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The association between reactive oxygen species and α-tocopherol treatment in a sample of infertile Iraq male

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Abstract
Male infertility estimated about 20-70% of couples or >30 million men worldwide. The goal of this study was to elucidate the interrelations between oxidative stress (O.S) in seminal fluid and antioxidant inducing disruption of spermatogenesis. Twenty four infertile patients aged (23-41) years were involved in this study. Which were received α- tocopherol (vitamin E). This group (vit E group) was sub classified according to the WHO criteria (2010) into three different subgroups: oligoasthenoteratozoospermia (OAT) Asthenoteratozoo- spermia (A.T), and Asthenozoospermia (Astheno.). The control group consist of samples obtained from healthy donors of proven fertility (n=23). The main parameters were determined in seminal fluid of infertile patients and healthy men are the sperm and seminal plasma Malondialdehyde (MDA) concentration, and vitamin E concentration in seminal plasma. The results showed that, there was a high significant decrease ($p \leq 0.001$) in sperm parameters (concentration, progressive motility and normal morphology), and vitamin E concentration in infertile group compared with that fertile group.After analyzing the statistically difference in the different measured parameters between the vit E group after treatment, in comparison with that of before treatment; the following was observed A high significant increase ($p \leq 0.001$) were found in progressive motility, sperm [MDA] (MDAp), vitamin E concentration, and significant increase ($P \leq 0.05$) in the sperm concentration. From all of these observations, it can be concluded that O.S contributes most likely to sperm damage and may be responsible for poor seminal fluid characteristic of the infertile patients involved in the present study. Antioxidants α-tocopherol (vitamin E) plays an important role in the improvement of seminal fluid parameters.

Keywords: Infertility, oxidative stress, ROS, α- tocopherol

Introduction
Infertility is a world health problem that affects an estimated 15% (1 out of 7) couples worldwide, and can be caused by abnormalities in generating of viable gametes, and defects in fertilization and embryonic development [1] Many studies in the area of male infertility have been focused on oxidative stress-related mechanisms of sperm damage, Oxidative stress occurs when cells no longer have the antioxidant capacity to manage the excess production of ROS [2]. However, high generation of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) by immature and abnormal spermatozoa, contaminating leukocytes, sperm processing (excessive centrifugation, cryopreservation / thawing), accompanied by low scavenging and antioxidant levels in blood serum and seminal plasma, will induce a state of oxidative stress. Therefore, seminal oxidative stress (OS) develops as a result of an imbalance between ROS generating and scavenging activities [3-4]. On the other hand, human spermatozoa are capable of generating controlled low amounts of endogenous ROS, which play a significant role inducing sperm capacitation/acrosome reaction and acquisition of sperm-fertilizing ability [5]. The tocopherols, specifically α-tocopherol (vitamin E), have been studied extensively in mammalian researches as membrane stabilizers and antioxidants that scavenge oxygen free radicals, lipid peroxy radicals, and singlet oxygen. The antioxidant properties of α-tocopherol are the result of its ability to quench both singlet oxygen and peroxides [6-7].

Eight fat-soluble substances, including tocopherols and tocrerinol, make up vitamin E. [8]. Tocopherol, one of these types, is popular in North American diet and is present in maize oil, soybean oil, ghee, and dressings. The second type of vitamin as a fat-soluble antioxidant, alpha-tocopherols is one of the most physiologically active tocopherols in North America because they prevent the formation of reactive oxygen species when lipids are subjected to oxidation [9]. The eight vitamin E forms are divided into two categories; four of them are tocopherols and the other four are tocotrienols, and they are identified by the prefixes α, β, γ- and δ [10].

Materials and Methods

Sample collection: The current study involved a control group (n=23), who consisted of sample obtained from healthy donors of proven fertility and normal genital examinations, and a patients group (n=24), who had been unable to initial pregnancy during a period of at least one year of unprotected sexual intercourse and diagnosed according to the protocol described by WHO (WHO, 2010). Both groups their ages ranged from (23-41) years. The patient' group (vit E group) were given a tablet of vitamin E (α- tocopherol) 400 mg/daily for three months. Which was sub divided into three different subgroups: oligoasthenoteratozoospermia (OAT), Asthenoterato-zoospermia (A.T) and Asthenozoospermia (Astheno.). The exclusion criteria which were considered excluding patients with diabetes, high blood pressure or leucopenia in this study.

Standard Semen Analysis

Freshly ejaculated semen samples were obtained by masturbation after (3-5) days of sexual abstinence. The specimens were placed in an incubator at 37 °C for (15-30) minutes to allow liquefaction, after liquefaction, semen samples were evaluated for semen volume, appearance.pH and viscosity. Then specimens were analyzed for sperm concentration, progressive motility and normal morphology according to WHO criteria (WHO, 2010).

Measurement of Malondialdehyde (MDA) in Semen Fluid.

Malondialdehyde (MDA) levels were measured in both sperm and seminal plasma according to method described by Rao *et al*., 1989; MDA was assessed using the thiobarbituric acid reaction (TBAR) method. [11], as follows:

Sperm MDA assay. (MDAp)

A volume of 1 ml of seminal plasma was centrifuged at (400xg) for sperm 10 minutes. After washing twice in a solution of sodium chloride (Nacl), (0.9%), the sperm pellet was re-suspended in 1ml of distilled water into a glass tube. Another 1ml of distilled water was transferred to another glass tube that served as a blank. To each of the tubes (blank and assay) 500µl of TBA (Thiobarbituric acid,) reagent was added. Each tube was then covered with foil and heated for 1h in a boiling water both. After cooling, each tube was centrifuged to 10 minute at (4000xg) and the absorbance of the supernatant was read on a spectrophotometer at wavelength of 534nm.

Seminal plasma MDA assay. (MDAs)

MDA level were analyzed using (Reo *et al*, 1989); method with a slight modification. This modification involved using 0.5 ml of seminal plasma rather than 0.1ml as suggested in the original paper. A volume of 0.5 ml of seminal plasma was added to 0.5 ml distilled water in a glass tube. To each tube, 0.5 ml of thiobarbituric acid reagent was added and then heated for 1 hour in a boiling water bath. After cooling, each tube was centrifuged for 10 min. at 4000xg and the supernatant absorbance was measured at of 534 nm.

Vitamin E (α-tocopherol) Measurement

In this study, a simple and rapid high performance liquid chromatography (HPLC) was used for determination of vitamin E (α-tocopherol) concentration in human semen plasma a according to Catignani and Bieri, 1983 method [12]. To prepare a 5g / one litter stock standard vitamin E solution. 0.05 grams of standard vitamin E (α-tococpherol) was dissolved in 10 ml of ethanol. The solution was thoroughly mixed using vortex until complete dissolution of the vitamin E. A working solution was prepared by dilution of each of above standard solution 100-fold with ethanol. The concentration of the working solution was then calculated by HPLC.A series of calibrators was then prepared with the appropriate volume of ethanol to final vitamin E (α– tocopherol) concentration of µmol/L. A standard curve was plotted between α-tocopherol standard concentration against peak area of each standard concentration.The concentration of vitamin E is generally reported in µmol/L after appropriate correction for sample size dilution.

Statistical Analysis

Statistical analyses were performed using the SSPS for windows release 21. All values were given as mean with corresponding standard error. (Mean±S.E) student's depended up on tests, (Tukey's test for multiple comparisons) by ANOVA table of variance were used for data analysis. Correlations between individual variables were examined by Pearson correlation coefficient.

Results

Conventional semen Analysis

The results of certain sperm function parameters between the infertile and healthy control groups as shown in Table (1-1).

Table 1: Comparison the certain sperm function parameters between infertile group and the control groups.

Group	Age(y) (Mean ± SE)	Semen volume (mL) (Mean ± SE)	Sperm concentration (M/mL) (Mean ± SE)	Progressive motility (%) (Mean ± SE)	Morphologically abnormal sperm (%) (Mean ± SE)
Control group	33.4±1.7	3.2±0.18	69.4±3.6	64.2±2.5	67±2.4
Infertile group	32.5±2.1	2.77±0.12	13.3±3.4	24.1±1.5	85.14±1.36
P- value	0.7	0.8	0.001	0.001	0.001

Data were expressed as Mean ± SE; Statistical analyses were performed by ANOVA (2-tailed) and followed by Tukey's test for multiple comparisons. * $p<0.05$ = Significant values. ** $p<0.01$ = Significant values.

The results of sperm function parameters in Vit E group before and after treatment present in (Table 1-2).

Table 2: Results of certain sperm function parameters comparison among the different Vit E subgroups before & after treatment.

sperm function parameters		O.A.T (n=7)	A.T (n=7)	Astheno. (n=10)
Age(y) (Mean ± SE)		32.4±2.8	31.9±2.4	29.9±2.2
semen volume (mL) (Mean ± SE)	Before	3.0±0.3	3.0±0.3	3.6±0.1
	After	2.9±0.4	2.9±0.2	4.1±0.2
	P- value	0.9	0.7	0.06
sperm concentration (M/mL) (Mean ± SE)	Before	10.5±2.4	35.8±3.6	63.6±6.2
	After	16.1±1.9	39.2±4.8	68.2±7.4
	P- value	0.03*	0.4	0.2
Progressive motility (%) (Mean ± SE)	Before	19.4±4.0	24.2±3.1	31±2.1
	After	24.8±4.7	39.7±3.2	44.2±4.0
	P- value	0.18	0.005**	0.05*
Morphologically abnormal sperm (%) (Mean ± SE)	Before	96.7±1.76	95.7±1.4	70.1±3.4
	After	91.4±1.1	82.8±2.6	74.1±3.9
	P- value	0.2	0.008**	0.75

Data were expressed as Mean ± SE; Statistical analyses were performed by ANOVA(2-tailed) and followed by Tukey's test for multiple comparisons. * $p<0.05$ = Significant values. ** $p<0.01$ = Significant values; OAT: oligoasthenoteratospermic, Asth: asthenozoospermic patients; AT: asthenoteratospermic, patients.

Measurement of Malondialdehyde (MDA)

Sperm and seminal plasma samples' MDA levels were assessed using the 2-thiobarbituric acid method (Rao *et al*., 1989), in order to evaluate the oxidative stress detected in patients with different types of infertility present in studied patients. The results of Total MDA, MDAp (In sperm) and MDAs (In semen plasma) in patients groups were (1.486±0.13,1.42±0.12 and 0.05±0.01 respectively) whereas their concentration were found in control group (0.25±0.016, 0.243±0.015 and 0.009±0.002 respectively). It is clear that, there were a highly significant decrease in total MDA, MDAp values and a significant decrease in MDAs values of patient's group comparison with that of the control group.

The results of vit E subgroups indicated presence of that no significant decrease in Total MDA, MDAp and MDAs in O.A.T. subgroup, while it was significantly decreased in Total MDA and a highly significant decrease of MDAp in A.T. of vit E subgroups after treatment when compared with that of before treatment. (Table: 1-3) Which the other subgroups of vit E group, no significant difference were found in Total MDA, and MDAs, whereas significant decrease was percent in MDAp in asthenospermia of vit.E subgroup after treatment compared with that of before treatment.

Table 3: comparison the result of Total MDA, MDAp, and MDAs (µmol/sperm) in seminal fluid among the different Vit E subgroups before and after treatment.

Vit.E (αtocopherol) Subgroups	Biochemical Parameters	Before (Mean ± SE)	After (Mean ± SE)	P value
OAT	Total MDA (µmol/sperm)	2.3±0.2	2.5±0.2	0.1
	MDAp ((µmol/sperm)	2.3±0.2	2.1±0.3	0.2
	MDAs (µmol/sperm)	0.1±0.04	0.07±0.003	0.1
A.T	Total MDA (µmol/sperm)	1.0±0.2	0.8±0.1	0.02*
	MDAp (µmol/sperm)	0.9±0.1	0.7±0.1	0.003**
	MDAs (µmol/sperm)	0.04±0.02	0.01±0.002	0.2
Astheno.	Total MDA (µmol/sperm)	0.9±0.1	0.5±0.08	0.07
	MDAp (µmol/sperm)	0.7±0.06	0.5±0.07	0.03*
	MDAs (µmol/sperm)	0.02±0.008	0.008±0.008	0.12

Data were expressed as Mean ± SE; Statistical analyses were performed by ANOVA (2-tailed) and followed by Tukey's test for multiple comparisons. * $p<0.05$ = Significant values. ** $p<0.01$ = Significant values; OAT: oligoasthenoteratospermic, Asth: asthenozoospermic patients; AT: asthenoteratospermic, patients. n= indicates the number of cases.

Percentage of MDA (%)

The result (Mean ± SEM) of MDAs percent in patients group were (3.42±0.8) and MDAp percent (96.5±0.8), whereas in control group the values of percent were found to be (3.52±0.5) in MDAs and (96.3±0.5) in MDAp. The results showed no significant difference between the percentage of MDA (%) of patients group and control group. The results of the MDAs and MDAp percentages of vit E group is presented in (table 1-4).

Table 4: Result of MDAp (%) percent and MDAs (%) percent in vit E group

Vit. E group	Before	After	P- value
MDAp%	95.9±1.17	97.9±0.9	0.9
MDAs%	4±1.17	2±0.9	0.9

Measurement of Vitamin E Concentration

the concentration of vitamin E was measure using HPLC, the results of concentration vit E in semen plasma of patients group was found to be (2.13±0.11), whereas (3.41±0.12) in control group. A highly significant increase was observed in concentration vit E (α-tocopherol) after treatment when compared with that before treatment (2.13±0.1, vs. 2.52±0.68; $p\leq0.001$ respectively).The results present in (Table 1-5), shows that no significant difference is present in concentration vit E of semen plasma between different subgroups of vit E group, except Astheno. Subgroups, it was found that a significant increase in concentration vit E values after treatment, compared with that before treatment.

Table 5: Result of concentration α-tocopherol (µmol/l) in seminal fluid comparison between infertile patients of vit E group before and after treatment.

Subgroup	Before (Mean ± SE)	After (Mean ± SE)	*P value*
O.A.T	**1.8±0.11**	**1.9±0.1**	**0.1**
A.T.	**2.1±2.18**	**2.5±2.5**	**0.07**
Astheno.	**2.2±0.15**	**2.8±0.12**	**0.04***

Data were expressed as Mean ± SE; Statistical analyses were performed by ANOVA (2-tailed) and followed by Tukey's test for multiple comparisons. * $p<0.05$ = Significant values. ** $p<0.01$ = Significant values; OAT: oligoasthenoteratospermic, Asth: asthenozoospermic patients; AT: asthenoteratospermic, patients. n= indicates the number of cases.

The correlations between biochemical parameters and different standard semen characteristic in Vit E group before and after treatment are show in table (1-6).

Table 6: a) Correlation between biochemical parameters and different standard semen characteristic in Vit E group before treatment.

Biochemical parameters	Sperm concentration (M/ml)	Progressive motility (%)	Aormal morphology (%)
	r p	r p	r p
Total MDA (µmol/sperm)	- 0.562 0.004**	- 0.429 0.03**	- 0.318 0.3
MDAp (µmol/sperm)	- 0.778 0.0001**	- 0.583 0.003**	- 0.405 0.05 *
MDAs(µmol/sperm)	- 0.562 0.004**	- 0.529 0.008**	- 0.231 0.2
Vitamin E concentration (µmol/l)	0.308 0.1	0.178 0.7	0.461 0.06

**Correlation is significant at the 0.01 level (2-tailed).; *Correlation is significant at the 0.05 level (2-tailed)

Table 6: b) Correlation between biochemical parameters and different standard semen characteristic in Vit E group after treatment.

Biochemical parameters	Sperm concentration (M/ml)	Progressive motility (%)	Normal morphology (%)
	r p	r p	r p
Tota MDA(µmol/sperm)	0.791 0.0001**	0.695 0.0001**	0.384 0.06
MDAp(µmol/sperm)	0.774 0.0001**	0.624 0.003**	0.395 0.07
MDAs(µmol/sperm)	0.629 0.01*	0.557 0.005**	0.202 0.3
Vitamin E concentration (µmol/l)	0.550 0.005**	0.454 0.001**	0.296 0.1

**Correlation is significant at the 0.01 level (2-tailed).; *Correlation is significant at the 0.05 level (2-tailed).

Discussion

In recent years the generations of ROS (reactive oxygen species) in male reproductive tracts have become a real concern because of their potential toxic effects at high levels. Which are short-lived, unstable, and highly reactive species containing at least one oxygen atom, are able to snatch electrons from other molecules to achieve an electronically-stable state. In this process, the other molecule loses an electron following which a new radical is formed. Subsequently, this radical reacts with another neighboring molecule, thus passing on the radical status via a reaction called 'radical-chain reaction' until two radicals react with one another forming a stable bond. These reactions amplify the degree of alterations in the cellular structures [13]. These free radicals induced oxidative damage to spermatozoa; this in turn causes poor sperm function and infertility [13-14]. Polyunsaturated fatty acids (PUFAs) have been hypothesized to make up a significant portion of the plasma membrane of spermatozoa and their cytoplasm has a low level of scavenging enzymes. They are consequently more vulnerable to the harm caused by oxidative stress [15]. Increased cell permeability, enzyme deactivation, structural DNA damage, and cell death are all consequences of lipid peroxidation (LPO), which causes the loss of membrane integrity [16]. Malondialdehyde (MDA), one of the byproducts of LPO, has been utilized in biochemical assays to measure the level of oxidative damage to spermatozoa [17].

When the MDA concentration was measured throughout the current study in the sperm (MDAp) and seminal plasma (MDAs), the results (Table 1-3) indicated that MDA concentration (represented as percentage) was higher in the sperm, than in the seminal plasma in both fertile and infertile groups. The sperm plasma membrane contains a high amount of PUFA s, which is vulnerable to attack by ROS [18-19]. ROS in the seminal fluid has been reported to originate from two sources leukocytes & immature spermatozoa [19]. Since the patients with leukocytspermia were excluded from the present study, it can be concluded that the source of ROS in the semen sample of the present study was the immature spermatozoa.

α-tocopherol (vitamin E) which is well –documented antioxidant and has been shown to inhibit free radical-inducing damage to sensitive cell membrane [21] In the other hand, the use of vitamin E *in vitro* has been also documented to improve sperm motility and viability [21], Whereas in other studies there was no effect of even higher level of vitamin E (600-1200) mg/dl for three weeks [22], alone or in combination with vitamin C [23].

To investigate the role of vitamin E in the O.S present in the studied infertile group (vit E group), the [MDA] level were measured in vit E group before and after oral administration of vitamin E in a dose of 400mg/dl for 3 months. Vitamin E is considered as the major chain breaking antioxidant in the sperm plasma membrane. Because of its presence within plasma membrane, It neutrale hydrogen peroxide H2O2, superoxide anion ($O2^{\cdot}$) and hydroxyl radical ($HO^{\cdot}$), and protect the plasma membrane from LPO by scavenging peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals there, by breaking the chain reaction [21-24].The observed highly significant increase ($p \leq 0.001$) in vitamin E concentration in vit E group and the significant increase observed ($p \leq 0.05$) in the Astheno. Subgroup (Table 1-3) after such treatment in comparison with that of before treatment improved semen parameters. Therefore, a highly significant increase in progressive motility, and significant increase in sperm concentration in vit E group was observed in the present study, with presence of non-significant difference in normal morphology. As well, positive correlation with a highly significant difference ($p \leq 0.001$) was detected between vitamin E concentration and each of: sperm concentration& progressive motility (Table: 1-5). Therefore, administration of vitamin E may enhance fertility by decreasing free-radical damage detected by the reduction of MDA concentration [25-27].

Conclusions

Oxidative stress (O.S) contributes most likely to the damaged sperm in the patients included in the present study and the conclusion achieved is based on the following results; The higher level of ROS (MDA concentration) is present in infertile patients than normal group.as well as highly significant decrease in antioxidant capacity α-tocopherol (vitamin E) in seminal plasma of infertile patients group when compared with that of control group, this contribution is through impairment of semen parameters

which include (sperm concentration, progressive motility and normal morphology) of infertile patients group. Oral administration α-tocopherol (vitamin E) contributed most likely to improved sperm motility and sperm concentration after treatment, by reducing MDA concentration in infertile patients.

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