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Effect of catalase on the liquid storage of mithun (*Bos frontalis*) semen

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ABSTRACT

Objective: To assess the effect of catalase (CAT) on sperm motility, viability, total sperm abnormality, acrosomal and plasma membrane integrity, enzymatic profiles such as aspartate amino transaminase (AST), alanine amino transaminase (ALT), biochemical profiles such as cholesterol efflux and malonaldehyde (MDA) production and antioxidant profiles such as reduced glutathione (GSH), superoxide dismutase (SOD) and total antioxidant capacity (TAC). **Methods:** 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), group 2 to group 4: semen was diluted with 50 U/mL, 100 U/mL and 150 U/mL of catalase, respectively. These seminal, enzymatic, biochemical and antioxidant profiles were assessed at 5 ℃ for 0, 6, 12, 24 and 30 h of incubation. **Results:** Inclusion of catalase 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, CAT at 50 and 150 U/mL were inferior to CAT 100 U/mL treatments as regards to these characteristics and CAT at 100 U/mL has significant improvement in quality of mithun semen in *in- vitro* stored for up to 30 h. **Conclusions:** It was concluded that the possible protective effects of CAT on sperm parameters are it prevent efflux of cholesterol from cell membrane, MDA production and protect the function of antioxidants during preservation.

1. Introduction

Mithun (*Bos frontalis*) is a semi-wild free-range, rare bovine species present in the North-Eastern hill (NEH) region of India. It is believed to have originated more than 8 000 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 these regions. Greater efforts are required from all quarters to preserve the mithun population to enhance the socio-economic status of this region. Since mithuns are semi wild 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, namely a superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2) [2, 3] as the sperm membrane has high poly unsaturated fatty acids. The effects of lipid peroxidation include irreversible a loss in motility, damage to the sperm DNA and fertility [2, 4]. Mithun semen normally contains natural anti-oxidants, including GSH, cysteine hydrochloride, CAT, SOD that can offset lipid peroxidation (author's unpublished data). But the concentration of these antioxidants is reduced during dilution and storage that affect the semen quality during storage in semen preservation. Natural antioxidant system and synthetic antioxidants have been described as a defense functioning

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mechanism against lipid peroxidation (LPO) in semen [5]. 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 [6, 7].

The addition of anti-oxidants such as CAT to bull sperm [7], buffalo semen [8], ram semen [9] and boar semen [10] has been shown to protect sperm against the harmful effects of ROS and improve sperm motility and membrane integrity during liquid storage or in the unfrozen state.

Further, perusal of literatures revealed no information on the effect of addition of this anti-oxidant CAT, 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, biochemical, enzymatic and antioxidant profiles of mithun semen to pursuit future sperm preservation protocols.

2. Materials 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. Semen was collected from the animals through rectal massage method. 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 Institute Animal Care and Use Committee regulations.

2.2. Semen collection and analysis

Total numbers of 50 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 ℃ 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 ℃) Tris egg yolk citrate extender (TEYC). The partially diluted samples were then brought to the laboratory in an insulated flask containing warm water (37 ℃) 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 CAT. Group 1: semen without additives (control), group 2 to group 4: semen with 50 U/mL, 100 U/mL and 150 U/mL of CAT, 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 ℃, at a rate of 0.2-0.3 ℃/min in a cold cabinet and maintained at 5 ℃ during liquid storage for up to a 30 h period of the experiment. The percentage sperm motility, viability, total sperm abnormality, acrosomal integrity and the plasma membrane integrity by hypo-osmotic swelling test (HOST) were determined as per standard procedure in samples during storage of semen at 5 ℃ for 0, 6, 12, 24 and 30 h, respectively. The enzymatic profiles such as AST, ALT activity, antioxidant profiles such as SOD, GSH and TAC and biochemical profile such as cholesterol efflux of the seminal plasma were estimated by commercial available kit. Lipid peroxidation level of sperm and seminal plasma was measured by determining the MDA production, using thiobarbituric acid (TBA) as per the method of Buege and Aust [11] and modified by Suleiman *et al*. [13].

2.3. Statistical analyses

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 *i.e.* with additives or no additive for 0, 6, 12, 24 and 30 h of storage on the sperm parameters using the SPSS/PC computer program (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 CAT on sperm motility (Table 1), viability (Table 2), total sperm abnormality (Table 3), acrosomal (Table 4) and plasma membrane integrity (Table 5) at different hours of incubation in liquid storage (5 ℃) were presented in tables. Results also revealed that the inclusion of CAT 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, CAT at 50 and 150 U/mL were inferior to CAT 100 U/mL treatments as regards to these characteristics, and there were significant differences between CAT at 50 and 150 U/mL

Table 1

Motile sperm percentage for mithun semen following storage at 5 ℃ for different storage times.

Additives	Storage time				
	0 h	6 h	12 h	24 h	30 h
Control	67.86±1.98*	64.73±1.95*	53.82±1.99*	41.89±1.83*	33.49±1.51*
Catalase 50 U/mL	71.92±1.71△	69.29±1.77△	60.93±1.74△	50.56±2.14△	40.71±1.71△
Catalase 100 U/mL	75.34±1.51△	72.48±1.66△	64.18±2.07△	42.59±2.40△	42.59±2.40△
Catalase 150 U/mL	70.79±1.53*	66.06±1.37*	51.27±1.50*	32.54±1.35△*	29.86±1.15△*

△P<0.05 comparing with the control group. *P<0.05 comparing with group treated with CAT 100U/mL.

Table 2

Viable sperm percentage for mithun semen following storage at 5 ℃ for different storage times.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	72.79±1.51*	68.25±1.24*	58.91±1.83*	43.68±1.44*	37.60±1.63*
Catalase 50 U/mL	74.64±1.51$^{\triangle *}$	71.29±1.56$^{\triangle *}$	63.83±1.57$^{\triangle *}$	45.69±1.77*	39.44±1.94*
Catalase 100 U/mL	78.47±1.41$^{\triangle}$	74.71±1.46$^{\triangle}$	67.33±1.55$^{\triangle}$	54.37±1.95$^{\triangle}$	44.89±1.59$^{\triangle *}$
Catalase 150 U/mL	71.57±1.25*	66.76±1.34*	52.43±1.94$^{\triangle *}$	36.79±1.52$^{\triangle *}$	31.18±1.38$^{\triangle *}$

$^{\triangle}P<0.05$ comparing with the control group,$^{*}P<0.05$ comparing with group treated with CAT 100U/mL.

Table 3

Total abnormal sperm percentage for mithun semen following storage at 5 ℃ for different storage times.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	6.20±0.75	7.48±0.73*	11.03±1.07*	13.16±0.83*	14.57±088*
Catalase 50 U/mL	5.98±0.80	6.58±0.72$^{\triangle}$	10.63±0.99*	11.97±1.13$^{\triangle *}$	13.20±1.23$^{\triangle *}$
Catalase 100 U/mL	5.71±0.85	6.66±0.88$^{\triangle}$	7.41±0.79$^{\triangle}$	8.47±0.90$^{\triangle}$	9.60±0.94$^{\triangle}$
Catalase 150 U/mL	6.73±0.94	8.17±1.03*	10.46±0.90*	12.54±1.07*	14.00±1.07*

$^{\triangle}P<0.05$ comparing with the control group,$^{*}P<0.05$ comparing with group treated with CAT 100U/mL.

Table 4

Acrosomal Integrity (%) in semen of mithun for different storage times at 5 ℃.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	72.79±1.24*	64.98±1.66*	57.61±2.01*	48.73±1.58*	39.19±1.56*
Catalase 50 U/mL	79.64±1.85$^{\triangle *}$	76.85±1.75$^{\triangle *}$	67.32±1.96$^{\triangle *}$	52.27±1.44$^{\triangle *}$	42.00±1.48$^{\triangle *}$
Catalase 100 U/mL	84.91±1.60$^{\triangle}$	81.57±1.56$^{\triangle}$	72.30±1.36$^{\triangle}$	59.62±1.51$^{\triangle}$	48.12±1.57$^{\triangle}$
Catalase 150 U/mL	75.53±1.98$^{\triangle *}$	70.59±1.94$^{\triangle *}$	59.14±2.00*	39.62±1.80$^{\triangle *}$	35.47±1.93$^{\triangle *}$

$^{\triangle}P<0.05$ comparing with the control group,$^{*}P<0.05$ comparing with group treated with CAT 100U/mL.

Table 5

HOST positive sperm percentage for mithun semen following storage at 5 ℃ for different storage times.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	74.88±1.31*	70.59±1.59*	60.86±1.88*	47.19±1.64*	39.72±1.57*
Catalase 50 U/mL	76.77±1.48*	73.01±1.61$^{\triangle *}$	62.55±2.46*	48.97±1.78*	42.12±1.40$^{\triangle *}$
Catalase 100 U/mL	83.17±1.48$^{\triangle}$	78.34±1.62$^{\triangle}$	71.38±1.21$^{\triangle}$	57.07±1.60$^{\triangle}$	50.37±1.65$^{\triangle}$
Catalase 150 U/mL	73.10±1.55*	69.68±1.51*	56.70±1.66$^{\triangle *}$	36.66±1.36$^{\triangle *}$	32.96±1.66$^{\triangle *}$

$^{\triangle}P<0.05$ comparing with the control group,$^{*}P<0.05$ comparing with group treated with CAT 100U/mL.

Table 6

Cholesterol efflux for extended mithun semen containing additive at different storage times at 5 ℃.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	94.32±2.01	100.23±2.00	106.60±1.91	118.08±2.42	126.99±2.44
Catalase 100 U/mL	76.42±1.92$^{\triangle}$	80.44±2.03$^{\triangle}$	95.76±1.90$^{\triangle}$	102.28±1.95$^{\triangle}$	110.65±1.91$^{\triangle}$

$^{\triangle}P<0.05$ comparing with the control group.

Table 7

MDA production in the extended mithun semen containing additive at different storage times at 5 ℃.

Additives	Storage time				
	0 h	6h	12 h	24 h	30h
Control	2.71±0.80	3.05±0.67	3.35±0.66	3.77±0.70	4.15±0.64
Catalase 100 U/mL	1.91±0.33$^{\triangle}$	2.07±0.33$^{\triangle}$	2.31±0.38$^{\triangle}$	2.51±0.37$^{\triangle}$	2.85±0.43$^{\triangle}$

$^{\triangle}P<0.05$ comparing with the control group.

in relation to these features. The enzymatic profiles revealed that mean AST (Figure 1) and ALT (Figure 2) activity was lower in CAT treated semen than control group and were significantly (P<0.05) differed between groups. Similarly cholesterol efflux (Table 6) and MDA production (Table 7) was significantly differed between the CAT treated and control. Antioxidant profiles revealed that significantly (P<0.05) higher GSH (Figure 3), SOD (Figure 4) and TAC (Figure 5) in CAT treated semen than control group. It was obvious from the data of this experiment that the addition of CAT especially at the concentrations of 100 U/mL to the semen diluent resulted in significant improvement in quality, reduction of cholesterol efflux, MDA production and protection of antioxidant profiles of mithun semen in stored *in-vitro* for up to 30 h.

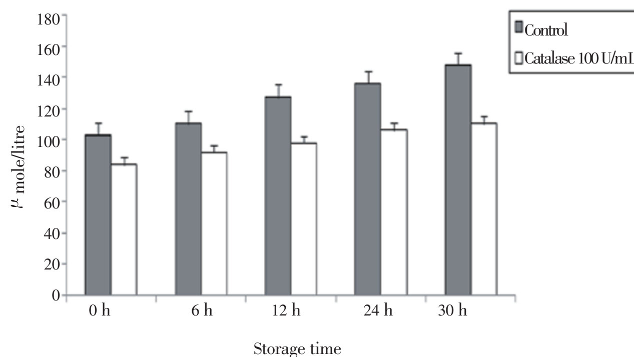

Figure 1. SGOT (AST) (μ mole/litre) level in the extended mithun semen containing additive at different storage.

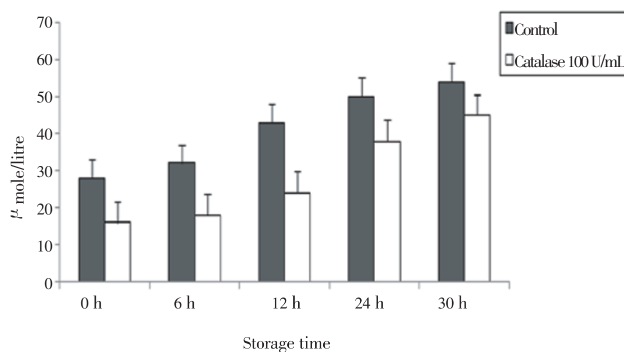

Figure 2. SGPT (ALT) (μ mole/litre) level in the extended mithun semen containing additive at different storage times.

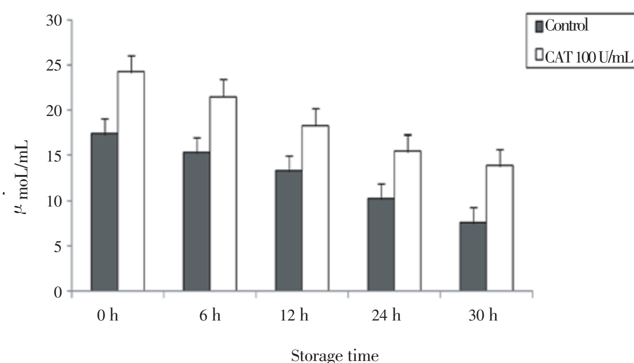

Figure 3. GSH (μ mole/mL) level in the extended mithun semen containing additive at different storage times.

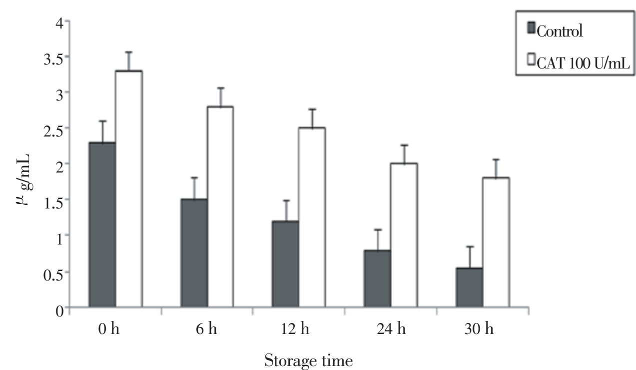

Figure 4. SOD (μg/mL) level in the extended mithun semen containing additive at different storage times.

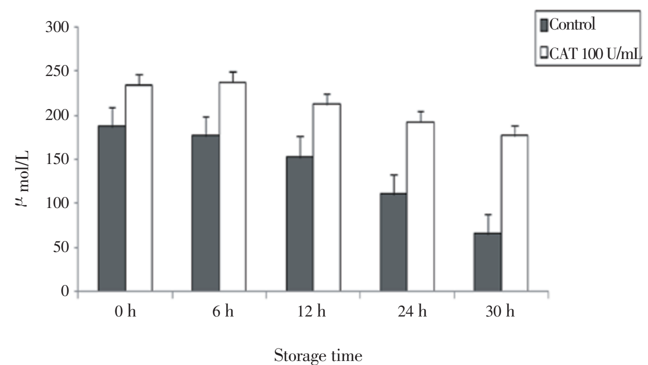

Figure 5. TAC (μ mole/L) level in the extended mithun semen containing additive at different storage times.

4. Discussion

In the present study, the results revealed that addition of CAT has improved the seminal parameters, enzymatic, biochemical and antioxidant 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 CAT on seminal parameters in mithun and to the best of our knowledge this is the first report of the effect of CAT on seminal parameters, enzymatic, biochemical and antioxidant 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. In the present study, CAT supplementation on these parameters revealed significant difference between the treatment groups. The beneficial effects of CAT in semen preservation are due to it is a very potent antioxidant [7, 8, 10, 13].

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 [6, 7]. Lipid peroxidation of the sperm membrane ultimately leads to the impairment of sperm

function due to the attacks by ROS, altered sperm motility, membrane integrity, damage to sperm DNA and fertility through oxidative stress and the production of cytotoxic aldehydes[14]. In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing [15]. These results are in agreement with works of Maxwell and Stojanov [9] that indicated addition of CAT to the extender improved survival of liquid stored ram spermatozoa. Therefore, inclusion of exogenous antioxidants may modulate the antioxidant system of semen [6, 7].

The results of the present study showed that addition of 100 U/mL of CAT improves the keeping quality of mithun semen preserved at 5 ℃ [7]. 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 of CAT or without CAT. 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 [16]. The different effects of the three levels of CAT might be explained according to the report of Shoae and Zamiri [5], who 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 CAT added to extender increases. However, CAT at dosage higher than 200 U/mL was toxic to ram spermatozoa [9]. Similarly, in the present study, increasing dosage of CAT, 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 bull semen [7] and boar semen [10]. Moreover, the addition of exogenous CAT was significantly improving the percentages of sperm viability and intact plasma membrane (swelling tails) especially at a level of 100 U/mL. The highest percentages of intact plasma and acrosomal membranes which were found in the present experiment due to 100 U/mL CAT may be the reason for better motility in these samples [7].

CAT helps maintaining the integrity of normal acrosome [9] and stabilizes the plasmalemma of spermatozoa and so increase motility. CAT, in sperm cells is able to react with many ROS directly for protecting mammalian cells against oxidative stress, and hence maintaining sperm motility [17]. Therefore, as seen by this study, attempts to improve the motility and viability of the sperm cells by incorporating catalase in liquid storage [9,10,13] and frozen semen form have been investigated [7,8].

Moreover, it maintains plasma and mitochondrial membrane integrity and cytoskeleton structure of flagella of sperm as cell protecting effects. CAT has also protects SOD, GSH and TAC level in the semen extender [18], which helps to maintain membrane transportation [2, 3, 15] and fertility of the spermatozoa.

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. 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 [19]. 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 [20]. 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 CAT will have high cryoresistance power than untreated control group. In the present study, it was observed that sperm parameters that received at 100 U/mL of CAT were significantly higher than those of the other and control group [7].

The enzyme such as AST and ALT levels in seminal plasma are very important for sperm metabolism as well as sperm function [21], 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 [22]. 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 [23]. 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 [24]. In the present study, AST and ALT levels were lower in semen preserved at 100 U/mL of CAT 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 non-protein 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 (GRD), glutathione peroxidase (GPD) and glutathione - s - transferase. GRD stimulates the reduction of GSSG to GSH. This ensures a steady supply of the reductive substrate (NADPH) to GPD. Glucose -6- phosphate dehydrogenase (G6PD) is required for the conversion of NADP to NADPH, is called as GSH oxidizing-reducing cycle in sperm and seminal plasma. In the present study GSH was higher in the seminal plasma of CAT added semen as it maintains the antioxidant system in liquid storage of mithun semen.

Similarly 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 hyper activation and capacitation induced by superoxide radicals before ejaculating [25]. In the present study, the concentration of SOD was higher in CAT treated semen. But normally, seminal plasma is a potent source of this antioxidant, CAT [26]. 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. CAT, when applied at a dose of 100 U/mL, has improved sperm motility during preservation, and displayed antioxidative

properties, elevating the SOD level, in association with GSH concentration. Further, CAT, a permeating cryoprotectant act as an antioxidant and cause 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 [27]. This could be one of the reasons for improved motility, viability and membrane integrity of spermatozoa, diluted in presence of CAT in the semen extender.

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

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Simoons FJ. Gayal or mithun. In: Manson IL.(ed.) *Evolution of domesticated animals*. London: Longman; 1984, p. 34–36.

[2] Perumal P, Selvaraju S, Selvakumar S, Barik AK, Mohanty DN, Das S, *et al*. Effect of pre-freeze addition of cysteine hydrochloride and reduced glutathione in semen of crossbred Jersey bulls on sperm parameters and conception rates. *Reprod Dom Anim* 2011a; **46**(4): 636-641.

[3] Perumal P, Selvaraju S, Barik AK, Mohanty DN, Das S, Mishra PC. Role of reduced glutathione in improving post-thawed frozen seminal characters of poor freezable Jersey crossbred bull semen. *Indian J Anim Sci* 2011b; **81**(8): 807-810.

[4] Maxwell WMC, Watson PF. Recent progress in the preservation of ram semen. *Anim Reprod Sci* 1996; **42**: 55–65.

[5] Shoae A, Zamiri MJ. Effect of butylated hydroxytoluene on bull sperm frozen in egg yolk-citrate extender. *Anim Reprod Sci* 2008; **104**: 414 – 418.

[6] Dandekar P, Nadkarni GD, Kulkarni VS, Punekar S. Lipid peroxidation and antioxidant enzymes in male infertility. *J Postgrad Med* 2002; **48**: 186-189.

[7] Asadpour R, Jafari R, Tayefi-Nasrabadi H. Effect of various levels of catalase antioxidant in semen extenders on lipid peroxidation and semen quality after the freeze-thawing bull semen. *Vet Res Forum* 2011; **2**(4): 218-221.

[8] El-Sisy GA, El-Nattat WS, El- Sheshtawy RI. Effects of superoxide dismutase and catalase on viability of cryopreserved buffalo spermatozoa. *Global Vet* 2008; **2**(2): 56-61.

[9] Maxwell WMC, Stojanov T. Liquid storage of ram semen in the absence or presence of some antioxidants. *Reprod Fertil Dev* 1996; **8**: 1013–1020.

[10]Roca J, Rodriguez MJ, Gil MA, Carvajal G, *Garcia* EM, Cuello C, *et al*. Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and/or catalase. *J Androl* 2005; **26**: 15–24.

[11]Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52**: 302-310.

[12]Suleiman SA, Ali ME, Zaki MS, Malik EMEA, Nast MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996; **17**(5): 530-537.

[13]Thiangtum K, Hori T, Kawakami E. Effect of catalase and superoxide dismutase on motility, viability and acrosomal Integrity of canine spermatozoa during storage at 5℃. *Thai J Vet Med* 2012; **42**(4): 447- 453.

[14]Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil* 1995; **103**: 17–26.

[15]Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a model of sub lethal cryo damage to human sperm during cryopreservation. *J Androl* 1992; **13**: 232-241.

[16]Urata K, Narahara H, Tanaka Y, Gashiru T, Takayama E, Miyakaw I. Effect of endotoxin - induced reactive oxygen species on sperm motility. *Fertil Steril* 2001; **76**: 163-166.

[17]Bilodeau JF, Blanchette S, Gagnon C, Sirard MA. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology* 2001; **56**: 275–286.

[18]Halvorsen B, Holte K, Myhrstad MCW, Barikmo I, Havattum E, Remberg SF. A systematic screening of total antioxidants in dietary plants. *J Nutr* 2002; **132**: 461 – 471.

[19]Witte TS, Schäfer-Somi S. Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Anim Reprod Sci* 2007; **102**: 181-193.

[20]Moore AI, Squires EL, Graham JK. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. *Cryobiology* 2005; **51**: 241-249.

[21]Brooks DE. Biochemistry of the male accessory glands. In:Lamming GE.ed. *Marshall's physiology of reproduction*. 4th ed. Edinburgh: Churchill Livingstone; 1990, p. 569-690.

[22]Corteel JM. Effects du plasma séminal sur la survie et la fertilité des spermatozoids conservés *in vitro*. *Reprod Nut Dev* 1980; **20**: 1111-1123.

[23]Gundogan M. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi Rams. *Turkish J Vet Anim Sci* 2006; **30**: 95-100.

[24]Buckland RB. The activity of six enzymes of chicken seminal plasma and sperm. 1. Effect of *in vitro* storage and full sib families on enzyme activity and fertility. *Poult Sci* 1971; **50**: 1724-1734.

[25]De Lamirande E, Gagnon C. Impact of reactive oxygen species on spermatozoa: A balancing act between beneficial and detrimental effects. *Human Reprod* 1995; **10**: 15-21.

[26]Kobayashi M, Kakizono T, Nagai S. Astaxanthin production by a green alga, Haematococcus pluvialis accompanied with morphological changes in acetate media. *J Ferm Bioeng* 1991; **71**:335–339.

[27]Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology* 20005; **3**: 47–58.