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**INVESTIGATING THE PROTECTIVE EFFECT OF VITAMIN E AND SELENIUM ON
SPERMATOGENESIS IN ADULT MALE RATS RESISTANT TO INSULIN**

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ABSTRACT

Diabetes is from metabolic diseases group and is a multifactorial disorder characterized by increase of chronic hyperglycemia and is due to secretion disorder or insulin function or both. In this study, the protective role of vitamin E and sodium selenite in prevention of adverse effects of insulin resistant (type 2 Diabetes) on spermatogenesis were studied.

Adult male Wistar rats (180-200 gr) were divided into 5 groups of N=7 (control group, sham group and 3 experimental groups). For 110 days, the rats daily received fructose 10% solution in water and also 200 mg/kg vitamin E (gavage), 0.5 mg/kg sodium selenite (injected intraperitoneally) or both. Sperm parameters, testosterone level, LH, daily sperm production (DSP), testicular histopathology and the rate of malondialdehyde (MDA) in the testes were examined. Sperm count, percent of sperm motility and viability and DSP decreased in rats resistant to insulin significant decrease in the number of leydig cells, spermatogonia, spermatids, and spermatozoa in testes of insulin-resistant animals was observed. MDA level and testosterone

in testes of insulin resistant animals were increased. Vitamin E and sodium selenite decreased MDA level, harmful effects of fructose on testicles, sperm parameters and testicular histology. Receiving Vitamin E and sodium selenite created the most protective effect. These findings indicate that prescribing vitamin E and sodium selenite protects the testes of rats against oxidative stress induced by diabetes type 2.

Keywords: Diabetes type 2, Vitamin E, Sodium Selenite, Spermatogenesis

INTRODUCTION

Investigating causes of infertility in men and how to prevent creation of infertility in men in a community is a valuable aid to patients who are suffering from infertility. Diabetes is from metabolic diseases group and is a multifactorial disorder characterized by increasing chronic blood sugar or hyperglycemia and results from secretion disorder or insulin function or both. In this study, the protective role of vitamin E and sodium selenite in preventing the harmful effects of resistance to insulin (Diabetes type 2) on spermatogenesis were studied. At this time, more than 170 million people worldwide suffer from diabetes and it is predicted that this number in 2025 will reach 300 million and by 2030, will effect 366 million people. Epidemiological studies indicate that the prevalence of mellitus diabetes is increasing globally. Diabetes type 1, due to specific destruction of insulin producing beta cells in pancreatic islands is created by T Iron reactive cells and produces mediated materials by these cells in

inflammation of these islands. Diabetes type 2 occurs because of the body's resistance to insulin action along with gradual disorder in beta cells function which leads to progressive loss of metabolic control. Resistance to insulin is the first stage of diabetes type 2 (diabetes independent of insulin: NIDDM).

Resistant to insulin causes an increase in serum insulin, disorders in glucose and hyperglycemia metabolism, and creates diabetes type 2. Diabetes type 2 is associated with many disorders including hypertension, elevated blood lipids, kidney and reproductive disorders. Up to now, many studies have been done on the effect of diabetes type 1 on spermatogenesis in human and laboratory animals but there are few studies on diabetes type 2 in human and laboratory animals. In the only study accomplished on rats that were resistant to insulin, it was shown that resistance to insulin causes reduction in the number of sperm, sperm motility and testosterone hormone; however there is no change in the amount of LH. One of the

mechanisms that is resistant to insulin and can have an effect on spermatogenesis is the increase of free radicals and creating induced oxidative stress [1]. Free radicals and reactive oxygen species play an important role in reducing the quantity and quality of semen and causes loss of sperm life [2, 3]. Effect of free radicals on spermatogenesis of rats resistant to insulin has not been studied yet. Vitamin E and selenium are antioxidant compounds. Vitamin E is a fat soluble vitamin that has Cleaner free radicals [4]. Selenium is a component of antioxidant enzymes such as glutathione peroxidase that neutralize free radicals and play a role in removing oxidative stress. However, resistant to insulin through the increase of free radicals, it causes an effect on spermatogenesis, and vitamin E consumption and selenium must be able to prevent these effects [5]. According to the above, the purpose of this study is to investigate the protective role of vitamin E and selenium on the effects of resistance to insulin on spermatogenesis in rats.

MATERIALS AND METHODS

In the current experimental study, 35 adult male Wistar rats with approximate weight 180 to 230g were used. The animals were maintained in Jahrom animal's house, located at the Islamic Azad University

Branch, Jahrom in standard conditions (12 hours light, 12 hours dark and $22\pm 2^{\circ}\text{C}$). These animals were randomly divided into 5 groups as follow:

Control group (A): They did not receive any medication.

Sham Group: They received no drug and drinking water containing fructose 10%. Dose was chosen based on a previous study that investigated fructose effects on induced sterility oxidative stress.

First Experimental Group (C₁)

Received Sodium selenite injected intraperitoneally and drinking water containing fructose 10%.

Second Experimental Group (C₂)

Received Vitamin E as gavage, sodium selenite injected intraperitoneally and drinking water containing fructose 10%.

Third Experimental Group (C₃)

Vitamin E as gavage and drinking water containing fructose 10%. The above-mentioned materials were fed to animals daily in the morning between 10-11am and for 110 days through gavage or intraperitoneal injection. It should be mentioned that during this study all rooting behavior treatment of animals based on rules of protecting animals (SPCA) approved in America in 2006 was considered.

Body Weight and Proportion of Testis Weight to Body Weight

Rat's weight on the first day before gavage and injection was measured using a digital scale. On the last day, rats were weighed again. Then by anesthetizing the animal directly, a 5 cc syringe blood sample was collected from heart, and after centrifuging blood (10 min and 3500 rpm), the serum was separated and maintained at -20°C. Aseptically, by creating fissure in the lower abdomen, the left and right testis and right epididymis were removed. Testis were weighted by use of Sartorius scale separately, with /0001 carefully. To investigate the possible effect of the drug on testicular weight, gained weight was divided by animal body weight and the resulting number was multiplied by 100. Right testicle until starting experiment, was frozen at -20° C for daily sperm production and malondialdehyde enzyme, the left testis for histological examination was placed in Bouin fixative solution.

Viability

One centimeter distal epididymis, immediately after removal from the body was cut with sterile scissors and placed in the isotonic solution (4ml phosphate-buffered saline) and with homogenizer machinethis tissue was homogenized and put for 5 minutes

at a room temperature of 37°C. This time is for complete removal of sperm from duct. Sample preparation was done using eosin-negruzyn dye and a drop of sperm suspension in the above steps. According to dye uptake by dead cytoplasmic cells, the produced was examined using light microscope and magnification of $\times 40$, and percentage of live sperms in comparison with total number of available sperms over the of view in 10 times field was calculated [4].

Evaluate Percentage of Sperm Motility

A drop of sperm suspension that had been obtained in the previous step was put on a slide, then by light microscope, two binoculars made by America LABOMED company equipped with a camera filmed 5 field microscope with a magnification of $40\times$ and, in a review of the film on computer, the movement of sperm was checked and the average movement was recorded as motility percent.

Counting of Sperm

A 10 microliter drop of sperm suspension was obtained during previous steps was taken and put on a slide and the total number of spermatozoa in four large slides (four slides white blood cells count) were counted and the average sperm count was obtained on one slide. Finally, the total number of voided sperms from one centimeter of distal

epididymal duct were obtained from the following formula,

$$A = BC+D$$

Where; A= total number of voided sperms from one centimeter of epididymal duct; B= number of counted sperms in 0/1 cubic millimeter of solution (big home consists of 1mm long , 1 mm wide and 0/1 mm depth, as a consequence, volume of solution filling one large home is 0/1 cubic millimeter); C= factor of depth 10; D= the dilution factor is 4000, because the sperm were placed in 4 ml of solution with saline pherphosphate

Daily Sperm Production (DSP)

After removing the right testis from the freezer and deleting capsule, a piece of testicular parenchyma was homogenized for 4 minutes in 2 ml of normal saline in a homogenizer machine at low rate. From the fluid of homogenized testis a dilution of 0/1 was prepared and a drop of the resulting solution was placed on a slide. Then, by using a light microscope and with magnification of 40× the number of sperms was counted. Since in rats spermatozoons growth takes almost 6/3 day during spermatogenesis, after counting total number of spermatozoons it was split up on 6/3 number to achieve production in one day [4].

Investigating Amount of Malondialdehyde Enzyme in Testis Tissue

With spectrophotometer device (model UNICO making America) and Hadley and Darper method, and based on the reaction

with thiobarbituric acid, malondialdehyde enzyme was measured. The remaining piece of testis tissue was placed in 6 cc buffer phosphate homogeny and then placed in a homogenizer machine with low rpm for 4 minutes. The tube containing the suspension (first examination tube) was then placed in centrifuge machine at 1000 rpm for 5 minutes. Next, 5 cc of this solution was placed in the second tube and 2/5 cc trichloroacetic acid solution was added. Test tube number 2 was placed in boiling water for 15 minutes. After cooling the was tube centrifuged at 1000 rpm again and 2 cc of supernatant solution was removed. Tube number 3, with 1 cc thiobarbituric acid solution at a concentration of 6/7 grams per liter was added and this tube was then placed in boiling water for 15 minutes. After cooling the tube at a wave length of 532 nm. A concentration of malondialdehyde enzyme was obtained using a spectrophotometer machine. Concentration of the above enzyme by absorption coefficient of complex of malondialdehyd ethiobarbituric acid equal to $1/56 \times 10^5$ as Nano gram per milligram of protein was calculated.

Measurement Method of Hormones Testosterone and LH

Testosterone and LH were measured using ELISA kit (DRG German Company). In brief,

the samples were dumped in a well and solution was added, it was incubated for 90 minutes at 37°C. The wells were then washed with deionized water and the substrate was added to each well and incubated for 20 minutes. Reaction was obtained by adding stop solution, the reaction was stopped and absorbance at wavelength of 450 nanometer was measured.

Histological Examination of Testis

Left testicle placed in bouin fixative solution was extracted from solution and the tissue molded with paraffin, then using cut slice thickness of 5 microns was produced and stained with hematoxylin-eosin method. Stained slides were investigated using light microscope and by a person who did not know about group: Number of cells of spermatogonia, primary spermatocytes, sertoli, leydig, spermatids, and spermatozoa. In each sample 12 seminiferous tubes with magnification×100 under light microscope were examined. Obtained values were expressed as mean number of cells per tube.

Statistical Analysis

For data analysis SPSS software version 16 was used, and one way ANOVA and Duncan test were used to compare groups. Based on Duncan test, if in each group there is a common goal, there is no significant difference. $P \leq 0.05$ was considered significant.

The means and standard deviations of data were calculated.

RESULTS

Body Weight and Proportion of Testis Weight to Body Weight

Findings related to body weight and proportion of testis weight to body weight is shown in **Table 1**. Based on these findings and comparing body weight average and also average of weight changes of right and left testis in comparison with body weight indicates that resistance to insulin, vitamin E, sodium selenite, causes a decrease in weight in different groups, but this decline does not show significant difference.

Average of Sperm Count

Insulin resistance causes significant decrease in the number of sperms, but vitamin E and sodium selenite both alone and also together could cause significant increase in number of sperms in comparison with control group (**Table 2**).

Viability, Daily Sperm Production (Dsp)

Insulin resistance causes significant decreased percent of live spermatozoa and DSP, but vitamin E and sodium selenite together could cause significant increase in percent of live spermatozoa and DSP in comparison with control group (**Table 2**). Also, statistical analysis shows significant decrease in the percentage of live sperms compared with total

number of available sperms in visual field (Table 2).

Percentage of Sperm Motility

Based on Table 2, resistance to insulin causes significant decrease, but vitamin E and sodium selenite together cause significant increase in the percentage of sperm motility compared with control group.

Average Tissue Concentration of Malondialdehyde Enzyme

Insulin resistance causes significant increase in tissue concentration of malondialdehyde enzyme, while a significant decrease in enzyme concentration of fructose group in combination with vitamin E seen sodium selenite (Figure 1).

Mean Serum Concentration of Hormones Testosterone and LH

Based on Figure 1, significant change in serum concentration of testosterone hormone and in

hormone LH was pointless.

Histological Examination of Testis

According to Table 3, it can be seen that insulin resistance causes significant decrease in number of spermatogonia cells, sertoli and leydig, but vitamin E and sodium selenite together could cause significant increase in the number of spermatogonia, sertoli and leydig cells in comparison with control group. Also, it is observed that insulin resistance causes significant decrease in the number of primary spermatocytes, spermatids, and spermatozoa cells, but vitamin E and sodium selenite both alone and also together could increase the number of primary spermatocytes, spermatids, and spermatozoa cells compared with control group (Figures 2-6).

Table 1: The Values (Mean \pm SEM, n=7 Each) of Body and Testes Weights of Control Rats, and Rat Streated with Fructose10 % (Sham), Sodium Selenite, Sodium Selenite + Vitamin E, or Vitamin E

Treatment	Initial Body Weight (g)	Final Body Weight (g)	Testis Weight Left / Body Weight (%)	Testis Weight Right/ Body Weight (%)
Control	205 \pm 2.2	269 \pm 2.3	1.38 \pm 0.02	1.36 \pm 0.02
Sham	188 \pm 10.5	229 \pm 12.1	1.38 \pm 0.03	1.33 \pm 0/03
Sodiu	224 \pm 3.4	259 \pm 7.2	1.47 \pm 0.04	1.38 \pm 0.05
Sodiu+ vit E	170 \pm 3.0	196 \pm 5.5	1.28 \pm 0.00	1.28 \pm 0.00
vit E	195 \pm 4.5	226 \pm 6.0	1.31 \pm 0.00	1.30 \pm 0.00

NOTE: Sodiu:Sodium selenite; vit E: vitamin E

Table 2: The Values (Mean \pm SEM, n=7each) of Sperm Parameters of Control Rats, and Rats Treated With Fructose 10% (Sham), Sodium Selenite, Sodiumselenite+Vitamin E, or Vitamin E

treatment	Sperm Count(%)	Viability (%)	DSP (No. \times 10 ⁶ /gr testis)	Sperm motility (%)
Control	32.5 \pm 0/66	77/57 \pm 1.75	115.57 \pm 2.60	28.71 \pm 0.97
Sham	24.84 \pm 1.2*	63.57 \pm 1.55*	89.71 \pm 1.01*	24.46 \pm 1.42*
Sodiu	35.19 \pm 0.57#	120.85 \pm 2.95#	112.85 \pm 2.63#	27.90 \pm 1,02#
Sodiu +vit E	35.91 \pm 0.72#	176.14 \pm 3.19#	205.14 \pm 4.29#	27.07 \pm 0.50#
vit E	32.97 \pm 1.11#	99.28 \pm 2.47#	139.85 \pm 3.63#	26.23 \pm 1.00#

NOTE: Sodiu:Sodium Selenite; Vit E: Vitamin E; DSP: Daily Sperm Production;*Indicate Significant Difference From the Control Group; #Indicate Significant Difference From Treated With Sham Alone

Table 3: The Values (Mean \pm SEM, n=7 Each) of Testes Cells of Control Rats, and Rats Treated With Fructose 10 % (Sham), Sodium Selenite, Sodium Selenite+Vitamin E, or Vitamin E

Treatment	Spermatogonia	Primary spermatocytes	sertoli	spermatids	spermatozoa	leydig
Control	87.85 \pm 1.80	189.00 \pm 4.30	40.14 \pm 0.91	118.28 \pm 1.30	77.57 \pm 1.75	34.42 \pm 1.23
Sham	73.57 \pm 1.81*	68.14 \pm 3.88*	39.71 \pm 1.5*	99.28 \pm 2.36*	63.57 \pm 1.55*	33.28 \pm 1.30*
Sodiu	80.28 \pm 0.83#	186.67 \pm 1.10#	40.42 \pm 2.43#	133.00 \pm 0.92#	120.85 \pm 2.95#	35.42 \pm 0.94#
Sodiu+ vit E	94.57 \pm 1.44#	08.14 \pm 2.06#	43.42 \pm 1.97#	265.43 \pm 2.76#	176.14 \pm 3.19#	38.28 \pm 0.71#
vit E	84.14 \pm 1.40#	187.85 \pm 1.58#	41.42 \pm 1.26#	165.28 \pm 3.21#	99.28 \pm 2.47#	34.85 \pm 1.10#

NOTE: Sodiu:Sodium Selenite; Vit E: Vitamin E;*Indicate Significant Difference From The Control Group; #Indicate Significant Difference From Treated With Sham Alone

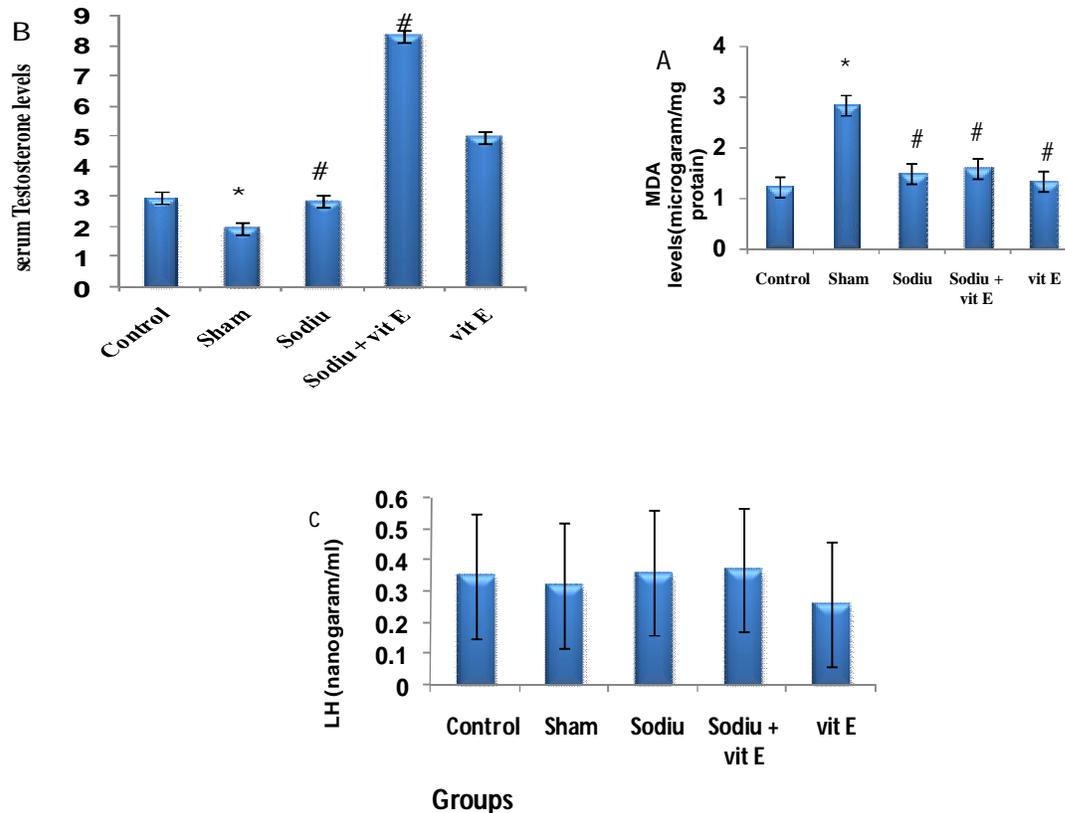


Figure 1: Concentrations (mean \pm SEM, n=7 of Malondialdehyde (MDA) (A), Serum Testosterone (B), Lutein Hormone (LH) (c) in the Control Rats and Rats Treated With Fructose10% (Sham), Sodium Selenite, Sodiumselenite + Vitamin E, or Vitamin E.*Indicate Significant Difference From the Control Group; #Indicate Significant Difference From the Group Receiving Sham Alone

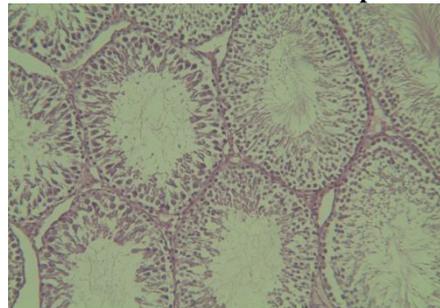


Figure 2: Photo Micrograph of the Seminiferous Tubul in Control Group, Staining With Hemotoxylin- Eosin, Magnification of \times 100, Wave Mode and the Reproductive Corrugated Epithelium of the Tubes 4 to 8 Cell Layer

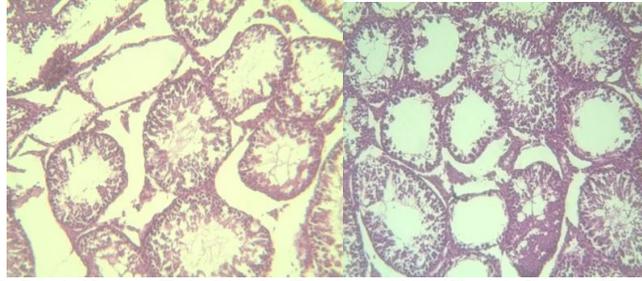


Figure 3: Photo Micrograph Seminiferous Tubul in the Fructose 10% (Sham) Group, Hematoxylin and Eosin Staining, Magnification $\times 100$, Spermatogenesis is Stopped at the Stage of Primary Spermatocytes, Thickening of the Interstitial Space is Visible Due to Fluid Retention (Edema)

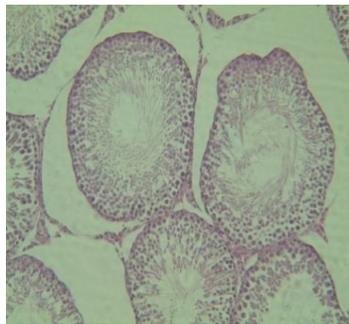


Figure 4: Photo Micrograph Seminiferous Tubular in Sodium selenite (Experiment 1), Stained with Hematoxylin and Eosin, Magnification of $\times 100$, Most of the Tubes are Rich in Reproductive Epithelium and Sperm Cells

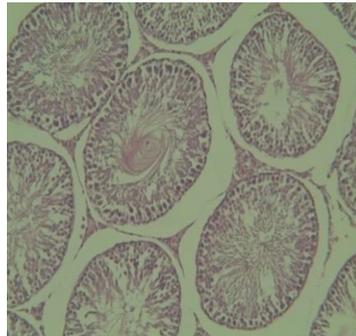


Figure 5: Photo Micrograph Seminiferous Tubular in Sodium Selenite+ Vitamin E (Experimental 2), Hematoxylin and Eosin Staining, magnification $\times 100$, about of 7 Tubes Seminiferous, Perfectly Normal Epithelium in About 6 to 8 Layers

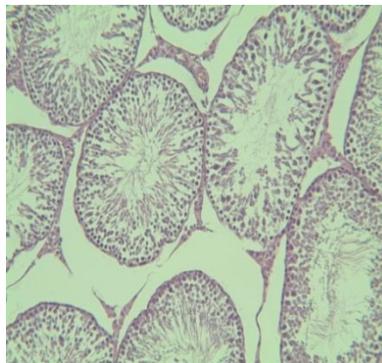


Figure 6: Photo Micrograph of Seminiferous Tubular in Vitamin E (Experiment 3), Hematoxylin and Eosin staining, Magnification of $\times 100$, Most Tubes Contains Multiple Layers of Reproductive Epithelium, Maturity Until the Final Stage of Sperm Production Continues so That all tubes Containing the Slides of the Lumen are Filled by These Cells

DISCUSSION AND CONCLUSION

According to studies, insulin resistance and increase in oxidative stress [6], and consequently, increase in malondialdehyde enzyme is the final product of lipid peroxidation by reactive oxygen species ROS [7] It can be concluded that production of ROS causes the cell cycle to stop and an increase in apoptosis process, thus causing a decrease in daily sperm production and also a decrease in total number of sperm [8-10]. According to this research and the fact that insulin resistance can cause the production of free radicals (ROS) in cells [11], it can be a reason why productions of free radicals in testis cells that are so sensitive cause their loss and a decrease in testis weight. Furthermore, one of the possible causes in atrophy of testes is unknown factors that may interfere in spermatogenesis, consequently, with reducing number of sexual cells, testes weight will decrease. In addition, other studies have found that production of ROS causes reduction in the quantity and quality of semen [11, 12], and by increasing penetration of cell cause loss of sperm life [13-14]. Other studies found that ROS is produced from two different sources in sperm liquid called damaged spermatozoa cells and active white leukocytes, the high amount of which causes disorder in DNA structure, reducing the

percentage of live sperms, and non-sperm binding to the egg surface causes infertility in men [15, 16]. Kobayashi *et al.*, 2001, showed that a progressive reduction in the number of live sperm cells is associated with increase in ROS [17]. Reactive oxygen species and malondialdehyde (MDA) increased enzyme activity in the cell membrane is impaired and order so that the concentration gradient of the ion transport processes in the membrane is disturbed, as well as transfer chemical messenger

[16, 18]. Sperm is sensitive to oxygen of lipid peroxidation and can damage the membrane and reduce its fertility [15, 19]. Studies show that reactive oxygen species produced by leukocytes and spermatozoa in infertile men have a lethal effect on sperm activity. Lipid peroxidation causes abnormality in central part of sperm and loss of acrosome capacity in fertilization [20]. Malondialdehyde molecules with intrusion in membrane structures of the cell causes asymmetry in the distribution of the lipid components, and in addition to creating a strong bond to the DNA of cells, causes damage and breakage of the chromosome [18]. The inverse relationship between serum MDA levels and sperm adhesion to the oocyte cell has been established in 1992 by Letken *et al.*, [21]. So ROS and malondialdehyde cause reduction of

sperm quality which has been observed with insulin resistance and oxidative stress [15, 18]. According to these researches and also publications published by The Center of Disease Control and Protection, and the expressed chemical materials disruption of endocrine system (EDC), by producing free radicals (ROS) are capable of oxidative damage to biomolecules such as DNA and proteins [11]. It is likely that insulin resistance caused free radicals (ROS) and an increase in mutation of testis tissue, especially sensitive cells such as spermatogonia, primary spermatocytes, spermatids, and spermatozoa, causing serious injury and loss of these cells. However, given that in this research insulin resistance causes a decrease of testosterone hormone (it is not significant decrease), the possibility of loss of germ cells exists, so there is a risk of damage and loss of interstitial cells and sertoli in this way. Also, leydig cells function is affected by sertoli cells [10]. Vitamin E or alpha-tocopherol due to lipid solubility can prevent destructive effects of ROS on sperm parameters [20]. Because alfa-tocopherol molecules as a breaking antioxidant of chain can inhibit two lipid proxils, it inhibits two potential reactions of peroxidation [22]. Selenium is a component of antioxidant enzymes such as glutathione peroxidase that

play a role in neutralizing free radicals and removing oxidative stress

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