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RESEARCH ARTICLE

Evaluation of enzymatic and non- enzymatic antioxidant status in seminal plasma of Iraqi Infertile Men

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Abstract

This study was conducted to demonstrate the effect of seminal oxidative stress (OS) on male infertility with different infertility potentials. To achieve this aim, the levels of malondialdehyde (MDA),reduced glutathione (GSH) ,superoxide dismutase(SOD), glutathione peroxidase (GP_X) and catalase (CAT) activity in addition to levels of vitamin E and vitamin C were determined in three groups: normospermic who were considered as controls (n=30), asthenospermic (n=27) and oligospermic(n=23).The seminal plasma MDA levels were significantly high in infertile patients ($p<0.001$)compared to the normospermic subjects ,while GP_X, SOD and CAT activities were significantly low in oligospermic and asthenospermic patients respectively in addition ,levels of seminal plasma vitamin E and vitamin C were reduced also significantly in astheno and oligospermic patients compared to normospermic subjects. Our results obviously emphasize the association of OS level in seminal plasma with the incidence and progression of the idiopathic infertility in infertile patients. Thus, seminal reactive oxygen species (ROS) would be used as a specific and sensitive biomarker for idiopathic male infertility. These findings indicate a protective role for antioxidant enzymes of seminal plasma against lipid peroxidation of spermatozoa in normospermic samples.

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INTRODUCTION

Infertility is defined as the failure to conceive after one year of regular, unprotected intercourse with the same partner. The physiopathology of male infertility could be explained by a cascade of molecular and biochemical events which represents itself in most of cases by abnormal semen parameters (Hesham et al .,2008).The causes of infertility have been attributed to a variety of anatomical, genetic, endocrinological and immunological factors (WHO (1999). It is clear that it is a common problem affecting young couples, and equally clear that it results in considerable distress for those couples affected. The feelings experienced by infertile couples encompass anger, depression, anguish, denial, guilt, shame, inadequacy, shock, isolation and embarrassment (Irvine D.,1998).Factors causing high rates of infertility in parts of the developing world are varied, but tubal infertility due to sexually transmitted, postpartum, post-abortive, and iatrogenic infections is widely regarded as the primary form of preventable infertility in the world today (Sciarra J .,1997).A large proportion of male infertility cases are associated either with systemic defects such as diabetes, obesity, varicocele, cystic fibrosis or with infections for mumps, herpes or else with imbalance in levels of gonadal steroids and trophic hormones [example, testosterone, dihydrotestosterone, follicle stimulating hormone, leutinizing hormone, and androgen receptor]. However, in nearly 25% cases of male infertility no organic cause is identified (idiopathic infertility) (Ambasudhan et al.,2003).
Traditionally, the diagnosis of male infertility is based upon the conventional semen profile, constructed according to recognized guidelines (WHO 1992 Van den Eede B (1995). Though semen analysis is the first diagnostic step routinely employed in the evaluation of the male infertility, it fails to predict the exact cause behind impaired

fertility (Anonymous (1996). This profile incorporates information on the volume of the ejaculate, the concentration of spermatozoa, their motility and their morphological appearance. However, sperm count and sperm motility are the first and most important predictors of fertility potential rather than sperm morphology. In half of the male infertile patients, the cause is not clear and hence such cases are diagnosed with idiopathic infertility. Moreover, idiopathic infertile cases are blindly treated and selected for assisted reproductive techniques without understanding the basic mechanism behind the fertility impairment (Venkatesh et al., 2009a).

Oxidative stress (OS), a condition where the production of reactive oxygen species (ROS) overwhelms antioxidant levels, has been considered as one of the major factors believed to be involved in idiopathic male infertility. Low levels of ROS are necessary for normal functions of
spermatozoa like capacitation, hyper activation, motility, acrosome reaction, oocyte fusion and fertilization (Agarwal et al .,2004; Venkatesh et al .,2009a). For the past two decades, the pathological role of ROS in the semen has been studied but not well established because of various possible sources associated with excess production of ROS including abnormal spermatozoa(Venkatesh et al., 2009b). It has been postulated that oxidants interfere with normal sperm plasma function via peroxidation of unsaturated fatty acids in the sperm plasma membrane which results in sperm dysfunction (Barroso et al., 2000). In addition, ROS are known to attack DNA inducing strand breaks and other oxidative based damage in spermatozoa. High levels of ROS endanger sperm motility, viability and increased midpiece sperm defects that impair sperm capacitation and acrosome reaction. The fertilizing ability of human spermatozoa is inversely related to the sperm ROS production (Gil-Guzman et al., 2001).

Since the pathophysiology of male infertility is still poorly understood and various diagnostic tests are unable to determine the underlying cause of sperm dysfunction, the aim of the present study was to investigate the correlation between OS markers and incidence of idiopathic infertility and subfertility in Iraqi men. Lipid peroxidation, enzymatic and non enzymatic antioxidants enzymes were tested.

Material and Methods

The study was conducted in Al-Kut governorate, from October 2012 to May 2013.Fivty infertile men with different infertility potential who were referred to Al-Zahra teaching hospital were subjected to this study. Those selected infertile men were divided into two groups according to infertility type: The oligospermic group include 23 patients with age range (23-41) years with primary infertility and sperm count less than 4 million/milliliter while asthenospermic group include 27 patients with age range (24-45) years with primary infertility and the sperm motility less than 50% (grade A according to World Health Organization (1999).The control group include thirty apparently healthy fertile volunteers with normal seminal parameters according to WHO (1999).Exclusion Criteria: Those patients were admitted to hospital for further investigations, monitoring, and treatment. Worthy to mention that those patients were not smokers, not alcoholics and not suffering from any other serious systemic illnesses like diabetes mellitus, cardiac diseases, renal diseases and hepatic diseases, not taking any drug in the last year and not have any hormonal disturbances, so as not to interfere with the result of measured parameters and the outcome of the study.

Measurement of Seminal Plasma Malondialdehyde (MDA)Concentration

Malondialdehyde levels were measured as per thiobarbituric Acid (TBA) method described by Yao-Yuan Hsieh et al.,. (2006). Semen sample was centrifuged at 3000xg for 10 minutes after liquefaction to get the seminal plasma. Then 0.1 ml of seminal plasma was added to 0.9 ml of distilled water in a glass tube, to it 0.5 ml of TBA reagent (0.67 gm of 2–thiobarbituric acid dissolved in 100 ml of distilled water with 0.5 gm of NaOH and 10 ml of glacial acetic acid) was added and then heated for 1 hr in a boiling water bath. After cooling the tube was centrifuged for 10 minutes at 4000 Xg and the supernatant absorbance was read on a spectrophotometer at 534 nm.

Measurement of Seminal Plasma SOD activity

Superoxide dismutase (SOD) activity was measured by colorimetric assay (Ukeda H, et al (1997). We used commercially available colorimetric method (Randox Laboratories Ltd, UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride (I.N.T) to form red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD inhibits reduction of INT by 50% under the conditions of the assay. After thawing, the seminal plasma was diluted 30-fold with 10 mM phosphate buffer, pH 7.0. Assay was performed at 37°C. Phosphate buffer was used as blank. Mixed substrate and xanthine oxidase were added into standards and sample tubes and vortexed well. With spectrophotometer adjusted at a wavelength of 505 nm, the initial absorbance (A_1) was read. Final absorbance (A_2) was read exactly after 3 minutes. Percentages of inhibition of standards and

samples were calculated. The SOD activity was measured using calibration curve of percentage inhibition for each standard against Log10 of standards and SOD activity was expressed as U/ml.

Measurement of Seminal Plasma Catalase activity

Catalase activity was estimated by the method of Aebi (1984). Catalase can degrade hydrogen peroxide which can be measured directly by the decrease in the absorbance at 240 nm. The hydrogen peroxide was diluted with phosphate buffer pH 7.0 and its initial absorbance was adjusted between 0.5 to 0.6 absorbance unit at 240 ml.The decrease in the absorbance was measured. One unit of catalase activity was defined as the amount of catalase which absorbed in 30 sec at 25°C. The catalase activity was then calculated from the change in absorbance and finally expressed as U/ml.

Measurement of Seminal Plasma Glutathione content

The GSH concentration in seminal plasma was measured according to the method of Beutler et al (1967). 0.2 ml of seminal plasma and 1.8 ml of distilled water were mixed with 3 ml of precipitating solution (metaphosphoric acid 1.67gm,disodium EDTA 0.2 gm, NaCl 30 gm in 100 ml distilled water). After allowing to stand for 5 minutes, the solution was filtered. 1 ml of clear filtrate, 4 ml of freshly made disodium hydrogen phosphate (4.6 gm/L) solution and 0.5 ml of DTNB reagent (5,5'dithiobis-2-nitro benzoic acid: 20 mg in 100 ml of citrate buffer) were added. Absorbance of the yellow colour developed was read in spectrophotometer at 412 nm.

Measurement of Seminal Plasma Glutathione peroxidase activity

Seminal plasma GP_X was measured by a Ransel kit (Randox Laboratories Ltd., London, U.K.). GP_X catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm was measured(Paglia, et al., 1967). The GP_X activity was expressed as specific activity (mU/mL seminal plasma) and total activity (mU/total seminal plasma). hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm was measured. The GP_X activity was expressed as specific activity (mU/mL seminal plasma) .

Measurement of Seminal Plasma Vitamin C:

Vitamin C was determined using ELISA kit for Human Vitamin C from Cusabio. Company (Catalog No. CSB-E08090h) which allows for the specific measurement of human Vitamin C concentrations in Serum or plasma. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Vitamin C has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Vitamin C present is bound by the immobilized antibody. An enzyme-linked monoclonal antibody specific for Vitamin C is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Vitamin C bound in the initial step. The color development is stopped and the intensity of the color is measured spectrophotometrically at a wavelength of 450 nm ±2 nm. The concentration of vitamin C in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Measurement of Seminal Plasma Vitamin E:

Vitamin E was determined using ELISA Kit for Human Vitamin E from Uscn Life Science Inc. Wuhan,(china(Cat. No. E0922Hu) which allows for the specific measurement of human Vitamin E concentrations in Serum or plasma. The ELISA is based on the competitive binding enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with an antibody specific to vitamin E. During the reaction, vitamin E in the sample or standard competes with a fixed amount of biotin-labeled vitamin E for sites on a pre-coated Monoclonal antibody specific to vitamin E Excess conjugate and unbound sample or standard are washed from the plate. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ±2 nm. The concentration of vitamin E in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Statistical analysis:

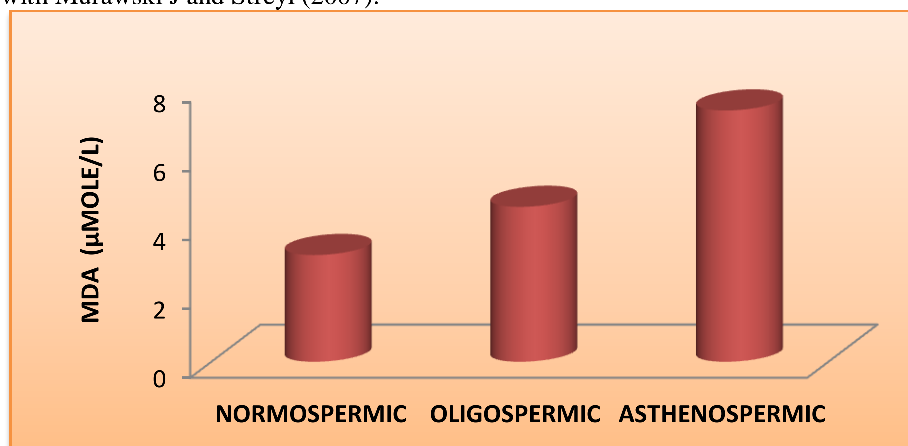
Differences between groups were assessed using Mann-Whitney U test and Kruskal-Wallis test. Coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value < 0.05 level with 95% confidence intervals. The data were expressed as the Mean ± SEM. Statistical computations were calculated using SPSS 11.5 for windows software (SPSS Inc, Chicago,IL, USA).

Results

The concentration of seminal MDA was significantly increased in oligospermic subjects 3.7±0.72µmole/L, while it was 7.3 ± 0.83 µmole/L in asthenospermic subjects against 82±0.22µmole/L in normospermic males. So the concentrations of MDA in both groups were significantly different (p<0.001) as seen from table 1 and graph 1. Individuals with oligo and asthenospermia are related with the elevated MDA concentration. Rise in MDA could be due to increased generation of reactive oxygen species(ROS) due to the excessive oxidative damage generated in these infertile men .These oxygen species in turn can oxidize many other important biomolecules including membrane lipids.

Assessment of seminal plasma enzymatic antioxidants activities .

Superoxide dismutase is an important antioxidant of seminal plasma which has superoxide scavenging capacity and plays an essential role in maintaining the balance between ROS generation and degradation (De lamirande et al., 1993). Several toxic substances including chemical reagents, drugs, heavy metal ions or nicotine decrease semen quality as well as SOD activity in seminal plasma. We found significantly lower seminal SOD activity in oligospermic and asthenospermic compared to normospermic men. In present study, SOD activity showed significantly positive correlation with sperm count(r=0.88P<0.001) and sperm motility(r=0.69,p<0.001)which was compatible with Murawski J and Streyl (2007).



Figure(1):lipid peroxidation marker, malondialdehyde(MDA) levels in oligo, astheno and normospermic men ...

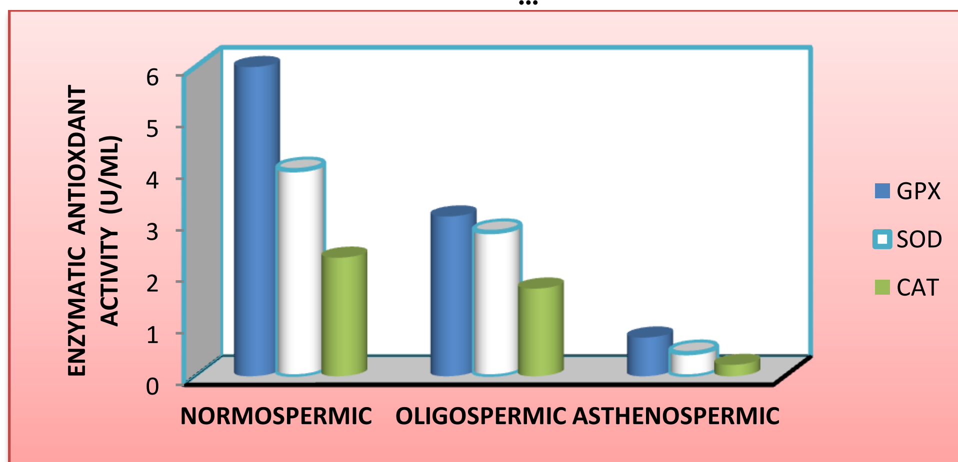


Figure (2): Enzymatic antioxidant activities (SOD &GPx) in seminal plasma of oligo, astheno and normospermic men .

Table 1:Levels of some biomarkers in normospermic, oligospermic and asthenospermic men (Mean ± Standard deviation)

parameter	**Normospermic group(N=30)**	**Severe -Oligospermic group(N=23)**	**Asthenospermic group(N=27)**
Sperm Count (Million/ml)	80± 17.2	3.2±1.3**	30.5±15.2 ***
Motility %	60.0±10.2	31.6±11.8**	25.4±18.2***
Abnormal morphology%	12 ±5.8	17 ±5.8	22 ±8.8**
MDA(µmole/L)	0.82±0.22	3.7±0.72**	7.3 ± 0.83***
GSH(µmole/L)	5.2 ±1.2	3.8±1.3*	2.7±1.7**
Vitamin E (µmol/L)	0.7±0.20	0.40±0.15*	0.20±0.15**
Vitamin C (µmol/L)	360±108	166±112**	222±117**
SOD(U/ml)	3.1±0.56	2.2±0.62*	1.7±0.78**
GPx(U/ml)	12±1.6	8.2±1.9*	6.3±1.7**
Cat(U/ml)	**0.34± 0.16**	0.22± **0.13***	**0.18 ± 0.08***

*** Significant P<0.05. ** significant P<0.01 , *** significant P<0.00**

Assessment of seminal plasma non Enzymatic antioxidant levels

A significant decrease in all the three non enzymatic antioxidant defense system levels of seminal plasma GSH , vitamin E and vitamin C as observed in figure 3,4 and 5.but the results were observed that levels of seminal plasma vitamin C more decreased in patients with oligospermic(166±112µmol/L) than asthenospermic (222±117µmol/L) when compared to controls(360±108µmol/L). The decrease in the levels of these non-enzymatic antioxidant parameters may be due to the increased turnover, for preventing oxidative damage in these patients suggesting an increased defense against oxidant damage in infertility men . The decreased concentrations of the GSH and antioxidant vitamin status supports the hypothesis that lipid peroxidation is an important causative factor in the pathogenesis of infertility .Therefore, the treatment with antioxidants in the initial stages of the disease may be useful as secondary therapy to prevent the oxidative damage.

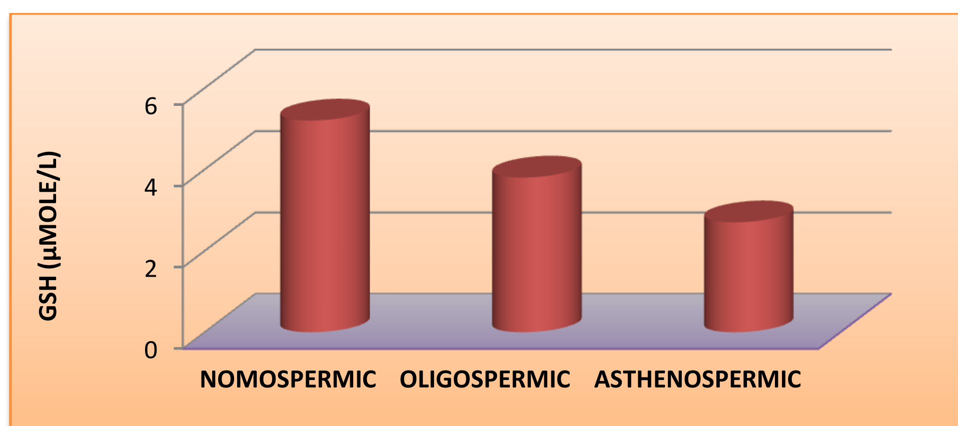


Figure 3: Glutathione levels in levels in oligo, astheno and normospermic men .

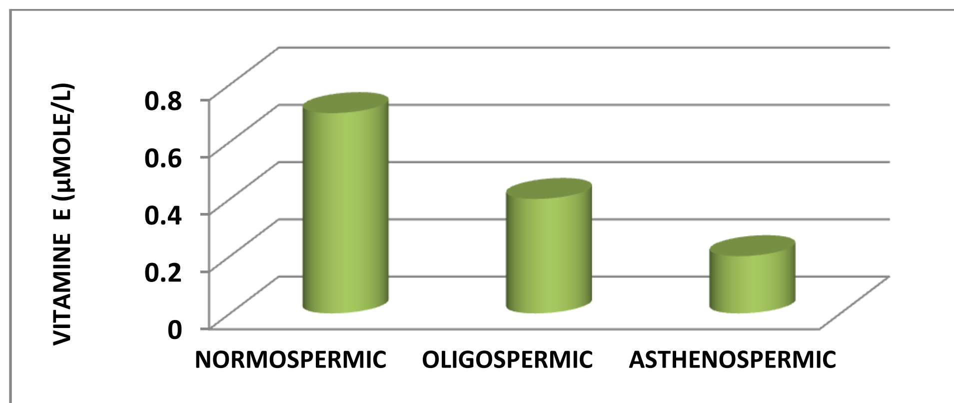


Figure 4: Vitamine E levels in oligo, astheno and normospermic men

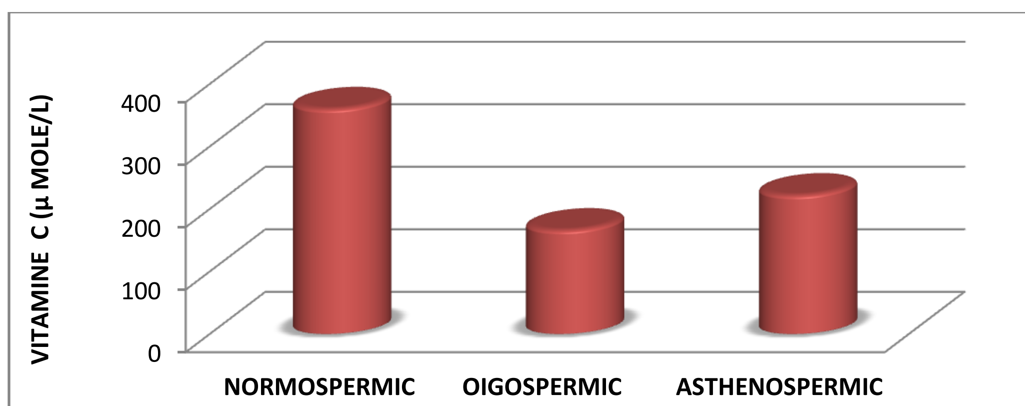


Figure 5: Vitamine C levels in oligo, astheno and normospermic men .

Discussion

It is well-known that spermatozoa themselves contain negligible levels of antioxidants, and thus render the cells particularly reliant to their immediate environment for protection (Geva et al., 1996). Human seminal plasma contains several antioxidants compromising enzymatic and nonenzymatic systems that play an important role in the normal function of sperm. Many studies suggested that decreased levels of antioxidants in seminal plasma might be a potential cause of infertility but there were always contradictory between reports (Geva et al., 1996).It is important to make a note of contradictions and controversial outcomes found and cited in articles. In point of fact, these differences can be due to several variables like the criteria included and excluded for patient selection. In actual fact, effects of abstinence time on the results of semen analysis were eliminated in this study by the abstinence time of subjects. The abstinence time was not different at either of the collection times.

Superoxide dismutase, glutathione peroxidase and glutathione and trace elements are important indicators of antioxidant status. Since it is difficult to measure the effectiveness of SOD in isolation of another antioxidant, we chose to determine SOD activity in seminal plasma of infertile men associated to GP_X activity, CAT level and GSH amount (Agarwal et al., 2006). The analysis of our results indicated a significantly lower seminal SOD activity detected in infertile groups compared to normospermic men. This outcome confirms previously published observations of the other authors (Murawski et al., 2007; Marzec-Wróblewska et al.,2010). Our study like several studies (Murawski et al .,2007; Marzec-Wróblewska et al., 2010) showed also highly significant and positive correlations between seminal SOD activity and semen parameters, sperm concentration and overall motility, which are regarded as the most important criteria for normal fertilizing ability of the spermatozoa. We noted also a negative association of seminal SOD to abnormal morphology. However, some investigators suggested that the

beneficial impact of SOD activity concerns only sperm movement, whereas no influence on sperm count has been noticed (Kurpisz et al., 1996;, Sanocka et al., 1996). The important activity of seminal SOD in control group and the noted associations between this enzyme and sperm quality proved the ability of this enzymatic antioxidant to remove $O_2^{\circ -}$ and its important biological role in controlling the fertilizing potential of this highly specialized cell. On the other hand, decrease of the capacity can result in accumulation of O_2°-and indirectly in excessive membrane lipid per-oxidation which is responsible of abnormal sperm motility determined as sperm hyper activation which was confirmed by the important amounts of seminal MDA found in seminal plasma of infertile patients and the high negative correlation ($P<0.001$; r=-0.490**) noted between this enzyme and MDA in our study. SOD is also considered as a pro-oxidant by the conversion of the superoxide anion into a quite stable and invasive free radical, H_2O_2. To efficiently recycle H_2O_2, two enzymatic activities are available: CAT and GP_X. In our research we tried to explore the seminal activity of GP_X and CAT in seminal plasma of our collected samples(Drevet JR(2006) .

Conversely to Tamer et al(2004), we noted that the activity of seminal GP_X was lower in abnormal groups than normospermic patients. Giannattasio et al(2006) showed also that the seminal GP_X activity from healthy subjects was 10 times greater than that from infertile males. Efficiently, the increased activity of GP_X in seminal plasma of normospermic men suggested that higher activity of this enzyme catalyzes the ROS which might protect sperm against per-oxidative damage (De lamirande et al .,1993) and also plays role in sperm maturation from the early events up to the onset of fertilization (Vernet et al .,1997). Reduction of GP_X in seminal plasma may lead to reduce fertilizing capacity and defective sperm quality(Hall et al., 1998). Dandekar et al.,(2002) and Hsieh et al., (2006)demonstrated that GP_X activities in seminal plasma correlate positively with Sperm motility which is in agreement with our findings. Successfully, we established strongly positive relation-ships between seminal GP_X activity, sperm motility (P=0,04; r= 0,263*) and sperm concentration (P<0,001 ; r= 470** Data not shown). However, we observed a negative but not significant correlation among this enzyme and percentage of abnormal sperm morphology. These associations reinforce the benefit effects of GP_X in scavenging of ROS and consequently in the safeguard of good sperm quality. Positive and strongly relationship which was noted between seminal SOD and GP_X activities confirms that it is difficult to measure the effectiveness of one antioxidant in isolation of another because there appear to be in cooperation(Agarwal et al.,2006). This association supported the concomitant effect of the two enzymes against deleterious effects of lipid per-oxidation and oxidative stress affecting sperm quality (Alvarez JG and Storey BT(1989).). But, the absence of significant correlation among GP_X activity and MDA content in seminal plasma suggested that the seminal GP_X is still not a useful tool in determining sperm fertilizing potential and this may be due to the case limitation or the complex interactions between the ROS and numerous antioxidants. In fact, the regulation of sperm function and the mechanisms involved may be complex and multi-factorial.

Glutathione peroxidase activity is related with the balance between the GSHr (reduced form of glutathione) and GSSG ,oxidized form of glutathione (Sharma et al.,1994). But, little information about the effects of seminal glutathione on male fertility potential is adequate till date. Moreover, there are lot of controversy and variability in the findings. In fact, as regards our investigation, we confirmed the presence in human seminal plasma of detectable amounts of different forms of GSH, While other studies found GSH levels below the limit of detection (<2.5µM) in seminal plasma of infertile men(Ochsendorf et al., 1998). We however found higher levels of Total GSH in seminal plasma of normospermic com-pared to any other abnormal groups, but the difference was statistically significant only after comparison with asthenospermic group (P=0.045). The important amounts of seminal GSH in control group confirmed the positive contribution of this free thiol as an antioxidant to maintain the good quality and motility of sperm. While stating that, other investigations could not observe any difference in GSH concentration between fertile and subfertile men (Wong et al., 2001). Ochsendorf et al., (1998) found out moderate reduction of GSH in oligospermic compared to normospermic. Others have noted that GSH levels must be significantly reduced in seminal plasma of infertile males compared to fertile ones (Raijmakers et al., 2003) and this notes corroborated with our findings. Hesham et al (2008), were also in accord with us, they noted a highly significant de-crease of mean GSH level in both azoospermic and oligospermic groups compared to normospermic one . In effect, as we indicated GSH exists into two forms, reduced (GSHr) and oxidized (GSSG) forms. It is well known that GSHr is the most abundant form and this evidence was confirmed by our results. Effectively, we noted that the most important form was the GSHr in seminal plasma of all groups included in this study. In actual effect, we found significant de-crease of seminal GSHr content in asthenospermic compared to control men (P=0.007). These data suggested that a lower level of seminal GSHr in asthenospermic subjects can be associated to a decline of sperm motility, however higher levels of this element in seminal plasma can guide to an enhanced sperm movement. Effectively, the presence of low amounts of GSHr in samples with abnormal sperm motility remains to be explained. While asthenospermic patients showed decreased levels of seminal GSHr, in addition to oligospermic group in comparison with controls, showed an elevated content of seminal GSHr sustained with a significant decline in GSSG levels (P=0.035). This

elevation of GSHr in oligospermic group may be due to the contribution of excessive ROS produced by the abnormal spermatozoa leading to up-regulation of thiol synthesis in order to protect sperm from oxidative damage. Even glutathione therapy was found to improve the semen quality (Murawski et al.,2007). For this reason we tried to estimate correlations that can be exist between the seminal GSH levels and the sperm criteria. We noted a significant and negative correlation among seminal GSSG and the percentage of abnormal morphology. This result provides evidence that GSSG in seminal plasma seem to protect the quality of sperm cell membrane and morphology (Garrido et al., 2004). This result was compatible with those observed by Bhardwaj A et al., (2000) and Chaudhari A.R et al (2008) who also observed positive correlation between seminal content of GSH and normal sperm morphology. We also found a significant negative correlation between GSSG and MDA levels of seminal plasma which was in agreement with Chaudhari A.R et al (2008). Therefore, GSH might have some fertility enhancing role by reducing lipid per-oxidation. On the other hand, there were no correlations between seminal GSHt and GSHr with the sperm parameters. This outcome which was in agreement with Garrido et al (2004) not negotiate the beneficial role of GSH to minimize oxidative damage to the sperms but more extended series of clinical trial will be needed.

Conclusion

To summarize, in this study we investigated whether the antioxidant status and the extent lipid peroxidation in seminal plasma would be the best predictor of sperm function. Successfully, it was found that the MDA level was more important in infertile men than normospermic and it was negatively correlated with sperm motility and concentration. In contrast, antioxidant status was significantly increased in seminal plasma of control group and positively associated to sperm motility and count. This could provide the database about the effects of MDA and antioxidants upon sperm. Finally, future research may include the studies using oxidative markers and antioxidant system on the large scale; the genetic susceptibility and their repercussions on IVF outcomes might be explored with respect to semen quality.

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