Lipid Peroxidation and Antioxidant Enzyme Activities in Infertile Men: Correlation With Semen Parameter

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The aim of this study was to investigate the oxidative stress status and antioxidant enzyme activities in infertile men's semen and to determine their relationship with spermatozoa characteristics. Four groups of infertile men, normozoospermic ($n=9$), azoospermic ($n=13$), oligoasthenozoospermic ($n=38$), and asthenozoospermic ($n=42$), were tested for malondialdehyde (MDA) concentration and catalase (CAT) and superoxide dismutase (SOD) activities in semen using spectrophotometric methods. We found that MDA levels in semen and SOD activity in seminal plasma (SP) were significantly higher in oligoasthenozoospermic and asthenozoospermic groups compared with normozoospermic group. In contrast, the mean values of CAT activity

were not significantly different in all groups. We found positive correlations between semen MDA concentration and SOD activity ($P<0.01$). MDA levels in sperm pellet and in SP were also positively correlated with mobility grade b ($P<0.01$), acrosome anomalies ($P<0.01$), and residual cytoplasmic droplets ($P<0.05$). In contrast, SOD activity in SP was negatively correlated with sperm concentration and normal morphology ($P<0.05$). Similarly, CAT activity in SP was negatively correlated with sperm and leukocyte concentrations ($P<0.05$). In conclusion, our results suggest that the evaluation of oxidative status and antioxidant defenses may be taken as an important tool for diagnosis and treatment of male infertility. *J. Clin. Lab. Anal.* 23:99–104, 2009. © 2009 Wiley-Liss, Inc.

Key words: infertility; sperm; lipid peroxidation; SOD activity; catalase activity

INTRODUCTION

The reactive oxygen species (ROS) play an important role in the pathogenesis of many reproductive processes and especially in pathological mechanism of male infertility.

One of the most important causes of defective sperm function is oxidative stress (OS) that is demonstrated by excessive ROS generation by spermatozoa (SPZ) and/or by the disruption of the antioxidant defense systems in the male reproductive tract. The maximum ROS production was observed in immature SPZ, especially with abnormal head morphology and cytoplasmic retention; ROS production is the lowest in immature germ cells and in mature SPZ (1).

SPZ are particularly susceptible to OS-induced damage, because their plasma membranes contain high concentration of polyunsaturated fatty acids (PUFA) (2) and their cytoplasm contains low concentrations of scavenging enzymes (3). However, these cells are naturally protected from such injury by the antioxidant properties of seminal

plasma (SP) (4). The latter contains a number of enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). In addition, semen contains a variety of nonenzymatic antioxidant molecules: vitamin C, vitamin E, pyruvate, glutathione, and carnitine, which can neutralize the potentially toxic effect of ROS (5). Moreover, a recent study proves a protective antioxidant role of estrogens in protecting the reproductive functions from the adverse effects exerted by ROS produced in large quantities in the aged testis of male rats (6).

The imbalance between ROS production and total antioxidant capacity (TAC) corresponding to OS is correlated with male infertility and the exposure of SPZ

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to ROS has been associated with cellular injury that includes DNA damage and lipid peroxidation (LP) (3). In fact, the excess of LP as represented by high levels of malondialdehyde (MDA) product may cause changes in the sperm characters, decreased fluidity and flexibility of the sperm membrane, and diminishing of the fertilization potential (7). This indicates that LP could be harmful to male sperm and reproductive system.

The aim of this study was to evaluate the concentration of MDA and antioxidant enzymatic defense in semen of infertile men and to determine their relationship with sperm parameters.

MATERIALS AND METHODS

Semen Samples and Sperm Preparation

Semen specimens were provided by 102 patients attending the Laboratory of Histo-Embryology for semen analysis. All semen samples were collected by masturbation after 3 days of abstinence. After liquefaction, semen volume, sperm concentration, motility of SPZ, their viability, and morphology were determined using standard procedures. Motility was assessed at room temperature according to WHO (8); four characteristics of the sperm cells were classified into four groups as grade a (Rapid progressive motility), b (Borderline progressive motility), c (Nonprogressive motility), and d (Immotility). Sperm density was determined by diluting a semen sample in immobilizing solution (9 g NaCl and 100 ml formol 40% made up to 1,000 ml with distilled water); after that, some quantity of the diluted sample was transferred to Neubauer counting chamber manufactured in Germany and the number of sperm counted under the microscope. Vitality was assessed by eosine–nigrosine test. Seminal smears were prepared from liquefied specimens and stained by Shorr for assessment of sperm morphology by reviewed David et al. method (9). Samples were divided into four categories: normozoospermia (concentration $\geq 20 \times 10^6$ sperm/ml, total motility $\geq 50\%$), azoospermia (lack of SPZ in semen), oligoasthenozoospermia (concentration $< 20 \times 10^6$ sperm/ml, total motility $< 50\%$), and asthenozoospermia (concentration $\geq 20 \times 10^6$ sperm/ml, total motility $< 50\%$). All samples were screened for leukocytes using Hendzt test (to assess positive peroxidase cells).

After semen analysis, SPZ were immediately separated from SP by centrifugation ($100 \times g$ for 10 min at room temperature). Supernatants were collected and stored at -20°C for later assessment of antioxidant activity and pellets were resuspended in phosphate buffer saline (pH 7.2).

Measurement of LP

Mean concentrations of SPZ in samples prepared for measurement of LP were adjusted at $10^7/\text{ml}$. MDA

levels were assessed using thiobarbituric acid (TBA) method according to Yagi (10). Briefly, 1 ml of each sample was added with 2 ml of TBA reagent (15% v/v trichloroacetic acid and 0.25 N HCL). The mixture was treated in a boiling water bath for 15 min. After cooling, preparation was centrifuged at $4,000 \times g$ for 10 min. Supernatant was then removed, and absorbance of the pellet (containing SPZ and round cells) was measured at 534 nm using spectrophotometry analysis.

Determination of CAT Activity

CAT activity was estimated by the method of Aebi (11). This enzyme 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 and its initial optical density was adjusted between 0.5 and 0.6 absorbance units at 240 nm by spectrophotometry method. The decrease of the absorbance was measured. One unit of CAT activity was defined as the amount of CAT absorbance in 30 sec at 25°C . The CAT activity was then calculated from the change in absorbance and expressed as U/ml.

Determination of SOD Activity

SOD activity was measured according to the method of Marklund and Marklund (12). Superoxide anion radical is involved in the auto oxidation of pyrogallol. At alkaline pH, SOD dismutates superoxide thereby inhibiting the auto oxidation of pyrogallol. The absorbance of each sample was measured at 420 nm before the addition of pyrogallol. The increase in the absorbance was measured at 30 sec interval up to 3 min. The SOD activity was expressed as U/ml.

Statistical Analysis

Data re expressed as mean and standard deviation (mean $\pm$ SD). The one-way analysis of variance and the Student–Newman–Keuls post hoc tests were performed on the data for intergroup comparisons. Database management and statistical analysis were performed using SPSS version 11 (SPSS Inc, Chicago, IL) statistical software package. The nominal statistical significance level was set at 0.05. Coefficients of correlation were calculated using Pearson's correlation analysis.

RESULTS

The mean concentration of SPZ, the percentage of normal morphology, acrosome abnormality, cytoplasmic retention, and motility (grades a and b) in the different groups are given in Table 1. Normal morphology and motility (grade a) in oligoasthenozoospermic

TABLE 1. Basic Parameters of Semen Samples in All Groups of Study ($n = 102$)

Semen parameters	Normozoospermic ($n = 9$)	Oligoasthenozoospermic ($n = 38$)	Asthenozoospermic ($n = 42$)
Sperm concentration ($10^6/\text{ml}$)	86.91 ± 53.37	$12.24 \pm 5.61^*$	95.39 ± 55.31
Normal morphology (%)	19.71 ± 11.87	$6.97 \pm 4.95^*$	$11.57 \pm 5.99^*$
Acrosome abnormalities (%)	47.43 ± 13.10	62.41 ± 18.23	56.83 ± 17.80
Cytoplasmic retention (%)	3.57 ± 6.2	5.03 ± 4.49	4.07 ± 4.22
Motility grade a ^a (%)	27.14 ± 2.67	$9.21 \pm 7.02^*$	$11.79 \pm 5.92^*$
Motility grade b ^a (%)	19.29 ± 3.24	20.79 ± 7.02	22.14 ± 5.75

Results are presented as mean $\pm$ SD.

^aGrade of sperm movement according to WHO criteria: a = Rapid progressive motility and b = Borderline progressive motility.

* $P < 0.05$ compared with normozoospermic group.

TABLE 2. MDA Levels and SOD and CAT Activities in Semen Samples of the Four Groups of Patients ($n = 102$)

	MDA levels		CAT activity	SOD activity
	SP (nmol/ml)	Pellet (nmol/ 10^7 SPZ)	SP (U/ml)	SP (U/ml)
Normozoospermic ($n = 9$)	1.22 ± 0.06	0.80 ± 0.20	1.30 ± 0.22	0.27 ± 0.05
Azoospermic ($n = 13$)	1.55 ± 0.31	0.49 ± 0.22	1.26 ± 0.21	$0.60 \pm 0.04^*$
Oligoasthenozoospermic ($n = 38$)	$3.40 \pm 0.27^*$	$2.60 \pm 0.31^*$	1.23 ± 0.21	$0.63 \pm 0.12^*$
Asthenozoospermic ($n = 42$)	$2.50 \pm 0.30^*$	$1.60 \pm 0.30^*$	1.09 ± 0.26	$0.54 \pm 0.19^*$

Results are presented as mean $\pm$ SD. SP, seminal plasma; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; SPZ, spermatozoa.

* $P < 0.05$ compared with normozoospermic group.

TABLE 3. Correlations Between MDA Levels (in Pellets and SP) and SOD and CAT Activities and the Basic Parameters of All Semen Samples ($n = 102$)

	MDA (pellet) (nmol/ 10^7 SPZ)		MDA (SP) (nmol/ml)	
	<i>r</i> -Value	<i>P</i> -value	<i>r</i> -Value	<i>P</i> -value
MDA SP (nmol/ml)	0.83	< 0.01	—	—
SOD SP (U/ml)	0.29	< 0.01	0.29	< 0.01
Motility grade b (%)	0.48	< 0.01	0.36	< 0.01
Retention cytoplasmic (%)	0.24	< 0.05	—	—
Acrosome abnormalities (%)	0.63	< 0.01	0.59	< 0.01
Sperm density ($10^6/\text{ml}$)	—	—	-0.21	< 0.05
	CAT (PS) (U/ml)		SOD (PS) (U/ml)	
	<i>r</i> -Value	<i>P</i> -value	<i>r</i> -Value	<i>P</i> -value
Sperm count ($10^6/\text{ml}$)	-0.24	< 0.05	-0.22	< 0.05
Leukocytes ($10^6/\text{ml}$)	-0.23	< 0.05	—	—
Normal morphology (%)	—	—	-0.32	< 0.05

SP, seminal plasma; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; SPZ, spermatozoa.

and asthenozoospermic groups were significantly lower than those in normozoospermic semen (Table 1). There is no significant difference in leukocyte count between different groups of patients.

The means $\pm$ SD of MDA concentrations in the pellets and in SP of oligoasthenozoospermic and asthenozoospermic groups were significantly greater compared with normozoospermic groups (Table 2). The mean level of

MDA in the azoospermic patients was not significantly higher in SP and not significantly lower in the pellet than that in normozoospermic groups (Table 2). In all samples, positive and significant correlation was established between MDA levels in the pellet and MDA levels in SP ($r = 0.83$, $P < 0.01$), SOD activity ($r = 0.29$, $P < 0.01$), motility grade b ($r = 0.48$, $P < 0.01$), cytoplasmic retention ($r = 0.24$, $P < 0.05$), and acrosome abnor-

malities ($r = 0.63$, $P < 0.01$) (Table 3). We also observed a significant correlation between one hand MDA levels and another hand SOD activity in SP ($r = 0.297$, $P < 0.01$), motility grade b ($r = 0.36$, $P < 0.01$), acrosome abnormality ($r = 0.59$, $P < 0.01$), and sperm concentration ($r = -0.21$, $P < 0.05$) (Table 3).

The mean of seminal SOD activity was significantly increased in the oligoasthenozoospermic, asthenozoospermic, and azoospermic patients compared with normoazoospermic patients (Table 2). This activity was negatively and significantly correlated with sperm concentration and percentage of normal morphology in different groups (Table 3). In contrast, no significant difference in the CAT activity measured in SP from all samples was seen, but this seminal CAT activity was negatively correlated with sperm concentration and leukocyte production in all samples (Table 3).

DISCUSSION

In the recent years, numerous studies demonstrated that ROS play a major role in the etiology of defective sperm function. Generation of ROS in the male reproductive tract has become a real concern regarding their potential toxic effects at high levels on sperm quality and functions.

SPZ are particularly susceptible to OS because their plasma membranes contain large amounts of polyunsaturated fatty acids (PUFA) (13) and their cytoplasm contains low concentrations of scavenging enzymes (3). It is known that the LP of PUFA in sperm membranes is one of the most important effects from ROS-induced cell damage, which may lead to persistent infertility (14). It was also reported that the sperm from normozoospermia had lower unsaturated fatty acid content than the sperm from asthenozoospermic individuals (15,16). In addition, it was suggested that SPZ from normozoospermic samples are less susceptible to ROS-induced peroxidative damages than SPZ from pathological samples.

In this study, we evaluated the OS status in infertile men by assessing the levels of LP product (MDA) in semen. Our results showed a significant increase in MDA levels in SP and pellet (containing mainly the SPZ) of oligoasthenozoospermic and asthenozoospermic men, in comparison with normozoospermic groups. We also found that MDA concentration in the semen of nonazoospermic groups was positively correlated with motility grade b. In an earlier study on infertile men (17), we found that MDA concentration in SPZ was much lesser than that in SP and that motility and viability were inversely correlated with semen MDA levels. In the literature, Jones and Mann (18) were the first to describe increased LP in SPZ with reduced motility; the relationship between movement charac-

teristics and LP has also been reported by Aitken et al. (19). Later, similar results have demonstrated the involvement of LP in the pathophysiology of male infertility (3). In fact, Huang et al. (20) found that the MDA concentrations in asthenozoospermia and oligoasthenozoospermia were significantly higher than those in normozoospermia. In contrast, Suleiman et al. (21) demonstrated that the seminal levels of MDA were not correlated with sperm concentration and motility. In other reports, the authors observed negative correlation between MDA concentration and sperm motility or concentration (14).

In our study, we found that in azoospermic groups, the MDA level was higher in SP than in pellet, but lower than that in the SP of other groups. These results would be related to the absence of SPZ, which are the main source of ROS production, and the low levels of MDA in the pellet originate from the immature germ cells and leukocytes. Dandekar et al. (22) suggested that the discrepancy in the levels of LP in azoospermia, in spite of the lack of sperm and leucocytes, may be owing to the lower antioxidant enzymes found in infertile men.

Recently, a significant increase of testicular MDA levels in testicular tissue from sertoli cell only (SCO) and maturation arrest patients compared with controls was reported. In addition, the elevation of testicular MDA level between SCO and moderate hypospermatogenesis and moderate hypospermatogenesis and maturation arrest groups was also significant (23). Some other authors reported an increase of LP substance levels in obstructive infertile patients (24). The mechanism of infertility in these males is the direct generation of oxygen radicals by leucocytes, sperm damage as a result of epididymal obstruction, and presence of immature sperm as a result of testicular damage owing to prior obstruction (25).

We found that MDA concentration in semen of all patients was also positively correlated with acrosome abnormality and cytoplasmic retention. In the literature, it was reported that high levels of OS were correlated with impairment of the acrosome reaction owing to protein cross-linking and LP (26) and abnormal sperm morphology (27). Recently, Mohammad et al. (28) showed a significant and negative correlation between sperm morphology and ROS production.

Normally, a balance is maintained between the amount of ROS produced and antioxidants. Cellular damage arises when this equilibrium is disturbed, especially when the cellular scavenging systems cannot eliminate the increase in ROS. The scavenging potential in gonads and seminal fluid is normally maintained by adequate levels of SOD, CAT, and probably GPX and reductase (5), which are mainly produced by the male accessory sex glands. In fact, it is admitted that SOD is

expressed in prostate, seminal vesicles, and epididymis, and it is possible that in these tissues SOD expression is upregulated in response to defective spermatogenesis or hormonal deficiency. It is also admitted that the presence of CAT activity in ROS producing semen samples may be a compensatory mechanism that produces lower-molecular-weight H_2O_2 scavengers (29).

In this study, we measured CAT and SOD activities in SP of infertile men to determine the relation between sperm characters and antioxidant defenses. Our findings are similar to those reported by Zini et al. who showed that SOD activity in infertile men is significantly greater than that in fertile men, whereas CAT activity is not different between these groups (30). However, Hsieh et al. (31) reported that SOD activities of SPZ and SP in infertile men with oligoasthenozoospermia were not significantly different from normozoospermic patients and these activities were positively, but not significantly, correlated with sperm motility and concentration. Same authors investigated in another study the activities of SOD and CAT in men with asthenozoospermia, teratozoospermia, and oligozoospermia in comparison with normozoospermic males; they showed a significant increase of intracellular activity of SOD and a decrease of CAT activity in infertile samples (29). Other studies have shown that low antioxidant capacity was related to male infertility. In fact, Alkan et al. (32) noted that infertile men have lower SP antioxidant activity (CAT, SOD, and GPX) than fertile men. More recently, Khosrowbeygi and Zarghami (33) found that the levels of CAT and total antioxidant capacity (TAC) were significantly lower in asthenozoospermic, asthenoteratozoospermic, and oligoasthenoteratozoospermic patients than in the control group; no significant changes were seen in SOD activities and levels of CAT and TAC were positively correlated with sperm motility and morphology. They concluded that decreasing SP antioxidant levels, especially CAT and TAC, could have significant role in the etiology of impaired sperm function. These results support the concept that the determination of MDA levels and the status of antioxidant activity may be taken as one of the important markers for diagnosis and treatment of male infertility induced by excessive production of ROS.

Recently, conflicting data were reported about the relationship between OS and sperm DNA damage. Smith et al. (34) found that nuclear DNA damage (TUNEL-positive cells) was significantly greater in patients with oligozoospermia compared with controls and suggested the involvement of seminal OS in the pathogenesis of sperm DNA damage. Inversely, Verit et al.'s (35) report did not find any relationship between sperm DNA damage and OS in normozoospermic infertile men. They suggested that the pathophysiology of idiopathic infertility cannot be explained by sperm DNA damage or seminal OS.

In conclusion, we demonstrated in this study that MDA levels in pathological infertile men's semen are higher than those in normozoospermic sperm, and that these levels are correlated with some semen parameters. In addition, seminal antioxidant enzyme (SOD and CAT) activities were correlated negatively essentially with sperm concentration. All these findings, similar to numerous results reported in other studies of literature, let us to suggest that the evaluation of oxidative status might have an indicative value on the diagnosis of male infertility and may be taken as a useful tool for determining sperm fertilization potential.

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