activities also increases fertility rate [26]. So the semen samples treated with melatonin will have high cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that received at 3 mM of melatonin were significantly higher than those of the other and control group. These results are basically consistent with the results previously reported [69]. Moreover, inclusion of melatonin in the semen extender increased the TAC. This may be due to the stimulatory effects of melatonin on the activity of enzymes involved in antioxidant defence [30]. In the present study, based on the result the effect of melatonin on the seminal and biochemical parameters are dose dependent [27, 28, 30, 47, 56] and at 3 mM melatonin was optimum dose for mithun semen preservation at liquid stage. Moreover, 1 and 2 mM of melatonin were low and 4 mM of melatonin was over dosage for mithun semen preservation at liquid storage.

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 melatonin. It was concluded that the possible protective effects of melatonin supplementation are it enhances the antioxidant enzymes content and preventing efflux of cholesterol and phospholipids from cell membrane and MDA production in dose dependent manner. Thus it may protect the spermatozoa during preservation and enhancing the fertility in this species at 3 mM. Future, ultralow temperature sperm preservation/cryoprotective studies are warranted to confirm the present findings.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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