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Effect of manganese on bovine sperm motility, viability, and lipid peroxidation *in vitro*

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Abstract

Manganese (Mn^{2+}) is a chain-breaking antioxidant in biological systems. The objective of the present study was to determine the optimal dose of Mn^{2+} to reverse free radical-mediated oxidative damage on motility, viability, and lipid peroxidation (LPO) of sperm from five bulls (local crossbred cattle). Fresh semen was suspended in 2.9% sodium citrate, divided into equal fractions, and subjected to Mn^{2+} treatment (0, 60, 100, or 200 µM) in the presence or absence of an oxidative stress inducer (ferrous ascorbate, FeAA; comprised of 150 µM $FeSO_4$ and 750 µM ascorbic acid). All sperm suspensions were incubated (37°C) for 2 h. Treatment with FeAA decreased motility and viability but increased lipid peroxidation. All doses of Mn^{2+} increased motility and viability but decreased LPO; however, 60 µM Mn^{2+} was most effective. For sperm motility, viability, and LPO level, there were significant main effects of bull, treatment, and interval, as well as their interactions. In conclusion, Mn^{2+} reduced the oxidative stress (LPO) caused by FeAA and improved sperm motility and viability under *in vitro* conditions as well as under induced oxidative stress.

Keywords: bovine, drug effects, lipid peroxidation, Mn^{2+}, sperm motility.

Introduction

Among numerous factors that influence male fertility, oxidative stress has elicited the greatest interest in recent years (Agarwal and Prabakaran, 2005). Oxidative stress in the reproductive system is thought to have an effect on the fertilizing ability of sperm (Aydemir *et al*., 2006). It occurs as a consequence of an imbalance between production of reactive oxygen species (ROS) and their removal by antioxidant defenses (Sikka, 1996). Several reactive oxygen species (ROS), including superoxide anion (O_2), hydroxyl radical (OH), and hypochlorite radical (OHCl) produced by both sperm and leukocytes contaminating the seminal fluid, adversely affect sperm motility and impair fertilizing ability (Verma and Kanwar, 1999; Fraczek *et al*., 2007). Such ROS-induced damage increase in the absence of seminal plasma, which carries several antioxidant systems (Verma and Kanwar, 1999).

In assisted reproduction, poor sperm motility rather than low sperm concentration is most often the cause of male infertility. Therefore, an antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility (Verma and Kanwar, 1999). Antioxidative mechanisms protect the sperm from the damage caused by free radicals (Gallardo, 2007). The antioxidative action of Mn^{2+} on various peroxidizing systems (sperms, neurons) has been studied. It inhibits lipid peroxidation (LPO) produced by a free-radical-producing system, but not LPO induced by a single oxygen (Cavallini *et al*., 1984). Manganese in very small amounts affects human health, and deficiency may cause symptoms such as impaired or depressed reproductive functions (Barber *et al*., 2005; Singh, 2008). It is an essential component of several enzymes, some of which (superoxide dismutase, pseudo-catalase and the photosynthetic oxygen evolving centre) are involved in redox processes (Campanella *et al*., 2005). Manganese has also been designated as a chain-breaking antioxidant, as it is able to quench peroxyl radicals (Coassin *et al*., 1992). The objective of the present study was to determine the optimal dose of Mn^{2+} to reverse free-radical-mediated oxidative damage on motility, viability, and LPO *in vitro* in sperm from local crossbred cattle.

Materials and Methods

Reagents

All chemicals were purchased from Sisco Research Laboratories (SRL; Pvt., Ltd., Mumbai, India).

Sperm

Ejaculates with more than 80% motility and 1.2 to 1.4 × 10^9 sperm/ml were collected (using an artificial vagina) from five local crossbred bulls (HHS, Holstein Friesen x Sahiwal; FC, Fresian crosses; 1F and 4F, first and fourth generation of inter-breeding) maintained at the dairy farm of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Three ejaculates from each bull were used.

Fresh semen was centrifuged (800 x g) at 37°C for 5 min, seminal plasma removed, the sperm pellet washed two or three times with 2.9% sodium citrate (pH 7.4), re-suspended in 2.9% sodium citrate, and divided into eight equal fractions in eight test tubes

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(concentration, 120 x 10^6 sperm/ml). In one tube (control), only 2.9% sodium citrate was added and the remaining seven tubes (experimental fractions) were subjected to Mn^{2+} treatment (0, 60, 100, or 200 μM) in the presence or absence of an oxidative stress inducer (i.e, ferrous ascorbate; FeAA; comprised of 150 μM FeSO4 and 750 μM ascorbic acid; Bansal and Bilaspuri, 2008). All sperm suspensions were incubated (37°C) for varying incubation periods.

Evaluation of sperm motility and viability

Every 30 min, sperm motility was estimated (37°C, in increments of 10%) by examination of a wet mount using bright-field microscopy (400X). Sperm viability (at 0 and 2 h) was determined by preparing an eosin-nigrosin smear (37°C) and assessing at least 100 sperm under bright-field microscopy (1000X; Blom, 1950).

Determination of lipid peroxidation (LPO)

At the end of the 2 h of incubation (37°C), the level of malondialdehyde (MDA) in control and experimental fractions was assessed by the determination of thiobarbituric acid reactive substances (TBARS; Buege and Steven, 1978). For this assay, a known volume of sperm suspension was incubated with 0.1 ml of 150 mM Tris-HCl (pH 7.1) for 20 min at 37°C. Subsequently, 1 ml of 10% TCA and 2 ml of 0.375% TBA were added followed by incubation in a boiling water bath for 30 min. Thereafter, the suspension was centrifuged for 15 min at 100.71 g. In the blank tube, sample was replaced by 2.9% sodium citrate (pH 7.4). The absorbance was read at 532 nm. The molar extinction coefficient for MDA is 1.56 x 10^5 M/cm. The results were expressed as moles MDA/μg protein.

Determination of total proteins

Total proteins in the control and experimental fractions were determined spectrophotometrically (Lees and Paxman, 1972). To each 0.1 ml of the sample (sperm suspension) in each test tube, 0.9 ml of 5% SDS in 0.5N NaOH was added, which was allowed to remain at room temperature for at least 3 h before agitating 2 to 3 times using a vortex mixer to make sure that sample was dissolved thoroughly. To this solution, 2.5 ml of copper carbonate solution was added and tube was allowed to stand for 15 to 20 min. Subsequently, 0.25 ml of Folin-phenol reagent was added; the sample was mixed immediately and allowed to stand for 45 min. The absorbance was read at 740 nm. The reference standard used was 20 to 100 μg/ml of bovine serum albumin (BSA).

Statistical analyses

Data of sperm motility and viability (Table 1 and 2) were arc-sine transformed (Snedecor and Cochran, 1980) and LPO data (Table 3) were submitted to the square root transformation. Factorial analysis in a completely randomized block design (CRD; software programme from the Department of Mathematics, Statistics and Physics, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, India) was performed on the transformed data to evaluate the significance levels between the parameters studied. The critical difference (CD) of three independent variables: A (bull), B (treatments), and C (interval) and the interactions between AB/AC/BC/ABC were determined statistically. For all analyses, $P < 0.05$ was considered significant.

Results

Sperm motility

Motility (%) among all the five bulls was studied at 30 min intervals up to 2 h in control as well as treated samples (Table 1). Significant differences in motility were observed among all bulls. With each successive examination (interval), a decrease in motility was observed among all the bulls. Data showed a significantly higher percentage of motility for Bulls 1 and 2 and lower for Bulls 3 and 4 (Table 1).

Corresponding to treatments, supplementation of Mn^{2+} increased motility significantly with all three doses (60, 100, and 200 μM) as compared to the control. Among all three doses of Mn^{2+}, there were no differences (Table 1). Furthermore, FeAA treatment decreased motility ($P < 0.05$) as compared to the control. Subsequently, supplementation of Mn^{2+} to FeAA-treated samples increased motility ($P < 0.05$) with all three doses as compared to the samples treated with FeAA only. Comparing the increase in motility among the three doses of Mn^{2+} in FeAA-treated Mn^{2+}-supplemented samples, motility was different ($P < 0.05$) between 60 μM and 100 μM, as well as 100 μM and 200 μM, but not different between 60 μM and 200 μM. Data analysis showed that 60 μM Mn^{2+} supplementation increased motility ($P < 0.05$) in both FeAA-treated as well as untreated samples. With the increase in interval (0.5 h) a gradual decrease ($P < 0.05$) in motility was observed (Table 1). Statistical analyses showed significant interactions between the variables bull and interval, treatment and interval, but no significant interactions between treatment and bull, as well as bull, treatment, and interval were found (Table 1).

Table 1. Effects of various concentrations of Mn^{2+} (µM) on sperm motility (%) of ferrous ascorbate (FeAA; Ferrous sulfate + ascorbic acid) treated or untreated bull spermatozoa.

Treatments (Factor B)	Intervals (h; Factor C)	Bull (Factor A)					Combination means for treatment factor (n = 8)
		1	2	3	4	5	
Control	0	63.4	63.4	56.8	53.7	60.0	50.1^{b}
	0.5	56.7	55.2	47.8	50.7	53.8	
	1	53.7	50.7	43.5	44.9	46.4	
	2	44.9	44.9	33.1	39.2	39.1	
60 µM	0	63.4	63.4	55.3	53.7	60.0	51.7^{a}
	0.5	56.7	55.2	34.9	50.7	53.8	
	1	56.7	53.7	49.3	46.4	47.8	
	2	47.8	52.2	43.5	44.9	44.9	
100 µM	0	63.4	63.4	56.8	53.7	60.0	51.5^{a}
	0.5	57.3	55.2	52.7	53.2	53.8	
	1	52.2	50.7	44.9	46.4	47.8	
	2	47.8	44.9	39.1	42.1	44.9	
200 µM	0	63.4	63.4	56.8	53.7	60.0	51.0^{a}
	0.5	57.3	55.5	49.3	50.7	50.7	
	1	53.7	50.7	44.9	49.3	46.4	
	2	44.9	44.9	39.1	44.9	40.6	
FeAA	0	63.4	63.4	56.8	53.7	60.0	46.1^{c}
	0.5	50.7	50.7	44.9	43.5	49.2	
	1	44.9	45.9	35.2	35.2	44.9	
	2	39.2	36.2	29.9	35.2	39.1	
FeAA+ 60 µM	0	63.4	63.4	56.8	53.7	60.0	51.7^{a}
	0.5	63.4	55.2	49.3	50.7	55.2	
	1	53.7	52.2	46.4	44.9	49.3	
	2	44.9	44.9	42.1	39.2	39.1	
FeAA+ 100 µM	0	63.3	63.3	56.8	53.7	60.0	49.7^{b}
	0.5	50.7	55.2	47.8	49.3	53.7	
	1	50.7	47.8	43.5	44.9	47.8	
	2	44.9	44.9	36.2	39.2	39.1	
FeAA+ 200 µM	0	63.4	63.4	56.8	53.7	60.0	51.3^{a}
	0.5	56.7	63.4	52.7	53.7	60.0	
	1	52.2	47.8	44.9	43.5	44.9	
	2	44.9	43.5	39.2	39.2	42.1	
Combination means for Bull factor (n = 5)		54.2^{a}	53.4^{a}	46.5^{c}	47.2^{c}	50.6^{b}	
Combination means for interval factor (n = 4)		59.4^{a}	52.7^{b}	47.5^{c}	41.9^{d}		

Each value represents a transformed mean after applying arc sine transformation with minimum 20% and maximum 82.5% original values. Any two means in a row or column having different superscripts (a, b, c, d) are different ($P < 0.05$). CD (5%) for various factors and their interactions: A-1.0, B-1.26, C-0.89, AC-2.0, BC-2.53, AB-NS, ABC-NS. NS, Non-significant.

Sperm viability

Morphology of viable and non-viable sperm remained unaffected by various doses of Mn^{2+}. Viability among the five bulls was studied at the 0 and 2 h intervals in control samples (Table 2). Significant differences in viability (%) were observed among bulls. Viability of Bulls 1 and 2 was higher ($P < 0.05$) and Bull 3 was lower than the other bulls (Table 2).

Corresponding to treatments regarding the supplementation of three doses of Mn^{2+}, viability increased ($P < 0.05$) with 60 µM, but did not ($P > 0.05$) with 100 µM and 200 µM as compared to the control. Among the three doses of Mn^{2+}, significant differences were observed between 60 µM and 100 µM, as well as 60 µM and 200 µM, but no significant differences were found between doses 100 µM and 200 µM. Ferrous ascorbate treatment decreased viability ($P < 0.05$) as compared to the control. Subsequently, supplementation of Mn^{2+} to FeAA-treated samples increased viability ($P < 0.05$) with all three doses. In response to interval, viability decreased ($P < 0.05$) from 0 to 2 h (Table 2). Statistical analysis showed a significant interaction between the factors bull and treatment, bull and interval, as well as treatment and interval, but there were no interactions ($P > 0.05$) among bull, treatment, and interval (Table 2).

Table 2. Effects of various concentrations of Mn^{2+} (µM) on sperm viability (%) of ferrous ascorbate (FeAA; Ferrous sulfate + ascorbic acid) treated or untreated bull spermatozoa.

Treatment (Factor B)	Interval (h; Factor C)	Bull (Factor A)					Combination means for treatment factor (n = 8)
		1	2	3	4	5	
Control	0	73.7	76.1	74.0	65.3	66.8	66.1^{b}
	2	60.7	63.6	65.3	56.9	58.1	
60 µM	0	76.5	76.3	74.8	65.4	69.4	69.2^{a}
	2	66.9	64.5	70.0	62.9	66.0	
100 µM	0	73.9	77.1	71.8	65.4	67.7	67.0^{b}
	2	63.1	63.6	66.0	58.2	62.9	
200 µM	0	73.7	76.1	74.0	62.2	65.8	66.9^{b}
	2	63.5	63.8	70.6	57.4	62.1	
FeAA	0	65.5	63.4	63.6	62.7	59.3	57.6^{e}
	2	53.4	52.0	56.7	50.2	50.9	
FeAA+ 60 µM	0	74.7	72.2	67.8	70.7	68.3	63.9^{c}
	2	58.1	56.9	59.8	53.5	56.6	
FeAA+ 100 µM	0	72.6	73.0	75.5	63.9	65.9	63.0^{c}
	2	56.2	55.4	59.2	53.0	55.0	
FeAA+ 200 µM	0	72.6	70.9	69.9	66.5	65.7	61.5^{d}
	2	53.8	52.6	59.1	50.7	53.2	
Combination means for bull factor (n= 5)	- -	66.1^{b}	66.1^{b}	57.3^{a}	60.3^{c}	62.0^{d}	- -
Combination means for interval factor (n = 2)	- -	69.7^{a}	59.1^{b}	- -	- -	- -	- -

Each value represents transformed mean after applying sine arc transformation with minimum 53.23% and maximum 5.55% original values. Any two means in a row or column having different superscripts (a, b, c, d, e) are different ($P < 0.05$). CD at 5% level of significance for various factors and their interactions: A-0.98, B-1.3, AB-2.77, C-0.61, AC-1.38, BC-1.75, ABC-NS. NS, Non-significant.

Lipid peroxidation (LPO)

Lipid peroxidation level among the five bulls studied at 2 h intervals in treated as well as in untreated samples are shown in Table 3. Data showed that MDA production was significantly higher in Bulls 3 and 4, but significantly lower for Bulls 1 and 2. Corresponding to treatments, Mn^{2+} supplementation decreased LPO level significantly as compared to the control. Among the three Mn^{2+} doses, significant differences were observed between 60 µM and 100 µM, as well as 100 µM and 200 µM; the maximum decrease in LPO was observed with 60 µM (Table 3).

As compared to the control, FeAA treatment significantly increased the MDA production, whereas supplementation of Mn^{2+} (all three doses) decreased it significantly in FeAA-treated samples. Among the three doses of Mn^{2+}, differences were significant between 60 µM and 100 µM, as well as 100 µM and 200 µM, but were not significant between 60 µM and 100 µM. The maximum decrease was observed with 60 µM. Analysis of the data showed that the decrease in MDA production was maximal with supplementation of 60 µM Mn^{2+} in both FeAA-treated as well as untreated samples (Table 3). Statistical analysis showed significant interactions between bull and treatment. Therefore, it is suggested that MDA production in different bulls is affected by different treatments (Table 3).

Table 3. Effects of various concentrations of Mn^{2+} (µM) on lipid peroxidation (LPO) of ferrous ascorbate (FeAA; Ferrous sulfate + ascorbic acid) treated or untreated bull spermatozoa.

Treatments (Factor B)	Interval (h; Factor C)	Bull (Factor A)					Combination means for treatment factor (n = 8)
		1	2	3	4	5	
Control	2	2.0	2.0	2.7	2.8	2.1	2.3^b
60 µM	2	1.3	1.2	2.1	1.6	1.5	1.5^e
100 µM	2	1.7	1.7	2.47	2.1	1.8	1.9^d
200 µM	2	1.2	1.2	2.0	2.0	1.6	1.6^e
FeAA	2	2.4	2.5	3.6	3.2	2.8	2.9^a
FeAA+60 µM	2	1.6	1.5	2.3	2.1	1.6	1.8^d
FeAA+100 µM	2	2.0	1.8	2.6	2.6	2.0	2.2^c
FeAA+200 µM	2	1.7	1.6	2.3	2.3	1.6	1.9^d
Combination means for bull factor (n = 5)		1.7^a	1.7^a	2.5^b	2.3^b	1.9^c	- -

Each value represents transformed mean after applying square root transformation with minimum 0.4 and maximum 12.6 original values. Any two means in a row or column having different superscripts (a, b, c, d, e) are different ($P < 0.05$). CD at 5% level of significance for various factors and their interactions: A-0.07, B-0.09, AB-0.21.

Discussion

The bull to bull variation in LPO levels, loss of motility, and viability were significant during different intervals. All factors (bull, treatment, and interval) had significant effects on the various parameters (motility, viability, and LPO). Similar observations were made by Singh *et al.* (1989).

Regarding motility, viability, and LPO levels of all bulls after 2 h of incubation, Bulls 1 and 2 had significant higher motility and viability but a lower LPO level. Therefore, sperm with higher motility and viability were less prone to oxidative stress (manifested by lower LPO). Similar observations were reported for buffalo bulls (Singh *et al.*, 1989).

Supplementation with Mn^{2+} improved sperm motility in both FeAA-treated as well as untreated samples; at 2 h, motility was maximal in sperm treated with 60 µM $Mn^{2+.}$ Similar observations were made in bull sperm (Lapointe *et al.*, 1996), where a low dose of Mn^{2+} (0.1 mM) maintained better motility than 0.5 mM (53% vs 26%, respectively). Magnus *et al.* (1990) had also reported that Mn^{2+} (0.2 to 1.0 mM) stimulated the progressive motility of human sperm. Furthermore, 60 µM Mn^{2+} resulted in maximal sperm viability in both FeAA-treated as well as untreated samples, consistent with previous reports (Garbers and Kopf, 1980; Lapointe *et al.*, 1996).

It has been suggested that Mn^{2+} supplementation stimulated adenylate-cyclase (membrane bound enzyme) activity in sperm, which in turn enhanced the level of cyclic adenosine monophosphate (cAMP; Tash and Means, 1983; Magnus *et al.*, 1990). This increase in concentration of cAMP through a cascade of events phosphorylated the axonemal proteins, which are involved in sperm movement. Therefore, the increased motility in response to Mn^{2+} supplementation in the present study may have been mediated through a signal transduction pathway.

All doses of Mn^{2+} decreased the MDA production, but 60 µM Mn^{2+} decreased it maximally and significantly in both FeAA-treated and untreated samples, indicating the potent antioxidative action of $Mn^{2+.}$ In previous studies, Mn^{2+}inhibited LPO both *in vitro* (Tam and McCay, 1970) and *in vivo* (Shukla and Chandra, 1981). Although the mechanism of its antioxidant effect has not been completely elucidated, Mn^{2+} was a potent radical scavenger as compared to Zn^{2+}, Ni^{2+}, and Fe^{2+}, which were almost completely ineffective (Coassin *et al*., 1992). The most plausible mechanism regarding the inhibitory effects of Mn^{2+} on lipid peroxidation are an interaction with superoxide anions and hydroxyl radicals to produce MnO^{2+} and $Mn(OH)^{2+}$. Furthermore, Mn^{2+} can also reduce the superoxide anion to H_2O_2 with the concomitant formation of Mn^{3+} (Kono *et al*., 1976; Cavallini *et al*., 1984). It is suggested that according to the following reaction, the chain breaking antioxidant capacity of Mn^{2+} is related to effective quenching of peroxyl radicals (Coassin *et al*., 1992).

$$ROO + Mn^{2+} + H^{+} \longrightarrow ROOH + Mn^{3+}$$

Manganese significantly inhibited the potential peroxidation of brain phospholipids (Cavallini *et al*., 1984). Sziraki *et al*. (1998) suggested the atypical antioxidative properties of Mn^{2+} (0 to 10 µM) protect the nigral neurons from iron-induced (0 to 5 µM) oxidative injury and lipid peroxidation. Manganese had also proved to be the best antioxidant in reducing the ferrous-ascorbate-induced lipid peroxidation in human placental membranes (Anand and Kanwar, 2001). As Mn^{2+} is a cofactor of enzyme Mn-SOD (the enzyme which dismutates O_2), we inferred that it protected the membrane from peroxidative damage produced by the superoxide radicals (O_2).

In the present study, Mn^{2+} inhibited LPO in FeAA-treated bull sperm, perhaps due to Mn^{2+} competing with Fe^{2+} for iron binding sites (Cavallini *et al*., 1984). In that regard, Mn^{2+} deficiency in rats resulted in loss of Mn-SOD activity, which in turn stimulated LPO *in vivo* (Tampo and Yonaha, 1992). Similar observations were reported by Paynter (1980) and Sheri and Keen (1987). Campanella *et al*. (2005) had also reported the active role played by Mn^{2+}against free radicals and consequently the important role of this metal ion in protecting *Unio* against oxidative stress.

In conclusion, bull, treatment, interval, and their interactions, significantly affected sperm motility, viability, and LPO. Furthermore, bulls with higher MDA production had lower sperm motility and viability, perhaps due to the deleterious effects of LPO on the integrity, fluidity, and flexibility of the sperm plasma membrane. Although all doses of Mn^{2+} increased motility and viability and decreased the LPO level, 60 µM Mn^{2+} was most effective. We concluded that Mn^{2+} was a useful antioxidant, reducing the oxidative stress (LPO) caused by FeAA in addition to improving sperm motility and viability under *in vitro* conditions as well as under induced oxidative stress.

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