Antioxidant effect of Mn^{2+} on capacitation and acrosome reaction of fresh and chilled cattle bull semen

Amrit Kaur Bansal, Ranjna Sundhey Cheema, Vinod Kumar Gandotra

Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India

Abstract

The aim of this paper was to investigate the antioxidant effect of Mn^{2+} (200 μM) on the sperm capacitation and acrosome reaction of fresh and chilled cattle bull semen. It has been found that Mn^{2+} supplementation improves ($P \leq 0.05$) the motility at 0, 2, 4 and 6 h of incubation. MDA (malondialdehyde), end product of lipid peroxidation, decreases significantly ($P \leq 0.05$) with the supplementation of manganese at 0- and 6-hr of incubation both in fresh and chilled semen. Manganese also increases acrosome reaction significantly ($P \leq 0.05$) both in fresh and chilled semen at 0, 4 and 6 h of incubation. Therefore, our findings suggest the role of Mn^{2+}supplementation in improving the quality of cattle bull semen by its scavenging property *i.e.* reduction in the production of reactive oxygen species during its storage at 4°C or incubation at 37°C for capacitation.

Introduction

Chilled-frozen thawed semen is the predominant form of semen used in the dairy industry.[1] In some species (bull, human, mouse, ram, Siberian tiger) chilled, rewarmed or frozen thawed spermatozoa are able to penetrate the oocyte in a short time after rewarming, while for fresh semen several hours of pre-incubation are needed.[2-8] Cooling/freezing/thawing of semen is routinely performed in cattle breeding industry in order to perform artificial insemination (AI). These procedures are known to produce reactive oxygen species (ROS) in the sperm samples.[9] Mammalian spermatozoa are highly susceptible to ROS attack. Controlled generation of ROS has a physiological role in sperm's functions such as hyperactivation, capacitation and acrosome reaction.[10-12] However, cold shock arising from cooling of spermatozoa generates an imbalance in the production of ROS that leads to lipid peroxidation (LPO) /oxidative stress resulting in decreased sperm motility, viability and increased mid-piece defects that impair sperm capacitation and acrosome reaction.[10,13,14]

A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation.[15] The antioxidative action of Mn^{2+} on various per oxidizing systems (sperms and neurons) has been studied.[15] Manganese is an essential ultra trace element;[16] it is needed for a variety of physiological processes ranging from the regulation of reproduction to normal brain functions.[16] It is an essential element required in living organisms both as activator and a constituent of several enzymes.[17] Manganese is an important cofactor of mitochondrial superoxide dismutase (SOD); an antioxidant enzyme which scavenges oxygen free radicals.[18] At low doses, it acts as a potent antioxidant in the protection against oxidative stress.[17,19] Supplementation of Mn to the broiler breeder semen improved the semen quality by enhancing sperm quality index (SQI) and sperm viability.[20] Therefore, present study was planned with an objective to determine the antioxidant effect of Mn^{2+} (200 μM) on sperm capacitation and acrosome reaction of fresh and chilled cattle bull semen.

Correspondence: Amrit Kaur Bansal, Senior Research Fellow, Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, India.
E-mail: bansal2amrit@yahoo.co.in

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Materials and Methods

Procurement of semen

Semen samples (n=5) with more than 80% motility and 1200×10^6-1400×10^6 mL sperm count were obtained from healthy local crossbred cattle bulls (HHS, Holstein-Friesian×Sahiwal; FC, Friesian crosses; IF and 4F first and fourth generation of interbreeding) maintained at Dairy Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India. Each parameter was analyzed by using three replicates of a single ejaculate of each bull; total five bulls were studied. A known volume of semen sample (pre-warmed at 37°C) was divided into four equal fractions. First two fractions were diluted with egg yolk citrate extender supplemented/un-supplemented with 200 μM Mn^{2+} (optimum dose of Mn^{2+}; antioxidant suggested by Cheema *et al.*[33]). Subsequently they were kept in refrigerator (4°C, overnight) for cooling. Other two fractions (containing fresh semen) were centrifuged (×800 g, 5 min, 37°C); seminal plasma was removed, pellet was washed 2-3 times with TALP (Tyrode's modified solution) medium (NaCl, 92.9 mM; KCl, 4 mM; $NAHCO_3$, 25.9 mM; $CaCl_2.2H_2O$, 10 mM; $MgCl_2.6H_2O$, 0.5 mM; sodium lactate, 7.6 mM; sodium pyruvate, 1.3 mM; HEPES, 20 mM; glucose, 0.25%; heparin, 200 μg/mL; BSA, 0.6%). Pellet was dissolved in TALP medium (pH 7.4) to prepare sperm suspension which was divided into two equal fractions (two tubes) with or without 200 μM Mn^{2+}. Both fractions were incubated at 37°C for 6 h. Chilled fractions were centrifuged for complete removal of the diluter and were processed as before for capacitaion. All the fractions were evaluated for the following parameters at varying incubating periods.

Percentage motility or hyperactivity

It was observed at 0, 2, 4, 6 h of incubation by direct light microscopy.

Morphology and percentage acrosome reaction

Smears of each fraction were prepared at 0, 4 and 6 h of incubation, stained with Giemsa and examined under oil emulsion using binocular microscope and percentage acrosome reaction and morphology of spermatozoa of all fractions were compared.

Lipid peroxidation

A known volume of fresh and cooled sperm suspension treated/untreated with 200 μM Mn^{2+} was incubated for 1 h at 37°C. Then, 0.1 mL of 150 mM Tris-HCl (pH 7.1) was added to each test tube, further incubation was performed for 20 min at 37°C. After the completion of the incubation, 1 mL of TCA (10%) and 2 mL of TBA (0.375%) were added and then kept for 15 minutes on the boiling water bath. Thereafter, it was centrifuged for 15 min at 3000 rpm. In the blank tube, sample was replaced by TALP medium.[21] The absorbance was read at 532 nm.

The molar extinction coefficient for MDA is 1.56×10^5 $M^{-1}.cm^{-1}$. The results were expressed as n moles of MDA/mg protein.

Statistical analysis

The *Analysis of Factorial Experiment in CRD* (computer software programme) *or one way variance analysis* was used to evaluate the significance levels between the parameters studied. Bull-to-bull variations was negligible, therefore, the effect of factor bull was not considered. The critical difference (CD) of three factors- A (incubation period), B (treatments) and AB (interaction between incubation periods and treatments) 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

Capacitation and acrosome reaction

Head-to-head sperm agglutination was observed after half an hour of incubation in TALP medium. After 1 h of incubation, agglutination decreased in all the samples. During this period, spermatozoa showed normal morphology (Figure 1). After 3 hrs of incubation, spermatozoa started showing a typical hyperactive motility in a zig-zag manner showing whiplash beating with very fast circular movements indicating the occurrence of capacitation. Three stages of acrosome reaction *i.e.* swelling of acrosome, vesiculation and shedding of acrosome were observed after 2, 4 and 6 h respectively (Figure 1).

Percentage motility or hyperactivity

Corresponding to the incubation period, percent hyperactivity of freshly ejaculated spermatozoa, incubated at 37°C decreased significantly (P≤0.05) from 0 h (66.9%) to 6 h (45.1%) (Table 1). Whereas, hyperactivity in the spermatozoa, pre cooled at 4°C for 24 h also decreased significantly (P≤0.05) from 62.4 to 47.6%. However, supplementation of Mn^{2+} (200 μM) improved it significantly (P≤0.05) both in fresh (62.4 to 64.9%) and chilled spermatozoa (47.6 to 51.6%)(Table1). Statistical analysis has shown non-significant (P≥0.05) interactions between treatments and incubation period. Thus, increase or decrease in percent hyperactivity with various treatments is not affected by incubation period or vice-versa (Table 1).

Lipid peroxidation

With the increase in incubation period, lipid peroxidation (LPO) increased significantly (P≤0.05) from 34.5 to 79.8 nmoles of malondialdehyde (end product of LPO, MDA /mg protein) (Table 2). Cooling of semen at 4°C for 24 h increased MDA production significantly (P≤0.05) in spermatozoa from 29.6 to 96.49 n moles MDA/mg protein. However, supplementation of 200 μM Mn^{2+} decreased MDA production non-significantly (P≥0.05) in fresh semen and significantly in chilled semen (Table 2). Statistical analysis showed non-significant interaction between treatments and incubation period, thus increase or decrease in LPO with treatments is not affected by incubation period or *vice-versa* (Table 2).

Acrosome reaction

An increase of 5.7% total acrosome reaction *i.e.* swelling, vesiculation and shedding of acrosome was observed in the presence of Mn^{2+} in freshly ejaculated spermatozoa, whereas, there was an increase of only 2.5 % in chilled semen (Table 3). Percentage of acrosome reacted spermatozoa increased significantly from 4 h (22.63%) to 6 h (30.8%) of incubation.

Corresponding to treatments, cooling of the semen samples decreased the percentage of total acrosome reacted spermatozoa significantly (40.3% to 31.1%). Supplementation of Mn^{2+} (200 μM) significantly improved the percentages of total acrosome reacted spermatozoa both in fresh (40.3% to 45.2%) and in chilled semen (31.1% to 36.5%) (Table 3).

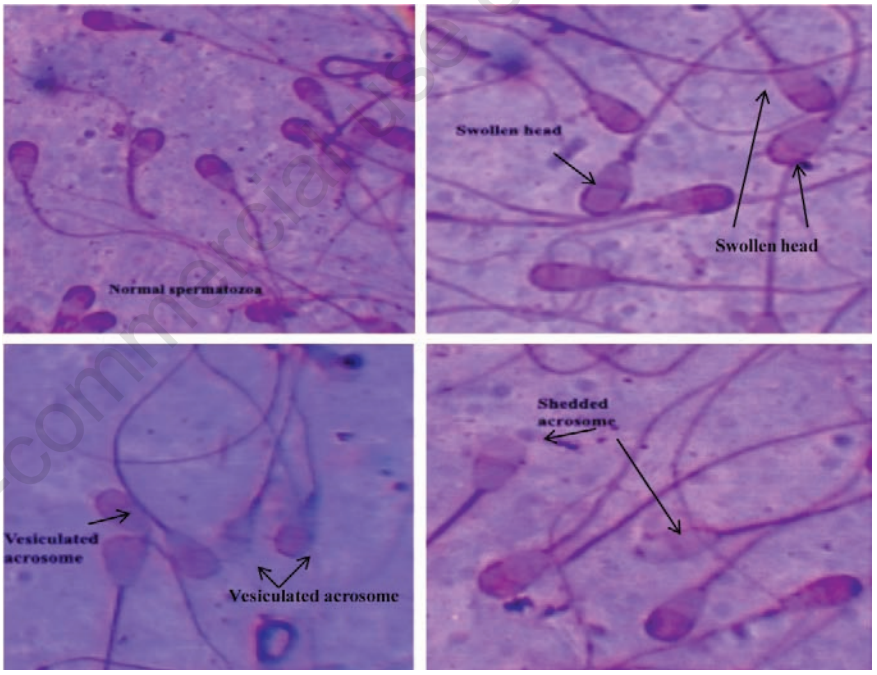

Figure 1. Different stages of acrosome reaction og cattle bull spermatozoa.

Table 1. Effect of Mn^{2+} supplementation on hyperactivity percentage during capacitation of freshly ejaculated and chilled cattle bull semen.

Incubation period (h)	Fresh semen		Chilled semen		Combination factor mean
	TALP	TALP + Mn^{2+}	TALP	TALP + Mn^{2+}	
0	72.18±1.33	72.64±1.21	60.48±2.786	62.42±2.33	66.9[a]
2	66.83±1.18	68.98±0.83	51.83±1.20	56.56±1.29	61.0[b]
4	59.61±1.54	63.26±1.63	43.47±1.786	48.15±2.48	53.6[c]
6	51.04±1.91	55.10±1.52	34.79±1.26	39.47±1.27	45.1[d]
Combination factor mean	62.4[a]	64.9[b]	47.6[c]	51.6[d]	

TALP, Tyrode's modified solution; [a-d]means in a row with different superscripts are significantly different at 5% level of significance.

Mn^{2+} supplementation to the fresh and chilled semen improved the percentages of acrosome reacted spermatozoa. Statistical analysis showed significant interaction between treatments and incubation period, thus increase or decrease in % acrosome reaction with treatments is affected by incubation period or *vice-versa* (Table 3).

Discussion

In this study, the sperm hyperactivity percentage decreased with increase in incubation period, both in fresh and chilled semen incubated in TALP medium at 37°C. Similar observations have been made by Dhanju *et al.*,[22] who reported a 15 to 21% and 55 to 62% decline in the percentage motility during incubation of spermatozoa in TALP medium and 0.85% saline, respectively. Bilaspuri and Bansal[14] found a significant decrease in percent hyperactivity (73.4 to 60.41%) in fresh semen samples during capacitation of the cattle bull spermatozoa. In the present study, during capacitation, the decrease in the percentage of hyperactive spermatozoa in chilled semen in comparison to fresh indicated that cooling decreased hyperactivity of the spermatozoa. This may be due to the production of reactive oxygen species (ROS) during cooling of the semen at 4°C for 24 h. ROS produced in such a way cause oxidative stress in chilled semen which deteriorates the quality of spermatozoa and decreases hyperactivity percentage during capacitation. Thuwant *et al.*[23] also observed that ROS production increases after cooling of the spermatozoa (from 22 to 4°C) over a 2 h interval. Preservation of semen at low temperature disturbed the antioxidant/pro-oxidant equilibrium and lead to oxidative damage to sperm plasma membrane which results in minimizing the level of cyclic adenosine monophosphate (cAMP) and decrease sperm motility and longevity.[24] Supplementation of 200 μM Mn^{2+} both in fresh and chilled semen improves hyperactivity percentage by activating a signal transduction cascade. It has been found that Mn^{2+} supplementation stimulated adenylate cyclase (membrane bound enzyme) activity in sperm, which in turn enhanced the level of cAMP.[25,26] This increase in concentration of cAMP through a cascade of events phosphorylated the axonemal protein, which are involved in sperm motility. Therefore, in the present study, increase in hyperactivity with Mn^{2+} (200 μM) supplementation is associated with adenylate cyclase activity. Lapointe *et al.*[27] also found increase in post thawed motility for a short period by increasing intracellular content of Mn^{2+} (200 μM), thereby stimulating adenylate cyclase activity.

The present study showed that MDA production was more in chilled semen as compared to fresh semen due to enhancement in production of ROS during cooling which lead to oxidative stress. Bailey *et al.*[28] has also found that lower cholesterol level in bull sperm make it sensitive to cooling and causes the generation of oxidative stress.

In this study, Mn^{2+} (200μM) possess antioxidative property as it decreases lipid peroxidation both in fresh and chilled semen. Eybl and Kotyzova[17] found that at lower doses Mn^{2+} act as potent antioxidant in the protection against oxidative stress. *In vitro* experiments revealed that the ability of Mn^{2+} to scavenge oxygen free radicals generated in differently mediated lipid peroxidations.[29-31] It also decreased the production of thiobarbituric acid reactive substances (TBARS) by quenching the superoxide anion and hydroxyl radicals generated during ferrous ascorbate induced LPO in bull spermatozoa.[32,33] Nair *et al.*[34] found that in cattle bull MDA production was increased from 1.17 (0 h) to 7.5/10^8 spermatozoa (72 h) and in buffalo bull from 1.99 (0 h) to 8.7/10^8 spermatozoa (72 h) during storage of semen at 4°C.

Sperm capacitation is a necessary prelude to fertilization and constitute a set of changes in the plasma membrane and enables sperm to undergo acrosme reaction.[1] In this study cooling decreased the percentage of total acrosome reacted spermatozoa. This may be due to chilling and thawing of bull semen suppressed the functions of post thawed spermatozoa, generate ROS and induce LPO in sperm. The sperm cell is easily damaged by LPO as its membrane

Table 2. Effect of Mn^{2+} supplementation on lipid peroxidation (LPO nmoles MDA/mg protein) during capacitation of freshly ejaculated and chilled cattle bull semen.

Incubation period (h)	Fresh semen		Chilled semen		Combination factor mean
	TALP	TALP + Mn^{2+}	TALP	TALP + Mn^{2+}	
0	14.37 ±1.41	8.99 ±0.98	65.41 ±2.55	47.49 ±4.39	34.5[a]
6	44.91 ±7.71	42.06 ±8.49	127.45 ±1.71	104.98 ±1.58	79.8[b]
Combination factor mean	29.6[a]	25.5[a]	96.4[b]	77.2[c]	

TALP, Tyrode's modified solution; [a-c]means in a row with different superscripts are significantly different at 5% level of significance.

Table 3. Effect of Mn^{2+} supplementation on percentage of acrosome reaction of freshly ejaculated and chilled cattle bull semen.

Incubation period (h)	Fresh semen fractions								Chilled semen fractions								
	TALP				TALP + Mn^{2+}				TALP				TALP + Mn^{2+}				
	Stages of acrosome reaction																
	S	V	Sh	TAR	S	V	Sh	TAR	S	V	Sh	TAR	S	V	Sh	TAR	CFM
0	3.87± 0.32	1.65± 0.20	0.45± 0.04	5.98± 0.28	3.52± 0.24	1.10V 0.14	1.56± 0.24	6.18V 0.38	6.20V 0.45	1.54V 0.30	0.62V 0.21	8.37V 0.61	6.38V 0.48	1.74V 0.19	1.54V 0.06	9.66V 0.50	3.77[a]
4	26.07± 0.29	11.68 ±0.25	8.14± 0.30	45.90 ±0.57	27.64 ±0.67	14.43 ±0.58	12.76 ±0.32	54.84 ±1.16	14.48 ±0.72	5.77± 0.89	14.55 ±0.94	34.81 ±0.21	25.27 ±0.46	7.48± 0.27	14.45 ±0.51	47.21 ±0.91	22.63[b]
6	20.23± 0.44	22.64 ±0.59	26.23 ±0.50	69.17 ±1.03	18.18 ±0.35	25.45 ±0.36	31.21 ±0.49	74.85 ±0.21	9.48± 0.51	19.23 ±0.37	21.41 ±0.69	50.13 ±1.26	7.04± 0.43	17.48 ±0.57	28.11 ±0.43	52.64 ±1.06	30.84[c]
CFM	16.7[e]	11.9[c]	11.6[c]	40.3[h]	16.4[de]	12.5[c]	15.1[d]	45.2[i]	10.0[b]	8.8[a]	12.1[c]	31.1[f]	12.9[c]	8.0[ab]	14.7[d]	36.5[g]	

TALP, Tyrode's modified solution; S, swollen; V, vesiculated; Sh, shedded; TAR, total acrosome reacted; CFM, combination factor mean; [a-i]means in a row with different superscripts are significantly different at 5% level of significance.

has high content of polyunsaturated fatty acids (PUFAs) resulting in deleterious changes in sperm membrane fluidity, membrane integrity and sperm motility,[35] which indicates the lower percentage of total acrosome reacted spermatozoa in chilled semen samples as compared to that of fresh sperm in this study. Cold shock also destroys the selective permeability of sperm membrane to calcium thus leading to excessive intracellular calcium which reduces hyperactivity and thus minimizes capacitation.[28] Whereas, Mn^{2+} (200 μM) enhanced the rate of acrosome reaction both in fresh and chilled semen samples by significantly increasing the percentage of total acrosome reacted spermatozoa. Increase in capacitation and acrosome reaction with Mn^{2+} supplementation is related to the increase in the concentration of intracellular calcium ions (Ca_i^{2+}) and adenylate cyclase activity but lowering the rate of LPO. Kuroda *et al.*[36] found that 2.25 mM Mn^{2+} supplemented to the cattle bull sperm results in increase in intracellular calcium both by cAMP and calcium signaling pathways, which leads to progress of sperm capacitation state.

Therefore, our findings suggest the role of Mn^{2+} supplementation in improving the quality of cattle bull semen by its scavenging property *i.e.* reduction in production of reactive oxygen species during its storage at 4°C or incubation at 37°C for capacitation.

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