Research Article

Effect of Mn^{2+} on Cryocapacitation, *in vitro* Acrosome Reaction, Hypo osmotic Swelling Test, Lipid Peroxidation, Superoxide Dismutase and Glutathione Enzymes Activity during Cryopreservation of Buffalo Bull Semen

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Abstract

Oxidative stress has been considered a major contributing factor to male infertility, which occurs during cryopreservation/ freeze-thawing process of semen. The supplementation of semen extender with antioxidants has been shown to provide a cryoprotective effect on semen quality. Therefore, the aim of the present study was to determine the effect of Mn^{2+} (200µM) on hypo-osmotic swelling (HOS) test, cryocapacitation of spermatozoa, lipid peroxidation (LPO), *in vitro* acrosome reaction, superoxide dismutase (SOD) , glutathione peroxidase (GPX), and glutathione reductase (GR) enzymes activity during different stages of cryopreservation. Fresh semen samples, diluted with tris citric acid egg yolk (TCY) extender with or without Mn^{2+} were cooled (pre-frozen), cryopreserved and post-thawed as per standard procedure. Five replicates of each of three bulls were evaluated for estimation of HOS, AD, LPO, SOD, GPx, GR and *in vitro* acrosome reaction. Percentage of HOS positive spermatozoa significantly ($p \leq 0.05$) decreased during prefreezing (PF) and post-thawed (PT) stages of cryopreservation, but, Mn^{2+} supplementation improved the percentage of HOS positive spermatozoa. However, non significant ($p \geq 0.05$) differences were observed in cryocapacitation, *in vitro* acrosome reaction, GPx and GR enzyme activity of spermatozoa irrespective of Mn^{2+} supplementation during cryopreservation. Malondialdehyde (MDA-end product of LPO) production showed significant ($p \leq 0.05$) increase after freezing- thawing of semen, which was non-significant ($p \geq 0.05$) in pre-frozen semen. Supplementation of Mn^{2+} reduced the MDA production significantly ($p \leq 0.05$) in post thawed semen, but, non-significantly ($p \geq 0.05$) in pre-frozen semen. The activity of SOD decreased significantly ($p \leq 0.05$) both in pre-frozen and post thawed semen. Whereas, supplementation of Mn^{2+} further improved the SOD activity in all pre-frozen as well as post –thawed semen samples. Therefore, it is concluded that Mn^{2+} proved as a potent antioxidant by reducing cryocapacitation of spermatozoa, LPO level and enhancing HOS, GPx, GR & SOD activity in pre-frozen and post-thawed semen.

Keywords: Buffalo, semen, HOS, LPO, SOD, Mn^{2+}

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Introduction

In order to perform artificial insemination (AI), freezing and thawing of semen samples are routinely performed [1], but cryopreservation also induces extensive biochemical and biophysical changes in the membranes of spermatozoa that ultimately decrease the fertility potential of spermatozoa [2]. Cryopreservation increases premature capacitation of spermatozoa [3]. These alterations may not affect only the motility, but, reduces the life span, ability to interact

with female reproductive tract and fertility potential of the spermatozoa. Cryopreservation is known to produce reactive oxygen species (ROS) in semen [4,5]. Numerous studies have shown that ROS play a significant role in male infertility [6, 7]. ROS, such as hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), and hydroxyl radicals ($OH^{\cdot}$), are formed as natural by-products of the normal metabolism of aerobic organisms. During metabolism, ROS, which are unstable and highly reactive, become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease [8]. ROS are a double-edged sword as these are involved in physiological functions of sperm including capacitation, acrosome reaction, and binding to the zona pellucida at physiological concentrations [9,10]. Under normal conditions, scavenging molecules known as antioxidants convert ROS to safe by-products to prevent damage caused by ROS. However, when the balance between ROS production and detoxification is disrupted, excess ROS create oxidative stress which can damage the sperm cell membranes [11], adversely affect DNA integrity [12], block oxidative metabolism[7], reduce sperm–oocyte fusion [13], and reduce sperm motility and viability membrane integrity, antioxidant status and fertility [14, 15].

In recent years, research on the application of antioxidants to improve cryopreservation of mammalian spermatozoa and improve quality of post-thaw semen has been extensively studied [6]. Damages due to oxidative stress may be reduced by supplementation of antioxidants [16]. Superoxide dismutase is one of the key enzymes to regulate the oxidative stress in sperm. SOD plays a major role in decreasing LPO and protecting spermatozoa under oxidative damages [17]. Generation of ROS can be quenched by the interaction of glutathione peroxidase (GPx) and glutathione reductase (GR) enzymes of glutathione cycle [18]. Out of many trace elements, manganese is one of useful antioxidant in reducing the oxidative stress/LPO and improving the viability of cattle bull spermatozoa [19, 20]. Manganese is a chain breaking antioxidant and quenches peroxyl radicals [21] Therefore, the present study was done to evaluate the potent effect of Mn^{2+} (as antioxidant) on hypo-osmotic swelling test (HOS) , cryocapacitation , lipid peroxidation (LPO) , *in vitro* acrosome reaction, superoxide dismutase (SOD) , glutathione peroxidase (GPX), and glutathione reductase (GR) enzymes activity during different stages of cryopreservation of buffalo bull spermatozoa.

Experimental

Materials and Reagents

Procurement of semen

Semen samples with more than 80 % initial motility and 1200-1400 × 10^6/ml sperm count were obtained from healthy buffalo bulls(n=3) maintained at Dairy Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Each parameter was analyzed using five replicates of each of three bulls.

Cryopreservation of semen

Freshly ejaculated semen sample was collected and a small part of it was kept for further estimations and rest was diluted with Tris citric acid egg yolk (TCY) extender (pH 7.4) up to a concentration of 120× 10^6 cells/ml. Diluted semen divided into two equal fractions, supplemented/ un-supplemented with 200µM Mn^{2+}, was incubated at 37^0C for 20 minutes. Subsequently, these fractions were transferred to a cold handling cabinet (4^0C) for four hours. After checking the motility of these pre-frozen/cooled samples, a part of these samples was kept for further analysis; rest was filled in 0.25ml French straws and cyopreserved at -196^0C for 24 hrs. After cryopreservation, these straws were thawed at 37^0C and semen was evaluated.

Hypo-osmotic swelling test [22]

Briefly, semen at three steps of freezing i.e. freshly ejaculated (FES), pre frozen (PFS) and post-thawed (PTS) with or without Mn^{2+} was incubated with 1.0 ml of 100µM of HOS solution and 0.85 % saline separately for 30 minutes.

After 30 minutes, spermatozoa with swollen and coiled tail were observed under 40×10X. A total of 200 coiled/uncoiled spermatozoa were counted. Percent of HOS positive spermatozoa was calculated by subtracting number of sperms in normal saline from that in HOS solution.

CTC (Chlorotetracyclin) staining for cryocapacitation

Effect of manganese on cryocapacitation was assessed by staining the sperm smears with CTC staining at different stages of cryopreservation [23]. At least 200 spermatozoa with normal (whole sperm head with bright fluorescence) and cryo capacitated sperm (acrosome-intact sperm with fluorescence on the acrosomal region and acrosome-reacted sperm with fluorescence on postacrosomal region) were counted in different fields at 10 x 40 X and percentage of cryocapacitated spermatozoa was calculated.

Lipid peroxidation (LPO) [24]

Membrane LPO was measured by the end point generation of malondialdehyde (MDA) determined by thiobarbituric acid (TBA) assay. 0.2 ml of all the five types of semen samples (FES, PFC, PFMn, PTC, PTMn) were incubated with 0.2 ml of 150 mM Tris-HCl (pH 7.1) in five tubes at 37^0C for 20 minutes. After the completion of the incubation, 1 ml of 10 % TCA and 2 ml of 0.375 % TBA were added and then kept for 20 minutes in the boiling water bath. Thereafter, samples were centrifuged for 15 minutes at 5000 rpm and supernatants were taken out. The absorbance was read at 532 nm.

Protein was measured by the method of Lowry *et al.* [25]. The molar extinction coefficient for MDA is 1.56×10^5 $M^{-1}.cm^{-1}$. The results were expressed as n moles of MDA/µg protein/ml.

Superoxide dismutase (SOD) [26]

Total superoxide dismutase activity was measured by the inhibition of nitro blue tetrazolium (NBT) reduction in the presence of the superoxide anion. Samples were incubated with NBT and nicotinamide adenine dinucleotide (NADH) at 25^0C for 10 minutes. Thereafter, phenazonium methosulphate (PMS) was added and colour developed was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme capable of decreasing NBT reduction by 50%.

Glutathione peroxidase (GPx)

GPx activity was measured by the method of Necheles *et al.* [27]. The reaction mixture consisted of 0.4 ml phosphate buffer (0.4 M, pH 7.0), 0.1 ml sodium azide (10 mM), 0.2 ml GSH (8 mM), 0.1 ml enzyme source, 0.1 ml H_2O_2 and 1.1 ml double distilled water. This was incubated at 37^0C for 5 minutes. After incubation, 0.5 ml of chilled trichloroacetic acid (TCA; 10%) was added to it. The reaction mixture was then centrifuged at 3000 rpm for 15 minutes. After centrifugation, 0.5 ml supernatant was taken and 3.0 ml disodium hydrogen phosphate (Na_2HPO_4, 0.3 M) was added to it. This was followed by the addition of 1.0 ml DTNB (20 mg/50ml of 1% sodium citrate, freshly prepared), absorbance was recorded at 412 nm within 5 minutes of addition of DTNB. Appropriate blank and standard were also run. The results were expressed as (IU/ 10^9/ min).

Glutathione reductase (GR)

The activity of glutathione reductase was measured by the method of Krohne-Ehrich *et al.* [28]. The reaction mixture (2 ml, pH 7.0) consisted of 0.25 ml potassium phosphate buffer (50mM), 0.5 ml potassium chloride (20mM), 0.5 ml EDTA (1mM), 0.5 ml GSSG (1mM); 0.25 ml bovine serum albumin (0.5 mg/ml) was incubated at 25^0C for 5 minutes. Thereafter, 100 µl of enzyme solution (1 mg protein/ml) was added to this mixture. Added 50 µl reduced NADP (Nicotine amide dinucleotide phosphate) to initiate the reaction. The decrease in absorbance was measured at 340 nm at 1 minute interval for 5 minutes. The results were expressed as (IU/ 10^9/ min).

In vitro Acrosome reaction

Smears of freshly ejaculated semen, post-thawed semen with and without Mn^{2+} were prepared at 2 hr interval, stained with Giemsa stain and counted in different fields at 10 x 100 X under phase contrast microscope and percentage acrosome reaction was calculated.

Statistical analysis

'Analysis of Factorial Experiment in CRD' (software programme) or 'One Way Variance Analysis' was performed to evaluate the significance levels between the parameters studied. The critical difference (CD) of two factors A (bull), B (treatments) and AB (interaction between) obtained were used to find the level of significance. A 'P' value of 0.05 was selected as a criterion for statistically significant differences.

Results and discussion

Effect of Mn^{2+} on hypo-osmotic swelling (HOS) test

During cryopreservation of buffalo bull spermatozoa, percentage of HOS positive spermatozoa was significantly ($p \leq 0.05$) decreased at PF and PT stages in comparison to FES, but, supplementation of Mn^{2+} improved the HOST non-significantly ($p \geq 0.05$)**(Table 1)**. Percentage of HOS positive spermatozoa was highest in bull number 3 irrespective of the stage of cryopreservation, but differences among the three bulls were non-significant ($p \geq 0.05$). Statistical analysis showed non- significant interaction between bull (A) and treatment (B) factors **(Table 1**).

Table 1 Effect of Mn^{2+} on Hypo osmotic swelling (%) test of buffalo bull semen

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	71.3 ±3.5	88.3 ±3.8	89.2 ± 2.5	**82.9^{c}**
PFC	51.9 ± 5.5	66.7 ±2.8	76.1 ± 6.1	**64.9^{b}**
PF-Mn	59.0 ± 6.3	71.1 ± 2.3	74.1 ± 5.2	**68.06^{b}**
PTC	46.0 ± 4.3	53.8 ± 6.2	55.4 ± 5.7	**51.7^{a}**
PT-Mn	52.3 ± 5.0	59.6± 5.8	62.5 ± 4.6	**58.1^{ab}**
Combination mean for Bull (n=3) factor	**56.1^{a}**	**67.9^{a}**	**71.4^{a}**	

Each value represents mean ± SE; ^{a,ab,b,c}Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn;; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

Effect of Mn^{2+} on cryocapacitation (%)

CTC staining showing bright fluorescence on the whole head indicated the status of non-capacitated spermatozoa, whereas fluorescence only on acrosome region indicated capacitation stage of spermatozoa. Capacitated, acrosome-reacted sperm showed the fluorescence only on postacrosomal region **(Figure 1)**. Non-significant ($p \geq 0.05$) differences were observed in percentage of cryocapacitated spermatozoa among the three bulls irrespective of Mn^{2+} supplementation during PF and PT stages of cryopreservation **(Table 2)**. Statistical analysis showed non- significant interaction between bull (A) and treatment (B) factors, which indicated percentage of cryocapacitation of spermatozoa in different bulls was not affected by cryopreservation **(Table 2).**

Table 2 Effect of Mn^{2+} on cryocapacitation (%) of buffalo bull semen

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	7.8 ± 0.7	10.2 ± 4.5	26.6 ± 5.6	**14.86[a]**
PFC	1.9 ± 0.2	23.7 ±8.8	40.8 ± 3.1	**22.13[a]**
PF-Mn	5.6 ± 1.4	6.2 ± 8.5	25.6 ± 4.6	**12.46 [a]**
PTC	32.1 ± 14.3	16.6 ± 8.3	55.8 ± 2.0	**34.8 [a]**
PT-Mn	19.2 ± 11.5	8.6 ± 4.9	30.9 ± 3.2	**19.5 [a]**
Combination mean for Bull (n=3) factor	**13.32[a]**	**13.06[a]**	**35.94[a]**	

Each value represents mean ± SE; [a,]Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn;; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

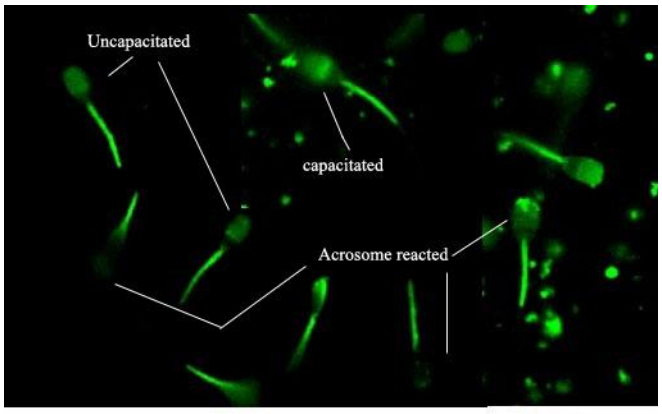

Figure 1 Different stages of cryocapacitation (CTC stain) of cattle bull spermatozoa during the process of cryopreservation

Effect of Mn^{2+} on lipid peroxidation (LPO)

There was variation in LPO level in freshly ejaculated (FES), pre freezing (PF) and post thawed (PT) semen irrespective of manganese supplementation in three tested bulls **(Table 3)**. Data showed that MDA production was highest in bull no. 3, but the differences among the three bulls were non- significant ($p \geq 0.05$). Further, as compared to freshly ejaculated semen, PF and PT showed an increase in level of LPO, but, this increase was significant ($p \leq 0.05$) only in PT stage. Supplementation of Mn^{2+} decreased the LPO level at both PF and PT stage, but was significant ($p \leq 0.05$) only at later stage. Statistical analysis showed significant ($p \leq 0.05$) interaction between bull factor (A) and treatment factor (B), which indicated that MDA production in different bulls was affected by different cryopreservation **(Table 3)**.

Table 3 Effect of Mn^{2+} on lipid peroxidation (n moles MDA/ mg protein/ml) of buffalo bull semen

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	138.9 ± 23.1	123.9 ± 36.5	190.5 ± 149.3	**151.10^{a}**
PFC	428.2 ± 111.5	209.5 ± 32.1	561.8 ± 267.4	**399.86^{ab}**
PF-Mn	274.6 ± 117.5	180.2 ± 58.2	433.4 ± 160.4	**296.09^{ab}**
PTC	452.7 ± 114.7	1351.7 ± 92.3	818 ± 337.8	**874.13^{c}**
PT-Mn	229.5 ± 61.5	528.9 ± 266.3	459.1 ± 136. 4	**405.83 ^{b}**
Combination mean for Bull (n=3) factor	**304.78^{a}**	**478.84^{a}**	**492.56^{a}**	

Each value represents mean ± SE; ^{a,ab,b,c} Any two means in a column or row having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

Effect of Mn^{2+} on superoxide dismutase (SOD) activity

Superoxide dismutase activity with or without Mn^{2+} supplementation was also studied in undiluted semen and two stages of cryopreservation **(Table 4)**. A significant ($p \leq 0.05$) decrease in SOD activity was observed in PF and PT samples as compared to FES. However, supplementation of Mn^{2+} increased the SOD activity both at pre freezing and post-thawed stage, which was significant ($p \leq 0.05$) only at later stage **(Table 4)**. With regard to bull factor, there was non-significant ($p \geq 0.05$) difference in SOD activity in bull nos. 1 and 2, but increase in SOD activity was significant ($p \leq 0.05$) in bull 3. Statistical analysis showed non- significant interaction between bull (A) and treatment (B) factors. Therefore, SOD activity in different bulls was not affected by different stages of cryopreservation and supplementation of manganese **(Table 4)**.

Table 4 Effect of Mn^{2+} on superoxide dismutase (IU/ mg protein/ml) enzyme of buffalo bull semen

Each value represents mean ± SE; ^{a,ab,b,c} Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn;; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	545.4 ± 265.4	227.8 ± 23.9	1480.6 ± 950.4	**751.3^{c}**
PFC	209.4 ± 167.5	256.6 ± 121.8	356.3 ± 152	**274.13^{ab}**
PF-Mn	374.4 ± 137.8	139.9 ± 127.1	539.7 ± 190.7	**351.38^{ab}**
PTC	91.9 ± 25.1	105.4 ± 53.5	70.4 ± 16.3	**89.23^{a}**
PT-Mn	192.4 ± 93.7	189.7 ± 20.7	73.02 ± 310.1	**151.7^{b}**
Combination mean for Bull (n=3) factor	**282.7^{a}**	**183.8^{a}**	**504.00^{b}**	

Effect of Mn^{2+} on glutathione peroxidase (GPx) and glutathione reductase (GR) enzyme activity

GPx activity decreased both in PF and PT semen samples as compared to FES **(Table 5)**. Mn^{2+} supplementation improved the GPx activity non-significantly ($p \geq 0.05$) only after freezing-thawing. Non-significant ($p \geq 0.05$) differences in GPx activity were observed among the three bulls, but it was highest in bull no. 3. Statistical analysis showed non- significant ($p \geq 0.05$) interaction between bull (A) and treatment (B) factors. Therefore, GPx activity in different bulls was not affected by different stages of cryopreservation and supplementation of manganese **(Table 5)**.

Table 5 Effect of Mn^{2+} on glutathione peroxidase (GPx) (IU/ 10^{9}/ min) enzyme of buffalo bull semen
Each value represents mean ± SE

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	0.20	0.12	0.92	**0.41^{a}**
PFC	0.16	0.16	0.31	**0.21^{a}**
PF-Mn	0.19	0.07	0.25	**0.17^{a}**
PTC	0.13	0.09	0.08	**0.10^{a}**
PT-Mn	0.78	0.27	0.14	**0.39^{a}**
Combination mean for Bull (n=3) factor	0.29^{a}	0.14^{a}	0.34^{a}	

^{a}Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn;; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

GR activity was increased both in pre-freezing and post-thawed stage, but non –significant ($p \geq 0.05$) differences were observed as compared to FES **(Table 6)**. Supplementation of Mn2+ improved the GR activity non-significantly ($p \geq 0.05$) only at post-thawed stage. Non significant differences were observed among the three bulls, but bull no. 1 showed the maximum GR activity. Statistical analysis showed non- significant ($p \geq 0.05$) interaction between bull (A) and treatment (B) factors. Therefore, GR activity in different bulls was not affected by different stages of cryopreservation and supplementation of manganese **(Table 6)**.

Table 6 Effect of Mn^{2+} on glutathione reductase (GR) (IU/ 10^9/ min) enzyme of buffalo bull semen

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	0.42	0.27	0.30	**0.33[a]**
PFC	0.52	0.90	0.53	**0.65[a]**
PF-Mn	0.38	0.36	0.42	**0.38[a]**
PTC	1.21	0.57	0.57	**0.78[a]**
PT-Mn	1.49	0.81	0.46	**0.92[a]**
Combination mean for Bull (n=3) factor	**0.80[a]**	**0.58[a]**	**0.45[a]**	

Each value represents mean ± SE; [a,] Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen ;PFC, Pre-frozen control; PF-Mn, pre-frozen semen supplemented with Mn;; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

Effect of Mn^{2+} on *in vitro* acrosome reaction

Table 7 Effect of Mn^{2+} on *in vitro* acrosome reaction (%) of buffalo bull post- thawed semen

Samples (Factor B)	Bull Nos.(Factor A)			Combination mean for treatment (n=5)factor
	1	2	3	
FES	49.57	21.99	42.4	**37.98[a]**
PTC	26.67	42.43	59.16	**42.75[a]**
PT-Mn	36.33	31.05	56.68	**41.3[a]**
Combination mean for Bull (n=3) factor	**37.52[a]**	**31.82[a]**	**52.74[b]**	

Each value represents mean ± SE; [a,,b]Any two means in a row and column having different superscripts are significantly different at 5% level of significance.

FES, Freshly ejaculated semen; PTC, post-thawed control; PT-Mn, post-thawed semen supplemented with Mn

An enhanced AR was observed at post-thawed stage with or without Mn^{2+} in comparison to FES **(Table 7) (Figure 2)**. Non significant ($p \geq 0.05$) differences were observed among the three bulls, but bull no.3 showed maximum AR. Statistical analysis showed non-significant ($p \geq 0.05$) interaction between bull (A) and treatment (B) factors. Therefore, AR in different bulls was not affected by different stages of cryopreservation and supplementation of manganese **(Table 7)**.

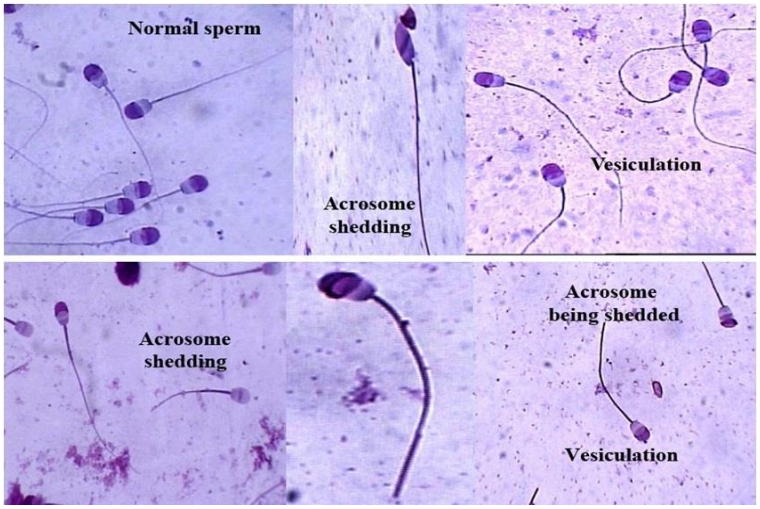

Figure 2 Different stages of cattle bull spermatozoa during the process of cryopreservation (Giemsa stain)

Correlation between different semen parameters

An attempt was also made to correlate different sperm parameters during the process of cryopreservation. A highly positive correlation of 0.99, 0.53, 0.90 was found between HOS and SOD in bull number 1, 2 and 3 respectively. In bull number 2, a positive correlation of 0. 88/ 0.36 were found between acrosome damage and LPO/ SOD.

Bull Nos 1, 3 and 1, 2 showed positive correlations between AR× GPx (0.008, 0.96) and GPx × GR (0.62, 0.73), respectively. A strong positive correlation was also found between AR × GR in bull nos. 2 and 3 (0.49, 0.96), respectively.

In the present study, level of HOS, cryocapacitation, *in vitro* acrosome reaction, LPO, SOD, GPx and GR enzyme activity varied from bull to bull irrespective of Mn^{2+} supplementation during different stages of cryopreservation of semen. Sperm membranes are the primary sites of injury during different stages of cryopreservation. In this study, percentage of HOS positive spermatozoa decreased and cryocapacitation increased at PF and PT stages, which may be due to loss in the membrane integrity during cryopreservation. Bell *et al.* [29] found that cryopreservation and thawing alone or in combination are likely to induce membrane damages, which is measured by degree of lipid peroxidation of polyunsaturated fatty acids (PUFAs) in cell membrane by free radicals. Neild *et al.* [30] also stated that this damage is caused by lipid peroxidation, which reduces membrane integrity. Supplementation of Mn^{2+} to the dilutor improved the percentage of HOS positive spermatozoa and decreased the level of cryocapacitation at PF and PT stages of cryopreservation, which may be due to its antioxidative property, as also observed in our earlier study on cattle bull semen [5].

Chatterjee and Gagnon [3] and Park *et al.* [31] observed an increased production of ROS during the process of freezing –thawing of semen. Cryopreservation of the buffalo semen also enhanced the level of LPO at PF and PT stages. Significant increase in MDA production was observed at PT semen in comparison to PF and FE semen. This may be due to more production of ROS during freezing–thawing process, which lead to more LPO/oxidative stress in PT samples. It was observed by Fraczek *et al.* [32] that polyunsaturated fatty acids (PUFAs) present in the sperm membranes are susceptible to ROS attack, which causes lipid peroxidation during cryopreservation of semen.

Supplementation of buffalo semen with Mn^{2+} decreased the level of LPO in PF and PT semen due to its antioxidative property. Studies have shown that Mn^{2+} significantly inhibited LPO in many cases such as brain phospholipids [33], nigron neurons [34], human placental membranes [19] and ferrous ascorbate treated fresh cattle bull sperm [20].

In the present study, SOD level was decreased in PF and PT semen, which was significant at PT stage. It indicated that freezing–thawing generates more oxidative stress/LPO and to counteract the harmful effects of ROS/LPO, there was a decline in the level of SOD. Orzolek *et al*. [35] also found a significant increase in LPO level after cryopreservation of boar semen, which was partially mediated by the loss of SOD activity. Freezing of fowl semen resulted in damage of plasma membrane, which caused leakage of proteins and loss of enzymes [36]. Therefore, decrease in the level of SOD observed during cryopreservation of buffalo bull semen may be due to freezing-thawing stress. The decrease in SOD activity after freezing –thawing was also found in bull [14] and human [37] sperm. Their studies suggested that partial loss of antioxidant enzymes in cell with damaged sperm plasma membrane caused them more susceptible to peroxidative damage after thawing. It was postulated by Bilodeau *et al*.[[14]and Surai *et al*.[38] that the impairment in antioxidant defense system of mammalian cryopreserved semen might be due to removal or high rate dilution of seminal plasma during freezing and thawing process.

GPx is a selenium containing and GR is the key enzyme of glutathione metabolism [39]. Both these enzymes constitute the glutathione cycle which acts as antioxidant in reducing the oxidative damages caused by cryopresevation. The antioxidant enzyme GPx of this cycle removes peroxyl ($ROO^{\cdot}$) radicals from various peroxides like H_2O_2, thus converts GSH (glutathione reduced) to GSSG (glutathione oxidized), whereas GR regenerates GSH from GSSG as shown below [40]:

$$2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+}$$

In this study, activity of GPx and GR enzymes was not significantly affected by freezing and thawing in untreated (PF & PT) as well as treated (PFMn & PTMn) semen samples. Bilodeau *et al.* [14] also observed that GPx and GR activities were less affected by cryopreservation in bull spermatozoa. However, GPx activity decreased and GR activity increased both in PF and PT samples in comparison to freshly ejaculated semen (FES). Mn^{2+} supplementation improved the GPx and GR activity both in PF and PT semen. It is suggested that GPX provided the most effective protection against cold shock and oxidative damages during cryopreservation process [41], so its level declined pre-freezing and post-thawed stage. In this study, during cryopreservation, to combat oxidative damages, GPx converted more of GSH to its oxidized form GSSG; to convert GSSG back in GSH form, the level of GR got increased, thus the cycle of glutathione remained maintained. Antioxidant response of spermatozoa is mainly due to the capacity of GPx to counteract ROS stress of spermatozoa and minimizes cryopreservation damages. Nair *et al*. [42] also reported the same in buffalo bull spermatozoa. Mn^{2+} as an antioxidant proved useful for the preventing loss of GPx and GR activity which was non-significantly affected by the freezing /thawing process.

In this study, Mn^{2+} supplementation improved *in vitro* acrosome reaction due to its antioxidative property. Manganese maintained the membrane integrity and viability of spermatozoa by decreasing LPO and enhancing HOS, which is pre-requisite for the *in vitro* acrosome reaction. Similar observations on acrosome reaction have been made, when bull sperms were incubated with 0.1 mM MnCl2 [43]. Another possible explanation for the increase in sperm capacitation and acrosome reaction is due to the increase in intracellular calcium (Ca^{2+}_{i}) content indirectly with Mn^{2+} supplementation, which is required for acrosome reaction [44].

In all the three bulls, a highly positive correlation between HOS and SOD indicated that membrane integrity of spermatozoa is required to prevent the leakage of SOD during cryopreservation; more the intactness in the membrane, more will be the enzyme activity. In bull no. 2, a positive correlation between cryocapacitation and LPO indicated that oxidation of polyunsaturated fatty acids in the membrane damaged the acrosome region. Neild *et al*. [30] has also observed that cryopreserved eqiune sperm are probably less able to cope with osmotic stress and have altered morphology as compared to fresh ones. A highly positive correlation between AR×GR and AR× GPx in bull nos.2, 3

and 3 indicated, glutathione content also facilitate the acrosome reaction by improving the membrane integrity. Glutathione depletion decreases the membrane integrity in cauda epdidymis of rat sperm [45].

Conclusions

It is concluded that although buffalo bull spermatozoa were exposed to lipid peroxidation during different stages of cryopreservation due to oxidative stress induced by the generation of ROS. But Mn^{2+} proved as a fruitful antioxidant in inhibiting cryocapcaitation of spermatozoa, LPO and improving, *in vitro* acrosome reaction, membrane integrity and the level of SOD, GPx and GR enzymes. As $Mn^{2+}$is a cofactor of Mn-SOD enzyme, so it maintained its level and also protected the sperm membrane from per oxidative damages produced by superoxide radicals and thus enhanced SOD activity. Further, studies are required to assess the effects of antioxidants, or a combination of antioxidants to reduce the causes of oxidative damages during cryopreservation of spermatozoa.

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