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Effect of vitamin E and Mn^{2+} on lipid and phospholipids contents of crossbred cattle bull spermatozoa under induced oxidative stress

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ABSTRACT

Many studies have been found that ferrous ascorbate (FeAA : $FeSO_4$ + ascorbic acid) is used as oxidative stress inducer . In this study, the effects of various doses of vitamin E (1mM, 2mM, 2.5mM) and Mn^{2+} (60 µM, 100 µM, 200 µM) on lipids and phospholipids contents have been determined in the local crossbred cattle bull spermatozoa. Fresh semen was suspended in 2.9% sodium citrate and this suspension was divided into eight equal fractions. All fractions; control (containing 2.9% sodium citrate + spermatozoa) and the experimental [treated / untreated with ferrous ascorbate (150 µM $FeSO_4$: 750 µM ascorbic acid), supplemented / un-supplemented with three doses of vitamin E (1mM, 2mM, 2.5mM) / Mn^{2+} (60 µM, 100 µM, 200 µM) were incubated for 2 hrs at 37°C. These fractions were assessed for determination of lipids and phospholipids contents. It is concluded that by inducing oxidative stress with FeAA, levels of lipid and phospholipids decreased. However, all doses of vitamin E and Mn^{2+} supplementation improved lipids and phospholipids contents under normal and oxidative stress conditions due to their antioxidative nature.

***Key Words*:** *Cattle , lipids, Mn^{2+}, phospholipids, spermatozoa , vitamin E*

I. INTRODUCTION

India has a vast resource of livestock which plays a vital role in improving the socio-economic conditions of the rural masses. Reproductive efficiency of farm animals is directly related to their productivity. Further, optimum reproduction leads to augmentation of economic returns to livestock owner [1].

Numerous factors influence male fertility, one of these being the oxidative stress (OS), which has elicited an enormous interest in the recent [2]. Oxidative stress occurs as a consequence of an imbalance between the production of reactive oxygen species (ROS) and the available antioxidant defenses against them [3].

Mammalian spermatozoa represent a growing list of cell types that exhibit a capacity to generate highly reactive oxygen species such as hydrogen peroxide (H_2O_2), the superoxide anion ($^{\bullet}O_2^-$), the hydroxyl radical ($^{\bullet}OH$) and the hypochlorite ($^{\bullet}OHCl$) when incubated under aerobic condition[4]. ROS are free radicals and peroxides that are derived from the oxygen metabolism. ROS are continuously produced by various metabolic and physiological processes [2].

Mammalian semen consists of different types of cells such as mature and immature spermatozoa, round cells from different stages of spermatogenic processes, leukocytes and epithelial cells. Of these, leukocytes and immature spermatozoa are the two main sources of ROS [2].

Lipid is an important constituent of cell membrane. Membrane lipid composition of spermatozoa has been correlated to different function. Many researchers have related membrane lipid with survival success after cryopreservation or cold shock[5]. 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) (fatty acids contain more than two carbon-carbon double bonds) [2]. ROS attack the PUFAs in the cell membranes leading to a chain of chemical reactions called lipid peroxidation (LPO). LPO can be broadly defined as oxidative deterioration of PUFAs[2]. Phospholipids are the most representative lipid fraction of the sperm cell membranes, of which phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are the major components [6]. In view of the economic importance of cattle bull and the gaps in the knowledge/researches, this systematic study was undertaken to meet the following objective:

- Monitor the role of vitamin E and Mn^{2+} (antioxidants) on lipid and phospholipid contents of crossbred cattle bull spermatozoa under induced oxidative stress

II. MATERIALS AND METHODS

2.1Sperm

Ejaculates with more than 80% motility and 1.2 to 1.4 $x10^9$ sperm /mL were collected (with an artificial vagina) from five healthy local crossbred cattle bulls (HHS, Holstein-Friesian x Sahiwal; FC, Friesian crosses; 1F and 4F first and fourth generation of inter-re-breeding) maintained at the Dairy Farm, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Three ejaculates were used for each bull. Fresh semen was centrifuged (800 x g) at 37°C for 5 min, seminal plasma and other contaminants were 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 x 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+} 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 treatment (0,1,2,2.5mM) 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 various cycles of freezing(- 100^0C) and thawing(37^0C) ,these fractions were evaluated for the following parameters.

2.2 Lipids [7]

2.2.1. Principle: This method is used for extraction of lipids when the moisture content of the tissue/sample is less than 50 per cent. The tissue/sample is homogenized and placed with solvents in the ratio of 1:10 for 24 hours so that complete extraction takes place. As the solvent gathers traces of sugar, amino acids and proteins along with lipids, their removal is carried out by Folch washings with 0.9 per cent NaCl (Saline solution).

2.2.2. Reagents: Chloroform : Methanol (2:1)

0.9% saline solution

2.2.3. Extraction of total lipids:

Total lipids from the sperm suspension treated/untreated with different concentrations of FeAA were extracted after Folch method (1957). A known volume of the semen sample was taken and then centrifuged at 800 g for 5 minutes. Pellet was resuspended in an equal volume of 2.9 per cent sodium citrate (pH 7.4) and after giving 2-3 washings with sodium citrate, a known volume of sperm suspension was made by adding 2.9 per cent sodium citrate. Sperm suspension was then divided into four equal parts in four test tubs. Three test tubes containing equal volume of sperm suspension were given different treatments of FeAA [100:500/150:750/200:1000 (µM) ($FeSO_4$ ascorbic acid)] and in the fourth test tube, equal volume of 2.9 per cent sodium citrate (pH 7.4) was added. Then, after incubating for 1 hour at 37°C, the tubes were centrifuged at 2500x g for 15 minutes. Pellet so obtained was mixed with chloroform : methanol (2:1 v/v) by intermittent shaking for 12 hours for extraction of lipids. The extract was filtered through sintered glass funnel (G-2) and the residue in the funnel was washed three times with chloroform : methanol solution for complete extraction of lipids.

2.2.4. Purification of Total Lipids

The crude total lipid extracted above was washed with 0.9 per cent NaCl (5:1 v/v) in a separating funnel. It was left undisturbed overnight for complete extraction of lipids into the chloroform phase. The lower chloroform phase was carefully removed into a small conical flask and the upper layer containing glycerol, soaps and lower fatty acids was washed with chloroform to completely remove the lipids, layers were allowed to separate and lower layer was collected again in the conical flask. Chloroform was evaporated to dryness. Total lipids were calculated as mg/10^9 spermatozoa. Lipids thus obtained were dissolved in known volume of chloroform to extract and estimate phospholipids.

2.3. Phospholipids [8]

2.3.1. Principle: The method relies on the release of organically bound phosphorus compounds as orthophosphate, by high temperature combustion at 450-500°C. The orthophosphate is then extracted with 0.5N HCl at 90°C. The liberated orthophosphate is reacted with a mixed reagent of molybdic acid, ascorbic acid and ammonium molybdate to form phosphomolybdic acid. This heteropoly acid is then reduced to the coloured molybdenum blue complex by ascorbic acid and the solution colour is measured spectrophotometrically. The amount of phospholipids is calculated by multiplication of the lipid phosphorus value with 25. These values are approximations since phosphorus does not exactly represents 4.0 per cent of each phospholipids molecule.

2.3.2. Reagents:

(i) 10% magnesium nitrate $6H_2O$ [$Mg(NO_3)_2$ $6H_2O$] in 95 per cent ethanol

(ii) 0.5N HCl

(iii) 10% ascorbic acid

(iv) Ammonium molybdate solution: (0.421% ammonium molybdate In 1N H_2SO_4)

2.3.3. Procedure

Extraction: A known volume of lipid sample, treated/untreated with different concentrations of FeAA was taken in test tube and evaporated to dryness in an oven at 60°C. To the test tube containing dried lipid sample, 0.18 ml of reagent (I) was added. The material was dried on a hot plate. The contents in the tube was ashed by shaking them over the hot plate until the brown fumes disappeared. The tube was then allowed to cool and 0.18 ml of reagent (II) was added. The stopper tube was then heated in a boiling water bath for 15 minutes, so as to hydrolyse any pyrophosphate formed during ashing to phosphate. Cooled the tubes.

2.3.4. Estimation of Phospholipids

To the above extract in 0.5N HCl, 4.2 ml of a mixture prepared by combining one part of solution (III) and 6 part of solution (IV) was added. Tube was then incubated at 45°C for 25 minutes. Per cent transmittance was noted at 820 nm against a blank prepared in a same manner but omitting the lipid sample.

The standard curve was prepared by using different concentrations (1-7 µg) of inorganic phosphate (KH_2PO_4). The phospholipids were calculated in mg phosphate/10^9 spermatozoa by multiplying the phosphorus content with 25 Fig.8).

2.4. 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

III. RESULTS

3.1. Effect of different doses of vitamin E on total lipids and phospholipids contents

FeAA treatment decreased the total lipids content non-significantly ($P \geq 0.05$) (Table 1). Vitamin E supplementation increased the total lipids content significantly ($P \leq 0.05$) with doses III but non-significantly ($P \geq 0.05$) with dose I and II as compared to vitamin E unsupplemented samples. Among the three doses, total lipid content increased non-significantly ($P \geq 0.05$) between doses I & III as well as doses II & III (Table 1).

FeAA treatment increased the total phospholipids content non-significantly ($P \geq 0.05$). Vitamin E supplementation increased the phospholipid contents gradually but non-significantly as compared to vitamin E unsupplemented samples. Among the three doses, phospholipid content decreased non-significantly ($P \geq 0.05$) (Table 1). Statistical analysis shows non-significant interaction between FeAA treatment and vitamin E supplementation. Thus, increase or decrease in lipid and phospholipid contents with vitamin E supplementation is not affected by FeAA treatment or vice-versa.

3.2. Effect of Mn^{2+} on lipids and phospholipids

Total lipids content decreased non-significantly (P≥0.05) with FeAA treatment (Table 2). Supplementation of various doses of Mn^{2+} to FeAA treated/untreated sperm samples increased the total lipid content non-significantly (P≥0.05) as compared to the Mn^{2+} unsupplemented samples. On comparing three doses of Mn^{2+}, lipid content increased non-significantly (P≥0.05). Increase in total lipids content was maximum with 200 µM Mn^{2+} (Table 2). FeAA treatment increased the phospholipids content significantly (P≤0.05) as compared to the control (Table 2). Supplementation of various doses of Mn^{2+} increased the phospholipids content non-significantly (P≥0.05). This increase was maximum with 200 µM Mn^{2+}. Statistical analysis has shown non-significant (P≥0.05) interaction between FeAA treatment and Mn^{2+} supplementation (Table 2). Thus, increase or decrease in lipid and phospholipid contents with Mn^{2+} supplementation is not affected by FeAA treatment and vice-versa.

IV. DISCUSSION

FeAA treatment decreased the lipid contents. It implies that under oxidative stress (induced by FeAA), there may be oxidative deterioration of the sperm membrane, which depletes the lipid content in spermatozoa. Another possible reason for decrease in lipid content is the peroxidation of polyunsaturated fatty acids that enhance fusogenicity, thereby, decreasing the integrity of sperm membranes. All these events may lead to leakage of lipids from the spermatozoa. Loss of viability is related to membrane leakiness which is induced by sperm phospholipids peroxidation [9,10].Further, vitamin E supplementation increases the lipid and phospholipid contents. If compared, maximal increase in lipid and phospholipid contents was observed with 2.5 mM and 1 mM vitamin E, respectively. This increase may be due to the decrease in oxidative stress by vitamin E as analysed in our previous studies[11]. Therefore, this study suggests that vitamin E is proved to maintain the lipid and phospholipid contents in bull spermatozoa due to its antioxidative nature. .

Data show that lipid and phospholipid contents of FeAA treated sperm samples are lesser than those of the control (Table 2). This implies that under oxidative stress conditions induced by FeAA, polyunsaturated fatty acids (PUFAs) of the sperm membranes get converted to lipid peroxides which in turn make the membrane more fusogenic and fragile[12].Thus, membrane integrity and flexibility decrease which ultimately leads to leakage of lipids and phospholipids from the membrane and, thereby, decreasing their contents. The major damage caused during cryopreservation is peroxidation of lipids especially phospholipid bound polyunsaturated fatty acids (PUFAs) [13,14]. But it is also established that membrane stress rather than lipid peroxidation is related to sublethal cryodamage [alvarez and storey, 1983]. The lipid composition of whole spermatozoa is well documented [16,17]. However, with the supplementation of $MnCl_2$, lipid and phospholipid contents increase non-significantly (P≥0.05). It may be correlated with the decrease in LPO by Mn^{2+}in our previous study[11] As, Mn^{2+} inhibits LPO [18] thus, increasing the membrane integrity and viability which are required for the storage of lipids and phospholipids. Therefore, Mn^{2+} supplementation to the samples reduced the leakage of lipid and phospholipid contents under normal and oxidative stress conditions.

V. CONCLUSIONS

Vitamin E and Mn^{2+} maintains the lipids and phospholipid contents in bull spermatozoa under normal and induced oxidative stress conditions due to their antioxidative nature .

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Table 1 Effects of various concentrations of vitamin E (mM) on total lipids and phospholipids content of Ferrous ascorbate (FeAA) (Ferrous sulphate + ascorbic acid) treated/untreated bull spermatozoa.

Concentrations of vitamin E(Doses)	mg/10^9 spermatozoa					
	Lipids			Phospholipids		
	Control	FeAA treated	Combination factor mean	Control	FeAA treated	Combination factor mean
0	0.326 ±0.071	0.457 ±0.074	0.392^{a}	0.207 ±0.025	0.116 ±0.023	0.161^{a}
1 (I)	0.524 ±0.070	0.382 ±0.124	0.453^{ab}	0.203 ±0.043	0.205 ±0.070	0.204^{ab}
2 (II)	0.878 ±0.146	0.536 ±0.156	0.707^{ab}	0.239 ±0.057	0.221 ±0.106	0.230^{ab}
2.5 (III)	1.028 ±0.149	0.823 ±0.125	0.925^{b}	0.286 ±0.012	0.374 ±0.139	0.330^{b}
Combination factor mean	0.689^{a}	0.550^{a}		0.233^{a}	0.229^{a}	

Each value represents mean ± SE of fifteen observations of atleast five animals.

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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Table 2 Effects of various concentrations of Mn^{2+} (µM) on total lipids and phospholipids content of Ferrous ascorbate (FeAA) (Ferrous sulphate + ascorbic acid) treated/untreated bull spermatozoa.

Concentrations of Mn^{2+}(Doses)	mg/10^9 spermatozoa					
	Lipids			Phospholipids		
	Control	FeAA treated	Combination factor mean	Control	FeAA treated	Combination factor mean
0	0.325 ±0.056	0.267 ±0.063	0.296[a]	0.412 ±0.061	0.323 ±0.052	0.367[a]
60 (I)	0.337 ±0.054	0.260 ±0.036	0.298[a]	0.474 ±0.085	0.341 ±0.042	0.407[a]
100 (II)	0.383 ±0.048	0.315 ±0.044	0.349[a]	0.398 ±0.048	0.357 ±0.059	0.378[a]
200 (III)	0.386 ±0.064	0.395 ±0.066	0.391[a]	0.593 ±0.112	0.373 ±0.081	0.483[a]
Combination factor mean	0.358[a]	0.309[a]		0.469[a]	0.348[b]	

Each value represents mean ± SE of fifteen observations of atleast five animals.

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