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ANTIOXIDANT POTENTIAL OF Mn^{2+} IN THE HUMAN EJACULATED SPERMATOZOA UNDER OXIDATIVE STRESS

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ABSTRACT: Reactive oxygen species (ROS) formation from fertile volunteer donor measured by thiobarbuteric acid reactive substances (TBARS). Spermatozoal suspension was treated or not with nicotine and supplemented or not with Mn^{2+}. The same protocol followed when we used ferrous ascorbate promoter system and calculates the C_{max} and k_c value of the reaction using Lineweaver-burk double reciprocal plot. For comparing the antioxidant status Mn^{2+} with other trace elements employing various concentrations (10-100µM), production of malondialdehyde (MDA) calculated. Effect of lipid peroxidation (LPO) upon membrane integrity monitored by performing hypo-osmotic swelling test (HOS). Extent of LPO was measured for one-hour intervals of 10 minutes each, TBARS production found to increase gradually. Supplementation of Mn^{2+} to nicotine treated or not spermatozoal suspensions significantly lower the TBARS production in different concentration of promoter system (ferrous sulphate and ascorbic acid; 1:5). According to Michaelis-Menten kinetic it was observed that there was linear and positive correlation between amounts of malon-dialdehyde produced (v_i) and increase in substrate concentration (S). Nicotine treated or not supplemented or not with Mn^{2+}, ferrous ascorbate promoter system the value of k_c was found to be 62.50µM FeSO4 and 312.5µM ascorbic acid. The value of C_{max} was found to be lower in Mn^{2+} supplemented treated or not with nicotine as compare to Mn^{2+} unsupplemented, nicotine treated or not sample. Supplementation of different concentration trolox, Mn^{2}+, Zn^{2+}, Co^{2+} and Ni^{2+} to assay mixture, amount of TBA-MDA complex was monitored and data has been transferred to Dixon plot (I/v) vs. [I]. Values of k_i of all trace metal were finding in the same order. The data follow a linear correlation with the C_{max} value of the reaction. Hypo-osmotic swelling test also show the significant result. On the basis of C_{max}, k_i values and other parameter we can conclude that Mn^{2+} is the most potent trace metal ion inhibitor of LPO in the human ejaculated spermatozoal suspension.

INTRODUCTION: Cryopreservation of sperm, common requirement and /or technique used in assisted reproductive technologies (ART), has the potential to exacerbate sperm oxidative stress [1, 2]. Human semen has a complex set of antioxidants (micronutrients, vitamins and enzymes) that prevent oxidative damage of the life-saving sperm components such as plasma cell membrane and nuclear DNA [3].

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Sperm preparation for cryopreservation involves the removal of seminal plasma and consequently the predominant source of antioxidant protection. Calamity occurs when production of reactive oxidative stress (ROS) exceeds this elimination by the antioxidant protective system or when the latter are damaged. Oxidative stress mediated altered sperm parameters like; impaired motility, impaired membrane integrity and impaired DNA content are playing a crucial role in ART.

The process of lipid peroxidation (LPO) is widespread in biology and is mediated through both enzymatic and non-enzymatic pathways [4]. Non-enzymatic or spontaneous LPO has been well characterized in human semen samples [5, 6].

Malondialdehyde (MDA) is an end-product of the radical initiated oxidative decomposition of poly-unsaturated fatty acids and, therefore, it is a frequently measured biomarker of oxidative stress [7, 8]. The Measurement of MDA (end-product of LPO) appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normo-zoospermic individuals [9, 10, 11].

The discovery of LPO as a causative mechanism in the etiology of defective sperm function is important because it leads to logical use of antioxidant strategy to reverse the damage caused by the oxidative stress [12, 13]. An effective antioxidant is sought that can be successfully be used to reverse the oxidative damage inflicted on human spermatozoa during sperm preparation procedure that involve centrifugation of ejaculated cells in the absence of protective environment normally provided by seminal plasma [14]. Antioxidant treatment which can also enhance the endogenous antioxidant defense system within the cell can inhibit a verity of signaling pathways.

Transitions metals such as manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni) copper (Cu), and zinc (Zn) are essential cofactors in the physiology of all organisms [15]. In fact, recent estimates suggest that over half of all proteins in every organism are metalloproteins [16]. Although essential in trace amounts, at higher levels these metals can be toxic to cells because they directly or indirectly compromise DNA, protein, and membrane integrity and function [15]. Sperm preparation media may also be supplemented with a variety of antioxidants to guard against oxidative stress [2], however, at the present moment commercial sperm preparation media does not contain any antioxidants aside from albumin and amino acids. It is reported that, among trace metal supplement, Mn^{+} is potent radical scavenger as compare Zn^{+}, Ni^{+}, Co^{+} [17].

Mn^{2+} has been reported to inhibit LPO both *in vitro* and *in vivo* [18]. Mn^{2+} is known to be required for mitochondrial superoxide dismutase synthesis and activate enzyme like hydrolase's and carboxylases [19]. Mn^{2+} can be easily transformed to Mn^{3+} by superoxide anion. Mn^{2+} complexes are also reported to be important catalyst of superoxide dismutation in some lactobacillus [16]. It has also been assigned as a chain breaking antioxidant that is able to quench the proxyl radicals [17]. It inhibits LPO produced by a free-radical-producing system, but not LPO induced by single oxygen [20].

Manganese (Mn^{2+}) has been suggested to be a potential antioxidant candidate. Considering above mentioned facts the present study was designed to find out antioxidant potential of Mn^{2+} in the human ejaculated spermatozoa under oxidative stress

MATERIALS AND METHODS:

Chemicals:

Nicotine, thiobarbuteric acid (TBA), trolox, bovine serum albumin (BSA) Cohn fraction V, sodium dodesyl sulphate (SDS) was purchased from Sigma. Fructose, sodium citrate, folin phenol reagent, $MnCl_2$, $CoCl_2$, $ZnCl_2$, and $NiCl_2$ trichloro acetic acid (TCA) was procured from Himedia Chemical Pvt. Ltd and other chemical used were analytical grade. Nicotine (Mol.wt.162; bp 246.1C) solution was prepared by dissolving in 0.2M phosphate buffer saline (PBS) solutions. 5% SDS solution were prepared in 0.5 N NaOH.

Collection of Semen Samples:

Semen sample was collected from healthy nonsmoker volunteer donors; students of Lala Lajpat Rai Bhawan (Boys Hostel No.-5), Panjab University, Chandigarh between age group (20-25). Semen analyses were performed after complete liquefaction and sperm concentration motility forward progression were evaluated subjectively at room temperature. Sample having final concentration between 70-80 X 10^6 sperm cell/ ml and with more than 70% motile sperm were selected. Seminal plasma discarded by centrifugation at 300 X g. The pallet so obtained was suspended in equal volume of 0.2M PBS.

Measurement of LPO:

The lipid peroxidation was monitored by using modified methods of Buege and Steven [21] as suggested by Anand and Kanwar [22]. The LPO was monitored in 1.0 ml of assay mixture containing 150 mM tris-HCl (pH 7.2), 200µM ferrous sulphate and 1000 µM ascorbic acid, appropriate amount of sperm suspension was added to assay mixture. In

nicotine treated series, 0.5 mM (final concentration) of nicotine was added to assay mixture. The supplemented series received 0.1 mM of $MnCl_2$.

To the untreated, unsupplemented sample an equal volume of PBS was added. Sample was mixed well and incubated at 37ºC for 20 minutes, 1 ml of 10 % child TCA was added to precipitate the protein. Tubes were centrifuged at 2500 X g for 10 minutes. 1 ml aliquot was taken out into another test tube. To this 0.25 ml of 2% TBA prepared in 0.05 N NaOH was added. Samples were vortexed and kept at 90ºC for 10 minutes. After cooling at room temperature the absorbance was read at 532 nm which is approximate $\lambda_{max.}$of malon-dialdehyde (MDA).

Kinetic Analysis:

For time kinetic LPO was monitored by incubating the assay mixture for different time point ranging from 10 minutes to 60 minutes. For substrate kinetic, different concentration of ferrous sulphate (25-200µM) and ascorbic acid (125-1000µM), all in ratio 1:5 were used. For comparing the antioxidant status of Mn^{2+} with different trace metal, different concentration of trolox, $MnCl_2$, $CoCl_2$, $ZnCl_2$, and $NiCl_2$ were supplemented to assay mixture and the extent of TBA-MDA complex formed was calculated.

Protein Estimation:

The extent of MDA production were calculated and expressed per mg protein. The amount of protein was estimated by SDS-Lowery method of Lee and Paxman [23] using BSA Cohn fraction V as standard, 0.9ml of 5% SDS in NaOH was added to 0.1 of sample in the test tube. Tubes are allowed to remain in room temperature for at least two hour and were agitated 2-3 times in vortex mixture. To make sure that the samples were dissolved thoroughly to this 2.5ml of copper carbonate solution were added and tubes were allowed to stand for 20 minutes. Then 0.25 ml of folin phenol reagent was added, sample were mixed immediately and allowed to stand for 45 minutes. The intensity of color developed was read at 740nm. BSA standard (20-100µM/ml) was also run simultaneously.

Statistical Analysis:

Statistical analysis was carried out employing SPSS (7.5) software. Data were expressed as Mean ± SD for observation in each group. The statistical significance of inter group difference of various parameters were determined by unpaired student's t-test. Comparison was made between untreated (without nicotine) supplemented with Mn^{2+} and nicotine treated supplemented or not with Mn^{2+} samples to that of the untreated and unsupplemented samples and the statistical significance was depicted employing symbol "*".

RESULTS AND DISCUSSION:

The process of lipid peroxidation is widespread in biology and is mediated through both enzymatic and non-enzymatic pathways [4]. The product of LPO has been analyzed in a wide variety of different matrices in numerous studies dealing with oxidative stress parameters or the modification of endogenous substances such as proteins, or DNA [8]. In the present investigation, the extent of lipid peroxidation in the human ejaculated spermatozoa suspended in PBS was monitored for one hour at an interval of 10 minutes each.

The MDA production was found to increase gradually. **Fig.1** show the average percent increase or decrease in the TBA–MDA complex formation in 0.5mM nicotine treated and untreated human ejaculated spermatozoa supplemented or not with 0.1mM Mn^{2+}. Upon 0.1mM Mn^{2+} supplementation, the extent of lipid peroxidation was lowered at all time points by an average of 19.23 ±0.49%. Nicotine, a known oxidant [22], raised the MDA level by about 2.5 times such that the average increase measured at all time point was 143.35 ±5.58%. When 0.1m Mn^{2+} was added to nicotine treated samples, the rate of increase of MDA production was lowered by 59.99 ±1.62%.

The measured intensity of the colored TBA-MDA complex was however, 46.07 ±6.88% more than that the control. It may be safely inferred that, spermatozoal lipid peroxidation is a gradual process under aqueous conditions. 0.1mM Mn^{2+} addition tends to lower the extent of lipid peroxidation and thus, render the spermatozoa active for a longer duration, as tested by the

spermatozoal motility that was found to increase upon Mn^{2+} addition [24].

In order to examine the dose response of promoter system, and calculate the kinetic parameters of lipid peroxidation analysis was performed with different promoter concentrations. All of these had same combination ratio of 1:5, ferrous sulphate: ascorbic acid, ranging from 25μM to 200μM $FeSO_4$, and 125μM to 1000μM ascorbic acid, respectively. This ratio (1:5) has been suggested by Aitken *et al.,* [25] and has been experimentally calculated by earlier workers of this laboratory. Subjecting the human spermatozoal suspension to increasing concentrations of ferrous ascorbate resulted in an increased lipid peroxidation (**Table 1**). The data has been treated according to Michaelis-Menten enzyme kinetics by extrapolating the combined promoter ration of the individual combination as substrate concentration [S] and treating the observed amount of malon-dialdehyde (MDA) produced equivalent to initial velocity (v_i).

TABLE 1: EFFECT OF 0.1mM Mn^{2+} SUPPLEMENTATION AND 0.5mM NICOTINE TREATED OR UNTREATED HUMAN EJACULATED SPERMATOZOA LIPID PEROXIDATION USING DIFFERENT FERROUS–ASCORBATE CONCENTRATION.

$FeSO_4$: Ascorbic Acid (uM)	Without Nicotine		With 0.5mM Nicotine	
	Without Mn^{2+}	With Mn^{2+}	Without Mn^{2+}	With Mn^{2+}
25:125	0.100	0.081	0.238	0.134
	0.035	0.014	0.026	0.004**
50:250	0.145	0.128	0.370	0.217
	0.043	0.005*	0.037	0.005**
100:500	0.182	0.171	0.500	0.294
	0.004	0.005	0.045	0.006**
150:750	0.227	0.200	0.616	0.334
	0.006	0.006*	0.082	0.008**
200:1000	0.250	0.213	0.555	0.371
	0.018	0.010*	0.072	0.008**

Each datum represent mean ± SD of six independent observation ach made in triplicate.
Mean value are n moles MDA. mg $prot^{-1}$. min^{-1}
*p<0.05 as compared to unsupplimented-untreated sampales
** p<0.01 as compared to nicotine treated-unsupplimented and supplemented samples.

The present observations imitate the first order of the Michaelis-Menton kinetics. The kinetic parameters k_m and V_{max} have been labeled as 'k_c' and 'C_{max}' as suggested by Anand and Kanwar [22] and Anand *et al.,* [24]. The 'k_c' and 'C_{max}' values **Table 2** were calculated from the Lineweaver-Burk double reciprocal plot **Fig. 2.** A liner increase, positively correlated with substrate concentration was observed in the Mn^{2+} supplemented- untreated and nicotine treated spermatozoal samples. At the highest dose of the promoter used (200 μM $FeSO_4$: 1000μM ascorbic acid), the rate of malon-dialdehyde production increased to 0.25 ±0.175 n moles MDA. mg $prot^{-1}$. min^{-1}. Nicotine was noted to ameliorate the process.

TABLE 2: KINETIC PARAMETERS OF THE 0.1mM Mn^{2+} SUPPLEMENTED AND 0.5 mM NICOTINE TREATED OR UNTREATED SPERMATOZOAL LIPID PEROXIDATION USING DIFFERENT FERROUS–ASCORBATE CONCENTRATION.

	Without Nicotine		With 0.5mM Nicotine	
	Without Mn^{2+}	With Mn^{2+}	Without Mn^{2+}	With Mn^{2+}
k_c (M) $FeSO_4$	62.50	62.50	62.50	62.50
k_c (M) Ascorbic Acid	312.5	312.5	312.5	312.5
C_{max} (n mole MDA. mg $prot^{-1}$.min^{-1})	0.323	0.283	0.820	0.476

This enhanced response to nicotine in the presence of promoter could be significantly suppressed by 0.1mM Mn^{2+} addition. The maximum amount of MDA production (C_{max}) in the Mn^{2+} supplemented-nicotine treated samples was found to be less than that of the nicotine treated samples unsupplemented

samples. Mn^{2+} supplementation to the untreated normal samples showed least C_{max}. Interestingly, the 'k_c' of the reaction remained unchanged. From the present study, it may be postulated that a higher 'k_c' and lower C_{max} value is favorable to the cell such that it indicates a lowered oxidative stress or in other words decreased lipid peroxidation. The present result also indicate that the lowering of MDA content by Mn^{2+} is not brought about by its binding to the same site as that of ferrous ascorbate, neither does it modify the ferrous ascorbate binding sites. Whatever mechanism Mn^{2+} might be following; the result is in nutshell benefiting the sperm cells, protecting them against oxidative damage.

TABLE 3: EFFECT OF EDTA ON HUMAN SPERMATOZOAL LIPID PEROXIDATION

Supplements	n mole MDA. mg $prot^{-1}.min^{-1}$	
	Without EDTA	With EDTA
Control	0.250 ±0.018	0.251 ± 0.011
Mn^{2+}	0.213 ±0.013*	0.249 ± 0.015
Trolox	0.202* ±0.012	0.203 ±0.011*
Co^{2+}	0.225 ±0.015	0.247 ± 0.017
Zn^{2+}	0.235 ±0.013	0.248 ±0.021
Ni^{2+}	0.253 ± 0.025	0.248 ±0.019

Each datum represent mean ± SD of six independent observation ach made in triplicate.
Mean value are n moles MDA. mg $prot^{-1}$. min^{-1}
*$p<0.05$ as compared to without EDTA-control sampales

Since the k_c of the reaction did not register any change where as the C_{max} was found to be lowered upon Mn^{2+} supplementation, reaction appear to mimic the non competitive type of enzymatic inhibition. The inhibitor constant, 'k_i' was calculated using following equation:

$$v_i = \frac{[S].V_{max}}{[[S] + K_m]\left[1 + \frac{[I]}{k_i}\right]}$$

The values of k_i calculated to be 0.6465 μM in untreated but Mn^{2+} supplemented spermatozoal samples and 0.1461μM in nicotine treated Mn^{2+} supplemented samples. This indicates the effectiveness of this trace metals ion as potent antioxidant that work well under extreme oxidative stress conditions.

A comparison has been made to compare the status of manganese with other reported antioxidants like α- tocopherol [26], cobalt [27], zinc [28, 29] and nickel [30]. In the present studies different concentration of Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and trolox ranging from 10 to 100mM were added to assay mixture and the extent of MDA produced in the presence of ferrous ascorbate promoter system was measured as n moles MDA mg $prot^{-1}min^{-1}$. Trolox registered minimum amount of TBA- MDA complex, however, if we compare the rate of LPO in different trace element supplementation series, the value of TBA-MDA complex was found minimum in Mn^{2+} and maximum in Ni^{2+} supplementations. The data was subjected to regression analysis and transformed in Dixon plot 1/v vs. [I] **Fig. 3**.

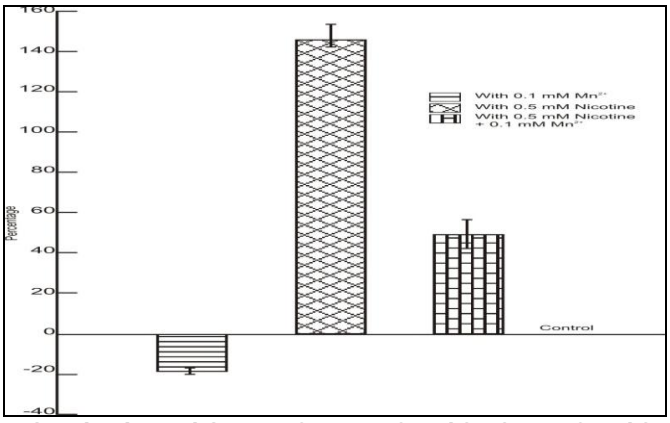

FIG.1: AN AVERAGE PERCENT INCREASE OR DECREASE IN TBA-MDA COMPLEX FORMATION IN 0.5mM NICOTINE TREATED AND UNTREATED HUMAN EJACULATED SPERMATOZOA SUPPLEMENTED OR NOT WITH 0.1mM Mn^{2+}.

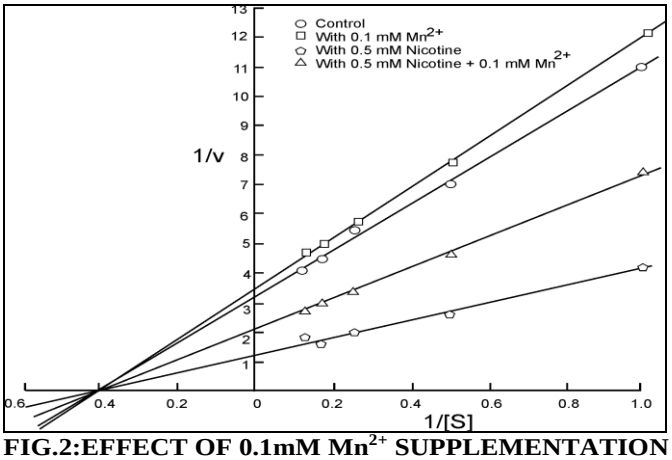

FIG.2:EFFECT OF 0.1mM Mn^{2+} SUPPLEMENTATION ON 0.5 mM NICOTINE TREATED AND UNTREATED SPERMATOZOL LIPID PEROXIDATION USING DIFFERENTFERROUS-ASCORBATE CONC.

v = n moles MDA.mg prot-1.min-1
[S] = substrate concentration (ferrous-ascrobate)

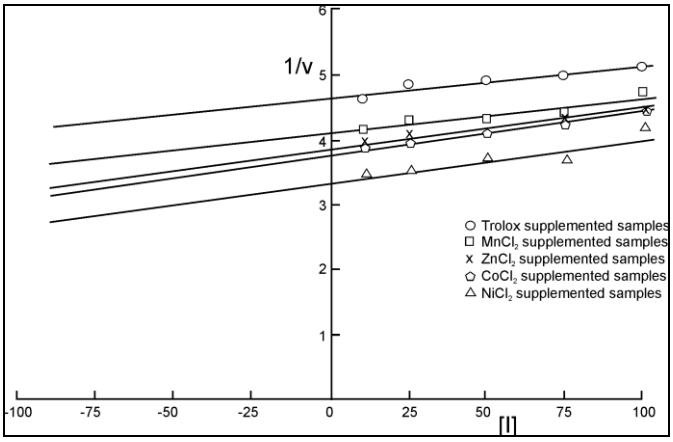

FIG. 3: THE EXTENT OF HUMAN SPERMATOZOAL LIPID PEROXIDATION USING DIFFERENT CONC. OF TROLOX, $MnCl_2$, $ZnCl_2$, $CoCl_2$ and $NiCl_2$

For the comparison the antioxidant status of Mn^{2+} with other trace element namely Co^{2+}, Ni^{2+}, and Zn^{2+}, the Inhibition constant (k_i) values were calculated. The k_i values of Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and trolox were 0.645, 1.088, 2.136, 6.218 and 0.465 respectively. Although Co^{2+} and Zn^{2+} showed some antioxidant potential, their ability to quench the ROS was not much as Mn^{2+}. The k_i value of Zn^{2+} was found to be more than that of Co^{2+} which was 1.7 times that of Mn^{2+}.

It may be noted that k_i is not equivalent to the concentration of the inhibitor which yield 50% inhibition (in the MDA formation). It is the concentration of inhibitor (metal ion) that doubles the slop of 1/v vs 1/[S] plot. In regular enzymatic convention, lower the value of k_i, greater is the degree of inhibition at any given [S] and [I]. Present observations reveal that Mn^{2+} is the most potent inhibitor of LPO in the human ejaculated spermatozoal suspension. In nutshell, the order of decreasing k_i was found to be $Ni^{2+}>Zn^{2+}>Co^{2+}>Mn^{2+}$ or increasing inhibiting potential was $Mn^{2+}>Co^{2+}>Zn^{2+}>Ni^{2+}$.Trolox, however, registered least k_i value and hence, showed the greatest inhibiting potential.

Trolox, a water soluble analogue of α- tocopherol as a potent antioxidant is also registered least ki value in the present investigation. It forms a relatively stable radical tocopheroxyl by breaking the free radical chain reaction [25, 31, 32.] Aitken and Clarkson [31] reported that damage caused by iron-catalyzed peroxidation is known to be prevented by including α- tocopherol. The protective effect of trolox was also reported in boar seminal plasma against the lipid peroxidation (Brezinska-Slebodzinska *et al.*,[26]. that the levels of α-tocopherol and ascorbic acid levels were also reported significantly decreased in semen plasma of cigarette smokers whether infertile or fertile compared to the corresponding data of the nonsmokers [3].

Vitamin E (α-tocopherol) is a powerful lipophilic antioxidant that is vital for maintenance of spermatogenesis. It suppresses lipid peroxidation in the testicular microsomes and mitochondria and reverses the detrimental effects of oxidative stress on testicular function [33, 34]. The ferrous ascorbate induced TBARS production was found to be inhibited by about 62% through the water-soluble vitamin E analogue (trolox), as compared to 57% inhibition by glutathione (GSH).

Investigation of the effect of zinc as an antioxidant is semen is of interest because human spermatozoa are endowed with zinc- rich prostatic fluid during the sequence of ejaculation [35], which implies that zinc may affect spermatozoa during the immediate post ejaculatory periods [36]. As zinc is loosely bound to human spermatozoa, washing during the isolation procedure causes an over 90% loss of intracellular Zn^{2+} [37]. Thus, spermatozoa may take up zinc from the artificial medium used in vitro experiments [38]. It has been reported that zinc inhibits LPO in cultured hepatocytes [37].

It has been suggested that zinc might be inhibiting the multi step reaction from hydroperoxide to malon-dialdehyde catalyzed by Fe^{2+}, competing with the later for the oxygen legends in the oxidized polyunsaturated fatty acids [28]. From the present investigation it is clear that, Zn^{2+} also stabilizes the spermatozoal membranes, though not as much as Mn^{2+}.

Cobalt (Co^{2+}) too, seems to follow Zn^{2+} like mechanism to inhibit the Fe^{+} induced lipid peroxidation. Such a mechanism for Co^{2+} has also been proposed, in liposomes by Tampo and Yonaha [27]. Cobaltous ions were found to inhibit the lipid peroxidation and also super oxide dismutase activity of the human ejaculated spermatozoa [13]. Nivsarkar and Patel [13] suggested that the lowered lipid peroxidation by very low concentration (10^{-9}) of cobalt results in masking of the sperm membrane sulphydryl groups. This result in reduction in membrane fluidity that is a prerequisite for normal sperm function.

Though Co^{2+} has shown antioxidant potential in the present study, it is however, not safe to recommend it as an additive to sperm media. Moreover, Cobaltous ion has been shown to exercise powerful sperm immobilizing properties at extremely low concentrations.

The loss of sperm surface thiol groups and the augmented production of superoxide anion radical have been stated to be the reason for the loss of motility [39]. During the present investigation, nickel (Ni^{2+}) did not register any antioxidant action. Coassin *et al.*, [17] have reported that Ni^{2+} is not reactive in the monoelectronic reduction of peroxyl radicals.

Out of the trace metal ions tested, Mn^{2+} has seems to be the best trace element antioxidant additive. The most plausible mechanism mediating the inhibitory effect of Mn^{2+} on the lipid peroxidation appears to be its interaction with superoxide and hydroxyl radicals to produce MnO_2^{2+} and Mn (OH) $^{2+}$. Kono *et al.*, [40] have also proposed a similar mechanism. Mn^{2+} has also been labeled as an effective chain breaking antioxidant like Co^{2+} [41]. Coassin *et al.*, [17] suggested that this chain breaking antioxidant capacity of Mn^{2+} is related to the effective quenching of peroxyl radicals, according to reaction:

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

These authors have further calculated the reduction potential of the redox couple [Mn^{3+}/Mn^{2+}] to be 1.51 whereas that of iron has been calculated to be +0.177. The difference in the reduction potential was therefore, suggested to be the key factor in the divergence between the anti-oxidant effect of Mn^{2+} and the pro-oxidant effect of Fe^{2+}. Although both metal ions react with hydroperoxyl radicals, only the more reducing Fe^{2+} is able to promote hydroperoxide hydrolytic O-O bond cleavage giving rise to alkoxyl radicals. Hydroperoxide infects, appear resistant to Mn^{2+}, which does not give rise to Fenton chemistry [42, 43].

To ensure that the inhibition of TBARS production noted in the metal ion treated sperm cells was actually due to the presence of these ions, a known divalent cation chelator, ethylenediamine tetra acetic acid (EDTA) was supplemented along with in the assay mixture. It was observed that the inhibiting action of Zn^{2+}, Co^{2+} and Mn^{2+} was abolished in the presence of EDTA (**Table 3**). In Ni^{2+} treated samples, where Ni^{2+} was found to support the peroxidation, EDTA rather lipid peroxidation. Such an action of EDTA has also been observed with Fe^{2+} treatment.

EDTA, desferrioxamine and bathophenanthroline were found to strongly inhibit lipid peroxidation thereby, indicating the involvement of endogenous iron in inducing LPO by binding to cell components in the rat brain homogenate [44]. Thus, it may be inferred that Ni^{2+} is involved in inducing spermatozoal lipid peroxidation while Co^{2+}, Zn^{2+} and Mn^{2+} inhibit it. In the trolox treated samples, no change was observed when EDTA was added to the assay mixture. It therefore, implies that trolox does not get chelated with EDTA and thus, acts independently.

Lapointe *et al.*, [45] reported that Mn^{2+} and Mg^{2+} are potent stimulators of bovine spermatozoa motility probably by stimulating the adenylate cyclase activity. Anand *et al.*, [24] also observed the 0.1mM Mn^{2+} supplementation to human ejaculated spermatozoal suspension stimulated the sperm

motility. They also reported that 0.1mM Mn^{2+} supplementation resulted in a increase in the Ca^{2+} and Mg^{2+} ATPases in the human ejaculated spermatozoal samples.

In order to complete the study before recommending Mn^{2+} as an in vitro antioxidant additive to the spermatozoal samples, the functional status of the spermatozoa under Mn^{2+} influence has been studied on the basis of hypo-osmotic swelling test (HOS). The percentage of swollen spermatozoa was calculated to be 51.9 +3.7% in the nicotine treated sample as compared to 61.8 +3.51% in the untreated control samples (manuscript under preparation). 0.1mM Mn^{2+} supplemented samples showed higher percentage value. Membrane integrity is not only important for sperm metabolism, but a correct change in the properties of the membrane is required for successful union of the male and female gametes, that includes sperm capacitation, the acrosome reaction, and the binding of the spermatozoon to the egg surface [46].

Since the integrity and functional activity of the sperm membrane is of fundamental importance in the fertilization process, assessment of membrane function may be a useful indicator of the fertilizing ability of spermatozoa.

The motility of spermatozoa also depends in part on the transport of compounds across the membrane in addition to large number of other biochemical functions such as sperm metabolism and the micro tubular action of the tail fibers [46]. Vanderven *et al.,* [47] have also reported much higher correlation of the *in vitro* fertilizing capacity of the spermatozoa with the hypoosmotic swelling test *(r = 0.56)* with standard sperm parameters *(r* varied from -0.04 to 0.25). Anand *et al.,* [24] have also reported that Mn^{2+} stimulates human spermatozoal motility in nicotine treated and untreated samples. Mn^{2+} protects the bull sperm against oxidative stress and facilitates the occurrence of capacitation and acrosome reaction [48].

Extracellular addition of Mn^{2+} ions also enhances the level of cAMP by stimulating Ca^{2+} or Mg^{2+} ATPase which leads to activation of calcium channel opening, thereby depositing more Ca^{2+} and promotes the acrosome reaction [49]. Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique for the goat breeding industry [50]. Present observations show a similar trend in Mn^{2+} action. Thus, it may be inferred that Mn^{2+} supplementation improves the overall functional ability of the spermatozoa and therefore, may be used as an antioxidant additive to spermatozoal samples.

CONCLUSION: On the basis of C_{max}, k_i values and other parameter we can conclude that Mn^{2+} is the most potent trace metal ion inhibitor of LPO in the human ejaculated spermatozoal suspension. Moreover, in continuation to the present work, 0.1mM Mn^{2+} is being tested on human semen samples to be used for assisted reproduction techniques like IVF, IUI, ICSI, etc.

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