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Research Paper

Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility

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Abbreviations: EYC, egg yolk citrate; EYC-G, egg yolk citrate-glycerol; HOS, hypo-osmotic swelling; LPO, lipid peroxidation; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid

Key words: cattle, lipid peroxidation, manganese, semen, oxidative stress

Reactive oxygen species (ROS) are generated by sperm metabolism. While, ROS are required for maturation, capacitation and acrosome reaction, they also modify many peroxidable cellular compounds. There is production of ROS during cryopreservation and frozen spermatozoa are highly sensitive to lipid peroxidation (LPO). Antioxidants exert a protective effect on the plasma membrane of frozen bovine sperm preserving both metabolic activity and cellular viability. Manganese (Mn^{++}) is proved to be a chain breaking antioxidant in biological system. Therefore, we examined the role of (Mn^{++}) during cryopreservation of cattle bull semen. Semen was divided into four parts and cryopreserved in egg-yolk-citrate extender + glycerol (EYC-G), EYC-G + 100 μM of Mn^{++}, EYC-G + 150 μM of Mn^{++} and EYC-G + 200 μM of Mn^{++}. After four hours of cooling and 24 hrs of freezing, the spermatozoa were examined for percentage motility, Hypo-osmotic swelling (HOS), LPO and protein leakage. Addition of manganese to the semen during cryopreservation showed a protective effect and accounted for an increase in semen quality parameters [percentage motility, HOS percent and decrease in malondialdehyde (MDA) production and protein leakage]. The effect of manganese on motility and HOS was non-significant ($p < 0.05$) in cooled spermatozoa but significant with 150 μM of Mn^{++} in frozen-thawed spermatozoa. MDA production and protein leakage decreased to a significant and maximum level ($p < 0.05$) on addition of 200 μM of manganese. The addition of manganese to EYC-G dilutor will improve the quality/fertility of semen, which will result in improvement of in vitro fertilization and artificial insemination success rate.

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Introduction

It is generally accepted that the consequences of sperm cryoinjury caused by the cryopreservation are impaired transport and poor survival in the female reproductive tract.[1] The sperm plasma membrane is the primary site of damage induced by cryopreservation.[2-4] Both freezing and thawing cause tremendous alterations in cell water volume, which confer considerable mechanical stress on the cell membrane.[2] Although a significant physiological role of ROS during normal sperm function has been reported,[5,6] such as they facilitate fusogenicity of the spermatozoa, which enables them to bind to the zona pellucida, undergo the acrosome reaction through membrane lipid peroxidation and phospholipase-A activity,[7] traverse through the zona pellucida and fuse with oocyte membrane,[8] but when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS create oxidative stress. Unsaturated fatty acids, predominant in sperm membrane are susceptible to peroxidation[9] and leads to membrane damage, inhibition of respiration and leakage of intracellular enzymes.[10] LPO increases after cryopreservation in bull semen.[11]

Improvement in sperm quality upon addition of antioxidants to semen indicates indirect evidences for the damaging effects of ROS in sperm function. The scavenging potential of the ejaculate is normally maintained by adequate levels of antioxidants, present in seminal plasma. Sperm oxidative damage is the result of an improper balance between ROS generation and scavenging activities. In domestic animals, defective and dead spermatozoa have been identified as a major source of ROS generation during cryopreservation. Radical scavenging activity of manganese related to the rapid quenching of peroxyl radicals has been demonstrated in the biological system viz, a typical antioxidative property of Mn^{++} may protect substantial nigra compact neurons from iron induced oxidative stress;[12] It could reduce the ferrous-ascorbate-mediated lipid peroxidation in placental membranes;[13] It induces an increase in the iron level, which provides direct evidence for Fe-mediated lipid peroxidation in the rat's brain that play an important role in the mechanism of Mn induced neurotoxicology;[14]

Mn-SOD transgene product protects frozen bone marrow cells/ irradiated at 24°C;[15] Mn mew-tetrakas porphyrin also results in significantly blunt nitration of renal proteins during renal ischemia/reperfusion by blocking oxidant production and inducing ATP-synthase-beta subunit and prevention of ATP depletion.[16] Therefore, the present study evaluated the potential effect of Mn^{++} in a dose dependent manner against cryopreservation induced damage to cattle bull spermatozoa. Spermatozoa were monitored for post thaw survival, HOS test, LPO and protein leakage.

Results

Effect of Mn^{++} on motility and HOS. Manganese added showed a protective effect and accounted for significantly higher values of motility and HOS-test response in cooled and frozen-thawed semen samples. A sperm motility declined to 63.3 ± 6.8% and 45 ± 2.4% in the cooled and frozen-thawed semen samples, diluted in EYC-G without Mn^{++} respectively. Whereas the percentage of motile spermatozoa declined to 67.5 ± 2.0, 45.0 ± 2.4, 66.6 ± 5.9, 66.6 ± 3.0 and 48.3 ± 4.9, 58.3 ± 3.6, 51.6 ± 3.0 in the cooled and frozen thawed semen sample, supplemented with 100 μM, 150 μM, 200 μM of Mn^{++} respectively (Table 1, Fig. 1).

There was 16.7% and 35% loss of sperm motility in the semen samples cryopreserved in EYC-G without Mn^{++} after cooling and freezing-thawing respectively. But the loss of motility decreased to only 12.5%, 13.4%, 13.4% and 31.7%, 21.7%, 28.4% on addition of 100 μM, 150 μM, 200 μM of Mn^{++} in the cooled and frozen-thawed semen samples respectively (Table 1 and Fig. 1). It indicates non-significant ($p < 0.05$) effect of Mn^{++} on percentage motility of cooled spermatozoa and a significant ($p < 0.05$) effect on frozen-thawed spermatozoa, but 150 μM concentration of Mn^{++} showed a maximum protective effect.

The percentage of HOS +ve spermatozoa was 55.7 ± 2.8% in the freshly diluted semen, which declined to 46.7 ± 2.8% and 19.17 ± 4.4% in the cooled and frozen-thawed semen samples, diluted in EYC-G without Mn^{++} respectively. The percentage of HOS +ve spermatozoa further declined to 45.1 ± 5.1%, 43.0 ± 6.4%, 44.2 ± 3.1% in the cooled semen samples and 23.19 ± 3.9%, 30.1 ± 6.9%, 24.25 ± 5.2% in the frozen-thawed semen samples, supplemented with 100 μM, 150 μM, 200 μM of Mn^{++} respectively (Table 2 and Fig. 2).

The percentage of HOS +ve spermatozoa declined to 8.97% in cooled semen samples, diluted in EYC-G without manganese. But this decline was increased to 10.60%, 12.70%, 11.50% in cooled semen samples supplemented with 100, 150 and 200 μM of Mn^{++}. A percentage of 27.56% in HOS was observed in semen samples, cryopreserved in EYC-G without Mn^{++}, whereas a decrease 21.91%, 12.84% & 19.95% in HOS +ve spermatozoa was observed in frozen thawed semen samples, supplemented with 100, 150 & 250 μM of Mn^{++} (Table 2 and Fig. 2).

The results indicate non-significant ($p < 0.05$) effect of Mn^{++} on sperm membrane integrity during the cooling process and significant ($p < 0.05$) effect during the freezing process, but to a maximum level on addition of 150 μM of Mn^{++}.

Effect of Mn^{++} on lipid peroxidation and protein-leakage. Only 45.0 ± 3.3 μmoles/10^9 cells of MDA were produced in the freshly diluted semen, which increased to 73.3 ± 1.6 and 122.5 ± 6.9 μmoles/10^9 cells in the cooled semen samples, diluted in EYC-G without Mn^{++}. MDA production was reduced to 66.96 ± 2.0, 63.43 ± 4.2, 56.26 ± 1.6 μmoles/10^9 cells in the cooled semen samples and to 101.8 ± 1.4, 96.8 ± 7.7, 75.3 ± 2.2 in the frozen-thawed semen samples, supplemented with 100, 150 and 200 μM of Mn^{++} respectively (Table 3 and Fig. 3).

It shows a significant increase in MDA production in the cooled as well as frozen-thawed spermatozoa irrespective of the addition of Mn^{++} to EYC-G (Table 3). The results indicate an increase of 38.82% and 40.16% in the cooled and frozen thawed spermatozoa, diluted in EYC-G without Mn^{++}. Comparatively the percentage of MDA production decreased to 30.82%, 29.08% and 19.96% in the cooled spermatozoa and to 36.05%, 34.47% and 25.18% in frozen-thawed spermatozoa, supplemented with 100 μM, 150 μM and 200 μM of Mn^{++} respectively. However, progressive decline in MDA production with an increase in concentration of Mn^{++} was observed. Anti-oxidant effect of Mn^{++} was significant ($p < 0.05$) in cooled as well as frozen-thawed semen samples but to a maximum level on addition of 200 μM of Mn^{++}.

The total protein content of freshly diluted washed spermatozoa was 18.72 mg/10^9 spermatozoa. The protein content of cooled spermatozoa declined to 15.53 ± 1.8, 16.35 ± 1.4, 16.73 ± 8.4 and 16.84 ± 8.0 mg/10^9 cells, diluted in EYC-G, EYC-G + 100 μM, EYC-G + 150 μM and EYC-G + 200 μM of Mn^{++} respectively. The protein content of frozen-thawed spermatozoa further declined to 8.65 ± 1.0, 10.71 ± 8.5, 9.68 ± 5.3 and 12.50 ± 6.2 mg/10^9 cells, cryopreserved in EYC-G, EYC-G + 100 μM, EYC-G + 150 μM and EYC-G + 200 μM of Mn^{++} respectively (Table 4, Fig. 4).

There was 17.04% and 44.30% leakage of total sperm proteins in the cooled and frozen-thawed semen samples, diluted in EYC-G without Mn^{++} (Fig. 5, Table 4).The leakage of total sperm proteins was reduced to 12.66%, 10.63% and 10.04% and 34.49%, 42.14% and 25.77% in cooled and frozen thawed semen, supplemented with 100 μM, 150 μM and 200 μM of Mn^{++} respectively. The results indicate that the

Table 1 Manganese increases sperm motility during cryopreservation of cattle bull semen

Concentration of Mn^{++} (μM)	Motility (Mean ± S.E.)			
	Pre-Freezing	Decrease in motility (%)	Post-Thaw	Decrease in motility (%)
Control	80.0 ± 0.00[a]	-	-	-
0	63.3 ± 6.8[bc]	16.7	45.0 ± 2.4[d]	35
100	67.5 ± 2.0[c]	12.5	48.3 ± 4.9[d]	31.7
150	66.6 ± 5.9[c]	13.4	58.3 ± 3.6[b]	21.7
200	66.6 ± 3.0[c]	13.4	51.6 ± 3.0[d]	28.4

Superscripts (a, b, c and d) indicate the difference at 5% level of significance within the columns. The table depicts non-significant protective effect of Mn^{++} on percentage motility of cooled spermatozoa and significant and significant ($p \leq 0.05$) effect on that of frozen thawed spermatozoa. 150 μM concentration of Mn showed a maximum protective effect.

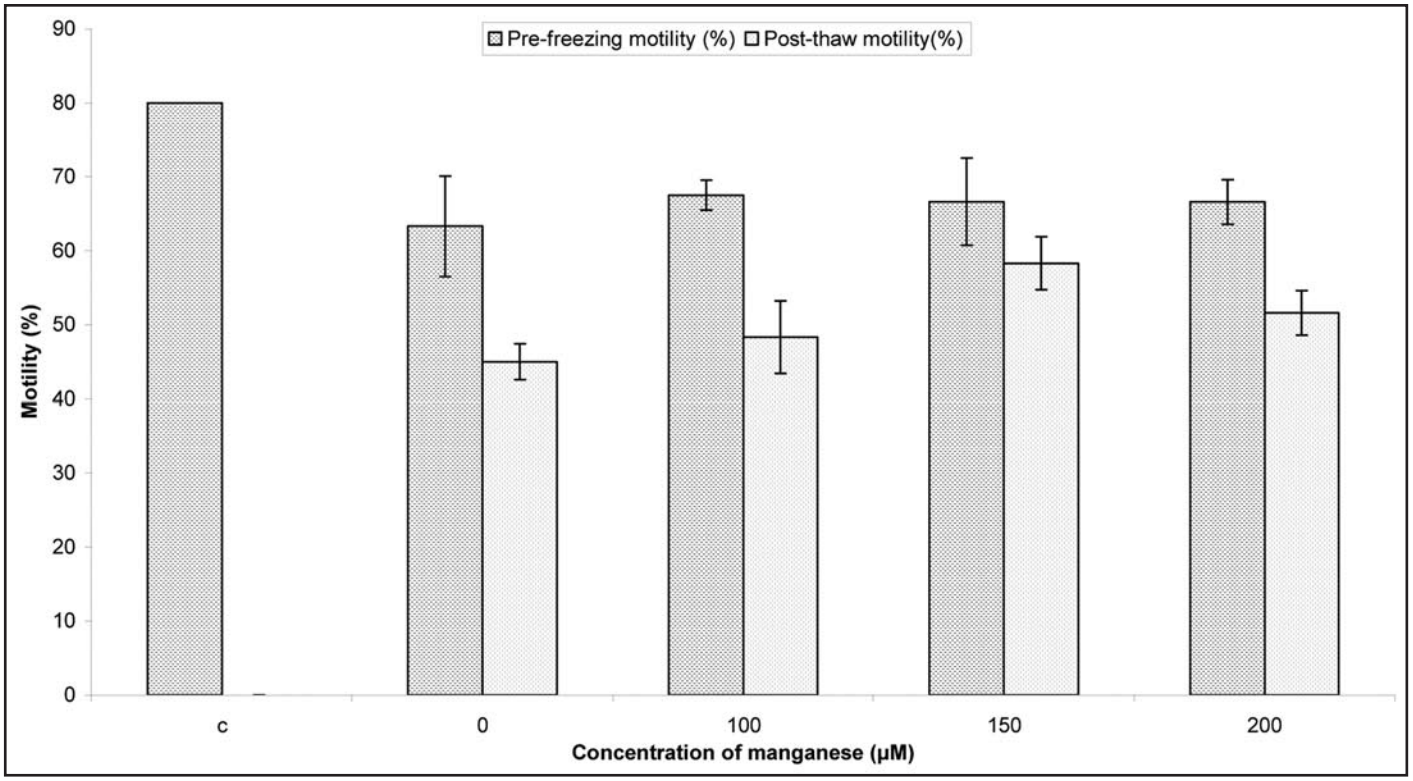

Figure 1. Manganese increases sperm motility (%, Mean ± SE) during cryopreservation of cattle bull semen. The figure depicts non-significant protective effect of Mn^{++} on percentage motility of cooled spermatozoa and significant and significant ($p \leq 0.05$) effect on that of frozen-thawed spermatozoa. 150 μM concentration of Mn^{++} showed a maximum protective effect.

Table 2 **Manganese increases HOS percentage (mean ± S.E) during cryopreservation of cattle bull spermatozoa**

Conc of Mn^{++} (μM)	Spermatozoa cooled at 4°C		Frozen-thawed spermatozoa	
	HOS (%)	Difference in HOS (%) between control & cooled spermatozoa	HOS (%)	Difference in HOS (%) between cooled and frozen-thawed spermatozoa
Control	55.7 ± 2.8[a]	-	-	-
0	46.7 ± 2.8[b]	8.97	19.17 ± 4.4[c]	27.56
100	45.1 ± 5.1[b]	10.60	23.19 ± 3.9[c]	21.91
150	43.0 ± 6.4[b]	12.70	30.1 ± 6.9[d]	12.84
200	44.2 ± 3.1[b]	11.50	24.25 ± 5.2[c]	19.95

Superscripts (a, b, c and d) indicate the difference at 5% level of significance within the columns. This table depicts non-significant ($p \geq 0.05$) effect of Mn^{++} on sperm membrane integrity in cooled semen samples and significant ($p \leq 0.05$) effect in frozen thawed semen samples, but to a maximum level on addition of 150 μM Mn^{++}.

addition of Mn^{++} to EYC-G could control the leakage of sperm membrane proteins to a significant level ($p < 0.05$) both in cooled and frozen-thawed semen, but to a maximum level on addition of 200 μM of Mn^{++}.

Discussion

Much of cryopreservation sperm damage depends on the structural stability of the plasma membrane.[17] The structural re-organization of sperm head plasma membrane after cryopreservation appears to disrupt the ability of the sperm to interact normally with cells of the female genital tract. Cryopreservation in the presence of cryoprotectants (typically glycerol) is considered to be a more moderate treatment than cold shock. Since impaired sperm membrane function due to cryopreservation inevitably affects capacitation, acrosome reaction, penetration into zona, fusion and penetration into the oolema and so diminishes successful fertilization in vivo. That is why when equal number of motile spermatozoa are inseminated, the fertility of fresh semen is superior to that of frozen semen. Manganese is an element of great importance in the life cycle of plants/animals and it plays an essential role as an activator of various enzymatic systems. Antioxidant effect of Mn^{++} or its protective effect against LPO has been studied in various biological systems.[12-14,18] In various organisms, high intracellular manganese provide protection against oxidative damage through unknown pathways and recently it has been found that manganese for oxidative protection is provided by the Nramp transporters.[19] The potential role of manganese in evaluation of infertile males has also been reported.[20] Elbetieha et al.[21] also postulated that ingestion of high dose of manganese chloride by

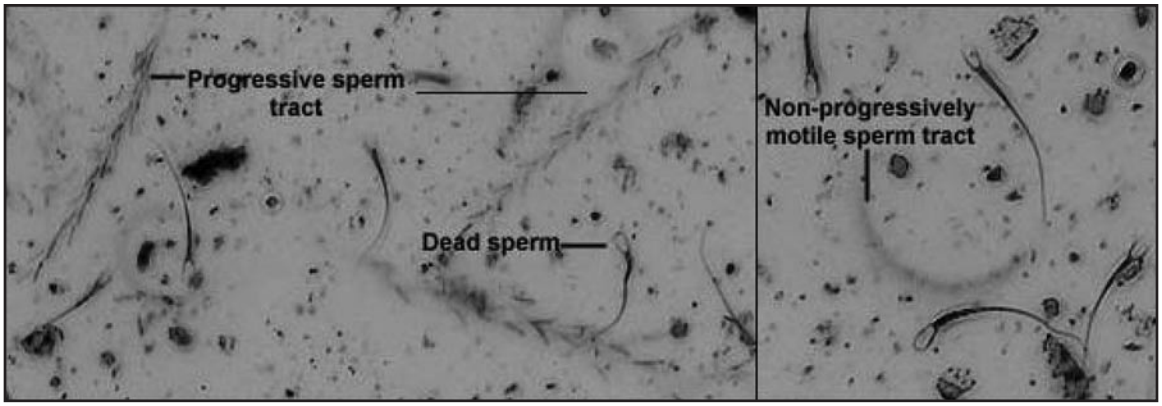

Figure 2. Cattle bull spermatozoa showing progressive and non-progressive motility.

male and female mice causes some adverse effects on fertility and reproduction. Mn^{++} has a peroxyl radical scavenging activity, which seems to be related to the rapid quenching of peroxyl radicals according to the reaction R-OO + Mn^{++} + H(+) → $ROOH^{+}$ + Mn.[22]

Effect of Mn^{++} on motility and HOS. A subpopulation of sperm survives and maintains fertilizing capacity not withstanding an overall reduction in the percentage of motile and viable cells. However, computer assisted motility of bovine sperm, immediately after thawing is not correlated with fertility.[23] Similarly Lapointe et al.[24] suggested the beneficial effect of 0.1 mM $MnCl_2$ for the maintainace of sperm motility without detrimental effects on mucus penetration and fertilizing ability and hypothesized that Mn^{++} would have an effect on sperm cyclase activity that lead to increased Ca^{++} concentration and motility.However, despite preservation of adequate motility, cryopreserved human sperm exhibit significant membrane damage as indicated by subnormal hypo-osmotic swelling tests.[25] During the present studies, although the post thaw motility ranged from 45.0% to 51.6% in all the experiments but the percentage of HOS +ve spermatozoa ranged only from 19.95% to 27.56%. It shows that even motile spermatozoa have damaged membrane. About 36–47% reduction in normal morphology i.e., motility, viability, mitochondrial function has been observed in frozen-thawed human spermatozoa.[26] It has also been reported that the release of oxidase from dead sperms in EYC reduces the motility and viability of remaining living bull spermatozoa.[27] Therefore, the addition of Mn^{++} to EYC-G resulted in significant improvement in the post-thaw motility and HOS of frozen cattle bull spermatozoa in a dose dependent manner, which was maximum on addition of 150 μM of Mn^{++}. Similarly the addition of Mn^{++} to human washed spermatozoa resulted in stimulation of progressive motility in a time and dose dependent manner.[28] The positive effects of antioxidants vitamin E and Mn^{++} have been studied on buffalo and cattle bull spermatozoa incubated with lipid peroxidation catalysts.[29,30]

Effect of Mn^{++} on lipid peroxidation and protein-leakage. Although a significant negative correlation between the ROS and IVF fertilization rate has been found,[31] controlled quantities of ROS have shown to be essential for the development of capacitation and hyper activation,[32] two physiological processes of the sperm that are necessary to ensure fertilization. The maintenance of a suitable ROS level is therefore essential for adequate sperm functionality.

When the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS such as H_2O_2, SO etc., creates oxidative stress and results in membrane damage. The activation of an aromatic amino acid oxidase following the death of ram and bull spermatozoa has been identified as major source of ROS production in the semen of these animals.[33] It has been reported that LPO in bull spermatozoa increases after cryopreservation.[11] The cryopreservation of cattle bull spermatozoa also resulted in an increase in LPO in terms of MDA production irrespective of the addition of Mn^{++}. Frozen-thawed bull spermatozoa are more easily per oxidized than freshly ejaculated spermatozoa.[34] The supplementation of Mn^{++} could reduce the level of MDA

Table 3 **Manganese decreases the malondialdehyde production (mean ± SE) during cryopreservation of cattle bull spermatozoa**

Conc of Mn^{++} (μM)	Spermatozoa cooled at 4°C		Frozen-thawed spermatozoa	
	MDA produced μM/10^9 cells	Difference in MDA production (freshly diluted & cooled sperms)	MDA produced μM/10^9 cells	Difference in MDA production (cooled & frozen-thawed sperms)
Control	45.03 ± 3.3[a]	-	-	-
0	73.33 ± 1.6[b]	38.55	122.5 ± 6.9[e]	40.16
100	66.96 ± 2.0[c]	30.82	101.8 ± 1.4[f]	36.05
150	63.43 ± 4.2[c]	29.08	96.8 ± 7.7[g]	34.47
200	56.26 ± 1.6[d]	19.96	75.2 ± 2.2[h]	25.18

Superscripts (a, b, c, d, e, f, g and h) indicate the difference at 5% level of significance within the columns. This table depicts significant antioxidant effect of Mn^{++} in cooled as well as frozen thawed semen samples but to a maximum level on addition of 200 μM of Mn^{++}.

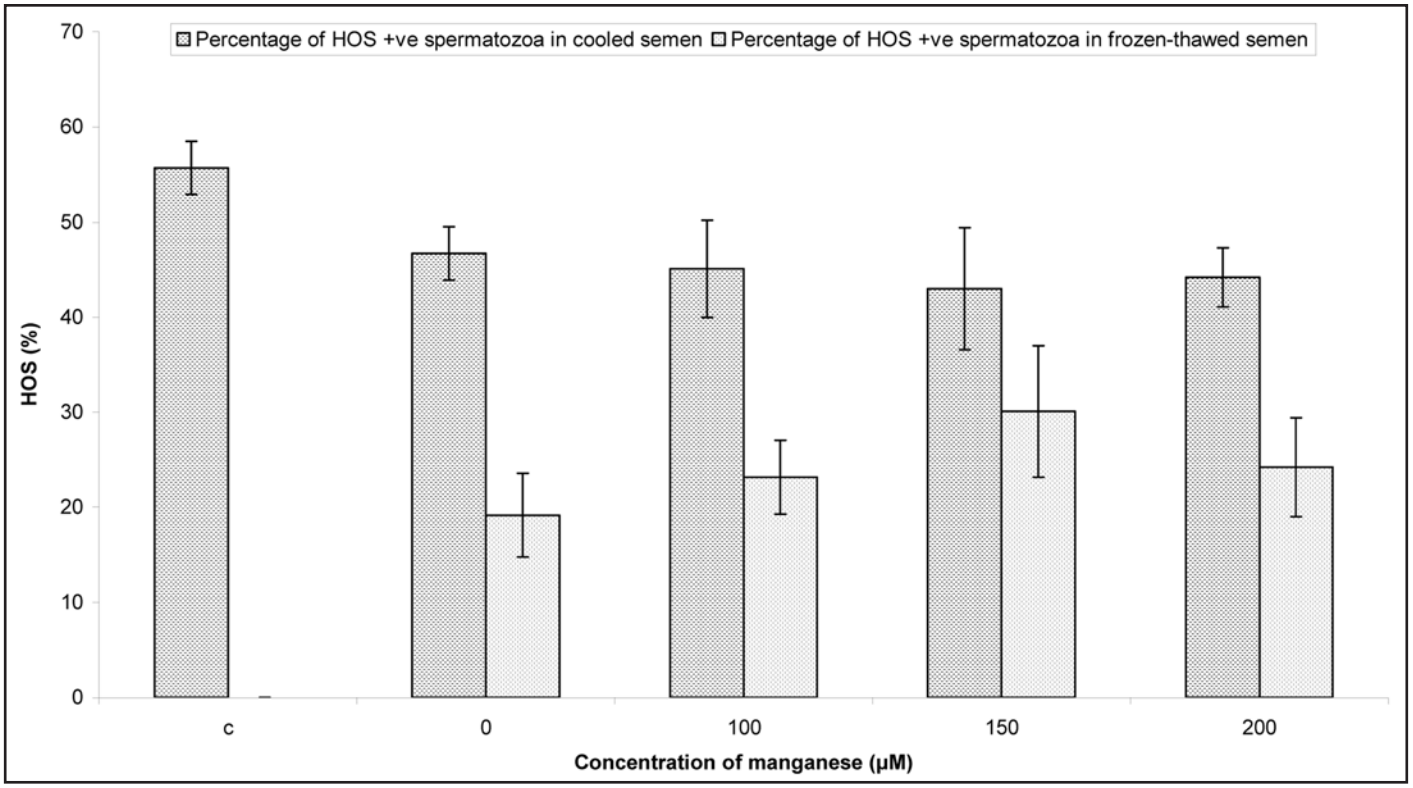

Figure 3. Manganese increases HOS percentage (Mean ± SE) during cryopreservation of cattle bull spermatozoa. The figure depicts non-significant ($p \geq 0.05$) effect of Mn^{++} on sperm membrane integrity in cooled semen samples and significant ($p \leq 0.05$) effect in frozen-thawed semen samples, but to a maximum level on addition of 150 μM of Mn^{++}.

Table 4 **Manganese decreases the leakage of total protein (mean ± SE) during cryopreservation of cattle bull spermatozoa**

Conc of Mn^{++} (μM)	Spermatozoa cooled at 4°C		Frozen-thawed spermatozoa	
	Protein leakage μM/10^9 cells	Increase in protein leakage as compared to control	Protein leakage μM/10^9 cells	Increase in protein leakage as compared to cooling
Control	18.72 ± 3.6[a]	-	-	-
0	15.53 ± 1.8[b]	17.04	8.65 ± 1.0[c]	44.30
100	16.35 ± 1.4[b]	12.66	10.71 ± 5.5[cd]	34.49
150	16.73 ± 5.4[b]	10.63	9.68 ± 2.3[c]	42.14
200	16.84 ± 5.0[b]	10.04	12.50 ± 4.2[d]	25.17

Superscripts (a, b, c, d, e, f, g and h) indicate the difference at 5% level of significance within the columns. This table depicts that the addition of Mn^{++} to EYC-G could reduce the leakage of total sperm proteins to a significant level both in cooled and frozen-thawed semen samples, which was maximum on addition of 200 μM of Mn^{++}.

production significantly in spermatozoa cooled at 4°C and frozen-thawed spematozoa, but to a maximum level on addition of 200 μM of Mn^{++}. Inclusion of natural oxidants (α-tocopherol and ascorbate) had a protective effect on metabolic activity and cellular viability of cryopreserved bovine sperm.[35,36] Similarly Mn^{++} as an antioxidant showed a protective effect on cattle bull spermatozoa during cryopreservation.

The presence of antioxidant enzymes, SOD, glutathione peroxidase (GPx) and catalase in human;[37] bull[38] and ram[39,40] semen and the effect of semen dilution results in reducing their protective capacity. Marti et al.[41] indicated a 65% decrease in SOD activity of ram spermatozoa after freezing-thawing and addition of seminal plasma proteins with oleic-linoleic acid & vitamin E accounted for an increase in enzyme activity levels. In our study, sperm membrane damage due to oxidative stress during cryopreservation of cattle bull spermatozoa also resulted in protein leakage irrespective of the addition of Mn^{++}. However, the addition of Mn^{++} could prevent the leakage of proteins to a significant level in a dose dependent manner and the effect was maximum with 200 μM of Mn^{++}. Bilodeau et al.[40] also observed a reduction in glutathione levels of bull spermatozoa during cryopreservation. Literature indicate that the enzyme system comprising SOD, glutathione peroxidase/reductase and catalase function as a defense against lipid peroxidation in mammalian sperm and defect of these enzyme activities could produce a loss to cell function. It can be further postulated from our results that supplementation of Mn^{++} as an antioxidant to EYC-G during cryopreservation may maintain the enzyme system by its scavenging activity for free radicals or by reducing the oxidative stress and could reduce the effect of cryoinjury to cattle bull spermatozoa.

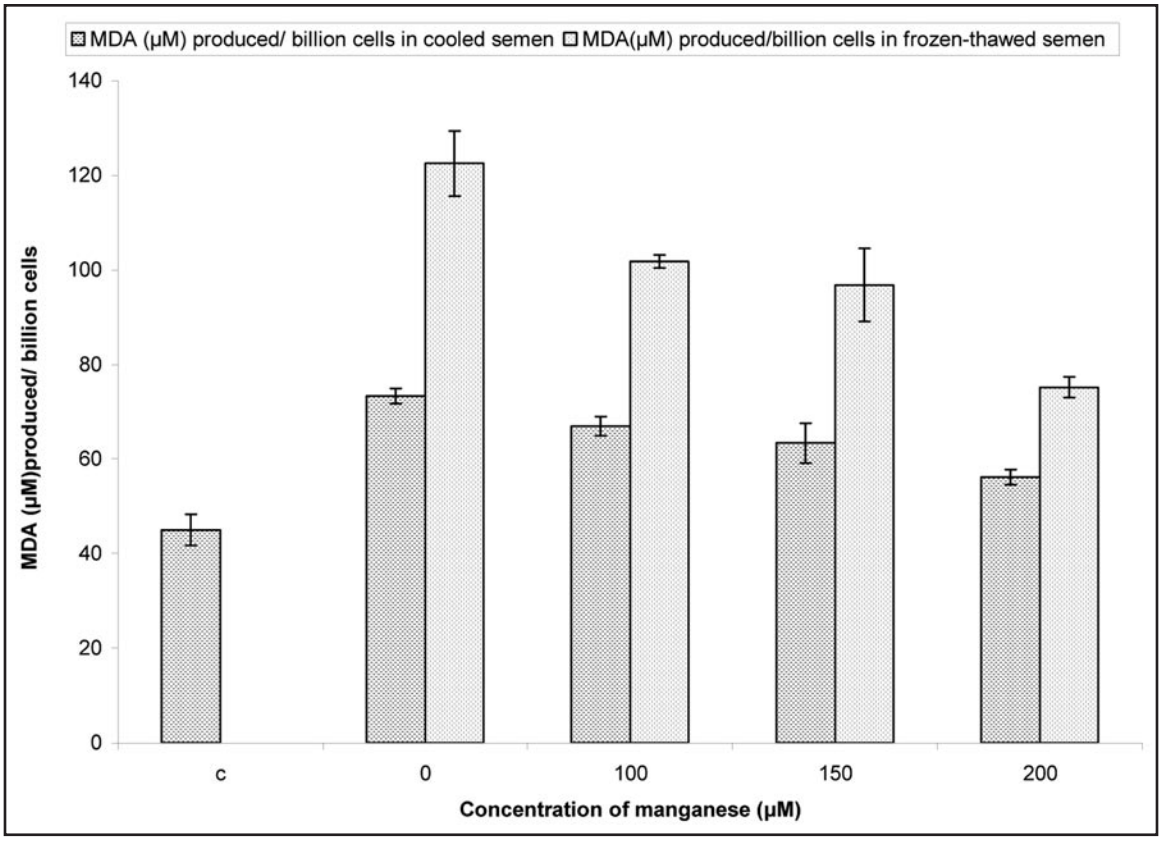

Figure 4. Manganese decreases the malondialdehyde (MDA) production (Mean ± SE) during cryopreservation of cattle bull spermatozoa. The figure depicts significant antioxidant effect of Mn^{++} in cooled as well as frozen thawed semen samples but to a maximum level on additon of 200 µM of Mn^{++}.

It can be concluded that 200 µM concentration of Mn^{++} is an optimum dose, which can be added to EYC-G during cryopreservation of cattle bull spermatozoa to reduce the oxidative stress/ improve the quality of semen.

Materials and Methods

Procurement and evaluation of semen. Freshly ejaculated semen was collected with the help of artificial vagina, immediately transported to the lab and evaluated for its mass activity. A drop of semen was placed on a clean glass slide and observed under microscope (10 x 10X) for waves, swirls and eddies. Grading was done on the basis of observations; Immotile/dead sperm (0), no waves but sperm movement (+), slow wave formation (++), relatively more wave formation with swirls (+++), waves with swirls and eddies (++++). The semen with only ++++ mass activity was used for freezing.

Experiment design. Three separate experiments were performed to evaluate the effect of adding different concentrations of Mn^{++} to EYC-G on the post thaw sperm survival performance (pre-freezing motility, post thaw motility, HOS-test, LPO, protein leakage) as follows:-

Experiment 1. Addition of 100 µM of $MnCl_2$.

Experiment 2. Addition of 150 µM of $MnCl_2$.

Experiment 3. Addition of 200 µM of $MnCl_2$.

In all experiments treatments were compared to a sample without Mn^{++} and to a control (freshly diluted semen).

Cryo-preservation of semen. Immediately EYC-G in the ratio of 1:1 was added to the semen and kept at 37°C for 5–10 minutes. Semen with 80% initial motility was further diluted to 1:8 at a sperm concentration of about 100 x 10^6 cells/ml. Diluted semen samples of experiments 1–3 and without Mn^{++} were kept at 4°C in a cold handling cabinet for four hours. After checking the pre-freezing motility, half of the semen was taken out to study various sperm functions. Second part of each experiment was cryopreserved using the straw freezing procedure. After 24 hours, semen was processed for post thaw motility and various sperm functions.

Sperm concentration. Sperm concentration was estimated spectrophotometrically by taking absorbance at 545 nm, a standard curve for which was prepared (Sperm concentration, calculated with haemocytometer versus absorbance at 545 nm.)

Percentage motility. Motility of pre-cooled and frozen-thawed semen was observed microscopically using CCTV. A total of 200 motile and non-motile sperms were observed on the monitor and percent of motile spermatozoa was calculated.

HOS-test.[41] HOS-test indicates the membrane integrity of the spermatozoa and damage is caused to the sperm membrane during cryopreservation. Therefore, HOS-test was performed to find out the protective effect of Mn^{++} on sperm damage. Briefly, 0.2 ml of semen was incubated with 1.0 ml of 100 µM of HOS solution and 0.85% saline separately for 30 minutes. After 30 minutes spermatozoa with swollen and coiled tails were observed under microscope

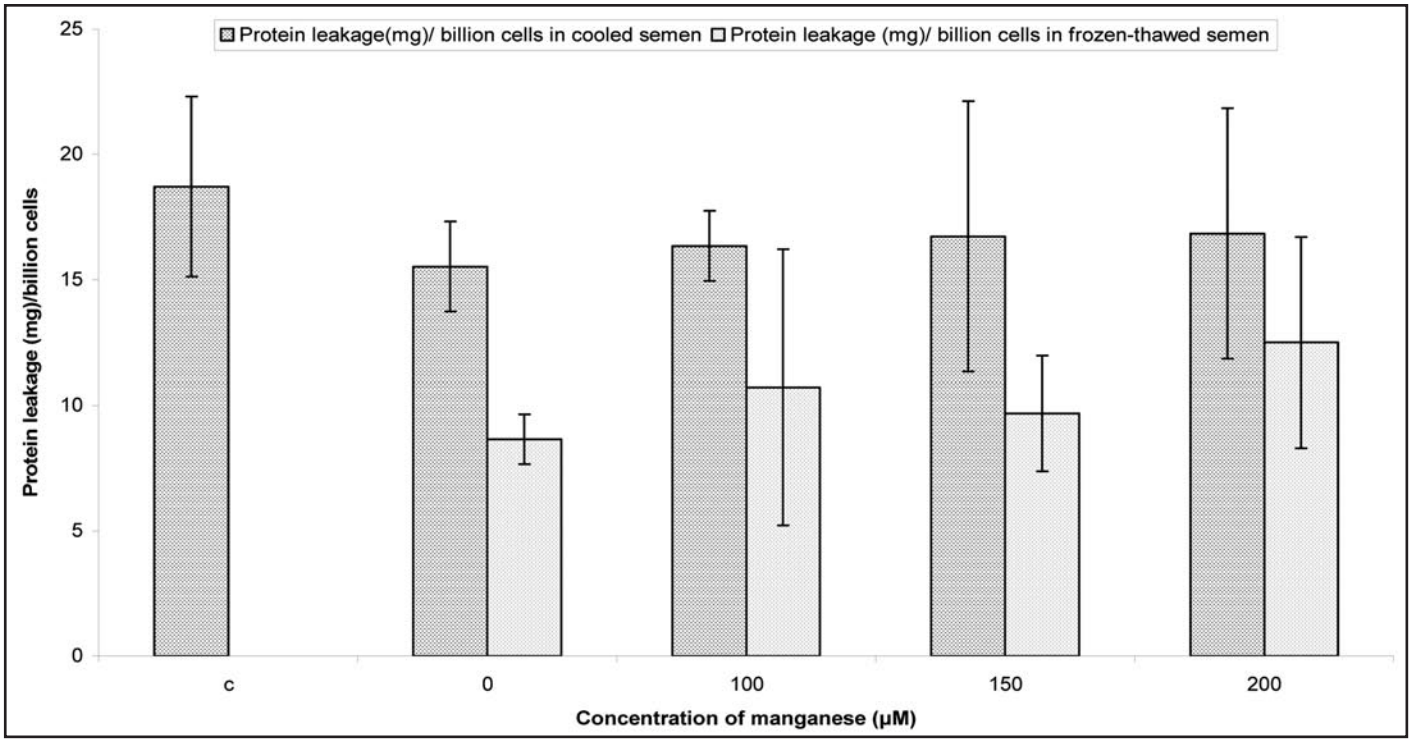

Figure 5. Manganese decreases the leakage of total protein (Mean ± SE) during cryopreservation of cattle bull spermatozoa.

at 10 x 40X. A total of 200 coiled/uncoiled sperms were counted and percent coiled spermatozoa were calculated. Percent of HOS positive spermatozoa was calculated by subtracting number of coiled sperms in normal saline from that in HOS solution.

Membrane lipid peroxidation. Oxidative stress is induced during cryopreservation of semen and as a result ROS are produced. Therefore, LPO was measured to find out an anti-oxidant effect of Mn^{++} on oxidative stress. Membrane LPO was estimated by the end point generation of MDA determined by the TBA test.[42] Briefly, extended spermatozoa [(40–70) x 10^6 cells] in 0.5 ml of cold 10% (wt/vol) chilled trichloroacetic acid to proliferate proteins. The precipitate was pelleted by centrifugation (6,000 rpm for 10 minutes) and 1 ml of 0.375% (wt/vol) TBA was added to the supernatant and kept in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was read at 532 nm (UV-VIS spectrophotometer, systronics, India). MDA production was calculated by the formula:

[O.D. at 532 nm x Volume of assay mixture]/[Molar extinction coefficient x Volume of sample]

($1.56 \times 10^5\ M^{-1}C^{-1}$)

Total proteins. There is leakage of sperm membrane enzymes and total proteins during cryopreservation. Therefore, total sperm protein was measured to find out the protective effect of Mn^{++} on protein leakage. It was estimated by the standard method[43] using 4–7 x 10^6 sperms/0.05 ml. Bovine serum albumin was run as a standard.

Increase in MDA production and protein leakage due to cooling and freezing-thawing of spermatozoa was calculated by subtracting the values of control from that of cooled and of cooled samples from that of frozen-thawed ones respectively.

Statistical analysis. The data were analyzed by using computerized soft ware programmed for analysis of RBD (Randomized block design).

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