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Mn2+: A potent antioxidant and stimulator of sperm capacitation and acrosome reaction in crossbred cattle bulls

Abstract

Manganese is a well known antioxidant and is a potent inhibitor of *in vitro* oxidative stress. The aim of this work was to study the influence of Mn^{2+} (60 µM) on capacitation and acrosome reaction of crossbred cattle bull spermatozoa. Fresh semen was centrifuged and pellet so obtained was suspended in TALP medium (pH 7.4). The sperm suspension was divided into four equal fractions(four tubes). In the control tube, only TALP was added; whereas the remaining three tubes were supplemented with ferrous ascorbate (FeAA – 150 µM $FeSO_4$: 750 µM ascorbic acid) , 60 µM Mn^{2+} and FeAA+ Mn^{2+}, respectively. All fractions were incubated incubated (37°C) for 6 hr in CO_2 incubator. These fractions were assessed for morphology, % hyperactivity, lipid peroxidation (LPO) and % acrosome reaction. Morphology of the capacitated spermatozoa remained unaltered with FeAA/Mn^{2+}/FeAA+Mn^{2+} treatments. FeAA treatment non-significantly ($p \geq 0.05$) decreased % sperm hyperactivity % acrosome reaction, but, increased the LPO level. Supplementation of Mn^{2+} increased % hyperactivity and % acrosome reaction significantly ($p \leq 0.05$) in FeAA untreated fractions, but, non-significantly ($p \geq 0.05$) in FeAA treated fractions. Malondialdehyde (MDA – end product of LPO) decreased significantly ($p \leq 0.05$) with the Mn^{2+} supplementation both in FeAA treated and untreated fractions. It is concluded that Mn^{2+} protects the bull sperm against LPO/oxidative stress and facilitates the occurrence of capacitation and acrosome reaction.

Key words**:** FeAA; Mn^{2+}; sperm capacitation; acrosome reaction; Lipidperoxidation

Zusammenfassung

Titel der Arbeit: **Mn^{2+} als Antioxidanz und Stimulator für die Kapazitierung und Akrosomenreaktion von Sperma bei Kreuzungsbullen.**

Magnesium ist bekannt als Antioxidanz und Hemmstoff für *in vitro* oxidativen Stress. Ziel dieser Studie war die Untersuchung des Einflusses von Mn^{2+} (60 µM) auf die Kapazitation und Akrosomenreaktion bei Sperma von Kreuzungsbullen. Das Frischsperma wurde zentrifugiert, pelletiert und anschließend TALP Medium (pH 7,4) ausgesetzt. Danach erfolgte eine Aufteilung in vier gleiche Fraktionen. Die Proben der ersten Fraktion enthielten nur TALP Medium, während den verbleibenden drei Proben Eisenascorbit (FeAA – 150 µM $FeSO_4$: 750 µM Ascorbinsäure), 60 µM Mn^{2+} beziehungsweise FeAA+ Mn^{2+} zugesetzt wurde. Alle Fraktionen wurden sechs Stunden bei 37° in CO_2 inkubiert und anschließend hinsichtlich Morphologie, prozentualer Hyperaktivität, Lipidperoxidation (LPO) sowie prozentualer Akrosomenreaktion bewertet. Morphologisch unverändert blieb die Spermienkapazitation der FeAA/Mn^{2+}/FeAA+Mn^{2+} behandelten Fraktion. Die FeAA Behandlung ergab keine signifikante (p=0,05) Verminderung der Sperma Hyperaktivität und der prozentualen Akrosomenreaktion, aber eine Erhöhung des LPO Spiegels. Ein Mn^{2+} Zusatz ergab eine signifikante Erhöhung der prozentualen Hyperaktivität und Akrosomenreaktion gegenüber der FeAA unbehandelten Fraktion, aber keine signifikante Erhöhung in der FeAA Fraktion. Malondialdehyd (MDA - Endprodukt von LPO) verminderte signifikant die Mn^{2+} ergänzten FeAA und nicht FeAA behandelten Fraktionen. Es wird geschlussfolgert, dass Mn^{2+} Bullensperma gegen LPO und oxidativen Stress schützen kann und die Vorgänge der Spermakapazitation und Akrosomenreaktion erleichtert werden.

Schlüsselwörter: FeAA, Mn^{2+}, Sperma, Kapazitation, Akrosomenreaktion, Lipidperoxidation

Introduction

For successful fertilization, mammalian spermatozoa must undergo a preparation period known as capacitation (O'FLAHERTY et al., 1999). In physiological terms, capacitation can be considered as the sum of biochemical and biophysical modifications that take place in sperm cell during its transport through the female genital tract (PARRISH et al., 1999). These modifications regulate temporary changes in the pattern of sperm motility referred to as hyperactivation. This process culminates in an exocytotic event called acrosome reaction, an essential step to fertilize oocytes (O'FLAHERTY et al., 1997).
Mammalian spermatozoa represents a growing list of cell types that exhibit a capacity to generate highly reactive oxygen species (ROS). Controlled generation of ROS has a physiological role in spermatozoal functions such as hyperactivation, capacitation and acrosome reaction (de LAMIRANDE et al., 1993;GRIVEAU et al., 1997;O'FLAHERTY et al.,2005). Uncontrolled and excessive ROS production or exposure of spermatozoa to fatty acidperoxides or the combination of ferrous ions(Fe^{2+}) and ascorbic acid (AA) at high concentrations induces lipid peroxidation (LPO) / oxidative stress, resulting in decreased sperm motility , viability and increased mid-piece defects that impair capacitation and acrosome reaction (de LAMIRANDE et al., 1993 & 1995; BANSAL and BILASPURI, 2007). Therefore, an antioxidant that reduces oxidative stress and improves spermatozoal functions could be useful in the management of male infertility (VERMA and KANWAR, 1999).Antioxidants are the agents, which break the oxidative chain reaction, thereby, reducing the oxidative stress (MILLER and SLEBODZINSKA, 1993; KUMAR and MAHMOOD,2001). A variety of biological antioxidants that attack ROS and LPO are presently under investigation (SIKKA, 1996).

The antioxidative action of Mn (II) on different peroxidizing systems has been studied. It inhibits LPO produced by free radical producing system but not the one induced by singlet oxygen (CAVALLINI et al., 1984). Manganese in very – very small amount affects human health and participates in many enzymatic activities(SINGH, 2008). It is an essential component of several enzymes and some of them (superoxide dismutase, pseudo-catalase and the photosynthetic oxygen evolving centre) are involved in redox processes. Manganese has also been assigned as a chain breaking antioxidant, as it is able to quench the peroxyl radicals (COASSIN et al., 1992).

The present study was undertaken to examine the effect of 60 µM Mn2+ on morphology, % hyperactivity, % acrosome reaction and changes in lipid peroxidation (LPO) level during capacitation and acrosome reaction of crossbred cattle bull spermatozoa.

Materials and methods

Semen samples (n = 5) with more than 80% motility and 1200 x 10^6 – 1400 x 10^6/ml sperm count were obtained from the healthy local crossbred cattle bulls (HHS, Holstein-Friesian x Sahiwal; FC, Friesian crosses; 1F and 4F first and fourth generation of interbreeding) maintained at the Dairy Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Each parameter was analysed by using three replicates of a single ejaculate of each bull;total five bulls were studied. A known volume of semen

sample was taken in a centrifuge tube (prewarmed at 37°C) which was centrifuged (x 800 g, 5 minutes); seminal plasma was removed, sperm pellet was washed 2-3 times with TALP medium (NaCl – 92.9mM; KCl – 4mM; $NAHCO_3$ – 25.9mM; $CaCl_2.2H_2O$ – 10mM; $MgCl_2.6H_2O$ – 0.5mM; sodium lactate – 7.6mM; sodium pyruvate – 1.3mM; HEPES – 20mM; glucose – 0.25%; heparin – 200 µg/ml and BSA – 0.6%). Pellet was dissolved in TALP medium (pH 7.4) to prepare sperm suspension which was divided into four equal fractions(four tubes). In the control tube, only TALP was added; whereas the remaining three tubes were supplemented with ferrous ascorbate (FeAA – 150 µM $FeSO_4$: 750 µM ascorbic acid) (best dose of oxidative stress inducer, suggested by BANSAL and BILASPURI,2008), 60 µM Mn^{2+} (best dose of antioxidant, suggested by BANSAL,2006) and FeAA+ Mn^{2+}, respectively. All fractions were incubated (37°C) for 6 hr in CO_2 incubator. Thereafter, control and experimental fractions were evaluated for the following parameters at varying incubating periods.

Percentage motility or hyperactivity:

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

Morphology and percentage acrosome reaction:

Smears of each fraction were prepared at 2 hr interval, 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 (LPO):

Malondialdehyde (MDA – end product of LPO) was determined by the thiobarbituric acid (TBA) assay (BUEGE and STEVEN, 1978).

'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 were negligible , therefore, the effect of factor bull was not considered. The critical difference (CD) of three factors- A (incubation period), B (control and 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

Morphology:

Morphology of bull spermatozoa remains unaffected with FeAA/Mn^{2+}/FeAA + Mn^{2+} treatments, however, the spermatozoa undergo normal process of capacitation and acrosome reaction. The stages during these processes observed in bull spermatozoa are clearly seen in Figs. 1 to 4.

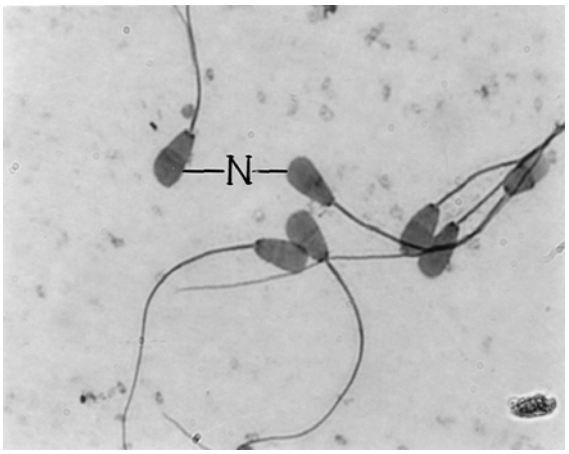

Fig. 1: Cattle bull spermatozoa incubated for 2hr in Mn^{2+} (60µM) at 37° C. The capacitation has not started yet and spermatozoa show normal (N)morphology.

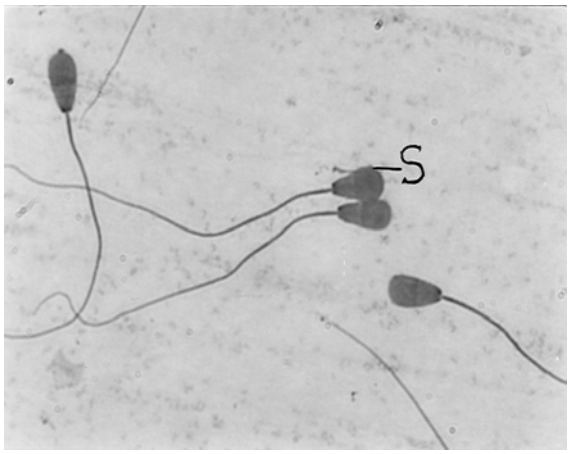

Fig. 2: Cattle bull spermatozoa incubated for 4hr in Mn^{2+} (60µM) at 37° C. The capacitation has has started as shown by swelling (S)in the head region.

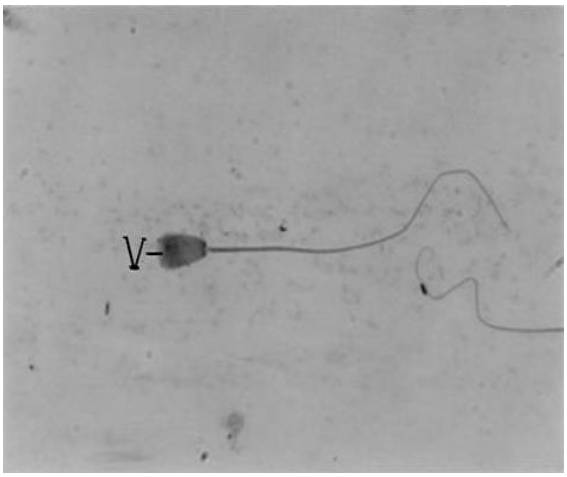

Fig. 3: Cattle bull spermatozoa incubated for 4 hr in Mn^{2+} (60µM) at 37° C. The capacitation has advanced further as shown by vesiculation (V) in the head region.

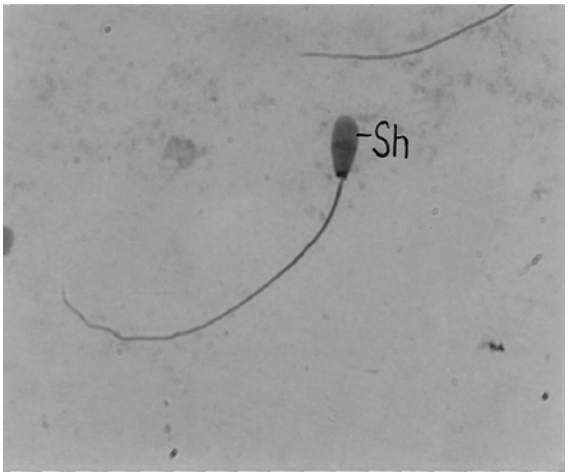

Fig. 4: Cattle bull spermatozoa incubated for 6hr in Mn^{2+} (60µM) at 37° C. The shedding (Sh) of the acrosomal cap show the occurrence of acrosome reaction.

Hyperactivity (%):

Corresponding to the incubation period, % hyperactivity of spermatozoa decreased non-significantly ($P \geq 0.05$) upto 2 hr, but, significantly ($P \leq 0.05$) upto 6 hr as compared to 0 hr (Table 1). Treatment of spermatozoa with FeAA decreased the hyperactivity non-significantly ($P \geq 0.05$). However, Mn^{2+} supplementation increased it significantly ($P \leq 0.05$) in FeAA untreated but non-significantly ($P \geq 0.05$) in FeAA treated samples (Table 1). Statistical analysis has shown non-significant ($P \geq 0.05$) interaction between treatments and incubation periods. Thus, increase or decrease in % hyperactivity with various treatments is not affected by incubation periods or vice-versa.

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Table 1
Effects of FeAA/Mn^{2+}/FeAA + Mn^{2+} on % hyperactivity of bull spermatozoa duringcapacitation and acrosome reaction**.** **(**Einfluss von FeAA/Mn^{2+} /FeAA + Mn^{2+} auf prozentuale Hyperaktivität von Bullensperma während Kapazitation und Akrosomenreaktion)

	% hyperactivity				
Incubationperiod (hr)	FeAA untreated		FeAA treated		Combination factor mean
	Control	Mn^{2+}	FeAA	FeAA + Mn^{2+}	
0	73.888 ±1.367	74.166 ±1.416	72.5 ±0.962	73.055 ±1.510	73.402[a]
1	72.222 ±1.0718	76.388 ±1.118	71.388 ±1.047	74.166 ±1.521	73.541[a]
2	72.166 ±0.881	73.055 ±1.228	71.944 ±1.609	72.5 ±1.360	72.416[a]
3	69.444 ±1.289	71.666 ±1.521	68.611 ±1.422	70.555 ±1.095	70.069[b]
4	67.5 ±2.187	69.444 ±1.458	66.111 ±1.928	68.333 ±1.360	67.847[c]
5	62.222 ±2.338	65.833 ±2.515	61.944 ±2.214	65.000 ±1.883	63.75[d]
6	59.166 ±2.545	63.055 ±2.349	57.777 ±2.705	61.666 ±2.319	60.416[e]
Combination factor mean	68.087[a]	70.515[b]	67.182[a]	69.325[a]	

Any two means in a row or column having different superscripts (a, b, c, d, e) are significantly different at 5 % level of significance.

Lipid peroxidation and % acrosome reaction:

Corresponding to the incubation period, LPO of all fractions increased significantly ($P \leq 0.05$) after 4 hr and 6 hr as compared to 0 hr of incubation (Table 2). FeAA treatment to the bull sperm increased the LPO non-significantly ($P \geq 0.05$). However, supplementation of Mn^{2+} decreased the MDA production significantly ($P \leq 0.05$) in both FeAA treated and untreated spermatozoal samples (Table 2). Statistical analysis shows non-significant interaction ($P \geq 0.05$) between treatments and incubation periods, thus, increase or decrease in LPO with treatments is not affected by incubation periods or vice-versa.

The % acrosome reaction of bull spermatozoa increased significantly ($P \leq 0.05$) from 4 hr to 6 hr (Table 3). FeAA treatment decreased the % acrosome reaction non-significantly ($P \geq 0.05$) as compared to the control. Subsequently, supplementation of 60 µM Mn^{2+} increased % acrosome reaction significantly ($P \leq 0.05$) in FeAA untreated, but, non-significantly ($P \geq 0.05$) in FeAA treated fractions (Table 3). Statistical analysis shows non-significant ($P \geq 0.05$) interaction between the treatments and incubation periods. Thus, increase or decrease in acrosome reaction with various treatments is not affected by incubation periods or vice-versa.

Table 2
Effects of FeAA/Mn^{2+}/FeAA + Mn^{2+} on lipid peroxidation of bull spermatozoa during capacitation and acrosome reaction. (Einfluss von FeAA/Mn^{2+} /FeAA + Mn^{2+} auf Lipidperoxidation von Bullensperma während Kapazitation und Akrosomenreaktion)

Incubationperiod (hr)	n moles MDA/µg protein				
	FeAA untreated fractions		FeAA treated Fractions		Combination factor mean
	Control	Mn^{2+}	FeAA	FeAA + Mn^{2+}	
0	2.146 ±0.473	2.016 ±0.551	3.385 ±0.541	2.491 ±0.694	2.510[a]
4	4.663 ±1.171	2.833 ±0.399	5.111 ±1.199	3.448 ±0.362	4.013[b]
6	4.956 ±0.352	1.672 ±0.311	6.336 ±0.721	2.993 ±0.564	3.989[b]
Combination factor mean	3.922[a]	2.174[b]	4.944[a]	2.977[b]	

Any two means in a row or column having different superscripts (a, b) are significantly different at 5% level of significance.

Table 3
Effects of FeAA/Mn^{2+}/FeAA + Mn^{2+} on % acrosome reaction of bull spermatozoa. (Einfluss von FeAA/Mn^{2+} /FeAA + Mn^{2+} auf prozentuale Akrosomenreaktion bei Bullensperma)

Incubationperiod (hr)	% acrosome reaction				
	FeAA untreated fractions		FeAA treated Fractions		Combination factor mean
	Control	Mn^{2+}	FeAA	FeAA + Mn^{2+}	
4	72.570 ±4.527	71.32 ±7.300	57.76 ±3.508	70.817 ±4.828	68.116[a]
6	74.367 ±4.572	86.141 ±5.117	72.662 ±4.469	77.951 ±1.016	77.780[b]
Combination factor mean	73.468[a]	78.730[b]	65.211[a]	74.384[a]	

Any two means in a row or column having different superscripts (a, b) are significantly different at 5% level of significance.

Discussion

In our study, % sperm hyperactivity decreases from 3 hr to 6 hr of incubation. It indicates that during capacitation, motility of spermatozoa decreases gradually and significantly ($P \leq 0.05$). Similar observations were made by DHANJU et al., 2006, 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. Corresponding to the treatments, by inducing oxidative stress, FeAA decreases the % hyperactivity. It may be explained on the basis of oxidative stress caused by FeAA which deteriorates the membrane

permeability and integrity, thereby, decreasing the % hyperactivity or motility (KODAMA et al., 1996). The present study assumes that supplementation of Mn^{2+} to FeAA treated/untreated samples increases the % hyperactivity by activating a single transduction cascade.

Following model of signal transduction for the role of Mn^{2+} in enhancing % hyperactivity is suggested based on our study and relevant literature (LAPOINTE et al., 1996; GARBERS and KOPF, 1980; ROJAS et al., 1992; TASH and MEANS, 1983). Messenger systems such as adenylate cyclase, guanylate cyclase and calmodulin are highly affected by intracellular salts, such as Mn^{2+}, Mg^{2+} and Ca^{2+}. Out of these, Mn^{2+} is a well known potent stimulator of adenylate cyclase activity, which in turn enhances the level of cAMP (LAPOINTE et al., 1996). Increase in the level of cAMP with the Mn^{2+} supplementation phosphorylates many proteins that are involved in the movements or flagellar beating of spermatozoa. Increase in the level of cAMP also stimulates the Ca^{2+} uptake by the cell, thus, increasing the level of intracellular calcium (Ca_i^{2+}) (GURAYA, 1999). At a higher level, Ca_i^{2+} increase the membrane integrity and viability. These properties of the sperm are required for its optimal functioning under normal and oxidative stress conditions. Elevation of Ca_i^{2+} in flagellum of spermatozoa drives hyperactivation and this action of calcium could be at the level of sperm flagella as revealed by experiments with demembranated rat (LINDEMANN and GOTZ, 1988) and bull (LINDEMANN et al., 1991) spermatozoa.

The previous literature suggest that the intake of Ca^{2+} is slow during capacitation; but rapid at the time of acrosome reaction. Adequate level of Ca^{2+} is required in sperm, once the capacitation gets completed (GURAYA,1999). LARSEN (1994) found that high concentration of Ca_i^{2+} is related to the cell death. Based on the present and above studies, it is suggested that supplementation of Mn^{2+} to the bull spermatozoa permits the rise in Ca_i^{2+} level without decreasing their viability. Further, Mn^{2+} has beneficial effects on sperm survival and % hyperactivity during capacitation and acrosome reaction.

The present study also shows that Mn^{2+} possess the antioxidative property as they decrease the MDA production significantly in both FeAA treated as well untreated samples. 60 µM Mn^{2+} supplementation increases the rate of acrosome reaction under normal and oxidative stress conditions. Thus, on correlating the results of LPO and acrosome reaction, it is suggested that supplementation of Mn^{2+} to the bull sperm enhances the % acrosome reaction by decreasing the oxidative stress. As manganese inhibits LPO both *in vitro* (TAM and McCAY, 1970) and *in vivo* (SHUKLA and CHANDRA, 1981), it is suggested that its antioxidative property stabilizes the plasma membrane, thereby, maintaining membrane integrity and viability. Therefore, Mn^{2+} enhance the rate of acrosome reaction in the present study. Similar observations on acrosome reaction have been made, when bull sperms are incubated with 0.1 mM $MnCl_2$ (LAPOINTE et al., 1996). Another possible explanation for the increase in sperm capacitation and acrosome reaction with Mn^{2+} supplementation is related to the increase in intracellular calcium (Ca_i^{2+}) content.

The following model for the role of Mn^{2+} in sperm capacitation and acrosome reaction is suggested based on our study and relevant literature (GARBERS and KOPF, 1980; TASH and MEANS, 1983;SIDHU et al., 1984; SIDHU and GURAYA, 1989). According to this model, calmodulin or calmodulin-like proteins loosely bind to the plasma membrane and/or Ca^{2+} or Mg^{2+}-ATPase. These bindings enhance the extrusion of intracellular calcium (Ca_i^{2+}) and interfere with the capacitation and acrosome reaction processes. However, Mn^{2+} supplementation stimulates the calmodulin removal from its receptors, thereby, enhancing the Ca_i^{2+} level. As more and more Ca_i^{2+} depositing leads to vesiculation of the acrosome, it causes the fusion of outer acrosomal membrane with the plasma membrane, thus, resulting in acrosome reaction. Extracellular addition of Mn^{2+} ions also enhance the level of cAMP by stimulating Ca^{2+} or Mg^{2+}-ATPase which lead to the activation of calcium channel openings, thereby, depositing more Ca_i^{2+}. Thus, Mn^{2+} promotes the acrosome reaction.

Further studies are required to understand the antioxidant strategies or the mechanisms whereby ROS and endogenous antioxidants produced in sperm cells influence the reproductive processes, and ,thereby, promote the fertility of cattle bull spermatozoa.

It is concluded that Mn^{2+} protects the crossbred cattle bull sperm against LPO / oxidative stress and facilitates the occurrence of capacitation and acrosome reaction.

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