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Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress

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Vitamin E is one of the major membrane protectants against reactive oxygen species (ROS) and lipid peroxidation (LPO). The study aimed at determining the optimum dose of vitamin E to reverse free radical-mediated oxidative damage on motility, viability and LPO of bulls'sperm. Fresh semen of five local crossbred bulls was suspended in 2.9% sodium citrate, divided into equal fractions and subjected to vitamin E treatment (0, 1, 2, 2.5 mM) in the presence or absence of oxidative stress inducer, *i.e* ferrous ascorbate (FeAA, containing 150 µM $FeSO_4$ and 750 µM ascorbic acid). All sperm suspensions were incubated at 37°C for 2 h. Treatment with FeAA reduced sperm motility and viability, but increased the LPO. All doses of vitamin E increased sperm motility and viability, but reduced LPO. However, 2 mM vitamin E was most effective. In conclusion, vitamin E reduced the LPO caused by FeAA, and improved sperm motility and viability *in vitro* under induced oxidative stress.

KEY WORDS: bulls / ferrous ascorbate / lipoperoxidation / oxidative stress / reactive oxygen species / sperm / vitamin E

Numerous factors affect male fertility, one of these being the oxidative stress (OS), which has elicited an enormous interest in the recent years [Agarwal and Prabakaran 2005]. Oxidative stress is known to play a major role in the sperm malfunctions *via* induction of lipid peroxidation (LPO) to biomembranes [Arabi *et al.* 2001].

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The sperm cells under aerobic conditions are always attacked by reactive oxygen species (ROS). This ROS-induced damage leads to LPO due to high polyunsaturated fatty acids (PUFAs) content in sperm plasma membrane which results in low membrane fluidity and loss of sperm × oocyte interaction. LPO is known to have an informative and diagnostic tool to evaluate the membrane integrity [Arabi *et al*. 2001] Supplementation of transition metal ions such as Fe^{2+} to the sperm suspension results in a sudden acceleration of LPO and loss of sperm functions such as motility and viability [Sharma and Agarwal 1996].

Against ROS attack, sperm cells are well equipped with a powerful defense system of antioxidants, but an imbalance between the production of ROS and the available antioxidant-defenses result in oxidative stress [Sikka 1996]. Therefore, antioxidants are supplemented extracellularly under *in vitro* conditions. Antioxidants are the main defense factors against oxidative stress induced by free radicals [Agarwal *et al*. 2005]. Vitamin E is believed to be the primary component of the antioxidant system of the spermatozoa and is one of the major membrane protectants against ROS and LPO attack [Yousef *et al*. 2003]. It appears to be the first line of defense against the peroxidation of PUFAs contained in the cellular and sub-cellular membrane phospholipids because of its lipid solubility [Horton *et al*. 2002]. It is a major chain-breaking antioxidant in membranes directly neutralizing superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) – Sharma and Agarwal [1996]. The present *in vitro* study aimed at finding out the efficacy of vitamin E, a biological antioxidant, in reversing the free radical-mediated oxidative damage on sperm motility, viability and lipid peroxidation.

Material and methods

Reagents

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

Sperm

Ejaculates showing more than 80% motility and 1.2 to 1.4×10^9 spermatozoa/mL were collected with artificial vagina from five local crossbred bulls maintained at the Dairy Farm, Guru Angad Dev, Veterinary and Animal Sciences University, Ludhiana, India. The three subsamples of a single ejaculate from each of the five bulls were used for the analysis of each indicator.

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 eight equal portions in eight test tubes (120×10^6 spermatozoa/mL). To one tube (control) only 2.9% sodium citrate was added and the remaining seven tubes (experimental portions) were subjected to Mn^{2+} treatment (0, 60, 100, or 200 μM) in the presence or absence of

oxidative stress inducer, *i.e.* ferrous ascorbate (FeAA) containing 150 µM FeSO4 and 750 µM ascorbic acid – Bansal and Bilaspuri [2008]. All sperm suspensions were incubated at 37°C for various periods.

Evaluation of sperm motility and viability

Every 30 min, sperm motility was estimated (37°C) by examination of a wet mount under bright-field microscope (400 X). Sperm viability (at hour 0 and 2) was determined by preparing an eosin-nigrosin smear (37°C) and assessing at least 100 sperms under bright-field microscopy (1000 X) – Blom [1950].

Determination of lipid peroxidation (LPO)

At the end of hour 2 of incubation, the level of malondialdehyde (MDA) in control and experimental portions was measured by determining the thiobarbituric acid reactive substances (TBARS) according to Buege and Steven [1978]. For this assay, a known volume of sperm suspension was incubated with 0.1 ml of 150 mM Tris-HCl (pH 7.1) for 20 min at 37°C. Subsequently, 1ml of 10% trichloroacetic acid and 2 ml of 0.375% thiobarbituric acid were added followed by keeping in boiling water bath for 30 min. Thereafter, it was centrifuged for 15 min at 3000 rpm. In the blank tube, sample was replaced by 2.9% sodium citrate (pH 7.4). The absorbance was read at 532 nm. The molar extinction coefficient for MDA is 1.56×10^5 $M^{-1}.cm^{-1}$. The results were expressed as n moles MDA/µg protein.

Determination of total protein

Total protein in the control and experimental portions was determined spectrophotometrically according to Lees and Paxman [1972]. Accordingly, 0.9 ml of 5% SDS in 0.5N NaOH was added to 0.1 ml of the sample (sperm suspension) in the test tube, kept at room temperature for at least 3 h before agitating 2-3 times in a vortex mixer to make sure that sample was dissolved thoroughly. To it, 2.5 ml of copper carbonate solution was added and tube was allowed to stand for further 15-20 min. Next, 0.25 ml of Folin-phenol reagent was added; sample was mixed immediately and allowed to stand for 45 min. The absorbance was read at 740 nm. Twenty to 100 µg/mL of bovine serum albumin (BSA) standard was used as a reference.

Statistical

'Analysis of Factorial Experiment in CRD' (software programme made by Department of Mathematics, Statistics and Physics, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, India.) or 'One Way Variance Analysis' were used to evaluate the significance levels between the parameters studied. The critical differences (CD) of three factors – A (incubation period), B (FeAA and vitamin E treatments) and AxB interactions 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

Percentage sperm motility

A gradual and significant ($P \leq 0.05$) decrease in sperm motility was observed after 0.5, 1 and 2 h of incubation (Tab. 1). Supplementation of vitamin E in FeAA-untreated samples significantly increased ($P \leq 0.05$) the sperm motility with vit. E doses II and III, but non-significantly with dose I. Among the three doses of vitamin E, increase in motility was significant between dose I and II as well as between I and III, but non-significant between dose II and III. Maximum increase in motility occurred when dose II was applied (Tab. 1).

Treatment of spermatozoa with ferrous ascorbate (FeAA – 150 µM $FeSO_4$, 750 µM ascorbic acid) resulted in significant ($P \leq 0.05$) and sudden reduction in motility as compared to the control (Tab. 1). Subsequently, as compared to vitamin E-unsupplemented (control) samples, supplementation of vitamin E improved the motility significantly with dose II, but non-significantly with doses I and III in FeAA-treated samples. Among the three doses of vitamin E, sperm motility increased significantly from dose I to dose II as well as from dose II to dose III, but non-significantly from dose I to III in FeAA-treated samples.

Percentage sperm viability

The morphology of viable and non-viable spermatozoa remained unaffected with various doses of vitamin E. Corresponding to incubation period, % viability dropped ($P \leq 0.05$) from hour 0 to hour 2 (Tab. 2). Supplementation with all the doses of vitamin E improved the per cent viability non-significantly as compared to the control. Upon treating with FeAA, viability of spermatozoa decreased ($P \leq 0.05$). Subsequently, as compared to vitamin E-unsupplemented / FeAA-treated samples, vitamin E supplementation improved % viability non-significantly with doses I, II and III. Non-significant increase in per cent viability was observed when all the three doses of vitamin E were applied (Tab. 2).

Statistical analysis showed non-significant interaction incubation periods x treatments. Thus, increase or decrease in motility (%) and viability (%) with different treatments is not affected by the incubation periods or *vice versa*.

Lipid peroxidation

Three doses of vitamin E were applied to reduce the production of malondialdehyde (MDA is the end product of LPO) in FeAA-treated and untreated samples. FeAA treatment increased the MDA production significantly as compared to the control (Tab. 3).

Supplement of vitamin E reduced the LPO ($P \leq 0.05$) as compared to vitamin E-unsupplemented samples treated or untreated with FeAA. Non-significant drop was observed in the production of MDA when all the three doses of vitamin E were

Table 1. Means and their standard errors (SE) for motility of bull spermatozoa (%) as affected by three doses of vitamin E, untreated *vs*. treated with FeAA

Incubation period (h)		Control	Untreated with FeAA			FeAA	Treated with FeAA			Combination factor mean
			vit. E dose I (1 mM)	vit. E dose II (2 mM)	vit. E dose III (2.5 mM)		vit. E dose I (1 mM)	vit. E dose II (2 mM)	vit. E dose III (2.5 mM)	
0	mean	73.75	73.75	73.75	73.75	73.75	73.75	73.75	73.75	73.75[a]
	SE	2.72	2.72	2.72	2.72	2.72	2.72	±2.72	2.72	
0.5	mean	67.50	63.13	70.63	70.63	56.88	68.13	70.00	67.50	66.80[b]
	SE	1.25	2.56	1.54	0.54	2.70	3.23	±3.95	±2.80	
1	mean	56.25	66.88	68.13	66.25	48.75	59.38	63.75	58.75	61.02[c]
	SE	2.58	1.62	0.54	1.40	3.70	2.23	±2.72	3.25	
2	mean	51.25	58.75	66.25	58.13	40.00	54.38	61.88	54.38	55.63[d]
	SE	4.46	2.17	2.58	4.27	±5.30	2.56	1.85	1.85	
Combination factor mean		62.19[a]	63.63[a]	69.69[b]	67.19[b]	54.84[c]	63.91[a]	67.63[b]	63.59[a]	

FeAA – ferrous sulphate + ascorbic acid.
Each value represents mean ± SE of fifteen observations of at least five animals.
[ab...]Two means in a row or column having different superscripts are significantly different at 5% level of significance.

Table 2. Means and their standard errors (SE) for viability of bull spermatozoa (%) as affected by three doses of vitamin E, untreated *vs*. treated with FeAA

Incubation period (h)		Control	Untreated with FeAA			FeAA	Treated with FeAA			Combination factor mean
			vit. E dose I (1 mM)	vit. E dose II (2 mM)	vit. E dose III (2.5 mM)		vit. E dose I (1 mM)	vit. E dose II (2 mM)	vit. E dose III (2.5 mM)	
0	mean	89.71	89.85	91.50	88.88	79.95	81.90	82.11	83.86	85.97^{a}
	SE	2.96	2.82	1.50	1.07	0.37	1.55	0.81	5.46	
2	mean	76.42	82.41	80.83	77.89	63.76	61.80	73.19	72.05	73.55^{b}
	SE	3.48	2.83	1.39	3.87	2.65	4.88	2.47	1.87	
Combination factor mean		83.07^{a}	86.13^{a}	86.17^{a}	83.39^{a}	71.86^{b}	71.85^{b}	77.65^{bc}	77.96^{bc}	

FeAA – ferrous sulphate + ascorbic acid.
Each value represents mean ± SE of fifteen observations of atleast five animals.
[ab...] Any two means in a row or column having different superscripts are significantly different at 5% level of significance.

Table 3. Effects of various concentrations of vitamin E (mM) on lipid peroxidation (LPO) on FeAA-untreated *vs*. -treated bull spermatozoa

Vit. E concentration		n moles MDA/μg protein		
		untreated with FeAA (control)	treated with FeAA	combination factor mean
0 mM	mean	3.23	5.51	4.37[a]
	SE	0.36	0.60	
1 mM (dose I)	mean	2.94	4.07	3.51[b]
	SE	0.38	0.36	
2 mM (dose II)	mean	2.77	3.16	2.96[b]
	SE	0.35	0.10	
2.5 mM (dose III)	mean	2.59	3.267	2.91[b]
	SE	0.39	0.15	
Combination factor mean		2.88[a]	4.00[b]	

FeAA – ferrous sulphate + ascorbic acid.
Each value represents mean ± SE of fifteen observations of at least five animals.
[ab]Any two means in a row or column having different superscripts are significantly different at 5% level of significance.

compared (Tab. 3).

A non-significant interaction was found of FeAA treatment x vitamin E supplementation. Thus, the effect of increase or decrease in LPO level on vitamin E supplementation is not affected by FeAA treatment *or vice versa*.

In the present study, ROS-mediated damage to sperm cell could be reduced by the supplementation of various doses of vitamin E. ROS are generally produced under oxidative stress conditions (induced by FeAA promoter system). Supplying of antioxidants to sperm samples can protect against the damaging effects of ROS on sperm movement and may be of clinical value in assisted conception procedure [Baker *et al*. 1996].

The present study on bull spermatozoa indicates that vitamin E improves the per cent sperm motility under normal and oxidative stress conditions. Inducing oxidative stress to spermatozoa with the treatment of FeAA causes a significant reduction in their motility. But, supplementing the incubation medium with all doses of vitamin E, *i.e*. 1mM, 2mM and 2.5 mM (treated/untreated with FeAA) increased the motility from hour 0 to hour 2 of incubation. Thus, it is suggested that vitamin E may be effective in preventing the rapid loss of motility that normally occurs during incubation of spermatozoa and maintains the motility under oxidative stress conditions.

This study shows that all doses of vitamin E improve the motility of cattle sperm, but the dose of 2 mM vitamin improves it significantly under both normal and oxidative stress conditions. These results may be explained based on the fact that vitamin E protects the spermatozoa by preventing from endogenous oxidative DNA and membrane damages, thereby helping the sperm to overcome the oxidative attack induced by FeAA (promoter system). Thus, by maintaining the membrane integrity

and optimum functioning of cattle sperm, vitamin E improves the per cent sperm motility. Supplementing the freezing medium with vitamin E improved the sperm motility in humans [Askari *et al*.1994] and in rabbits [Yousef *et al*. 2003].

All concentrations of vitamin E increased the % viability of sperm samples significantly in FeAA-treated, but non-significantly in FeAA-untreated samples. It may be explained based on the fact that vitamin E reduces the FeAA-induced oxidative damage in sperm cell membranes by disrupting the oxidative chain reaction (chain of LPO). Thus, vitamin E promotes sperm membrane integrity and increases the live sperm per cent. Similar observations have been made on humans [Verma and Kanwar 1999], buffalo [Singh *et al.* 1989] and boar [Slebodzinska *et al*. 1995].

Figures presented in Table 1 show a significant decrease in MDA production (n moles MDA/µg protein/ml) – from 4.367 to 2.925 – upon supplying various doses of vitamin E (1mM, 2mM and 2.5 mM) to sperm samples treated/untreated with FeAA (optimum dose). The data indicate that vitamin E addition inhibited the peroxidative damage in sperm as it does in other tissues. Singh *et al*. [1989] reported a drop ($P \leq 0.05$) in MDA production ($nM/10^9$ spermatozoa) from 35.2 to 29.3 upon addition of 2.5 µM vitamin E to buffalo sperm treated with 0.025 mM $FeSO_4$ / 0.125 mM ascorbic acid. Thus, this study confirms that vitamin E supplement inhibits the FeAA-induced lipid peroxidation in cattle sperm.

Data analysis shows that when sperm samples are incubated for 120 min, within all doses, 2.5 mM vitamin E reduces the TBARS (indicator of LPO) production maximum under control and oxidative stress (FeAA-induced) conditions (Tab. 1). Verma and Kanwar [1999] observed that among 0.1, 1 and 2 mM vitamin E doses the 2 mM dose reduced the MDA production maximum after hour 4 to hour 6 of incubation. Hence, the present study confirms a dose-dependent reduction in MDA production when various doses of vitamin E are added to sperm samples. Similar results have been reported in humans [Agarwal *et al*. 2004].

The present study shows the effectiveness of vitamin E in protecting sperm motility and viability by suppressing LPO. Similar observations have been made on humans [Aitken *et al*. 1989, Agarwal *et al*. 2004], boar [Slebodzinska *et al*. 1995], rabbit [Yousef *et al*. 2003] and buffalo [Singh *et al.* 1989].

Vitamin E has been shown to inhibit the free-radical-induced damage to sensitive cell membranes as it is a major chain-breaking antioxidant [Sinclair 2000]. The present study suggests that vitamin E may directly quench the free radicals such as peroxyl and alkoxyl ($ROO^{\bullet}$) generated during FeAA-induced LPO. Thus, by scavenging these radicals, it breaks free-radical chain reaction and forms a relatively stable complex such as tocopheroxyl radical. The present findings on LPO suggest that vitamin E is a major chain-breaking antioxidant in cattle sperm. It not only scavenges oxygen radicals from the membrane, but also intercepts peroxyl and alkoxyl radicals which are generated during the conversion of lipid hydroperoxides that fuel the peroxidative chain reaction, thereby, preventing this damaging process from propagating through

plasma membrane. Similar suggestions concerning humans have been made by Verma and Kanwar [1999].

It is concluded that all doses of vitamin E increased the percentage of sperm motility and viability, but reduced the lipid peroxidation (LPO) level. Most effective occurred the 2mM dose, though differences among the doses were non-significant ($P \geq 0.05$). Thus, vitamin E protects the bull spermatozoa against the damages caused by reactive oxygen species. Supplementing the samples with vitamin E could, therefore, be of clinical importance for extending the time of spermatozoa storage before artificial insemination (AI), *in vitro* fertilization (IVF) and intrauterine insemination (IUI).

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Antyoksydacyjny wpływ witaminy E na ruchliwość, żywotność i peroksydację tłuszczu plemników buhajów w warunkach oksydacyjnego stresu

S t r e s z c z e n i e

Witamina E jest jednym z głównych czynników chroniących błony komórkowe zarówno przed działaniem rekatywnych form tlenu, jak i przed peroksydacja lipidow (LPO). Celem badań było określenie optymalnej dawki witaminy E , która odwróciłaby szkodliwy wpływ oksydacji – wywołany działaniem wolnych rodników – na żywotność i ruchliwość plemników buhaja oraz na poziom LPO. Świeże nasienie pobrane od pięciu lokalnych mieszańcowych buhajów było zawieszone w 2.9% roztworze cytrynianu sodu, a następnie rozdzielone na równe porcje i poddane działaniu witaminy E (0, 1, 2, 2.5mM) w obecności lub braku induktora stresu oksydacyjnego, którym był askorbinian żelazawy (FeAA), zawierający 150 µM $FeSo_4$ i 750µM kwasu askorbinowego. Wszystkie porcje nasienia były inkubowane przez 2 godz. w temperaturze 37°C. Działanie FeAA obniżało żywotność i ruchliwość plemników przy jednoczesnym wzroście LPO. Natomiast wszystkie dawki witaminy E powodowały wzrost żywotności i ruchliwości plemników, a jednocześnie obniżenie poziomu LPO, przy czym najbardziej efektywne okazało się stężenie 2mM witaminy E. Podsumowując, działanie witaminy E obniża poziom peroksydacji lipidów wywołanej działaniem FeAA i podwyższa żywotność i ruchliwość plemników *in vitro* w warunkach indukowanego stresu oksydacyjnego.