Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

Document heading doi: 10.1016/S2305-0500(14)60028-2

Activity of antioxidative enzymes in fresh and frozen thawed buffalo (*Bubalus bubalis*) spermatozoa in relation to lipid peroxidation and semen quality

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ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form 12 May 2014
Accepted 14 May 2014
Available online 20 September 2014

Keywords:
Buffalo spermatozoa
Cryopreservation
Antioxidative enzymes activity
ROS
Lipid peroxidation and semen quality

ABSTRACT

Objective: To investigate the activity of antioxidative enzymes in fresh and frozen thawed spermatozoa in relation to lipid peroxidation and semen quality in buffalo (*Bubalus bubalis*) bulls. **Methods:** Forty two semen ejaculates from seven buffalo bulls were collected by artificial vagina method and were used for the study. Sperm motility, livability, plasma membrane and acrosomal integrity, buffalo cervical mucous penetration test were assessed in fresh and frozen thawed semen. Intracellular antioxidative enzymatic activity such as super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) and reduced glutathione (GSH), reactive oxygen species (ROS) and lipid peroxidation (LPO) were estimated in fresh and frozen thawed semen. **Results:** A significant ($P<0.01$) reduction in activity of antioxidative enzymes (SOD by 47.7%, GSHPx by 62.7% and GSH by 58.6%) in frozen thawed spermatozoa as compared to fresh spermatozoa was found. Although the catalase activity was varied from 0 to 3.8 IU/10^9 sperm in fresh semen, but after freezing and thawing this activity was not detectable. These enzyme activities had a strong positive association with sperm motility, membrane integrity and distance traveled by vanguard spermatozoa in buffalo cervical mucus and negative correlation with LPO and ROS. However, no significant correlation with acrosomal integrity was found. **Conclusion:** It was concluded that loss of activity of intracellular antioxidative enzymes was evident after freezing and thawing and there was a strong association between the antioxidative enzyme activities, ROS, lipid peroxidation and sperm function in buffalo semen.

1. Introduction

Reactive oxygen species (ROS) is one of the most studied molecules in biomedical sciences owing to its multiple roles in various physiological systems. The free radicals (super oxide anion, hydrogen peroxide and the hydroxyl radicals) have either physiological or pathological effects on sperm function depending on their nature, concentration and duration of exposure. Moderate amounts of ROS has significant physiological role in normal sperm function including hyperactivation, capacitation, acrosomal reaction and zona binding[1, 2]. However, excessive ROS adversely affect the sperm functions and fertilizing ability[3-5]. Mammalian sperm has rich poly unsaturated fatty acids and hence susceptible to lipid peroxidation, which causes loss of membrane integrity, increased permeability and leakage of intra cellular enzymes[3, 4]. Buffalo sperm seems to be more prone to oxidative damage as compared to bull sperm because of its rich amount of polyunsaturated fatty acids [6, 7], leading to relatively higher freeze- thaw associated damage, lower post thaw motility and conception rate[8].

Mammalian spermatozoa have evolved defense

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mechanisms through an enzymatic antioxidant system to protect themselves against oxidative stress. The best known enzymatic antioxidant system is super oxide dismutases (SOD), catalases (CAT) and glutathione peroxidase (GSHPx). The concentrations of these antioxidative enzymes are differed among species both in seminal plasma and spermatozoa[9-11]. The equilibrium between amount of ROS production and antioxidative enzyme system is essential for sperm stability and function[3, 4, 10]. Sperm cells are exposed to oxygen during various cryopreservation and artificial insemination procedures and this could lead to generation of ROS, resulting in oxidative damage to spermatozoa[12]. Frozen thawed bull spermatozoa are more easily peroxidized than fresh spermatozoa[13]. Bovine semen samples with the highest sperm viability after freezing and thawing were also characterized by high activity of antioxidative enzymes before freezing[12]. The balance between ROS production and their detoxification may be an important factor in sperm survival and function before and after cryopreservation. Therefore, the present study was focused on activity of antioxidative enzymes in fresh and frozen thawed buffalo spermatozoa in relation to ROS, lipid peroxidation and semen quality.

2. Materials and methods

2.1. Chemicals

Reduced glutathione and tris buffer were purchased from Merck (India) Ltd and all other chemicals used in the study were purchased from Sigma-Aldrich (New Delhi, India).

2.2. Semen collection and preservation

Seven healthy Murrah buffalo bulls (4-6 years age) with good body condition (score 5-6) maintained under uniform feeding, housing, lighting and management conditions at Germplasm Centre, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India, is located at an altitude of 564 feet above the sea level and at latitude of 28 ° north and a longitude of 79 ° east. The climate touches both the extremes of cold and hot weather experienced in the country and the relative humidity ranges between 15% and 85%. Each experimental animal was offered *ad libitum* drinking water and concentrate: 1 kg/100 kg BW, green fodder: 25 kg, dry roughage: 6 kg. Concentrate mixture consists of 30 parts of maize, 30 parts of soy bean meal, 37 parts of wheat bran which are fortified with mineral mixture and salt daily. Forty two semen ejaculates (Six ejaculates from each bull) were collected by artificial vagina method. Immediately after collection, semen samples were evaluated. 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 ejaculate was divided into two aliquots; one aliquot was used for fresh semen studies, second aliquot was used for cryopreservation at -196 ℃ in liquid nitrogen. The semen was extended in Tris-fructose-egg yolk-glycerol extender[14] to the extent that each mL of diluted semen contained 60 million spermatozoa and equilibrated at 4 ℃ for 2.5 h and exposed to liquid nitrogen vapor for 10 min. before plunging into liquid nitrogen. During the study, all the experimental protocols met the Institutional Animal Care and Use Committee regulations.

2.3. Semen analysis

2.3. 1. Motility and livability

The neat semen was diluted in tris buffer at 37 ℃ and a drop of the diluted semen was kept on a clean, grease free, pre-warmed glass slide, cover slip was placed and progressive motility was assessed under high power magnification (Nikon, Eclipse 80i; 400 × magnification) of a phase contrast microscope. The live sperm percentage was estimated by differential staining technique using Eosin-Nigrosin stain (NE)[15]. Acrosomal intactness was determined in fresh and frozen thawed semen by Giemsa stain[16]. A total of 200 sperm were counted in each smear for live sperm percent and acrosome integrity.

2.3.2. Plasma membrane integrity (Hypo-osmotic swelling test) and cervical mucus penetration test (CMPT)

Hypo-osmotic swelling test (HOST) was carried out according to the method described by Jeyendran *et al.* [17]. Cervical mucus penetration test (CMPT) was carried out according to Matouseket *et al.* [18]. Cervical mucus was collected aseptically from estrus buffalo and loaded in capillary tubes (8.0 cm in length). One end of the tube was sealed with haemoseal (Shandilya, Chemical Pvt. Ltd., Mumbai, India). The free end of capillary tubes were placed in 2.0 mL micro-centrifuge tube containing 0.5 mL semen sample and incubated at 37 ℃ for 60 min. After incubation, the distance (in mm) traveled by the vanguard spermatozoa in cervical mucus was measured under high power (Nikon, Eclipse 80i; 400 × magnification) of phase contrast microscope. Each sample was assessed in duplicate.

2.4. Sperm washing

Sperm cells in fresh and frozen thawed semen were washed using Percoll density gradient as described by Suarez *et al.* [19]. 1 mL layer of 40% percoll (v/v) in modified phosphate buffer saline (mPBS; 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose and 1.0 mM pyruvate, pH 7.4) was pipetted carefully over a 1 mL layer of 80% Percoll (v/v in mPBS) in a disposable 15 mL centrifuge tube. One ml of fresh or frozen-thawed semen

was gently layered on top of the two step Percoll column. The sperm pellet (total of $\times 10^9$) was washed twice with tris HCl buffer (pH 7.4) by centrifugation (500 g; 10 min.). Sperm cells suspended in the Tris-HCl were sonicated and the supernatant was collected for estimation of activity of antioxidative enzymes and LPO.

2.5. Activity of antioxidative enzymes estimation

2.5.1. Super oxide dismutase

Superoxide dismutase in sperm lysate was estimated as per the method of Madesh and Balasubramanian[20]. Freshly prepared 90 μL of pyrogallol solution (1 mM pyrogallol and 1 mM Ethylene diamine tetra acetic acid (EDTA) in PBS) was added to assay mixture, which consisted of 36 μL of MTT (1.25 mM), 774 μL PBS (pH7.4), 600 μL of sperm suspension. The mixture was incubated for 5 min. at room temperature and the reaction was terminated by addition of 900 μL of Dimethyl sulfoxide (DMSO). The tubes were shaken and optical density was measured at 570 nm wavelength using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). A reagent blank was also prepared simultaneously in which sample was replaced with same amount of distilled water. The amount of super oxide formed was calculated using the molar extinction co-efficient of MTT formazan E_{570} of 17000 $M^{-1}cm^{-1}$ at pH 7.4. Per cent inhibition by the presence of SOD was calculated from the reduction of MTT colour formation as compared to MTT formazan formed in the absence of SOD which was taken as 100%.

2.5.2. Catalase

Catalase activity was determined in sperm lysate using Aebi's method[21]. 5 mL of the lysate was added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity was measured at 240 nm for 1 min. using the spectrophotometer. The molar extinction coefficient of H_2O_2 43.6 M cm^{-1} was used to determine the catalase activity. One unit of activity is equal to one millimole of H_2O_2 degraded per mL.

2.5.3. Glutathione peroxidase

GSHPx activity was measured based on the principle that GSHPx catalyze the reaction between H_2O_2 and reduced glutathione (GSH) to form oxidized glutathione (GSSG) and water as per the method described by Hafeman *et al.* [22]. The GSHPx activity was the rate of oxidation of GSH by H_2O_2. The assay mixture was consisted of 0.2 mL sperm lysate, 1 mL GSH (2.mM), 1 mL sodium phosphate buffer (pH, 7.2), 0.5 mL sodium azide (0.01 M) and 1.3 mL distilled water was incubated at room temperature for 5 min. One mL H_2O_2 was added to assay mixture and incubated for 3 min at 37 ℃. Following incubation, 1 mL of the reaction mixture was added to 4 mL of meta-phosphoric acid precipitation solution and centrifuged at 500 g for 10 min. 2 mL of resultant supernatant was added to 2 mL of disodium hydrogen phosphate (0.4 M) and 0.1 mL of 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), mixed well and absorbance was measured at 421 nm using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). One unit of GSHPx activity was defined as the decrease in log (GSH) by 0.001 per minute after the decrease in log (GSH) per minute of the non-enzymatic reaction was subtracted.

2.5.4. Reduced Glutathione (GSH)

Reduced glutathione was estimated by DTNB method of Beutler *et al.* [23]. 1 mL of the clean sperm lysate was added to 2 mL of 0.3 M sodium phosphate buffer (pH 8.7) and 1 mL of DTNB prepared in 0.2 M phosphate buffer (pH 8.0) was added just before taking reading and mixed well. Simultaneously, a reagent blank was also prepared. When color development was complete (due to formation of 2-nitro-5-thiobenzoic acid) the optical density was measured spectrophotometrically at 412 nm against blank using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). Standard curves were made using reduced glutathione (5-50 nmol/L).

2.6. Estimation of reactive oxygen species

The present study used 2′, 7′ – dichlo dihydrofluorescein diacetate (DCFH-DA) fluorescent dye to estimate the ROS level in the spermatozoa with flowcytometry. The ROS level in fresh and frozen thawed spermatozoa was estimated as per the procedure described by Sharma *et al.* [24]. Briefly, Percoll washed spermatozoa was resuspended in 1 mL PBS at the concentration of 1×10^6/mL and incubated with DCFH-DA (final concentration of 1.0 μM) in dark at 37 ℃ for 15 min. before addition of PI (final concentration of 25 μg/mL). The labeled spermatozoa were washed with PBS and analyzed with flow cytometer. Flow cytometric analysis was performed using FACS Calibur (Becton Dickinson and Co., Frnalin Lakes, NJ). The DCFH-DA fluorescence was detected in FL1 filter and the fluorescence of PI was detected using a long pass filter of 620 nm (FL-2). Ten thousand sperm cells were acquired and analyzed in each sample at the rate of 50 to 500 events per second and analyzed further with Cell-Quest software (Becton Dickinson, Siteward).

2.7. Estimation of lipid peroxidation

The lipid peroxidation in spermatozoa was measured based on the malondialdehyde (MDA) concentration by adopting the procedures of Buege and Aust[25] and modified by Suleiman *et al.* [26]. 2 mL of TBA-TCA reagent (Tri-Chloro Acetic Acid 15% (w/v), TBA 0.375% (w/v) in 0.25N HCl) was added to 1 mL of sperm suspension. The mixture was treated in a boiling water bath for 15 min. After cooling,

the suspension was centrifuged (500 g; 10 min) and the supernatant was separated, absorbance was measured at 535 nm using Double beam UV-VIS Spectrophotometer (DBS; Model-UV5704SS, ECIL, India). The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5/mol/cm^3).

2.8. Statistical analysis

The statistical analysis of the data was done as per standard procedures[27]. The data recorded in percent values were subjected to angular transformation before the analysis. Analysis of variance (ANOVA) was performed to examine differences between and within bulls. Student 't' test was used to evaluate significant difference between fresh and frozen-thawed spermatozoa for various parameters. Pearson's correlation coefficient was calculated using standard procedure. All the analyses were carried out using SPSS 10.1 version (Statistical analysis software package, USA). Results are presented as mean ± standard error.

3. Results

3.1. Semen quality

The average sperm motility reduced from 81.45 % in fresh semen to 56.72 % in frozen thawed semen. The mean (±SEM) percent of live sperm was (88.40 ± 0.90) in fresh semen and (59.68 ± 4.20) in frozen-thawed semen. The mean (±SEM) percentage of sperm with intact acrosome in the fresh semen was (90.70 ± 1.20) and (71.84 ± 4.48) in frozen-thawed semen. There was a significant ($P<0.01$) reduction in sperm motility, live sperm percent and intact acrosome after freezing and thawing in the present study (Table 1).

3.2. Plasma membrane integrity (HOST) and cervical mucus penetration test (CMPT)

The mean (±SEM) percentage of spermatozoa responding to HOST in fresh and frozen thawed semen was (74.40 ± 0.80) and (47.75 ± 0.97), respectively and the difference was significant ($P<0.01$). The mean distance traveled in the cervical mucous by vanguard spermatozoa was (25.81 ± 0.65) and (19.45 ± 1.23) mm/60 min. in fresh and frozen-thawed semen, respectively. The traveled distance was significantly ($P<0.05$) higher in fresh semen as compared to frozen thawed semen (Table 1).

3.3. Activity of antioxidative enzymes

The SOD activity in fresh spermatozoa was varied from 14.34 to 29.34 IU/10^9 spermatozoa, while the corresponding values in frozen thawed spermatozoa was 6.86 to 19.56 IU/10^9. There was a significant ($P<0.01$) reduction in intracellular SOD activity after freezing and thawing. In fresh spermatozoa, the catalase activity was varied from 0 to 3.8 IU/10^9 spermatozoa with mean value of (0.80 ± 0.01). After freezing, the catalase activity was not detectable. The GSHPx activity level was varied from 3.80 to 13.20 nmol/NADPH oxidized per min/10^9 sperm in fresh semen. Similarly, the GSH activity was (23.46 ± 0.87) nmol/10^9 sperm in fresh and (15.36 ± 0.43) nmol/10^9 sperm in frozen-thawed semen. There was significant ($P<0.01$) reduction in intracellular GSHPX and GSH activity after freezing and thawing as compared to fresh semen (Table 1).

Table 1
Seminal parameters and intracellular antioxidative enzymes level in fresh and frozen-thawed buffalo spermatozoa (mean ± SE; n=42).

Parameters	Fresh semen	Frozen-thawed semen
Sperm motility (%)	81.45±2.40	56.72±4.80**
Live spermatozoa (%)	88.43±0.70	59.68±4.20**
Membrane integrity- HOST positive (%)	74.40±0.80	47.75±0.97**
Intact acrosome (%)	90.70±1.20	71.84±4.48**
CMPT (mm/ 60 min)	25.81±0.65	19.45±1.23*
SOD (U/10^9 spermatozoa)	23.34±0 .77	11.78±0.88**
Catalase (U/10^9 spermatozoa)	0.80±0.01	0.00±0.00
GSHPx (U/10^9 spermatozoa)	9477.32±24.78	5276.43±18.34**
GSH (nmole/10^9 spermatozoa)	23.46±0.87	15.36±0.43**
LPO (nmol MDA/ 10^9 spermatozoa)	278.78±18.28	364.67±22.40**

Significant * ($P<0.05$), ** ($P<0.01$)

3.4. Reactive oxygen species and lipid peroxidation

The ROS level in frozen thawed sperm cells was significantly ($P<0.05$) lower than fresh semen when the proposition of dead cells were included, however, the different was non- significant when the dead cells were excluded. The ROS in the viable sperm did not differ significantly (Table 3). Mean percentage of live spermatozoa with low and high ROS was 51.60% and 36.70% in fresh and 38.4% and 29.5% in the frozen thawed semen, respectively (Table 2). Similarly, the mean (±SEM) MDA concentration in the sperm extract of fresh semen was 278.78 ± 18.28 nmole/10^9 sperm (Table 1). The MDA concentration was significantly higher ($P<0.01$) in frozen-thawed spermatozoa than that in fresh spermatozoa (Table 1).

3.5. Relationship between activity of antioxidative enzymes, ROS, LPO and semen quality

The correlation coefficients between different parameters were shown in the Table 3 and 4. Strong association was existed among activity of antioxidative enzyme activity, ROS, LPO and semen quality. These enzymes activities had significant positive correlation with sperm motility, membrane integrity and distance traveled by vanguard spermatozoa and negative correlation with MDA level in both fresh and frozen-thawed semen. However, no correlation was existed with acrosomal integrity. The proposition

spermatozoa with high ROS was negatively correlated with SOD (r= −0.77), catalase (r= −0.63) and GSHPx (r= −0.72) in fresh semen, while these association was poor in frozen-thawed semen when compared to fresh semen (Table 4). The MDA concentration was negatively associated with distance traveled by vanguard spermatozoa (r = −0.87 at fresh and −0.91 at post thaw stage) and membrane integrity (r = −0.86 at fresh and −0.94 at post thaw stage).

Table 2
Proposition of buffalo spermatozoa with low and high intracellular ROS level in fresh and frozen-thawed semen (mean ± SE; n=42) .

Level of ROS	Fresh Semen	Frozen-thawed semen
Low ROS	51.6±2.9	38.4±3.2*
High ROS	36.7±2.6	29.5±2.1*
Dead	12.1±0.8	32.3±2.7**
Excluding dead cells		
Low ROS	57.5±3.5	56.5±4.3
High ROS	42.4±3.2	43.3±3.3

Significant * (P<0.05), ** (P<0.01)

Table 3
Correlation coefficient among different seminal parameters and antioxidative enzymes level(n=42) in buffalo spermatozoa.

Between Parameters	Fresh semen	Frozen-thawed semen
Sperm motility and SOD	0.79	0.87
Sperm motility and GSHPx	0.84	0.90
Sperm motility and Catalase	0.67	–
Membrane integrity and SOD	0.88	0.95
Membrane integrity and GSHPx	0.86	0.93
Membrane integrity and Catalase	0.74	–
Acrosomal integrity and SOD	0.34	0.27
Acrosomal integrity and GSHPx	0.32	0.28
Acrosomal integrity and Catalase	0.31	–
CMPT and SOD	0.78	0.81
CMPT and GSHPx	0.83	0.86
CMPT and Catalase	0.56	0.54

Table 4
Relationship among antioxidative enzymes, ROS and LPO in fresh and frozen-thawed buffalo spermatozoa (n=42).

Between Parameters	Fresh semen	Frozen-thawed semen
Sperm with high ROS and SOD	−0.77	−0.58
Sperm with high ROS and catalase	−0.63	–
Sperm with ROS and GSHPx	−0.72	−0.48
Sperm motility and sperm with high ROS	−0.47	−0.27
Membrane integrity and sperm with high ROS	−0.52	−0.37
High ROS and LPO	0.72	0.38
LPO and SOD	−0.75	−0.56
LPO and catalase	−0.62	–
LPO and GSHPx	−0.58	−0.43
LPO and sperm motility	−0.78	−0.56
LPO and membrane integrity	−0.86	−0.94
LPO and acrosomal integrity	−0.34	−0.28
LPO and CMPT	−0.87	−0.91

4. Discussion

Mammalian spermatozoa are highly susceptible to oxidative damage and the natural antioxidative enzymes in the seminal plasma and spermatozoa are the frontline defense mechanisms against the oxidative damage[2, 13]. The SOD, CAT, GSHPx and GSH are the major enzymes involved in detoxification of ROS in mammalian spermatozoa. These enzymes activity are widely differed among the different species. The SOD activity of buffalo spermatozoa was recorded in this study was slightly higher as compared to bull spermatozoa[28, 29], but was in agreement with earlier reports in buffalo[7]. Lower SOD activity was recorded in bull spermatozoa as compared to boar, ram, stallion and donkey spermatozoa earlier[9]. The present study observed that low catalase activity in fresh buffalo spermatozoa was as reported in bull[29]. Indeed, catalase activity was poor or absent in mammalian spermatozoa[9].

In the present study, low level of GSHPx activity in buffalo spermatozoa was observed, which was in agreement with results obtained by Nair *et al.* [7] in buffalo spermatozoa and Bilodeau *et al.* [29] in bull spermatozoa. Slaweta *et al.* [30] reported higher levels of total GSHPx activity in bull spermatozoa than the results of present study. Lower SOD, GSHPx and G6PD activities in buffalo spermatozoa were observed than in cattle spermatozoa[7] in the present study.

The balance between intracellular activity of antioxidative enzymes and ROS production is an important factor in sperm survival and function during freezing and thawing process. In the present study, sperm SOD activity by 47.7%, GSHPx activity by 62.7% and GSH activity by 58.6% were reduced after freezing and thawing process. These findings were in agreement with those reported in bull[29, 30], boar [31] Dog[32] and in human[33, 34] spermatozoa. The loss of intracellular antioxidative enzymes activity after freezing might be due to altered membrane integrity and increased permeability as a consequence of membrane damage during cryopreservation [35, 36]. The loss of intracellular activity of antioxidative enzymes following cryopreservation and thawing is the major cause for the susceptibility of frozen thawed sperm cells to peroxidative damage[29, 33].

The intracellular activity of antioxidative enzymes was positively correlated with sperm motility, livability and membrane integrity. These findings were in agreement with those reported by Nair *et al.* [7], who observed the SOD, GSHPx and G6PD activities of both bull and buffalo spermatozoa were to be positively correlated with motility

and livability. Similarly, a significant positive correlation between sperm SOD activity and motility has been observed in human[34] and in Dog[10]. Alvarez and Storey[33] reported that semen samples with highest viability after freeze-thawing were characterized by high SOD activity and a strong correlation between loss of SOD activity and loss of sperm motility and membrane integrity. In the present study, there was a negative correlation between the antioxidative enzymes and LPO production, which is in agreement with the earlier findings in dog[10] and in human[33].

The results of present study revealed that there was significantly higher LPO in frozen thawed spermatozoa than in fresh spermatozoa as reported in bull[30, 37], boar[35] and stallion [38]. This could be due to the fact that frozen thawed spermatozoa were more easily peroxidized than fresh spermatozoa[13]. The concentration of sperm MDA was negatively correlated with sperm motility, livability and membrane integrity in fresh as well as frozen thawed semen, this finding was in agreement with Slaweta *et al.* [30], Alvarez and Storey[33] and Kadirvel *et al.* [8]. A significant negative correlation between sperm motility and lipid peroxidation was reported in cattle and buffalo[7, 8], dog[10] and human[26]. Alvarez *et al.* [39] opined that cryopreservation might indirectly cause membrane damage by enhancing lipid peroxidation and induction of membrane leakiness. The freezing and thawing process induces oxidative stress, leading to LPO of sperm membrane, resulting in increased membrane permeability and loss of membrane integrity, successive leakage and reduction in the intracellular antioxidative enzymes. However, Alvarez and Storey[33] hypothesized two models of sub-lethal cryo damage. One relates to membrane damage through LPO and other relates to membrane embrittlement during phase transitions following freezing and thawing and is termed it as "stress-related". Recently, it has also been demonstrated that LPO and membrane damage are relatively independent processes[5, 40]. It is, therefore, understandable that freezing and thawing induces membrane damage and increase membrane permeability, leading to leakage and loss of these enzymes. This leads to increased susceptibility to oxidative stress and LPO. Further studies are required to rule out whether oxidative damages proceeds "stress-related" or vise versa during freezing and thawing process.

Many biochemical or mechanical factors have been found to stimulate the production of ROS by spermatozoa. The increased level of sperm ROS during cryopreservation in bovine sperm and cause impairment of sperm function has been reported[12]. However, the results showed a significant decrease in levels of ROS production by sperm cells following freeze-thawing as compared to fresh sperm cells. These findings were in close agreement with Guthrie and Welch[41]. Similarly, the basal ROS formation was remained relatively low and constant during cryopreservation process has been reported in boar[42] and in human spermatozoa[43]. The decreased ROS generation following cryopreservation was observed in the study might be due to reduction in the metabolic activity and cell viability during cryopreservation, since the formation of the free radicals is an integral part of the metabolic activity of living cells. Further, these increases in ROS production also may be associated with cellular exposure to decreasing temperatures as a result of increased enzymatic activity. However, as temperatures are further reduced, the ability to produce ROS gradually decreases during cryopreservation[44].

The distance traveled by vanguard spermatozoa in buffalo cervical mucous was positively correlated with the activity of antioxidative enzymes and negatively correlated with LPO. The association between the intracellular level of glutathione and ability to penetrate the cervical mucus in human sperm was reported in past[45]. This can be explained by the fact that spermatozoa with higher antioxidative enzymes had better protection against oxidative damage and LPO, maintain the membrane integrity better and survive longer duration in the cervical mucus than sperm with lower antioxidative enzymes. In the present study, there was not a significant correlation between the MDA concentration and acrosomal integrity. These results are in line with Baumber *et al.* [5] and Neild *et al.* [38], who reported that the acrosomal damage was independent from ROS and lipid peroxidation. Brouwers *et al.* [40] also observed that the localization of LPO was virtually absent in sperm head as assessed by fluorescent techniques using the LPO reporter probe (C11-BOIPY). It seems that the acrosomal damage during cryopreservation thawing might be related to stress-related.

It could be concluded from the findings of the present study that loss of activity of intracellular antioxidative enzymes was evident after freezing and thawing of buffalo semen and also there was a strong association between antioxidative enzymes activity, lipid peroxidation and sperm function in frozen thawed buffalo semen.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgement

The authors thankfully acknowledge the Director and the Head, Animal Reproduction Division, IVRI, Izatnagar, Bareilly, India for providing necessary facilities to conduct the research work.

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