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GAMETE BIOLOGY

Effects of reduced seminal enzymatic antioxidants on sperm DNA fragmentation and semen quality of Tunisian infertile men

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Abstract
Purpose To evaluate levels of sperm DNA fragmentation and enzymatic antioxidant status in seminal plasma of Tunisian fertile and infertile men in order to assess the effects of seminal oxidative stress on sperm DNA integrity and semen quality.
Methods Semen samples from 100 infertile patients (40 oligoasthenoteratozoospermics, 31 teratozoospermics and 29 asthenozoospermics) and 50 fertile men (controls) were analyzed for DNA fragmentation by TUNEL assay and biochemical parameters. Seminal antioxidant activities (Superoxide dismutase, Glutathione peroxidase and Catalase) and malondialdehyde concentrations were measured spectrophotometrically.
Results Sperm DNA fragmentation and malondialdehyde levels in infertile groups were more elevated than controls. Nevertheless, the activities of the antioxidant enzymes were significantly lower in abnormal groups compared to normozoospermics. Sperm DNA fragmentation was closely and positively correlated to malondialdehyde levels (r=0.37, P=0.008); meanwhile, reduced seminal antioxidant profile was negatively associated to sperm DNA fragmentation. Interestingly, we noted also that sperm DNA fragmentation was negatively correlated to sperm motility (r=−0.54, P<0.001) and positively associated to the abnormal sperm morphology (r=0.57, P=0.002).
Conclusions This report revealed that increased sperm DNA fragmentation can be due to the impaired seminal enzymatic antioxidant profile and increased Lipid peroxidation. Our results sustain that the evaluation of sperm DNA fragmentation and seminal oxidative biomarkers in infertile men is recommended as a consistent prognostic tool for male infertility assessment.

Keywords Male infertility · Sperm DNA fragmentation · Oxidative stress · Reactive oxygen species · Seminal plasma · Antioxidants

Capsule Infertile men have a reduced seminal antioxidant profile and increased sperm DNA fragmentation.

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Introduction

Infertility affects around 15 % of couples in reproductive age and male factor is a major contributor by approximately half of these cases [32]. Traditionally, the diagnosis of male infertility is based on microscopic assessment, including ejaculate volume, sperm concentration, motility and morphology, but the results of this conventional semen analysis are insufficient as a diagnostic tool in male infertility [17]. In fact, new tests have been developed to better investigate the physiopathology and etiology of male infertility [13].

Recently, sperm DNA integrity has been regarded as a more accurate and precise biologic marker of male fertility since an intact DNA is necessary for the correct transmission of genetic material to the next generation [40]. It is well known that DNA damage in spermatozoa, characterized by single or double stranded DNA breaks/fragmentation, occurs during late spermatogenesis as a consequence of endogenous factors present in the testis/epididymis, or due to exogenous factors present after ejaculation [21, 30]. The origins of this DNA damage are not fully understood, but several lines of evidence suggested that oxidative stress (OS) plays a key role in the underlying etiology [4, 26].

OS is caused by an imbalance between reactive oxygen species (ROS) production and antioxidant scavenging activities in semen. ROS are highly reactive oxidizing agents belonging to the class of free radicals containing one or more unpaired electrons which are continuously being generated through metabolic and physiopathologic processes. Unlike other cells, spermatozoa are more vulnerable to OS because their plasma membrane is rich of polyunsaturated fatty acids (PUFAs) and membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [6, 7].

In effect, ROS have been shown to correlate with reduced male fertility by causing membrane lipid peroxidation (LPO) and affecting sperm concentration, morphology and motility [6, 7]. LPO in the sperm cell membranes is an autocatalytic, self-propagating reaction, which can cause cell dysfunction associated with the loss of the membrane function, integrity and finally leads to decreased fertilizing ability of spermatozoa [5]. Malnodialdehyde acid (MDA) is the major product of LPO and studies have implicated increased MDA levels with poor sperm parameters in infertile men [6, 7]. Moreover, it was reported that ROS react with sperm DNA leading to oxidative base modifications and high frequencies of DNA fragmentation [3]. Defective spermatid protamination and disulfide bridge formation because the inadequate oxidation of thiols during epididymal transit, results in diminished sperm chromatin packaging, making sperm cells more vulnerable to ROS-induced DNA fragmentation [13]. As a result, spermatozoa carrying extensively fragmented DNA could make a significant contribution in the affected fertility potential and now is more and more clearly designated as an obvious parameter in early or late failures of assisted reproductive technologies (ARTs) [32].

To protect spermatozoa from the ROS-induced damage, seminal plasma is well equipped with an array of antioxidant systems comprising natural antioxidants (Vitamines A, C, E, ascorbate and glutathione) and enzymatic antioxidants [6, 7]. The enzymatic antioxidants include the following components: superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). These enzymes are constitutively expressed in human semen [1, 27]. The free radical scavenging activity of SOD, GPX and CAT protects the sperm membrane against the LPO, preserving its integrity and hence maintaining the normal sperm parameters of motility and morphology [6, 7]. Studies by [2, 8] have also implicated the role of antioxidants in protecting the sperm nuclear genome from oxidative strand breaks and base modifications. Therefore, the combination between antioxidant profile, determined by SOD, GPX and CAT activities, and LPO biomarker (MDA) was reported to be the better indicator of OS.

Since DNA fragmentation may be a crucial indicator of male infertility and keeping in view the main protection provided by seminal antioxidants against oxidative damages, the purpose of this study was to (1) assess the levels of enzymatic antioxidants (SOD, GPX and CAT) and MDA in the seminal plasma of Tunisian fertile and infertile men and to (2) evaluate their effects on sperm DNA fragmentation and associated sperm alteration. To our knowledge, this report which investigated the repercussions of the primordial antioxidant enzymes on sperm DNA fragmentation and semen quality constitutes the first one conducted in Tunisia.

Methods

Study design and subject selection

A total of 100 men (28–50 years) participating in the In Vitro Fertilization (IVF) program at our department of cytogenetic and Reproductive Biology, Farhat Hached University Hospital (Soussa, Tunisia) were included in this prospective controlled study. Furthermore, 50 healthy donors (24–38 years) with proven fertility were included in this study in order to evaluate possible differences between fertile and infertile men. General details and characteristics of the studied population were explored in Table 1.

A detailed medical history was obtained from all participants, including reproductive history and infertility evaluation. Inclusion and exclusion criteria for sample selection were as follows:

Inclusion criteria

Patients included in this investigation were belonging couples living together with regular unprotected coitus for a reasonable period of time but not less than 1 year without conception. Furthermore, healthy donors selected for our work were characterized by perfect semen analysis.

Exclusion criteria

Subjects with azoospermia were not qualified for our study. Patients currently on any medication or antioxidant supplementation were not included. Males exposed to radiotherapy/chemotherapy and those with testicular varicocele,

Table 1 General details of controls and patient groups

Parameters	Controls (n=50)	OAT (n=40)	Terato (n=31)	Astheno (n=29)
Duration of infertility (years)				
Means ± SD	–	5.85±3.12	3.77±2.28	4.77±2.46
Min-Max	–	1–15	1–8	1–13
Duration of sexual abstinence				
(days)	3	3	3	3
Race	Tunisian	Tunisian	Tunisian	Tunisian
Area (n)				
Urban	19 (38.00)	27 (67.5 %)	21(67.74 %)	18 (62.06 %)
Rural	31 (62.00 %)	13 (33.5 %)	10 (32.25 %)	11 (37.93 %)
Diet (n)				
Vegetarian	20 (40.00 %)	22 (55 %)	23 (74.19 %)	17 (58.62 %)
Mixed	30 (60.00 %)	18 (45 %)	8 (25.80 %)	12 (41.37 %)
Cigarette smoking (n)				
Yes	0 (0 %)	11 (27.50 %)	9 (29.03 %)	12 (41.37 %)
No	50(100 %)	29 (72.50 %)	22 (70.96 %)	17 (58.62 %)

OAT Oligoasthenoteratozoospermics, *Terato* Teratozoospermics, *Astheno* asthenozoospermics, *Max* maximum, *Min* minimum, *SD* standard deviation

genital infection, leukocytospermia (>1×10^6/ml), chronic illness and serious systemic diseases and alcoholics were also excluded from this study because of their well-known high seminal ROS levels and decreased antioxidant activity.

Study consent

A written consent of each subject was taken after explaining the aims and objectives of the study and its benefits on individual and society. Also, the study was approved by the Local Ethic Committee of the Farhat Hached University Hospital, Sousse (Tunisia).

Semen analysis

Semen samples were obtained in sterile containers by masturbation after 3 days of sexual abstinence. After liquefaction at room temperature (37 °C) for 30 min, basic semen parameters were immediately assessed according to the World Health Organization guidelines [37]. The variables taken in consideration were ejaculate volume (in milliliters), sperm concentration (count) ($n\times10^6$/ml), total motility (%) and morphology (abnormal morphology, %).

Sperm motility was classified into four categories: rapid progressive motile (Type a), slow progressive motile (Type b), non-progressive motile and immotile spermatozoa, and was assayed at exactly 0.5 and 2 h after liquefaction. Total progressive motility was defined as the combination of type a rapid motility and type b slow progressive (At least 50 % of sperm should have normal motility (categories a+b)). Sperm concentration was determined with an improved Neubauer Hemacytometer® counting chamber. Sperm morphology was evaluated using the Diff-Quick staining method. At least 100 spermatozoa per patient were examined at a magnificent of X 100 according to the David classification [16]. Sperm parameters were considered normal when sperm concentration was≥20 millions/ml, total sperm motility (a+b)>50 % and normal sperm forms≥30 % by WHO criteria [37]. Accordingly, selected subjects were categorized into four groups (Details of semen characteristics were described in Table 2):

- **Controls (Fertile donors) [50 cases]**: these subjects represented normozoospermic men (Normo) which were characterized by perfect semen analysis and normal values for all semen parameters (total sperm motility (a+b>50 %), sperm concentration (>20×10^6 sperm/ml) and abnormal sperm morphology (<70 %).
- **Oligoasthenoteratozoospermics (OAT) [40 cases]**: These patients represented a combination of all the sperm abnormalities (total sperm motility (a+b<50 %), sperm concentration (< 20×10^6 sperm/ml) and abnormal sperm morphology (>70 %).
- **Teratozoospermics (Terato) [31 cases]**: Patients with elevated percentage of atypical sperm forms (>70 %).
- **Asthenozoospermics (Astheno) [29 cases]**: Cases characterized by decreased total sperm motility (a+b< 50 %).

After semen analysis, patient's samples were divided into two parts. The first part was designated for IVF and the remainder was collected for our investigation. The second part was also divided into two aliquots; first one for DNA fragmentation evaluation with terminal desoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) assay and the rest was collected to obtain seminal plasma by centrifugation at 3,500 rpm for

Table 2 Descriptive statistics and comparisons of age and conventional semen parameters between controls and infertile groups

Parameters	Controls (n=50)	OAT (n=40)	Terato (n=31)	Astheno (n=29)	P-value		
					Controls vs OAT	Controls vs Terato	Controls vs Astheno
Age (years)							
Means ± SD	34.81±5.43	39.40±5.20	37.65±6.21	36.69±4.16	<0.001	0.02	NS
Min-Max	24.00–38.00	28.00–50.00	29.00–43.00	33.00–46.00			
Volume (ml)							
Means ± SD	3.39±1.23	3.34±1.22	3.14±1.24	3.39±1.27	NS	NS	NS
Min-Max	2.60–4.75	2.00–3.50	2.00–4.00	2.00–4.50			
Motility (%)							
Means ± SD	57.84±7.70	16.32±10.89	52.46±3.19	37.84±7.70	P<0.001	NS	P<0.001
Min-Max	50.00–62.50	10.00–38.00	46.00–60.00	8.50–39.00			
Sperm count (%)							
Means ± SD	92.45±41.63	13.85±6.27	45.17±4.47	50.07±13.71	P<0.001	P<0.001	P<0.001
Min-Max	30.25–250.00	5.00–19.80	20.00–55.00	20.00–100.00			
Abnormal morphology (%)							
Means ± SD	57.47±10.08	83.52±7.58	78.09±8.34	59.22±5.27	P<0.001	P<0.001	NS
Min-Max	30.00–66.88	77.00–96.00	70.22–100	36.12–67.92			

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15 min. Obtained seminal plasma was frozen at −80 °C until enzymes and MDA analyzes.

Measurement of DNA fragmentation by TUNEL assay

An aliquot of the fresh semen was washed twice in Phosphate Buffered Saline (PBS, pH7.4) and centrifuged at 400 g for 5 min. The sediment was then fixed in methanol/-acetic acid (3: 1) for at least 30 min at 4 °C. The fixed specimens were smeared on slides and stored at −20 °C until further processing.

The level of DNA breaks is conveniently expressed by the DNA fragmentation index (DFI). The presence of apoptosis-related DNA strand breaks in spermatozoa was evaluated by the TUNEL assay, using the ApopTag® Apoptosis Detection Kits (QBiogene, Paris, France) in controls and patients. For cell permeabilisation, slides were incubated in phosphate buffer saline (PBS) with a solution of 1 % Triton X100 (Sigma). The procedure was carried out according to the manufacturer's instructions. Briefly, the specimens were washed twice in PBS 1X, equilibrated with the equilibration buffer at room temperature for 10 s and incubated in a dark moist chamber at 37 °C, for 1 h, with the Terminal Desoxynucleotidyl Transferase (TdT) solution in order to allow DNA elongation. After stopping the enzyme reaction, the slides were washed twice in PBS and the DNA elongation was revealed by incubation of the cells with anti-digoxigenin antibody coupled to peroxidase, during 30 min in a dark moist chamber. The peroxidase was revealed with DiAminoBenzidine (DAB). Slides were then counterstained with Harris' haematoxylin (RAL, Martillac, France) and finally mounted using Faramount mounting (Dako, Carpinteria, CA, USA).

Controls were also included in every experiment: for the negative control TdT was omitted and we have include proteinase K digestion to control for nonspecific incorporation of nucleotides or for nonspecific binding of enzyme-conjugate, whereas positive controls were generated by incubating the sperm cells for 10 min at room temperature with DNase I.

Slides were observed under a microscope (Zeiss, Oberkochen, Germany) equipped with a 100 magnification lens. Spermatozoa with fragmented DNA had brown-colored nuclei, whereas the other cells were blue-gray (counter coloration with Harris's haematoxylin). On each slide, approximately 500 cells were counted, and the percentage of spermatozoa with fragmented DNA (DFI, %) was calculated.

Biochemical procedures

Chemicals

All reagents and chemicals used for biochemical analysis were of analytical grade or higher purity and obtained from standard commercial suppliers. Ultra-pure water was received from water purification Milli-Q system (Millipore Corporation, USA).

Antioxidant enzyme assay

Total SOD activity was determined using pyrogallol as a substrate by the method of [23]. This method is based on

pyrogallol oxidation by the superoxide anion ($O_2^{\circ-}$) and its dismutation by SOD. One Unit (U) of total SOD and Cu-Zn-SOD is defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50 %.

GPX activity was assayed by the method of [20]. It is based on the subsequent oxidation of NADPH at 240 nm with t-butyl-hydro-peroxide as a substrate. GPX units (U/g) were defined as μmol NADPH oxidized per gram of protein.

Seminal CAT activity was determined using the method described by [9] by measuring hydrogen peroxide (H_2O_2) decomposition at 240 nm. CAT activities were expressed Units per gram of proteins (U/g). They were determined as mmol of H_2O_2 consumed/g proteins.

Determination of protein amounts

The protein content in supernatant was estimated by the Biuret method [19] using Serum Albumin Bovine as standard (BSA).

Assessment of lipid peroxidation by measurement of MDA concentrations

LPO was measured by determining the MDA production, using the Thiobarbituric Acid (TBA) method [38]. After liquefaction, semen samples were centrifuged at 3,500 rpm for 15 min to get seminal plasma. Then, 0.1 ml of seminal plasma was added to 0.75 ml of TBA reagent (0.8 g of 2-TBA dissolved in 80 ml of distilled water with 0.5 ml of NaOH. Perchloric acid (7 %) was added to this mixture in order to adjust the pH=7.4). This mixture was heated for 1 h at 95 °C in a warm water bath. After cooling, the tube was centrifuged for 10 min at 4,000 rpm and the supernatant's absorbance was read on a spectrophotometer at 535 nm.

Statistical analysis

The Windows computing program Statistical Package for the Social Sciences "SPSS 11.0" (SPSS, Chicago, IL, USA) was used for analyzing the data. All data were expressed as mean ± standard deviation (SD). The differences that existed between the evaluated study groups were assessed by performing an analysis of variance (One-way ANOVA) and the post hoc test (Tukey test) to conduct pair-wise comparisons. The differences were considered to be significant at values of $P \leq 0.05$. Finally, Pearson's correlation was performed to examine relationships between DNA fragmentation levels, oxidative biomarkers and semen quality. All hypothesis testing were two-sided with a probability value of 0.05 deemed as significant ($P<0.05$=significant*; $P<0.001$=highly significant**).

Results

Age and conventional semen characteristics

Statistic description about the age and semen parameters of infertile groups and controls was given away in Table 2.

The age of the men varied from 24 to 38 years (34.81± 5.43) in the proven fertile men compared with 28–50 years (39.40±5.20) in OAT group, 29–43 years (37.65±6.21) in Terato group and 33–46 years (36.69±4.16) in Astheno group. As shown, age was significantly lower in controls than in OAT patients ($P<0.001$) and Terato group ($P=0.02$).

When conventional semen parameters were compared between controls and infertile groups, total sperm motility showed a significant increase in normozoospermics compared to OAT patients ($P<0.001$) and Astheno group ($P<0.001$). Sperm concentration was also increased significantly ($P<0.001$) in controls than the three patient groups. However, abnormal morphology was significantly increased in OAT and Terato groups when compared to controls ($P<0.001$).

Sperm DNA fragmentation levels and oxidative biomarkers

Results of TUNEL assay for the proven fertile men and the abnormal groups can be found in Table 3. The mean percentage of sperm cells with fragmented DNA was 12.23± 4.73 % in the control group compared with 28.88±5.87 % in the OAT group, 20.62±4.42 % in Terato patients and 25.18± 6.18 % in Astheno group. Indeed, the levels of DNA fragmentation signed highly significant increase between controls and patient groups ($P<0.001$).

Table 3 summarizes also the statistical comparisons of seminal antioxidant status and seminal MDA concentrations among controls and infertile men. In effect, we noted a significant increase for seminal SOD activity in the control group compared with OAT group ($P<0.001$), Terato group ($P=0.02$) and Astheno patients ($P=0.003$). Seminal GPX showed an elevated activity in controls when compared to the patient groups but the difference was significant only with OAT group ($P<0.001$). Seminal CAT activity demonstrated also a single significant increase noted between controls and OAT group ($P=0.001$). Nevertheless, LPO which was expressed by seminal MDA content was found to be significantly higher in the three abnormal groups than in fertile donors. Mean MDA levels in abnormal groups were found to be 2.91±1.15 μmol/l in OAT group, 2.19±0.72 μmol/l in Terato group and 2.39± 1.30 μmol/l in Astheno group.

Correlations between DNA fragmentation, seminal antioxidant status and classical semen parameters

The Pearson's correlation coefficients calculated between sperm DNA fragmentation, Age, seminal oxidative biomarkers

Table 3 Statistic comparisons of DNA Fragmentation Index and oxidative biomarkers between controls and infertile men

Parameters	Controls (n=50)	OAT (n=40)	Terato (n=31)	Astheno (n=29)	P-value		
					Controls vs OAT	Controls vs Terato	Controls vs Astheno
DFI (%)							
Means ± SD	12.23±4.73	28.88±5.87	20.62±4.42	25.18±6.13	P<0.001	P<0.001	P<0.001
Min-Max	0–23.40	16.41–44.80	10.80–28.41	12.24–49.01			
SOD ($\times10^{-3}$ g/P)							
Means ± SD	2.75±0.97	1.09±0.53	2.17±1.31	2.10±0.60	P<0.001	0.02	0.003
Min-Max	1.02–7.99	0.44–6.76	0.91–6.76	0.71–3.41			
GPX ($\times10^{-3}$ g/P)							
Means ± SD	9.58±2.44	3.89±2.21	9.56±2.81	8.79±2.31	P<0.001	NS	NS
Min-Max	3.21–20.65	0.11–7.83	0.98–15.03	2.21–9.87			
CAT ($\times10^{-5}$ g/P)							
Means ± SD	2.49±1.46	1.89±0.81	1.97±0.71	2.30±0.97	0.001	NS	NS
Min-Max	0.65–10.80	0.01–6.43	0.80–3.65	0.43–2.99			
MDA (μmol/l)							
Means ± SD	1.54±0.68	2.91±1.15	2.19±0.72	2.39±1.79	P<0.001	P<0.001	0.03
Min-Max	0.43–2.99	0.65–5.77	0.59–4.54	1.03–10.80			

OAT Oligoasthenoteratozoospermics, *Terato* Teratozoospermics, *Astheno* asthenozoospermics, *DFI* DNA Fragmentation Index, *SOD* superoxide dismutase, *GPX* glutathione peroxidase, *CAT* Catalase, *MDA* Malondialdehyde acid, *Max* maximum, *Min* minimum, *SD* standard deviation, *NS* Not significant. Data are expressed as means ± SD. $P \leq 0.05$=significant; $P \leq .001$=highly significant

and classical semen criteria were described in Table 4. Correlations between sperm DFI and the studied parameters were investigated in 150 semen samples. The degree of correlation was assessed with the coefficient correlation (r) and p-value. In fact, there was no significant correlation between patient's age and the different studied parameters (sperm DFI, seminal antioxidants and sperm parameters).

Interestingly, sperm DFI showed strongly significant correlations with routine semen parameters. We noted that sperm DFI decreased significantly with an elevated total sperm motility (r=−0.54, P<0.001) and augmented in parallel with the percentage of abnormal sperm morphology (r=0.57, P=0.002).

Concerning correlations between sperm DNA fragmentation and seminal oxidative biomarkers, we found strong negative relationships between DFI and seminal antioxidant enzymes (SOD [r=−0.46, P<0.001] and GPX [r=−0.48, P<0.001]). Indeed, seminal LPO was positively associated to sperm DFI (r=0.37, P=0.008).

Table 4 Correlations between sperm DFI, age, seminal antioxidant status, seminal MDA and conventional semen criteria

	DFI	Age	SOD	GPX	CAT	MDA	Total motility	Sperm count	Abnormal morphology
DFI	–	NS	r=−0.46**	r=−0.48**	NS	r=0.37*	r=−0.54**	NS	0.57*
			P<0.001	P<0.001		P=0.008	P<0.001		P=0.002
Age	–	–	NS	NS	NS	NS	NS	NS	NS
SOD	–	–	–	r=0.42**	r=0.16*	r=−0.23**	r=0.50**	r=0.34**	r=−0.33**
				P<0.001	P=0.05	P=0.002	P<0.001	P<0.001	P<0.001
GPX	–	–	–	–	NS	r=−0.25**	r=0.68**	r=0.49**	r=−0.42**
						P=0.001	P<0.001	P<0.001	P<0.001
CAT	–	–	–	–	–	NS	NS	NS	NS
MDA	–	–	–	–	–	–	r=−0.36**	r=−0.33**	r=0.34**
							P<0.001	P<0.001	P<0.001
Total motility	–	–	–	–	–	–	–	r=0.50**	r=−0.48**
								P=0.001	P=0.001
Sperm count	–	–	–	–	–	–	–	–	r=−0.60**
									P<0.001

DFI DNA Fragmentation Index, *SOD* Superoxide dismutase, *GPX* Glutathione peroxidase, *CAT* Catalase, *MDA* Malondialdehyde acid, *NS* Not significant. * Significant correlation P<0.05, ** Correlation strongly significant $P \leq 0.001$.

Correlations between seminal antioxidant status, seminal MDA and semen parameters were also evaluated in this study and presented in Table 4. In actual fact, only seminal SOD and GPX activities showed significant correlations with total sperm motility ([r=0.50, P<0.001], [r=0.68, P<0.001]; respectively) sperm concentration ([r=0.34, P<0.001], [r=0.49, P<0.001]; respectively) and abnormal morphology ([r=−0.33, P<0.001], [r=−0.42, P<0.001]; respectively). Seminal MDA was negatively associated to total sperm motility (r=−0.36, P<0.001) and sperm concentration (r=−0.33, P<0.001). Meanwhile, there was a positive significant correlation among seminal LPO and the abnormal morphology (r=0.34, P<0.001).

Discussion

In reproductive medicine, the molecular and biochemical bases of male infertility are still poorly defined. Research on the integrity of the sperm nuclear DNA has recently been the subject of intense study which reported that sperm DNA fragmentation index appears to a more stable criterion than do classical semen parameters [14]. Currently, it is becoming clearer that oxidative sperm DNA fragmentation forms an obvious parameter in male infertility and ART's failures [32]. In this study, we determined the sperm DFI and the seminal antioxidant status in fertile and infertile men in order to estimate the correlations between sperm DNA damage and seminal OS and to evaluate their effects on semen quality.

The most relevant findings of this study were: (*1*) significantly higher degrees of sperm DNA fragmentation were noted in infertile cases when compared to controls. (*2*) Conversely to the seminal antioxidant enzymes, seminal MDA amounts were significantly increased in infertile groups than controls. (*3*) Firm relationships were evident, particularly in relation to the negative effects of impaired antioxidant status and increased seminal LPO on sperm DNA fragmentation and semen quality. These findings indicated that some infertile men may be poorly equipped to deal with OS and oxidative DNA fragmentation due to the impaired seminal antioxidant defenses.

As we have previously indicated, our results reported highly significant increases of the sperm DNA fragmentation in the three infertile groups compared to controls. We also identified significant correlations between conventional semen parameters and sperm DNA fragmentation. In fact, total sperm motility showed a strong negative correlation with sperm DFI, however; abnormal sperm morphology was positively associated to the sperm DFI. Our data were in agreement with the observations of [25, 39, 40] who found that sperm DFI was significantly higher in Astheno and oligozoospermic patients than normozoospermics. Huang et al. [22] and [10, 11] evaluated also sperm DNA fragmentation in correlation with sperm parameters using TUNEL assay and they noted significant elevated levels in patients with sperm dysfunctions than in those with normal sperm criteria. Nevertheless, several other studies didn't corroborate with our findings and reported elevated rates of sperm DFI in normozoospermics compared to the abnormal groups [31]. These differences may be due to several variables among which are inclusion and exclusion criteria for patient selection, the particular abstinence time (3 days), the cigarette-smoking habits and the technique used in the sperm DFI determination. In our investigation, we have used TUNEL assay to evaluate the rates of sperm DFI; however [32], have used SCSA (Sperm Chromatin Structure Assay) test. Also, there were marked differences in the reported control population. In our work we used healthy donors with perfect semen parameters, while others have used idiopathic infertile patients with normozoospermia [34]. In keeping with relationships between sperm DNA fragmentation and classical sperm criteria, despite some studies have reported either only a weak or no correlation between semen quality and sperm DFI [14], several other investigations have found significant correlations. Zini et al. [39] demonstrated negative correlations between sperm DNA integrity and sperm quality in partners of couples attending IVF clinics. Additionally, in a small cohort of patients consulting for infertility investigation, prospective analyzes showed that both sperm motility and sperm morphology were inversely correlated to the rate of nuclear DNA fragmentation [10–12, 36]. These results corroborated with our findings and suggested that sperm motility and morphology defects may be in part due to a reduction of sperm DNA integrity which can be promutagenic and can adversely affect fetal outcome after IVF and has lifelong implications on health of child [34].

The origins of DNA fragmentation in spermatozoa is still a matter of debate, but many causes could be responsible for it and may be attributed to intra and/or extra-testicular factors [17, 34]. Recently, growing evidence has been postulated indicating the important physiological role of OS in sperm DNA fragmentation, male infertility and consequently the ART's outcomes [4, 26]. In this study, increased levels of sperm DFI observed in the infertile groups may be caused by the high levels of OS found in their seminal plasma. The link between seminal OS and sperm DNA damage may be also related to a defect in spermiogenesis that causes the release of immature spermatozoa that have high DNA damage [32]. Efficiently, we found that seminal MDA concentrations observed in OAT, Astheno and Terato groups were significantly increased than the seminal MDA amounts observed in controls. This significant raise of seminal LPO in abnormal groups can be explained by the significant decrease of the enzymatic antioxidant activities (SOD, GPX and CAT) in infertile groups. These observations corroborated with the findings of [6, 7, 18, 24] and highlighted the fundamental scavenging activity of these antioxidant enzymes against the

oxidation of membrane lipids and the loss of sperm membrane integrity [28, 34]. Moreover, we have clearly indicated that lowered antioxidant profile and elevated seminal MDA concentrations were closely associated with increased sperm DFI. These correlations confirmed also the important role of seminal antioxidant enzymes in protection of sperm DNA integrity against excessive ROS production. Effectively, the reduced antioxidant profile and the increased DFI in OAT and Terato groups demonstrated that morphologically abnormal spermatozoa, with residual cytoplasm, may be more susceptible to DNA strand breaks [17]. These spermatozoa have been shown to have a capacity to generate high levels of ROS that, on exceeding critical levels cause OS [32]. Increased OS in their seminal plasma may be attributed to NADPH, which is mediated by G6PD abundant in cytoplasmic residues [34]. Our results were consistent with previously reports that documented the presence of impaired DNA integrity in abnormal semen samples without leukocytospermia [17]. We showed also that sperm motility linked to the membrane fluidity was largely affected by the altered antioxidant activity and the oxidation of the membrane PUFAs. Alternatively, we have indicated that the Astheno samples with greater MDA levels have increased levels of DNA fragmentation. This observation confirmed that sperm DNA fragmentation can be a result of seminal OS and a relationship to sperm motility may be expected. Shamsi et al. [33] reported that ultra-structural defects in asthenozoospermics harbored a high level of mitochondrial DNA damage and had elevated MDA concentrations. This could be explained by OS induced mitochondrial DNA damage and LPO which disrupts ATP production and microtubule assembly [34].

Other factors which can induce sperm DNA fragmentation were cited like defective apoptosis before ejaculation ([29], Brahem et al. [11]), improper packaging and ligation during sperm maturation and the advanced age of infertile men [10, 11]. Accordingly, these mechanisms, co-dependently with OS, might be responsible for sperm DNA fragmentation [34]. In our study, no correlation was found between the age and the sperm DNA fragmentation. This was in agreement with the results of [15] who found no difference in DNA fragmentation rate according to age among 212 men aged between 25 and 70 years, but in disagreement with the results of others studies [10, 11, 35] who found a significant correlation between DNA fragmentation and patient's age.

Conclusions

In summary, associations of impaired seminal antioxidant activities and augmented LPO with DNA fragmentation and conventional semen parameters indicated that the decrease of seminal antioxidant profile can be a risk factor for sperm DNA damage and semen abnormality. Accordingly, these data suggested that the routine determination of sperm DNA fragmentation and oxidative biomarkers levels becomes recommended as a reliable prognostic tool for male infertility assessment. Therefore, large scale and randomized study might be explored in the future in order to determine the effects of oxidative sperm DNA fragmentation on the outcome of ARTs with respect to semen quality. This may help selection of semen samples with the least amount of sperm DFI and reduce the risks associated with the use of DNA-fragmented sperm for fertilization and avoid financial, social and emotional problems associated with failed IVF attempts.

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