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Effect of vitamin E and Mn^{2+} on the thiol status of cattle bull spermatozoa under induced oxidative stress

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Ferrous ascorbate – FeAA ($FeSO_4$ + ascorbic acid) – has been used in the past to induce oxidative stress. In this study, the effects of various doses of vitamin E (1 mM, 2 mM, 2.5 mM) and Mn^{2+} (60 µM, 100 µM, 200 µM) on total thiol (TSH), glutathione reduced (GSH), glutathione oxidized (GSSG), and redox ratio (GSH/GSSG) were determined in the local crossbred cattle bull spermatozoa. Fresh semen was suspended in 2.9% sodium citrate and the suspension was divided into eight equal fractions. All fractions, *i.e.* control (containing 2.9% sodium citrate + spermatozoa) and experimental, *i.e.* treated / untreated with ferrous ascorbate (150 µM $FeSO_4$: 750 µM ascorbic acid), supplemented / unsupplemented with three doses of vitamin E (1 mM, 2 mM, 2.5 mM) / Mn^{2+} (60 µM, 100 µM, 200 µM) were incubated for 2 h at 37°C. These fractions were used for thiol components assessment. By inducing oxidative stress, FeAA decreased the TSH and GSH levels and GSH / GSSG ratio, but increased the GSSG level. All doses of vitamin E and Mn^{2+} improved the TSH and GSH level and GSH / GSSG ratio, but decreased GSSG level under normal and oxidative stress conditions. It is concluded that oxidative stress treatment (FeAA) decreased ($P \leq 0.05$) TSH and GSH contents but maintained GSSG concentration and GSH/GSSG ratio unchanged ($P \geq 0.05$). However, supplementation with vitamin E increased ($P \leq 0.05$) both the GSH content and GSH/GSSG ratio, but Mn^{2+} increased ($P \leq 0.05$) the GSH content only.

KEY WORDS: cattle / Mn^{2+} / spermatozoa / thiol / vitamin E

Membrane sulfhydryl (-SH) groups are important entities of the sperm membranes involved in normal sperm functioning like motility and metabolism [Nivsarkar *et al*.

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1998]. Loss of these groups causes inhibition of superoxide dismutase (SOD) that dismutates superoxide radical activities both in human and rat caudal sperm, resulting in enhanced production of superoxide ($\cdot O_2^-$) radical and other reactive oxygen species (ROS) ultimately decreasing the spermatozoa motility [Kumar *et al.* 1990]. These ROS enhance membrane fluidity (which is not required for normal sperm functioning) *via* lipid peroxidation (LPO) – Nivsarkar *et al.* [1998], whereas the membrane -SH groups play an important role in sperm membrane modulation by reducing membrane fluidity. Decrease in motility and loss of sperm functions in unexplained male infertility can be attributed to the masking of active -SH groups thereby resulting in the loss of sperm functions. Membrane sulfhydryl groups can, therefore, be used as tools for infertility assessment in unexplained male infertility, and be targeted for contraceptive research [Nivsarkar *et al.* 1998].

Glutathione (GSH) is the major non-protein thiol in mammalian cells and is known to have numerous biological functions. It plays a prominent role in detoxication and antioxidation of exo- and endogenous compounds as well as in maintaining the intracellular redox status [Luberda 2005]. Cooling and freezing/thawing exert marked effects on the sulfhydryl groups by inducing oxidative stress in bull spermatozoa [Chatterjee *et al*. 2001]. Freezing and thawing is associated with ROS generation and reorganization of sulfhdryl groups on the sperm membranes. The scavenging action of GSH helps to counteract the effects of oxidative stress on sperm cells which could result in lipid peroxidation (LPO) of plasmalemma, irreversible loss of motility, leakage of intracellular enzymes and damage of chromatin [Luberda 2005].

GSH content of bull spermatozoa has been reported to be 2.93 nM/10^8 spermatozoa [Agarwal and Vanha-Perttula 1988] or 566+ 72 pmoles/mg protein [Bilodeau *et al*. 2000], or 246.5 to 776.2 pmoles/mg protein [Stradaioli *et al*. 2007]. Luberda [2005] reported the GSH content of spermatozoa in five breeding bulls to range from 295 to 691 pmoles/mg protein/ml thus showing wide interindividual variation. Glutathione exists in two forms: the reduced (GSH) and the oxidized (GSSG) – Luberda [2005]. The protective action of glutathione against ROS is facilitated by the interaction with its associated enzymes such as glutathione peroxidase and glutathione reductase [Luberda 2005].

Lipids are the most susceptible macromolecules to ROS attack and are present in the sperm plasma membrane in the form of polyunsaturated fatty acids (PUFAs) – Agarwal and Prabakaran [2005]. ROS attack the PUFAs in the cell membrane leading to a chain of chemical reactions called lipid peroxidation (LPO). Supplementation of sperm suspension with transition metal ions such as iron (Fe^{2+}) results in a sudden acceleration of LPO and loss of sperm functions [Sharma and Agarwal 1996, Bansal and Bilaspuri 2008a]. Propagation of LPO in the sperm is impeded as a result of antioxidant mechanisms activity [Sharma and Agarwal 1996, Bansal and Bilaspuri 2008c].

Numerous antioxidants have proven beneficial in treating male infertility [Sinclair 2000]. Antioxidants are the main defense factors against oxidative stress

induced by free radicals [Agarwal *et al*. 2005]. The antioxidative action of Mn^{2+} on different peroxidizing systems has been studied by Cavallini *et al.* [1984]. In cattle bull spermatozoa , Mn^{2+} is a useful antioxidant in reducing the oxidative stress (LPO) caused by ferrous ascorbate (FeAA). In addition, it improves motility and viability of spermatozoa *in vitro* as well as oxidative stress conditions [Bansal and Bilaspuri 2008c]. It has also has been assigned as a chain-breaking antioxidant as it is able to quench peroxyl radicals [Coassin *et al*. 1992].

Vitamin E is believed to be the primary component of the spermatozoa antioxidant systems [Surai *et al.* 1998]. It is a major chain-breaking antioxidant present both in seminal plasma and sperm membranes. Within the biological membranes, it is believed to provide protection to PUFAs [Agarwal *et al*. 2005]. Vitamin E inhibits peroxidative damage to buffalo spermatozoa [Singh *et al*. 1989] and other peroxidizing systems [Van-Haaften *et al*. 2003]. It also scavenges all the important types of ROS such as superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) – Agarwal and Prabakaran [2005]. Vitamin E supplement has been shown to increase total boar spermatozoa output and concentration [Brzezinska-Slebodzinska *et al.* 1995]. In rabbits, vitamin E has improved the sperm characteristics and reduced the production of ROS [Yousef *et al.* 2003].

The present *in vitro* study aimed ad assessing the antioxidative effects of various doses of vitamin E (α-tocopherol acetate) – 1 mM, 2 mM, 2.5 mM – and Mn^{2+} ($MnCl_2$) – 60 μM, 100 μM, 200 μM – on total thiol (TSH), glutathione reduced (GSH), glutathione oxidized (GSSG), and redox ratio (GSH / GSSG) of crossbred cattle bull spermatozoa under control *vs*. oxidative stress conditions.

Material and methods

Reagents

All chemicals were purchased from SISCO RESEARCH LABORATORIES (SRL) Private, Ltd., Mumbai, India.

Sperm

Ejaculates with more than 80% motility and 1.2 to 1.4 × 10^9 sperm /mL were collected (with an artificial vagina) from five healthy local crossbred cattle bulls of age 3-4 years, maintained at the Dairy Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Three subsamples of a single ejaculate from each of the five bulls were used for the analysis of each indicator. In total, five bulls were studied. Fresh semen was centrifuged (800 × g) at 37°C for 5 min, seminal plasma was removed, the sperm pellet was washed two or three times with 2.9% sodium citrate (pH 7.4), re-suspended in 2.9% sodium citrate and divided into two sets; each set containing eight equal fractions in eight test tubes(concentration , 120 × 10^6 sperm/mL). In the first set, one tube (control) was added with 2.9% sodium citrate and the remaining seven tubes (experimental fractions) were subjected to Mn^{2+}

($Mncl_2$)treatment (0,60, 100, or 200 µM) in the presence or absence of oxidative stress inducer, i.e, ferrous ascorbate (FeAA; comprised of 150 µM $FeSO_4$ and 750 µM ascorbic acid [Bansal and Bilaspuri 2008a]. In the second set, remaining seven tubes (experimental fractions) were subjected to vitamin E (α tocopherol-acetate) treatment (0,1,2,2.5 mM) in the presence and absence of oxidative stress inducer i.e, FeAA as mentioned above. All fractions of both the sets were incubated (37°C) for 2 hrs. After freezing(-20°C) for overnight and thawing(37°C) ,these fractions were evaluated for the following parameters.

Determination of thiols

Total thiols, glutathione reduced (GSH) and glutathione oxidized were determined with the standard methods of Sedlak and Lindsay [1968] with slight modifications.

Estimation of total thiols (TSH)

Assay mixture containing 0.2 M Tris-buffer (pH 8.2) and a known volume of sperm (120×10^6 sperm/mL) fraction (control/experimental) was incubated for 30 min at 37°C and 0.1 ml of 0.1 M DTNB was added. The volume was made up to 10 ml with absolute methanol and mixed thoroughly. The capped tubes were kept at room temperature for 30 min with occasional shaking and then centrifuged at 2500 × g for 15 min. The absorbance of the clear aliquot (after filtration through Whatman No.1) was measured spectrophotometrically at 412 nm. The molar extinction coefficient (ε) at 412 nm was taken as 131,00 $M^{-1}.cm^{-1}$.

Estimation of glutathione reduced(GSH)

Assay mixture containing 0.2M Tris-buffer (pH 8.2) and a known volume of sperm (120×10^6 sperm/mL) fraction(control/experimental) was incubated for 30 min at 37°C. The volume was made up to 2 ml with 20% chilled TCA. The tubes were vortexed and allowed to stand for 15 min with occasional shaking. The samples were centrifuged at 2500 × g for 15 min. Next, 1 ml aliquot was mixed with 2 ml of 0.4M Tris-buffer (pH 8.9) and 0.1 ml of 0.1M DTNB. The contents were mixed well and the absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank. The molar extinction coefficient (ε) at 412 nm was taken as 13,100 $M^{-1}.cm^{-1}$.

Estimation of glutathione oxidized (GSSG)

Glutathione oxidized (GSSG) was determined by subtracting the glutathione reduced (GSH) from total thiols (TSH) level.

Estimation of Redox ratio (GSH/GSSG)

Redox ratio of the FeAA treated/untreated and/or vitamin E/Mn^{2+} supplemented/ unsupplemented was calculated by dividing the reduced glutathione (GSH) content of each fraction by the oxidized glutathione (GSSG) content of its respective fraction.

Statistical

Analysis of Factorial Experiment in Completely Randomized Block Design' (CRD) – a software programme made by Department of Mathematics, Statistics and Physics, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, India – was used to evaluate the significance of differences between the parameters studied. Semen samples from five bulls were used. The three subsamples of a single ejaculate from each of the five bulls were used for the analysis of each parameter. The critical difference (CD) of three factors – A [FeAA (oxidative stress) treatment], B [vitamin E/ Mn^{2+}(antioxidant) treatment] and AB (interaction between FeAA and vitamin E/Mn^{2+} 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 and discussion

Total thiols (TSH)

FeAA treatment decreased the TSH content significantly ($P \leq 0.05$) – Tables 1 and 2. No significant differences were found in TSH content during vitamin E/ Mn^{2+} supplementation (Tab. 1 and 2).

Glutathione reduced (GSH)

FeAA treatment caused a reduction ($P \leq 0.05$) in GSH content (Tab. 1 and 2). All doses of vitamin E /Mn^{2+} supplementation increased the GSH content significantly ($P \leq 0.05$) compared to vitamin E / Mn^{2+} unsupplemented samples (Tab. 1 and 2).

Glutathione oxidized (GSSG)

A non-significant ($P \geq 0.05$) reduction occurred in GSSG content related to FeAA treatment as compared to the control (Tab. 1 and 2). Subsequently, all doses of vitamin E / Mn^{2+} decreased the GSSG content non-significantly ($P \geq 0.05$) as compared to the vitamin E / Mn^{2+} unsupplemented samples (Tab. 1 and 2).

Redox ratio (GSH/GSSG)

FeAA treatment decreased the redox ratio non-significantly ($P \geq 0.05$) – Tables 1 and 2. Redox ratio increased ($P \leq 0.05$) with increasing doses of vitamin E, but non-significantly ($P \geq 0.05$) with all doses of Mn^{2+} (Tab. 1 and 2).

No significant ($P \leq 0.05$) interaction was identified between FeAA treatment and vitamin E / Mn^{2+} supplementation. Supplementation of various doses of vitamin E / Mn^{2+} increases or decreases the thiol (TSH, GSH, GSSG, GSH/GSSG) contents, which are not affected by FeAA treatment (Tab. 1 and 2).

Thiol groups (-SH) are indispensable for reducing the oxidative stress in biological materials [Bansal and Bilaspuri 2008b]. Under oxidative stress induced by

Table 1. Effects of various concentrations of vitamin E (mM) on total thiols (TSH), non protein bound sulfhydryl groups (NP-SH) or glutathione reduced (GSH) and protein bound sulfhydryl groups (PB-SH) or glutathione oxidized (GSSG) and redox ratio (GSH/GSSG) of ferrous ascorbate ($FeSO_4$: ascorbic acid) treated/untreated bull spermatozoa

Concentrations of vitamin E (B)	n moles SH/10^8 spermatozoa											
	Thiol components											
	TSH			GSH			GSSG			GSH/GSSG		
	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean
0	5.322 ±0.611	4.474 ±0.448	4.898[a]	1.553 ±0.162	0.564 ±0.208	1.058[a]	3.769 ±0.558	3.910 ±0.242	3.840[a]	0.441 ±0.078	0.133 ±0.045	0.287[a]
1 (I)	7.137 ±1.073	4.798 ±0.426	5.967[a]	4.271 ±0.464	2.974 ±0.275	3.623[b]	2.865 ±0.792	1.823 ±0.504	2.344[a]	2.827 ±1.433	1.324 ±0.227	2.075[b]
2 (II)	7.829 ±0.937	6.250 ±0.426	7.039[a]	5.227 ±0.343	3.72 ±0.483	4.473[b]	2.601 ±0.713	2.53 ±0.543	2.565[a]	2.724 ±0.758	1.835 ±0.462	2.279[b]
2.5 (III)	7.494 ±0.658	5.959 ±0.347	6.726[a]	5.306 ±0.518	4.131 ±0.452	4.718[b]	2.187 ±0.566	1.828 ±0.656	2.007[a]	2.961 ±0.569	3.610 ±0.843	3.285[b]
Combination factor mean	6.945[a]	5.370[b]		4.089[a]	2.847[b]		2.855[a]	2.523[a]		2.238[a]	1.725[a]	

Each value represents mean±SE.
[a b]Means in a row or column with different superscripts differ significantly at P≤0.05.
Factor A: FeAA (oxidative stress) treatment.
Factor B: Vitamin E(antioxidant) treatment.

Table 2. Effects of various concentrations of Mn^{2+} (μM) on total thiols (TSH), non protein bound sulfhydryl groups (NP-SH) or glutathione reduced (GSH), protein bound sulfhydryl groups (PB-SH) or glutathione oxidized (GSSG) and redox ratio (GSH/GSSG) of ferrous ascorbate ($FeSO_4$: ascorbic acid) treated/untreated bull spermatozoa.

Concentrations of Mn^{2+} (B)	n moles SH/10^8 spermatozoa											
	Thiol compounds											
	TSH			GSH			GSSG			GSH/GSSG		
	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean	control	FeAA treated (A)	combination factor mean
0	4.363 ±0.715	3.229 ±0.116	3.796[a]	2.599 ±0.114	1.058 ±0.221	1.784[a]	1.854 ±0.693	2.065 ±0.295	1.959[a]	1.961 ±0.445	0.591 ±0.191	1.276[a]
60 (I)	4.665 ±0.377	3.422 ±0.972	4.044[a]	2.953 ±0.248	2.455 ±0.221	2.704[b]	1.721 ±0.419	0.967 ±0.235	1.339[a]	2.458 ±0.915	3.567 ±1.100	3.012[a]
100 (II)	5.274 ±0.705	3.226 ±0.122	4.250[a]	3.794 ±0.239	2.808 ±0.134	3.301[b]	1.721 ±0.598	0.606 ±2.003	1.164[a]	3.189 ±0.871	3.951 ±0.750	3.570[a]
200 (III)	4.665 ±0.653	3.503 ±0.273	4.084[a]	3.809 ±0.441	2.587 ±0.246	3.198[b]	1.439 ±0.435	0.915 ±0.339	1.177[a]	3.991 ±1.247	2.396 ±0.507	3.194[a]
Combination factor mean	4.742[a]	3.345[b]		3.266[a]	2.227[b]		1.681[a]	1.386[a]		2.900[a]	2.626[a]	

Each value represents mean±SE.
[ab]Means in a row or column with different superscripts differ significantly at P≤0.05.
Factor A: FeAA (oxidative stress) treatment.
Factor B: Mn^{2+} (antioxidant) treatment.

FeAA in cattle sperm, TSH, GSH, GSSG and GSH/GSSG decreased. Subsequently, introduction of various doses of vitamin E improved the TSH, GSH and GSH/GSSG ratio, simultaneously decreasing the GSSG level. It indicates that vitamin E has been able to maintain the sulfhydryl group content of FeAA treated / untreated samples.

The present findings may suggest that both the thiol components – GSH and GSSG – are related to each other through the glutathione cycle which is an effective intracellular defense mechanism against a variety of oxidative stresses [Li 1975].

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + H_2O$$

$$GSSG + NADP + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Results summarized in Table 1 may be explained on the basis of the fact that under oxidative stress induced by FeAA, lipid peroxidation (LPO) is enhanced resulting in oxidative deterioration of sperm membrane and damage of DNA [Sharma and Agarwal 1996]. Freezing and thawing reduced the superoxide dismutase (SOD) activity which could lead to LPO and ultimately to oxidative stress [Chatterjee *et al*. 2001]. To overcome these oxidative damages, GSH gets converted to GSSG, reaction being catalyzed by glutathione peroxidase (an antioxidant enzyme of glutathione cycle) – Bansal and Bilaspuri [2008b]. Consequently, the level of GSH decreased in FeAA-treated samples. A greater reduction in intracellular GSH level when bovine spermatozoa were incubated under aerobic conditions was also reported by Bilodeau *et al*. [2000]. During cellular oxidative stress, the preferential oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) protects cellular macromolecules and cell functions [Kaur *et al.* 2006]. GSSG thus formed may be leaked out from the sperm cell thereby decreasing its own content. Meister and Anderson [1983] suggested that the active transport of GSSG out of the cell may be an emergency mechanism to protect cell from toxic effects of GSSG. It has been suggested that GSH and GSSG could protect the sperm membrane from excessive LPO during cooling and freezing/ thawing procedures [Bildeau *et al.* 2000].

As the content of GSH decreases in FeAA-treated samples, the level of TSH also starts decreasing under oxidative stress conditions. These changes in GSH and GSSG contents ultimately cause reduction in GSH/GSSG ratio in FeAA-treated sperm samples as compared to the control. Normally, the GSH/GSSG ratio is precisely regulated for maintaining the cellular redox balance. Depletion of GSH or the generation of GSSG decreases the GSH/GSSG ratio reflecting an increase of oxidative stress [Kaur *et al*. 2006]. GSH/GSSG is an indirect indication of oxidative stress [Bansal 2000]. A high GSH/GSSG ratio will help the sperm to combat ROS or oxidative attack [Sikka 1996].

The study on cattle by Bansal [2006] shows that with the supplementation of various doses of vitamin E, LPO level decreases significantly in sperm under normal

and oxidative stress conditions. With this decrease in peroxidation process, the need of conversion of GSH to GSSG also decreased. That is why with the supplementation of vitamin E, GSH and TSH contents increase, but GSSG level decreases. Thus, this study suggests that vitamin E supplementation maintains the level of sulfhydryl groups so as to ensure the required membrane fluidity that is a pre-requisite for normal sperm functioning [Sikka 1996]. Supplementation with a 100 IU vitamin E /day significantly increased the GSH content and lowered LPO level of erythrocyte of type 1 diabetic patients [Jain *et al*. 2000].

This study correlates the level of lipid peroxidation with GSH content, as with the supplementation of vitamin E the susceptibility of lipid peroxidation decreases, resulting in increase in GSH and TSH contents. A number of investigations support the view that GSH protects cell membranes and proteins by maintaining essential sulfhydryl (-SH) groups by interacting with peroxides and free radicals (produced during LPO) – Meister and Tate [1976], Irvine [1996]. The scavenging function of GSH helps to counteract the effects of oxidative stress on sperm cells, its depletion resulting in irreversible loss of motility and damage of the chromatin; and hence affects the fertility status [Luberda 2001]. In the present investigation all doses of Mn^{2+} through their antioxidative property increased the GSH and TSH, but lowered the GSSG level. It is suggested that Mn^{2+} may stimulate the enzymes of glutathione cycle and affect the TSH, GSH and GSSG contents. Tampo and Yonaha [1992] reported that Mn^{2+} decreases the peroxidative damages caused by LPO, through conjugating with glutathione peroxidase enzyme in rat brain homogenate. Oral administration of high dose of Mn^{2+} (0,25 or 50 mg Mn^{2+} / kg body weight / day) significantly increased total cerebro- cortical GSH content in neonatal rats [Weber *et al.* 2002]. With the supplementation of Mn^{2+}, LPO level falls significantly ($P \leq 0.05$) in bull sperm under normal and oxidative stress conditions [Bansal and Bilaspuri 2008c]. Thus, the present study suggests that Mn^{2+} additive may decrease peroxidative process in bull spermatozoa. The need of conversion of GSH to GSSG gets, therefore, lowered. Levels of GSH begin to increase and GSSG level to decrease, thereby, maintaining the redox ratio (GSH / GSSG). Stradaioli *et al*. [2007] reported that in bull spermatozoa with the supplementation of commercial extender, GSH level increases. This may be due to reduction in oxidative stress which is evolved during freezing and thawing procedures.

It is concluded that oxidative stress treatment (FeAA) decreased ($P \leq 0.05$) TSH and GSH contents of bull spermatozoa, but maintained GSSG concentration and GSH/GSSG ratio at the unchanged level ($P \geq 0.05$). However, supplementation with vitamin E increased ($P \leq 0.05$) both the GSH content and GSH/GSSG ratio, whilst supplementation with Mn^{2+} increased ($P \leq 0.05$) the GSH content only.

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