Zinc supplementation attenuates lipid peroxidation and increases antiperoxidant activity in seminal plasma of Iraqi asthenospermic men

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Abstract: Oxidative stress and impaired antiperoxidant levels have been proposed as a potential factors involved in the pathophysiology of diverse male infertility types, including asthenospermia. The present study was conducted to study the effect of zinc supplementation on the quantitative and qualitative characteristics of semen along with antiperoxidant activity in the seminal plasma of asthenospermic patients. Semen samples were obtained from 55 fertile and 55 asthenozoospermic infertile men with matching age. The subfertile group was treated with zinc sulfate; every participant took two capsules per day for three months (each one 220 mg). Semen samples were obtained (before and after zinc sulfate supplementation). Total antiperoxidant activity and various sperm parameters were compared among fertile controls and infertile patients (before and after treatment with zinc sulfate). The mean antiperoxidant activity of fertile controls (59.4% in seminal plasma & 59.13% /10^6 spermatozoa in spermatozoa) was significantly higher than that of the infertile patient group (39.3% in seminal plasma & 47.74%/10^6 spermatozoa in spermatozoa) ($p<0.05$). Antiperoxidant activity levels were significantly elevated in the infertile group which treated with zinc sulfate (54.8% in seminal plasma & 68.69% /10^6 spermatozoa in spermatozoa) ($p<0.05$). On the other hand, antiperoxidant activity is positively correlated to sperm motility. Decreased antiperoxidant activity was associated with impaired sperm function. Conjugated diene hydroperoxide (CDH) was found to be increased significantly in the infertile patient group. Volume of semen, progressive sperm motility percentage and total normal sperm count were increased after zinc sulfate supplementation.
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Key words: zinc supplementation, oxidative stress, antiperoxidant activity, asthenozoospermia, seminal plasma.

1. Introduction

Male factor infertility is the character cause of subfertility in about 20% of infertile couples, and in 30% to 40% of couples both male and female factors contribute. Thus half of all infertility can be recognized in part or perfectly to the male factor (Patel and Niederberger, 2011). There are several causes leading to male infertility, like oxidative stress, and nutritional insufficiency of trace elements like, selenium and zinc (Wong *et al*., 2000; Olayemi, 2010). The zinc is the subsequent only to iron as the most abundant element in the body. Although, zinc is found in most types of meat and milk products; the World Health Organization (WHO) approximates that thirty percentage of world population is deficient in zinc (Khan *et al*., 2011). Zinc is a fundamental micronutrient essential for different biological functions in the human body. There are two types of zinc; the first is included in the muscle, most of which are inadequately exchangeable and closely bound to high molecular weight ligands such as metalloproteins, nucleoproteins and nucleic acids. The second type of zinc has full freedom to replacement and loosely linked to an amino acid and citrate (Outten and O'Halloran, 2001). Zinc plays an essential part in cell proliferation and differentiation by regulating several pathways like nucleic acid metabolisms, protein synthesis, growth hormone, prolactin and testosterone (Ozturk *et al*., 2005). Zinc is a structural component found in many enzymes necessary for DNA synthesis and transcription. It is key component in the zinc binding proteins of elementary transcription factors where these factors provide a platform for interaction with nucleic acids or proteins (Vallee *et al*., 1991).

The generation of reactive oxygen species (ROS) in the male reproductive tract has become an actual concern because of their probable noxious effects, at high levels, on physical properties of sperm quality (Sharma and Agarwal, 1996). Normal levels of ROS are essential to complete functionality of sperms. ROS play a vital role in sperm functions such as sperm maturation, sperm motility, sperm hyperactivation, sperm capacitation, acrosome reaction and sperm-Oocyte fusion (Kothari *et al*., 2010; Baumber *et al*., 2003; Roy and Atreja, 2008; Ickowicz *et al*., 2012; Keber *et al*., 2013). On the other hand, high levels of ROS can negatively affect sperm quality. Pathological roles of ROS include increment lipid peroxidation levels (Bhagavathy and

Sumathi, 2012), decrement sperm motility (De Lamirande and Gagnon, 1992), DNA damage (Shamsi *et al*., 2011; Gonzalez-Marin et al., 2012) and apoptosis (Suen *et al*., 2008; Izunya *et al*., 2010). The oxidative stress initiated sperm damage has been documented to be a significant contributing feature in about 80% of male subfertility cases (El-Tohamy, 2012). Subfertile males producing high levels of ROS are seven times less likely to develop a pregnancy compared with those who have low levels of ROS (Agarwal *et al*., 2006). The production of ROS can be aggravated by environmental, infectious, and lifestyle etiologies (Esteves and Agarwal, 2011). Figure (1) summarizes the positive and negative roles of ROS.

The lipid content of plasma membrane of mammalian spermatozoa consists of high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids therefore sperm cells are principally susceptible to the injure stimulated by excessive ROS production (Shinde *et al*., 2012; Pabst *et al*., 2012). All lipid components found in the sperm membranes are concerned with the regulation of developmental processes such as sperm maturation, spermatogenesis, capacitation, acrosome reaction and finally in membrane fusion (Tulsiani and Abou-Haila, 2012). The possible sources of ROS include activated leukocytes, immature spermatozoa, and morphologically abnormal spermatozoa. Undoubtedly, peroxidation of sperm lipids may also disturb all the particular sperm functions, and in severe cases even absolutely inhibit spermatogenesis (Tawfeek *et al*., 2006). On the other hand, every human ejaculate has enzymatic and non-enzymatic antioxidants to scavenge ROS as self-defense systems (El-Tohamy, 2012). The excessive ROS formation that arises in some pathological cases (e.g., Genital tract inflammation) results in seminal oxidative stress that may inhibit antioxidant levels. The result of peroxidation is determined among seminal lipids also by their elevated concentrations (Esteves and Agarwal, 2011).

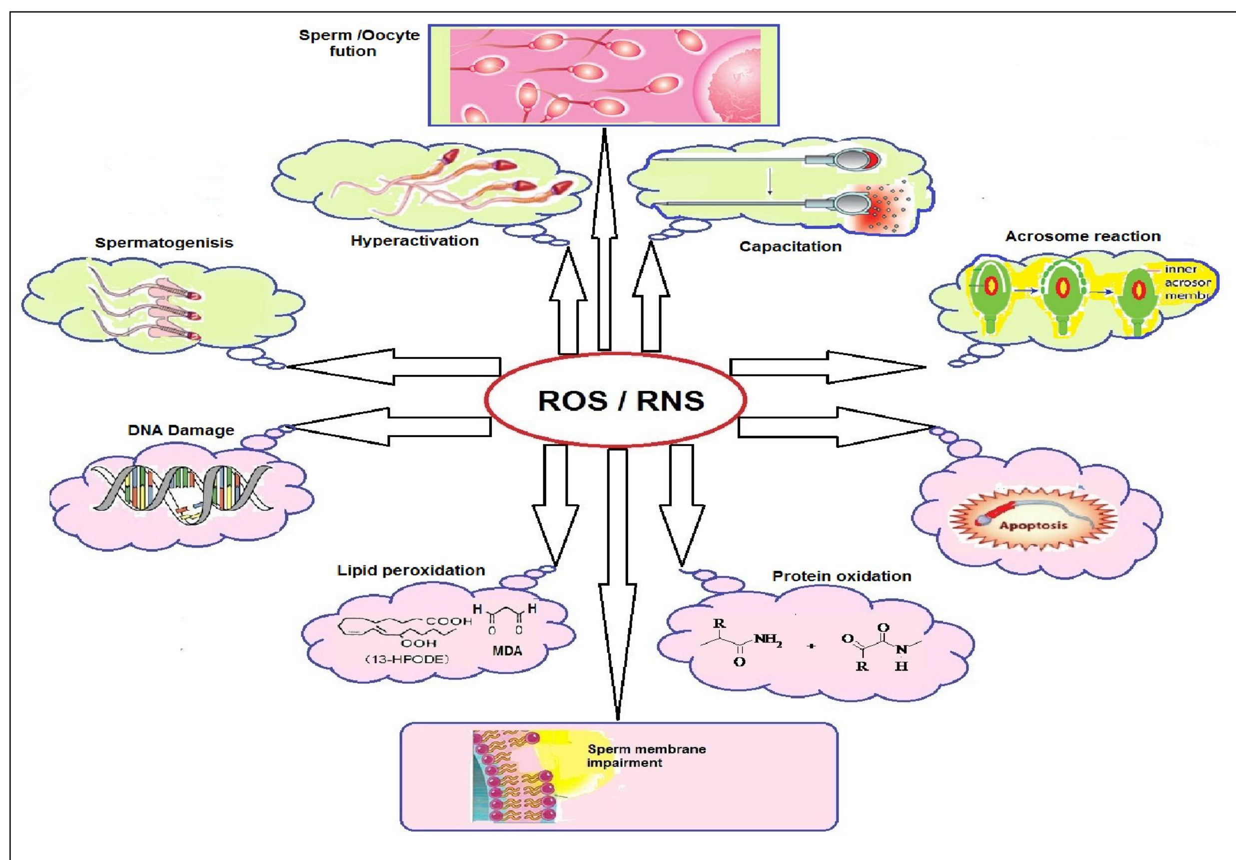

Fig. 1 Suggested Physiological and Pathological Roles of ROS.

The group of enzymatic and non-enzymatic systems that acts to prevent or to inhibit lipid peroxidation process called antiperoxidant (Strzezek *et al*., 2004). Table 1 shows enzymatic and non-enzymatic antiperoxidants.

Table 1: Constituents of Antiperoxidant Defense System, (A): Preventive antioxidants: act to convert peroxides to non-hazardous compounds such as water and alcohols. (B) Quenching of active oxygen: act to quench singlet oxygen. (C) Radicals-scavenging antioxidants: remove the harmful effects of reactive oxygen compounds.
(Palmier and Sbelendorio, 2007).

A) Preventive antioxidants:	
Glutathione peroxidase (cellular)	Decomposition of free fatty acid hydroperoxides: LOOH + 2GSH → LOH + H_2O + GSSG
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxides
Phospholipid hydroperoxide Glutathione peroxidase	PLOOH + 2GSH → PLOH + H_2O+GSSG
Glutathione-S-transferase	Decomposition of lipid hydroperoxides
Thioredoxin	Reduction of peroxides
B) Quenching of active oxygen:	
Carotenoids, vitamin E	Quenching of singlet oxygen
C) Radicals-scavenging antioxidants:	
Vitamin E, ubiquinol, carotenoids	Antiperoxidants scavenge radicals to inhibit the chain initiation and break chain propagation.

Although several studies have considered the relationship between infertility and semen lipid peroxidation levels, no study on the effects of asthenospermia treatments such as oral zinc supplementation on antiperoxidant activity which are important in fertility of the individual has been reported. The present study was conducted to study the effect of zinc supplementation on the quantitative and qualitative characteristics of semen along with antiperoxidant activity in the seminal plasma of asthenospermic patients.

2. Methods

2.1. Patients

This study includes 55 fertile (age 33.5±3.7 year) and 55 subfertile (age 32.8±3.57 year) men with asthenozoospermia between July 2011 to June 2012, from couples who had consulted the infertility clinic of the Babil hospital of maternity (Hilla city/ IRAQ). The approval of the institutional research ethics committee, and consent of every patient included in the study was obtained. A detailed medical history was taken and physical examination was performed. Subjects currently on any medication or antioxidant supplementation were not included. The inclusion criteria were asthenozoospermia, the absence of endocrinopathy, varicocele, and female factor infertility. Smokers and alcoholic men were excluded from the study because of their recognized high seminal ROS levels and decreased antioxidant levels. The selection criteria of fertile group were the absence of asthenozoospermia, endocrinopathy, varicocele, and have a birth in the last year. Samples were obtained (before and after zinc sulfate supplementation). After liquefaction seminal fluid at room temperature, routine semen analyses including semen volume, pH, concentration, sperm motility, normal sperm morphology and round cell were performed according to the 1999 (WHO 1999) recommendation.

2.2. Preparation of seminal plasma and spermatozoa for biochemical analysis

For each sample, seminal plasma was separated from the spermatozoa 1 h after semen collection by centrifugation of 2 ml of seminal fluid at 1500 g for 10 min at 4°C and maintained in -30°C until analysis. The pellet was resuspended in 10 volumes of suitable buffer (pH 7.4) (NaCl 113 mM, Tris 20 mM, EDTA 0.4 mM NaH_2PO_4 2.5 mM, Na_2HPO_4 2.5 mM, $CaCl_2$ 1.7 mM, D-glucose 1.5 mM) and centrifuged at 1500 g for 10 min at 4°C. This washing procedure was repeated three times. Triton X-100 (0.1%) was added to the pellets obtained and the samples centrifuged again at 8000 RPM for half an hour in a refrigerated centrifuge. This concentration of Triton X-100 does not affect enzyme levels. The supernatant was used for measurements in spermatozoa.

The samples were classified into three groups called group I (healthy donors), group II (patients before treatment) and group III (patients after treatment) respectively. After that, the samples were frozen (−20°C) until analyzed.

2.3. Chemicals

All reagents and chemicals were of analytical grade and obtained from standard commercial suppliers.

2.4. Biochemical procedures:

2.4.1. Total Antiperoxidant Activity:

2.4.1.1. Principle:

The levels of antiperoxidants activity were determined by measuring the capacity of sample antiperoxidants to prevent lipid peroxidation or slow this process after inducing lipid peroxidation by a free radical generating from the reaction of ascorbic acid with ferrous sulfate ($FeSO_4$). Lipid peroxidation (LPO) was measured as the production of malondialdehyde (MDA), with and without the sample, using the thiobarbituric acid (TBA) reaction (Rao *et al*., 1989; Strzezek *et al*., 1995, 2004). The absorbance was measured at a wavelength of 534 nm. Antiperoxidants activity was expressed as the percentage inhibition of MDA production.

2.4.1.2. Reagents:

1. Sodium phosphate buffer pH 7.4 (50 mM) was prepared by dissolving 0.13205 g of NaH_2PO_4 and 1.0864 g of $Na_2HPO_4.7H_2O$ in 100 ml distilled water.{1.1 g Na_2HPO_4 and 0.27 KH_2PO_4 in 100 ml distilled water}
2. Ferrous sulphate (0.01M) was prepared by dissolving 0.287 g of $FeSO_4.7H_2O$ in H_2O, and diluting to 100 ml with phosphate buffer pH 7.4.
3. Sodium ascorbate (0.1M) was prepared by dissolving 1.981 g sodium ascorbate in 100 ml phosphate buffer pH 7.4.
4. Ethylene diamine tetraaceticacid- disodium ($EDTA-Na_2$)(0.4M) 148.9gm of EDTA are dissolved in a final volume of 1 litter of DDW.
5. Tris-EDTA buffer (0.4) (pH = 8.0) 48.458 gm of tris is dissolved in 800 ml of DDW. 100 ml of (0.4M) EDTA solution is added and bring to a final volume of 1 liter with DDW. The pH adjusted to 8.0 by the addition of 1M of HCl (0.67 g of thiobarbituric acid dissolved in 100 ml of distilled)
6. Linoleic acid (1%) (1ml of linoleic acid dissolved in 100 ml phosphate buffer contain 0.1% Triton X-100).
7. Thiobarbituric acid reagent (0.67 g of thiobarbituric acid dissolved in 100 ml of distilled water with 20 gm of trichloroacetic acid and 2 ml glacial acetic acid added)

2.4.1.3. Procedure:

Reagents	Test	Control	Blank
Sodium linolate	1ml	1ml	1ml
Sample	50 µl	------	-------
Phosphate buffer pH 7.4	0.2ml	0.2ml	0.6ml
Ferrous sulphate ($FeSO_4$)	0.2ml	0.2ml	-------
Sodium ascorbate	0.2ml	0.2ml	-------
Test tubes were mixed by vortex and incubated for 60 minutes at 37°C, after that, the following solutions were added:			
Tris buffer (pH 8.0)	0.7ml	0.7ml	0.7ml
Thiobarbituric acid reagent	1.5ml	1.5ml	1.5ml
Sample*	------	50 µl	-----
Mix by vortex and then heated for 15 min in a boiling water bath. After cooling, each tube was centrifuged for 10 min at 4,000 x *g* and the supernatant absorbance was read on a spectrophotometer at 534 nm.			

* Sample was added to control to ensure the prevention of overlapping of MDA in the sample (seminal plasma) with that formed from the reaction induced lipid peroxidation reaction.

Table 2: Ejaculate Parameters:

	Volume (ml)	**Sperm count ($\times 10^6$)**	**Progressive sperm motility (%)**	**Normal sperm form (%)**
Healthy donors G1	2.5± 0.43	75 ± 6	65 ± 8	61 ± 7
Patients before treatment G2	1.72 ± 0.65 *	57 ± 11 *	21 ± 7 *	51 ± 9
Patients after treatment G3	2.37 ± 0.81 **	61 ± 11	37 ± 11 **	58± 7 **

Sn1 *: significance versus group I (Healthy donors).
Sn2 **: significance versus group II (Patients before treatment).

2.4.1.4. Calculations:

$$Test \quad MDA = \frac{Absorbance}{d * \in} X \ D.F$$

$$Control \quad MDA = \frac{Absorbance}{d * \in} X \ D.F$$

d = 1cm, ε =extinction coefficient = 1.56x 10^5 $M^{-1}cm^{-1}$

D.F = dilution factor

$$\text{Total antiperoxidant activity\%} = \frac{\text{Control MDA - Test MDA}}{\text{Control MDA}} * 100\%$$

2.4.2. Assay of conjugates diene hydroperoxide

Conjugates diene hydroperoxide measured spectrophotometrically according to method described by Buege and Aust, 1978.

3. Statistical Analysis

Data analysis was performed using the SPSS 16 for Windows Software Package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean, SD, SE and range, were evaluated by the one-way analysis of variance (ANOVA). The differences in mean values were calculated at a significance level of $P \leq 0.05$.

4. Results

The results in Table (2) indicate the baseline, characteristics of the semen parameters are depicted in the fertile **{G1}** and subfertile (before **{G2}** and after treatment **{G3}** with zinc sulfate) groups. These parameters were significant ($P < 0.05$) decreased in the infertile group compared with a healthy donor group. However, the level of the semen parameters were significant ($P < 0.05$) increased (return to normal value) after zinc sulfate supplementation.

Total Antiperoxidant and Lipid Peroxidation:

Previously Strzezek *et al*., 1995 documented the suitable method for the measurement of the total antiperoxidant in seminal plasma. This study describes an important development of Strzezek method. Strzezek and his Co-workers have used sperm suspension as a source of lipid. In the present study; it has been replaced by linoleic acid. This replacement produces efficient method, so that it can be used to measure the total antiperoxidants in the spermatozoa and seminal plasma, while the former method capable of measuring antioxidants in seminal plasma only. Additionally the use of linoleic acid removes overlaps resulting from the presence of antiperoxidants in sperm suspension. As a result, one can determine the levels of antiperoxidants activity by measuring the capacity of sample antiperoxidants to prevent lipid peroxidation or slow this process after inducing lipid peroxidation by a free radicals generating from the reaction of ascorbic acid with ferrous sulphate ($FeSO_4$). Lipid peroxidation (LPO) was measured as the production of malondialdehyde (MDA), with and without the sample, using the thiobarbituric acid (TBA) reaction (Rao *et al*., 1989; Strzezek *et al*., 1995). Antiperoxidants activity was expressed as the percentage inhibition of MDA production. Tables (3 and 4) show the Total antiperoxidant activity in the fertile {G1} and subfertile (before {G2} and after treatment {G3} with zinc sulfate) groups. Values are expressed as means, standard deviation; standard error; Confidence Interval, n= 55 for each group.

Table 3: Total Antiperoxidant Activity (%) in Seminal Plasma of Infertile and Healthy Donors Groups.

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	59.4	8.45	1.14	55.55	63.24	1	2	.000 *
							3	.270
G2	39.3	14.5	1.95	29.61	49.17	2	1	.000*
							3	.002*
G3	54.8	2.50	0.33	52.71	56.91	3	1	.270
							2	.002*

Table 4: Total Antiperoxidant Activity in Spermatozoa mmol/10^8 Spermatozoa of Infertile and Healthy Donors Groups.

N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	59.13	14.93	2.01	52.34	65.93	1	2	.050*
							3	.140
G2	47.74	19.57	2.63	34.59	60.90	2	1	.050*
							3	.005*
G3	68.69	6.72	0.90	63.07	74.319	3	1	.140
							2	.005*

Table 5: Conjugates diene hydroperoxide Levels (µmole/L)in Seminal Plasma of Infertile and Healthy men Groups.

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	7.69	6.56	0.87	3.72	11.65	1	2	0.048*
							3	0.259
G2	12.39	6.32	0.85	8.14	16.63	2	1	0.048*
							3	0.005*
G3	5.0	2.26	0.30	3.37	6.62	3	1	0.259
							2	0.005*

Table 6: Conjugates diene hydroperoxide Levels in Spermatozoa (µmol/10^8 Spermatozoa) of Infertile and Healthy Donors Groups.

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Compared groups		Sign.
				Lower Bound	Upper Bound			
G1	14.43	2.21	0.29	12.39	16.48	1	2	0.024*
							3	0.579
G2	18.73	2.60	0.35	16.32	21.13	2	1	0.024*
							3	0.007*
G3	13.45	4.48	0.60	9.307	17.60	3	1	0.579
							2	0.007*

* The mean difference is significant at the 0.05 level.

5. Discussion

In this research, the role of zinc supplementation on semen quality of infertile men with asthenozoospermia was considered. The results showed significantly (P< 0.05) higher values of ejaculate volume, sperm count, normal morphology and progressive sperm motility in-the group supplemented with zinc sulfate as evaluated to the same group before supplementation of zinc sulfate. These results may be attributed to the fundamental role of zinc in spermatogenesis (Madding et al., 1986). Zinc encourages spermatogenesis via regulating mitotic and meiotic cell divisions, along with synthesis of DNA and RNA by activation DNA polymerase and RNA polymerase (Bedwal and Bahuguna, 1994). Also, zinc attaches to the most important enzymes concerned in spermatogenesis such as sorbitol dehydrogenase and lactate dehydrogenase (Eggert et al., 2002). It participates in the synthesis and activation of testosterone (Netter et al., 1981).

The antioxidant property of zinc may be reliable for enhanced motility of sperm in Zn-supplemented groups via activation of Creatine Kinase. It's an enzyme responsible of cellular energy metabolism, which regulates the reversible transfer of the high energy N-phospho-group from phosphocreatine to ADP (Forstener et al., 1998), thus, maintains the stability of ATP concentrations. ATP is the principal donor of energy required by the sperm flagella for progressive motility (El-Masry et al., 1994).

Total antiperoxidant activity was significantly (p<0.05) decreased in the infertile group (39.3% in seminal plasma & 47.74%/10^6 spermatozoa in spermatozoa) when compared with a healthy donor group (59.4% in seminal plasma & 59.13% /10^6 spermatozoa in spermatozoa). The plasma membrane of the mammalian sperm is susceptible to ROS-related lipid peroxidation because the abundance of unsaturated fatty acids (El-Tohamy, 2012). Lipid peroxidation (LPO) of the sperm membrane is documented to be the predictable mechanism of ROS-induced sperm damage leading to infertility. Sperm, unlike other cells, are exceptional in composition, function, and susceptibility to damage by LPO (Agarwal and Prabakaran, 2005).

Reduced total antiperoxidant activity could be due to two causes. The first, increased generation of reactive oxygen species (ROS) that results in excessive oxidative damage generated in these patients. These oxygen species in turn can oxidize many other important biomolecules that act to prevent lipid peroxidation. The second cause could relate to inhibition the most cytoplasmic antioxidants-enzymes (Atig *et al.*, 2012) that might make spermatozoa highly susceptible to peroxidative damage. The antioxidants enzymes group includes glutathione peroxidase, a cytoplasmic seleno-protein, reduces lipidic hydroperoxides while oxidizing two molecules of GSH (Hsieh *et al*., 2006).

The level of total antiperoxidant was significantly (p<0.005) increased (return to normal value) after zinc sulfate supplementation (54.8% in

seminal plasma & 68.69% /10^6 spermatozoa in spermatozoa). Zinc supplementation improves the synthesis of zinc binding protein such as superoxide dismutase and metallothioneins (Low molecular weight- zinc binding protein) in seminal plasma of asthenozoospermic subjects (Di Leo *et al*., 2001; Canacci *et al*., 2011; Hadwan *et al*., 2012). Metallothioneins (MTs) protect biological tissues from damage of oxidative stress via capture harmful oxidant radicals like the superoxide and hydroxyl radicals (Suriya *et al*., 2012). Furthermore, Zinc has important characteristics such as anti-apoptotic (Chimienti *et al*., 2003) and antioxidant (Zago and Oteiza, 2001).

The found of zinc in proteins as an alternative of these highly reactive elements assists prevent the production of free radicals (HO, 2004). Zinc, for this reason, is an antioxidant. It sustains suitable folding, stability and activity of zinc-dependent enzymes by protecting these enzymes from ROS attacks (Coleman, 1992). Intracellular zinc also maintains the appropriate level of citrate in the prostate gland via removing unwanted oxidative stress (Omu *et al*., 1998). Two mechanisms for zinc antioxidant ability have been documented. In the first, zinc can reduce the effect of the oxidation by binding sulphydryl groups in proteins, while in the second; zinc is occupying binding sites for iron and copper in lipids, proteins and DNA (Zago and Oteiza, 2001; Bray and Bettger, 1990). To prove these antioxidant effects of zinc, many laboratory experiments were conducted for describing oxidative damage of biomolecules such as proteins, lipids and DNA in zinc-deficient laboratory animals (Oteiza *et al*., 1995; Bagchi *et al*., 1998). Zinc supplementation has been shown to protect against oxidative damage (Omu *et al*., 1998; Hadwan *et al*., 2012).

Conjugates diene hydroperoxide (CDH) is considered as a marker of oxidative stress and it was found that serum CDH increased significantly ($p<0.05$) in this study as shown in table (5&6). The elevated CDH level could be changed to lowering GPx activity, which can lead to a relative accumulation of 12-HpETE (12-hydroperoxy-eicosatetraenoic acid). 12-HpETE is the main hydroperoxide formed from arachidonic acid, and such an increase could activate signal transduction pathways leading to arachidonic acid release (Calzada *et al*., 2001). In addition, a tendency to decrease GPx activity in seminal plasma of patients with asthenospermia (Giannattasio *et al*., 2002; Crisol *et al*., 2012) could increase both the intracellular peroxide level and oxidative damage.

Conclusions

Zinc supplementation restores antiperoxidant activity and decrease conjugates diene hydroperoxide level in spermatozoa and seminal plasma of asthenozoospermic subjects to the normal ranges. Zinc supplementation enhances semen quantitative and qualitative properties.

Abbreviations:

ANOVA, Analysis of variance; CDH, Conjugates diene hydroperoxide; 12-HpETE, 12-hydroperoxy-eicosatetraenoic acid; WHO, World health organization; LPO, Lipid peroxidation; ROS, Reactive oxygen species; GSH, Glutathione; TBA, Thiobarbituric acid; MDA, Malondialdehyde.

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