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## In Vitro Capacitation of Bull Spermatozoa: Role of Vitamin E

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# In Vitro Capacitation of Bull Spermatozoa: Role of Vitamin E

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## Abstract

The aim of this work was to study the influence of vitamin E (2mM) on capacitation and acrosome reaction of crossbred cattle bull spermatozoa. Fresh semen suspended in TALP medium (pH 7.4) was divided into four equal fractions. All fractions; control (containing TALP + spermatozoa) and the experimental [treated / untreated with ferrous ascorbate (FeAA – 150  $\mu$ M FeSO<sub>4</sub> : 750  $\mu$ M ascorbic acid); supplemented / un-supplemented with 2mM vitamin E] were incubated for 6 h at 37°C. These fractions were assessed for % hyperactivity, morphology, lipid peroxidation (LPO), % acrosome reaction and changes in protein contents during sperm capacitation. FeAA treatment non-significantly ( $P \geq 0.05$ ) decreased % sperm hyperactivity, % acrosome reaction and protein contents, but, increased LPO level. However, supplementation of vitamin E to the FeAA treated / untreated fractions non-significantly ( $P \geq 0.05$ ) altered % hyperactivity, rate of acrosome reaction, protein content, but, significantly ( $P \leq 0.05$ ) decreased LPO level of bull spermatozoa during capacitation. It is concluded that vitamin E protects the sperm against LPO, and, alters its functions, though not effectively.

## Introduction

Mammalian spermatozoal membranes are rich in polyunsaturated fatty acids (PUFAs) and are sensitive to oxygen-induced damage mediated by lipid peroxidation (LPO), and, thus are sensitive to reactive oxygen species (ROS) attack which results in decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosome [1]. Antioxidants protect the plasma membrane against peroxidative damages. Vitamin E ( $\alpha$ -tocopherol) protects sperm membrane against oxidative damages [2]. It is a well known antioxidant and has been shown to inhibit free radical induced damage to sensitive cell membranes [3]. It acts as a scavenger of lipid peroxy (LOO $\bullet$ ) and alkoxy (LO $\bullet$ ) radicals [4]. Vitamin E may directly

quench the free radicals such as peroxy and alkoxy generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant [5]. Studies have found low vitamin E levels would allow for physiological production of ROS that are essential for membrane changes required for capacitation, acrosome reaction and in vitro fertilization (IVF) processes [6].

For successful fertilization, mammalian spermatozoa must undergo a preparation period known as capacitation [7]. 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 [8]. 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 [4].

Some studies show that sperm capacitation and acrosome reaction are oxidative processes; low concentration of ROS (especially H<sub>2</sub>O<sub>2</sub>,  $\bullet$ O<sub>2</sub><sup>-</sup>), exogenously added or minute amounts generated by spermatozoa are needed to trigger this phenomenon in vitro.

The present study was undertaken to examine the effect of 2mM vitamin E on morphology, % hyperactivity, % acrosome reaction, lipid peroxidation (LPO) level and changes in protein content during capacitation and acrosome reaction of local crossbred cattle bull spermatozoa.

## Materials and Methods

### Sperm samples

Semen samples (n = 5) with more than 80% motility and 1200 x 10<sup>6</sup> – 1400 x 10<sup>6</sup>/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. Three sub samples of each ejaculate of each bull (total five crossbred cattle bulls) were used to analyze each parameter. Thus, each parameter was repeated thrice using five animals. 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<sub>3</sub> – 25.9mM; CaCl<sub>2</sub>.2H<sub>2</sub>O – 10mM; MgCl<sub>2</sub>.6H<sub>2</sub>O – 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. This suspension was divided into four equal fractions. In first fraction (control), TALP was added, whereas, out of the remaining three fractions (experimental); two were treated with ferrous ascorbate (FeAA – 150 µM FeSO<sub>4</sub>: 750 µM ascorbic acid) and one (FeAA untreated) was added with equal volume of TALP. Subsequently, all experimental fractions were supplemented with 2mM vitamin E and incubated at 37°C for 6 h. All fractions were evaluated for the following parameters during varying incubating periods.

#### **Percentage motility or hyperactivity**

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

#### **Morphology and percentage acrosome reaction**

Smears of each fraction were prepared at 2 h interval, stained with Giemsa and examined under oil immersion at 100 X magnification 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 [9].

#### **Total proteins**

Total proteins in all the fractions were determined spectrophotometrically [10]; 20-100 µg/ml BSA standard was also run simultaneously.

#### **Statistical analysis**

'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' was used to evaluate the significance levels between the parameters studied. 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**

Spermatozoa undergo normal process of capacitation and acrosome reaction. Morphology of bull spermatozoa remained unaffected with the treatments of FeAA and vitamin E.

#### **% hyperactivity**

Corresponding to the incubation period, % hyperactivity of the sperm decreased non-significantly ( $P \geq 0.05$ ) after 1- and 2- h, but, significantly ( $P \leq 0.05$ ) after 3-, 4-, 5- and 6- h of incubation as compared to the control (Table 1). In response to treatments, FeAA treatment decreased the sperm hyperactivation non-significantly ( $P \geq 0.05$ ) as compared to the control. Subsequently, vitamin E supplementation improved % sperm hyperactivity non-significantly ( $P \geq 0.05$ ) both in FeAA treated as well as untreated fractions (Table 1).

Statistical analysis shows 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.

#### **Lipid peroxidation (LPO) and % acrosome reaction**

Lipid peroxidation of all fractions increased significantly ( $P \leq 0.05$ ) from 0 h to 6 h (Table 2). FeAA treatment to the bull sperm increased the LPO level non-significantly ( $P \geq 0.05$ ) as compared to the control. Subsequently, vitamin E supplementation significantly ( $P \leq 0.05$ ) decreased the MDA production in FeAA untreated samples, but, non-significantly ( $P \geq 0.05$ ) in FeAA treated samples as compared to the control. Statistical analysis shows non-significant ( $P \geq 0.05$ ) interaction between treatments and incubation periods. Thus, increase or decrease in MDA production with various treatments is not affected by incubation periods (Table 2).

% acrosome reaction of bull sperm significantly ( $P \leq 0.05$ ) increased at 6 h of incubation as compared to 4 h (Table 3). FeAA treatment non-significantly ( $P \geq 0.05$ ) decreased the rate of acrosome reaction as compared to the control. However, vitamin E supplementation non-significantly ( $P \geq 0.05$ ) increased % acrosome reaction both in FeAA treated as well as untreated samples (Table 3). Statistical analysis shows non-significant interaction between treatments and incubation periods, thus, increase or decrease in % acrosome reaction with various treatments is not affected by incubation periods.

#### **Total proteins**

Corresponding to incubation period, protein content of incubation medium (TALP + sperm suspension) increased non-significantly ( $P \geq 0.05$ ) from 0 h to 4 h (Table 4). Among 0-, 2- and 4- h of incubation, it was maximum at 4 h. After 6 h of incubation, the medium containing TALP and sperm suspension was

centrifuged and divided into two parts; supernatant and pellet. The protein content of the pellet was significantly ( $P \leq 0.05$ ) lesser than that of supernatant. Protein content decreased non-significantly ( $P \geq 0.05$ ) in FeAA, FeAA + vitamin E, vitamin E treated sperm samples as compared to the control (Table 4). Statistical analysis shows non-significant ( $P \geq 0.05$ ) interaction between treatments and incubation periods. Thus, increase or decrease in the protein content with various treatments is not affected by incubation periods.

## Discussion

In the present observations, % hyperactivity remains almost unchanged from 0 h to 2 h, but, subsequently upto 6 h, it significantly ( $P \leq 0.05$ ) decreases (Table 1). It is known that % hyperactivity decreases with the occurrence of capacitation process, during which spermatozoa start showing zig-zag movements [11]. Thus, this study shows that molecular changes related to the sperm capacitation begin after 2 h of incubation. Further, a significant ( $P \leq 0.05$ ) decrease in % hyperactivity from 4 h to 5 h shows the occurrence of acrosome reaction during which many metabolic and ionic changes occur in sperm membrane leading to the decrease in % hyperactivity. After 6 h of incubation, 15-20% decrease in % hyperactivity of the FeAA treated/untreated or vitamin E supplemented/ unsupplemented samples has been observed. Sidhu et al. [11] observed less than 20% decrease in % motility after 6 h of incubation of bull sperm [11]. Thus, present study confirms that % hyperactivity (motility) decreases with the increase in incubation period.

The results of the present study show that % hyperactivity is non-significantly ( $P \geq 0.05$ ) affected by FeAA treatment. On supplementation of vitamin E to the FeAA treated or untreated samples, % hyperactivity increases non-significantly ( $P \geq 0.05$ ). Similar observation was made by O'Flaherty et al. [7] in bull sperm. They supplemented the sperm (suspended in TALP medium) with 1 mg/ml of vitamin E and observed non-significant ( $P \geq 0.05$ ) increase in % hyperactivity as well as capacitation. Therefore, present results show that with the supplementation of vitamin E to bull spermatozoa (suspended in TALP medium), treated or untreated with FeAA, % hyperactivity increases slightly. This increase may be due to the role of vitamin E in inhibiting peroxidative damages in sperm membranes induced by ROS. Inhibition of membrane damages promotes membrane permeability and its integrity which allow the

spermatozoa to function optimally, and, thereby, maintaining its hyperactivity.

Data analysis shows a significant ( $P \leq 0.05$ ) increase in LPO from 0 h to 6 h of incubation, in bull sperm (Table 2). This implies that ROS production (measured by LPO) enhances during these hours. Thus, the present study shows that ROS are required for the capacitation and acrosome reaction as reported by a number of research workers [6,7,12,13,14].

Vitamin E is a well known antioxidant [15]. Therefore, it inhibits the production of ROS in FeAA treated and untreated sperm samples. Inhibition of ROS may lead to the decrease in capacitation and acrosome reaction, whereas, ROS are required to promote these processes. In the present study, rate of acrosome reaction is non-significantly ( $P \geq 0.05$ ) affected by vitamin E supplementation. A significant decrease in rate of acrosome reaction is found with vitamin E supplementation in the bull [7] and human [16] spermatozoa. Vitamin E is a major chain breaking antioxidant in biological system and its quenching property proves to be a potent action in decreasing certain ROS mediated processes [17,18]. Thus, it is suggested that vitamin E has negative effect on the acrosome reaction although it improves other functions of sperm such as motility/ hyperactivity.

The increase in the protein content from 0 h to 4 h indicates that during capacitation and acrosome reaction, membrane proteins leak out into the incubation medium due to membrane metabolic and ionic changes [7]. After 6 h of incubation, supernatant contains significantly more protein content than the sperm pellet. Thus, it confirms that maximum leakage of proteins takes place during acrosome reaction i.e. at 6 h of incubation.

Corresponding to the treatments, FeAA treatment to the bull spermatozoa decreases the protein content. 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 [19]. FeAA negatively affects the metabolic changes and membrane fusion events by decreasing the rate of acrosome reaction, resulting thereby, in the reduced leakage of proteins from the membrane. On the other hand, vitamin E supplementation to the FeAA treated samples improve the membrane integrity through its antioxidative property, which in turn causes leakage of proteins, and, hence increasing the protein content in all the fractions. In FeAA untreated or control fractions, the protein content remains unaltered on supplementation of vitamin E. In the discussion on the lipid peroxidation and acrosome reaction, it was suggested that vitamin E inhibits the rate of acrosome reaction. Therefore,

vitamin E suppresses the membrane fusion events, so that proteins do not leak out from the membrane. This explains the unaltered content of proteins in vitamin E supplemented samples as compared to the control. It is concluded that vitamin E protects the sperm against LPO, and alters its functions though not effectively.

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## Illustrations

### Illustration 1

Table 1. Effects of FeAA, vitamin E and FeAA vitamin E on hyperactivity (%) of bull spermatozoa during capacitation and acrosome reaction

Time (hrs)	% Hyperactivity				
	FeAA untreated fractions		FeAA treated fractions		Combination factor mean
	Control	Vitamin E	FeAA	FeAA+ Vitamin E	
0	73.888 ±1.367	74.722 ±1.593	72.5 ±0.962	73.888 ±1.367	<b>73.75<sup>a</sup></b>
1	73.611 ±1.071	74.444 ±1.458	71.388 ±1.047	72.777 ±1.269	<b>73.055<sup>a</sup></b>
2	72.166 ±0.881	73.334 ±1.576	71.944 ±1.609	72.222 ±1.776	<b>72.416<sup>a</sup></b>
3	69.444 ±1.289	70.833 ±1.842	68.611 ±1.422	69.722 ±1.141	<b>69.652<sup>b</sup></b>
4	67.5 ±2.187	68.611 ±1.624	66.111 ±1.928	67.5 ±2.187	<b>67.430<sup>b</sup></b>
5	62.222 ±2.338	65.277 ±1.640	61.944 ±2.214	64.722 ±2.057	<b>63.541<sup>c</sup></b>
6	58.611 ±2.545	61.388 ±2.456	57.777 ±2.705	60.277 ±2.498	<b>59.513<sup>d</sup></b>
<b>Combination factor mean</b>	<b>68.206<sup>a</sup></b>	<b>69.801<sup>a</sup></b>	<b>67.182<sup>a</sup></b>	<b>68.730<sup>a</sup></b>	

Each value represents mean ± SE. Any two means in a row or column having different

## Illustration 2

Table 2. Effects of FeAA, vitamin E and FeAA + vitamin E on lipid peroxidation of bull spermatozoa during capacitation and acrosome reaction

Time (hrs)	n moles MDA/ $\mu$ g protein				Combination factor mean
	FeAA untreated fractions		FeAA treated fractions		
	Control	Vitamin E	FeAA	FeAA + Vitamin E	
0	2.255 $\pm 0.473$	2.201 $\pm 0.692$	3.385 $\pm 0.541$	2.884 $\pm 0.597$	<b>2.681<sup>a</sup></b>
4	4.671 $\pm 1.171$	3.031 $\pm 0.501$	5.111 $\pm 1.199$	4.725 $\pm 0.541$	<b>4.384<sup>b</sup></b>
6	4.956 $\pm 0.352$	2.025 $\pm 0.423$	6.336 $\pm 0.721$	3.732 $\pm 0.719$	<b>4.262<sup>b</sup></b>
<b>Combination factor mean</b>	<b>3.961<sup>a</sup></b>	<b>2.419<sup>b</sup></b>	<b>4.944<sup>a</sup></b>	<b>3.780<sup>a</sup></b>	

Each value represents mean  $\pm$  SE. Any two means in a row or column having different superscripts (a, b) are significantly different at 5% level of significance.

Combination factor mean corresponds to interaction between incubation periods and

### Illustration 3

Table 3. Effects of FeAA, vitamin E and FeAA + vitamin E on % acrosome reaction of bull spermatozoa

Incubation period (hrs)	% Acrosome reaction				
	FeAA untreated fractions		FeAA treated fractions		<b>Combination factor mean</b>
	Control	Vitamin E	FeAA	FeAA + Vitamin E	
4	72.570 ±4.527	68.384 ±5.031	57.76 ±3.508	65.340 ±4.105	<b>66.013<sup>a</sup></b>
6	74.367 ±4.572	77.551 ±2.675	72.662 ±4.469	75.266 ±2.878	<b>74.961<sup>b</sup></b>
<b>Combination factor mean</b>	<b>73.468<sup>a</sup></b>	<b>72.968<sup>a</sup></b>	<b>65.211<sup>a</sup></b>	<b>70.303<sup>a</sup></b>	

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

Combination factor mean corresponds to interaction between incubation periods and

## Illustration 4

Table 4. Effects of FeAA, vitamin E and FeAA vitamin E on protein content of bull spermatozoa during capacitation and acrosome reaction.

Time (hrs)	Samples	mg/10 <sup>9</sup> spermatozoa				Combination factor mean
		FeAA untreated fractions		FeAA treated fractions		
		Control	Vitamin E	FeAA	FeAA + Vitamin E	
0	Incubation medium	4.954 ±1.294	5.168 ±1.08	3.637 ±0.721	4.680 ±0.839	<b>4.610<sup>a</sup></b>
2	Incubation medium	6.029 ±1.369	5.607 ±0.778	4.190 ±0.520	4.285 ±1.471	<b>5.028<sup>a</sup></b>
4	Incubation medium	7.397 ±0.705	6.178 ±0.708	4.414 ±0.573	5.142 ±0.712	<b>5.783<sup>a</sup></b>
6	Supernatant	3.574 ±0.520	4.488 ±0.597	4.731 ±0.588	5.156 ±0.334	<b>4.487<sup>a</sup></b>
6	Sperm pellet	1.335 ±0.191	1.838 ±0.357	1.527 ±0.207	1.393 ±0.033	<b>1.523<sup>b</sup></b>
<b>Combination factor mean</b>		<b>4.658<sup>a</sup></b>	<b>4.656<sup>a</sup></b>	<b>3.700<sup>a</sup></b>	<b>4.135<sup>a</sup></b>	

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

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