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Relationship between sperm DNA damage with sperm parameters, oxidative markers in teratozoospermic men

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ABSTRACT

Objective: This study was aimed at determining the extent of sperm nuclear DNA damage in patients with isolated teratozoospermia and examining its relationship with oxidative stress.
Study design: Semen samples from 60 patients with isolated teratozoospermia and 30 normozoospermic donors were examined. DNA damage was evaluated by the COMET assay. Seminal antioxidant activities (Superoxide dismutase; Glutathione peroxidase; Catalase), iron and malondialdehyde concentrations were measured spectrophotometrically.
Results: Sperm DNA damage; malondialdehyde and iron levels were more elevated in studied groups than controls. Nevertheless, the antioxidant enzyme activity obtained was significantly lower in the group of patients with teratozoospermia compared to the controls. Sperm DNA damage was positively correlated to malondialdehyde and seminal iron level while reduced seminal antioxidant status was negatively associated with sperm DNA breaks. Interestingly, we noted that sperm DNA damage; lipid peroxidation, iron level, and impaired antioxidant status were negatively correlated to normal sperm morphology.
Conclusion: These findings may explain the complex biological relationship between teratozoospermia, oxidative stress, and DNA damage. In fact, an impaired seminal antioxidant status and an increased seminal level of both lipid peroxidation and iron can affect sperm nuclear integrity resulting in DNA breaks and can be associated with poor sperm morphology.

Introduction

Human spermatozoa are professional generators of reactive oxygen species (ROS) which, when present in specific physiological concentrations, are thought to play an important role in many cellular processes such as sperm cell signaling events, sperm genomic stability and sperm fertilizing ability [1]. However, if the amount of ROS overwhelms their limited antioxidant defenses, a status of oxidative stress can occur. This oxidative attack can damage the DNA, RNA, proteins, and lipids which can alter the sperm fertility potential [2–4].The major ROS produced are O_2- and H_2O_2, the antioxidants such as GPX, CAT and SOD that neutralize them are present both within mitochondria and in the secretions of the reproductive tract [5].The ROS concentration can increase when there is an excess of their production, or in the absence of an adequate antioxidant system. It can also increase in the presence of Iron [6]. In fact, a high iron concentration has been detected in the seminal plasma of infertile men [7]. It has been hypothesized that iron plays a causative role in the spermatozoa dysfunction. In 2002, Agarwal et al [8] proved that the transition of metal ions such as iron can generate highly reactive hydroxyl radicals in the presence of $H_2O_2$and O_2- (Haber-Weiss reaction) or H_2O_2(Fenton reaction).

Both teratozoospermia and oxidative stress are strongly correlated with infertility [9–12]. Some studies have recently performed small comparisons between teratozoospermic and nonteratozoospermic groups [13,14] and showed a positive correlation between sperm ROS overproduction and the proportion of spermatozoa with abnormal morphology, which indicates a potential relationship between the two events.

ROS overproduction has been linked to DNA damage of the poorly protected sperm cells [15]. Despite the protective tight packaging of the sperm DNA, deoxyribonucleic acid bases and

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phosphodiester backbones are susceptible to peroxidation [16]. Nevertheless, the relationship between the defect in DNA integrity and the poor sperm morphology may not go undetected [17,18].

The exact mechanisms linking both oxidative stress and DNA damage within defects in sperm morphology have not been well understood. Therefore, in this study, we examined the relationships between oxidative stress biomarkers, fragmented DNA as well as morphologically abnormal spermatozoa. To investigate oxidative stress, we measured seminal levels of the following antioxidants enzymes; catalase, Superoxide Dismutase (SOD) and Glutathione peroxidase activity (GPx) and seminal levels of two prooxidants; iron and Malondialdehyde (MDA). We performed the COMET assay as an indicator of DNA damage.

Material and methods

Study population

Semen samples (n = 60) were obtained from patients attending our fertility clinic. This study group presented an isolated teratozoospermia

Additionally, 30 healthy donors with normal semen profiles and proven fertility were used as controls. None of the semen samples, included in our study had leucospermia or a necrozoospermia. All subjects in each group had no history of chemotherapy, radiotherapy, chronic illness or medication.

This protocol was approved by the local ethics committee of Fattouma Bourguiba University Teaching Hospital of Monastir, and all patients and controls had previously given informed consent for the study.

Semen analysis and preparation

Semen samples were collected by masturbation after 3–5 days of sexual abstinence. After sample liquefaction, standard semen parameters were evaluated according to the World Health Organization (WHO) guidelines [19]. According to the modified David classification [20], a detailed morphological assessment of the spermatozoawas performed to provide the specific type of morphological sperm abnormalities. An aliquot of the fresh semen was washed twice in Phosphate Buffered Saline (PBS) and centrifuged. Then the pellet was fixed in methanol / acetic acid (3:1). The supernatants and the fixed specimens were stored at −20 °C until further processing.

Assessment of oxidative stress biomarkers

Seminal oxidative stress biomarkers were measured spectrophotometrically '' Catalase [21]; Glutathione peroxidase activity (GPx) [22]; Superoxide dismutase (SOD) [23] and Malondialdehyde(MDA) [24]''.

Assessment of iron and protein level

The seminal plasma level of iron and Protein were determined spectrophotometrically using commercial reagent kits (Cobas IRON2, Roche Mannheim, Germany).

Assessment of DNA fragmentation by the comet assay

To detect the total DNA strand breaks, the alkaline comet assay was used according to the modified method of Singh et al. [25]. After electrophoresis, the slides were stained with ethidium bromide, and observed under a fluorescence microscope (Leica) at 40× magnification. Images of 50–60 individual cells per slide were acquired. These images were analyzed with open COMET ASSAY IV software and different parameters of DNA damage, such as tail length, tail percentage, tail moment and olive moment were obtained.

Statistical analysis

Statistical analysis was performed using SPSS 21 (SPSS Inc., Chicago, IL, USA). Statistical tests *t*-test and Spearman) were performed to determine whether the differences observed between each group were significant. They were perceived as such when the p value was less than 0.05.

Results

Semen characteristics and detailed morphology assessment

Seminal parameters and detailed morphological assessment for each group are reported in Table1. Both Abnormal sperm forms and head abnormalities were significantly higher in patients with teratozoospermia compared to controls, with a predominance of the microcephalic heads (42.66 ± 17.5) and the amorphous heads (29.84 ± 11.7).

For the tails abnormalities, a significant difference between the two groups (7. 11 ± 1.34 vs 8.29 ± 3.25 $p < 0.05$), particularly for, coiled tails (14.28 ± 6.35 vs 18.66 ± 9.01), and short tail (9.52 ± 4.19 vs 13.48 ± 7.47) was noted.

Sperm DNA comet parameters and oxidative biomarkers

The seminal plasma antioxidant status of our samples is shown in Table 2. We detected a significant decrease in seminal GPx activity in the group with teratozoospermia compared to controls (199.62 ± 168.92 vs 292.62 ± 148.2 μmol oxide GSH /min/mg of protein, $p < 0.05$). The seminal catalase activity was also significantly decreased in the same group (2633 ± 1404.5 vs 3605.29 ± 2549.4 μmol H2O2/min/mg of protein, $p < 0.05$). But

Table 1
Comparison of age, standard semen parameters and detailed morphology between control and study groups.

	Control group n=30 Mean ± SD	Study group n=60 Mean ± SD	*P* value
Age	35±5.65	34.56±7.09	*NS*
Volume	2.83 ± 0.94	3.86 ± 3.22	*NS*
pH	8.14 ± 0.21	8.1 ± 0.22	*NS*
Numeration (10^6spz/ml)	98.04 ± 58.88	96.89± 71.97	*NS*
leucocyte Concentration (10^6/ml)	0.54 ± 0.056	0.45 ± 0.2	*NS*
Sperm motility (%)	45.5 ± 8.13	41.25 ± 10.99	*NS*
Necrozoospermia	17.92 ± 8.37	22.77 ± 14.32	*NS*
Atypical forms (%)	81.63 ± 5.05	92.68 ± 3.37	0.001[b]
Head abnormalities (%)	1457 ± 3,94	20,77 ± 8,15	0.001[b]
Macrocephalic (%)	2.31 ± 2.85	2.03 ± 2.8	*NS*
Microcephalic (%)	33.51 ± 11.6	42.66 ± 17.5	0.012[a]
Amorphous head (%)	19.73 ± 6.34	29.84 ± 11.7	0.001[b]
Tapered head (%)	33.36 ± 11.01	38.95 ± 14.74	*NS*
Double head (%)	1.56 ± 1.81	1.78 ± 1.57	*NS*
Tail abnormilities (%)	711 ± 1,34	8,29 ± 3,25	*0.005*[b]
Two tailed (%)	1.26 ± 1.15	1.55 ± 3.03	*NS*
Coiled tail (%)	14.28 ± 6.35	18.66 ± 9.01	0.016[a]
Bent tail (%)	1.63 ± 2.34	1.53 ± 1.8	*NS*
Short tail (%)	9.52 ± 4.19	13.48 ± 7.47	0.007[b]
Cytoplasmic droplet (%)	1.63 ± 0.67	2.07 ± 2.05	*NS*
Multiple anomalies index	1.62 ± 0.17	1.82 ± 0.22	0.001[b]

Sperm parameters are expressed as mean ± standard deviation or median (interquartile range) depending on their normal distribution. Parametric data was analyzed by paired t-test.
NS Not significant.
[a] Significant difference with control group ($p < 0.05$).
[b] Highly significant difference with control group ($p < 0.01$).

Table 2
Statistic comparison of oxidative biomarkers between control group and study group.

	Control n = 30 Mean ± SD	Teratozoospermic n = 60 Mean ± SD	*P* Value
GPx (μmol oxide GSH/min/mg P)	292.62 ± 148.2	199.62 ± 168.92	0.05[a]
Catalase (μmol H2O2/min/ mg P)	3605.29 ± 2549.4	2633 ± 1404.5	0.05[a]
SOD (U/mg de protein)	2.61 ± 0.84	2.59 ± 0.83	*NS*
Iron (mg/ml)	4.7 ± 1.26	6.01 ± 2.8	0.001[b]
MDA (nmol/L)	0.58 ± 0.27	0,93 ± 0.83	0.05[a]

All values are expressed as mean ± standard deviation and analyzed using the paired t-test.
NS Not significant.
[a] Significant difference with control group ($p < 0.05$).
[b] Highly significant difference with control group ($p < 0.01$).

this difference was not significant for SOD activity (p = 0.94). On the other hand, the iron and MDA seminal concentration were significantly increased in the patients group ($p \leq 0.01$).

Using Open COMET IV, a robust software solution for COMET analysis, about 16 parameters were generated. Tail length (TL); Tail DNA percent (TP); Tail moment (TM) and Olive moment (OM) were chosen as measures to assess the extent of sperm DNA damage.

The results showed an appreciable and statistically significant increase of DNA damage in patients with morphologically abnormal spermatozoa compared to controls ($p < 0.001$) (Table 3).

Correlation between DNA COMET parameters with seminal oxidative biomarkers and standard sperm parameters

With Spearman test, correlation analysis was performed in both control and patient groups. Accordingly, the COMET DNA parameters in terms of Tail Moment (considered to be the optimum parameter representing DNA damage) showed statistically significant and positive correlations with the percentage of atypical forms ($p < 0.01$), and the multiple anomalies index ($p < 0.01$). This correlation is particularly evident with head abnormalities ($p < 0.001$) and tails abnormalities ($p < 0.05$). As for correlations between sperm DNA damage and seminal oxidative biomarkers, GPX was negatively correlated tothe Tail Moment(r=-0.376, $p < 0.01$). Whereas, MDA and Iron seminal levels were positively associated with the Tail Moment ($p < 0.01$) (Table 4).

Correlations between seminal antioxidant status, seminal MDA, Iron level and semen parameters were also investigated in this study and presented in Table 5.Significant correlations were found between the decreased level of GPx activity with morphological abnormalities (r=-0.301, $p < 0.01$) and the multiple anomalies index (r=-0.316, $p < 0.01$). Interestingly, Gpx activity was also correlated negatively with the total head abnormalities and the total tail abnormalities ($p < 0.05$).

In addition, there were positive significant correlations between seminal Lipid peroxidation (LPO), iron level and abnormal morphology ($p < 0.001$, $p < 0.05$) respectively. The multiple anomalies index was also positively correlated to the seminal LPO ($p < 0.01$).

Table 3
Statistic comparison of Comet DNA parameters between control group and study group.

	Control n = 30 Mean ± SD	Teratozoospermic n = 60 Mean ± SD	*P* Value
Tail DNA %	12.68 ± 3.2	20.76 ± 4.66	0.001[b]
Tail Length	26.54 ± 12.0	53.93 ± 15.26	0.001[b]
Tail Moment	9.07 ± 3.71	15.29 ± 4.94	0.001[b]
Olive Moment	5.6 ± 13.68	10.58 ± 11.17	0.001[b]

All values are expressed as mean ± standard deviation and analyzed using the paired t-test.
[b] Highly significant difference with control group ($p < 0.01$).

These correlations were evident with the incidence of head abnormalities (r = 0, 281 $p < 0.01$; r=0.23, $p < 0.05$) respectively.

Correlations between the studied oxidative biomarkers

Our study showed negative associations between Gpx activity and both MDA content ($p < 0.001$) and iron level ($p < 0.05$) in seminal plasma of teratozoospermic patients. In contrast, a positive relationship was found between seminal LPO and Iron level ($p < 0.01$). Furthermore, we noted another positive correlation between seminal Catalase and SOD activities ($p < 001$). On the other hand, we noted negative but not significant correlations between seminal MDA content, iron level among Catalase and SOD activities (Table 6).

Discussion

We analyzed important markers of oxidative stress and their eventual association with sperm DNA breaks, in order to better understand the pathology of nuclear defects leading to poor sperm morphology. In brief, we identified significantly higher specific degrees of sperm DNA damage in the group with isolated teratozoospermia when compared to the controls. Conversely to the seminal antioxidant enzymes, seminal MDA amounts and iron levels were significantly increased in the study group. Strong relationships were observed, particularly with the negative effects of impaired antioxidant status and increased seminal levels of iron and lipoperoxidation on sperm DNA breaks and abnormal sperm morphology. These findings were intriguing for many reasons as they may provide some insight into the mechanisms behind teratozoospermia. Oxidative stress resulting in DNA breaks has been recently reported in human spermatozoa, but the pathogenesis behind its occurrence and relationship with teratozoospermic samples remains unknown. There has been evidence that reactive oxygen species (ROS) remaining from defective seminal antioxidants machinery could be related to abnormal spermatozoa function and fertilization ability [1,26]. Nevertheless, seminal plasma is considered as the common source of enzymatic and non enzymatic antioxidants that protect sperm cells against oxidative insults and Superoxide dismutase (SOD), Glutathione peroxidase (GPX) and Catalase enzymes are important indicators of its antioxidant status. Since it is difficult to measure the effectiveness of SOD in isolation of the other antioxidants, we chose to determine SOD activity in seminal plasma of men with isolated teratozoospermia associated with GPX and Catalase activities. Our results analysis indicated lower but not significant seminal SOD activity detected in teratozoospermic group compared to the controls. This finding confirms previous observations published by other authors [27]. In contrast, the study of Calamera et al. [28] showed a highly significant seminal SOD activity in normozoospermic men compared to infertile groups. SOD as an antioxidant enzyme has an important biological role when removing superoxide anions ($O_2^{\cdot -}$) but it can also be considered as a

Table 4
Correlation between Comet DNA parameter (TM) with Sperm parameters and oxidative biomarkers.

	Atypical forms (%)	Head abnormalities (%)	Tail abnormilities (%)	Cytoplasmic droplet (%)	Multiple anomalies index	GPx	Catalase	SOD	Iron	MDA
Tail Moment (TM)	r = 0.774[b] $p<0.01$	r = 0.553[b] $p<0.01$	r = 0.213[a] $p<0.05$	*NS*	r = 0.446[b] $p<0.01$	r= -0.376[b] $p<0.01$	*NS*	*NS*	r = 0.404[b] $p<0.01$	r = 0.647[b] $p<0.01$

Statistical analysis was performed using the Spearman Rank Order correlation test.
NS Not significant.
[a] Significant difference with control group ($p < 0.05$).
[b] Highly significant difference with control group ($p < 0.01$).

Table 5
Correlation between Sperm parameters and oxidative biomarkers.

	GPx	Catalase	SOD	Iron	MDA
Atypical forms (%)	r= -0.301[b] $p<0.01$	*NS*	*NS*	r = 0278 [a] $p<0.05$	r = 0.511[b] $p<0.001$
Head abnormalities (%)	r= -0.213[a] $p<0.05$	*NS*	*NS*	r = 0.238 [a] $p<0.05$	r = 0.281[b] $p<0.01$
Tail abnormilities (%)	r= -0.213[a] $p<0.05$	*NS*	*Ns*	*NS*	*NS*
Cytoplasmic droplet (%)	*NS*	*NS*	*NS*	*NS*	*NS*
Multiple anomalies index	r= -0.316 [b] $p<0.01$	*NS*	*NS*	*NS*	r= -0.213[a] $p<0.05$

Statistical analysis was performed using the Spearman Rank Order correlation test.
NS Not significant.
[a] Significant difference with control group ($P < 0.05$).
[b] Highly significant difference with control group ($P < 0.01$).

pro-oxidant by the conversion of the superoxide anion into a stable and invasive free radical, hydrogen peroxide (H_2O_2). To efficiently recycle H_2O_2, two enzymes are available; Catalase and GPX. Conversely to Tramer et al. [29], we noted that the activity of seminal GPX was lower in the study group compared to normozoospermic patients, which can probably be attributed to a defective spermatozoa maturation process during their development.

Several studies suggested that the presence of an adequate activity of GPX in seminal plasma protects sperm against peroxidative damage and also plays a key role in sperm maturation from the early stages up to the onset of fertilization [30]. Decreased GPX activity can reduce thefertilizing ability and lower sperm quality. Even, specific inhibition of GPx in vitro can induce lipid peroxidation significantly, reinforcing its crucial contribution to counteract the deleterious effects of oxidants [30]. Additionally, we have demonstrated a reduction of the catalase activity in seminal plasma of the study group when compared to controls. This observation is in agreement with the reports of several studies [31]. The positive and strong relationship which was noted between seminal Catalase and SOD activities confirms that it is difficult to measure the effectiveness of one antioxidant in isolation of another because they appear to act in cooperation.

In order to estimate their contribution in generating good sperm quality, we tried to explore the association between the previously studied antioxidants enzymes and the rate of atypical forms as well as the rate of detailed morphological abnormalities in the semen of patients with isolated teratozoospermia. Although some studies have reported either weak or no associations between sperm morphology and GPx or/and Catalase activities [32,33], significant associations between poor sperm morphology and lower Gpx and Catalase levels were identified in our study. This result was in accordance with those observed by Giannatasio et al. [34]. Moreover, decreased levels of theseenzymes were strongly correlated to the total sperm head and tail abnormalities. These correlations suggest that sperm morphological defects may be due, in part, to a reduced seminal antioxidant capacity.

Impaired enzymatic antioxidant status detected in the seminal plasma of men with isolated teratozoospermia, may be an important risk factor for oxidative damage which leads to a highly susceptible spermatozoa to lipid peroxidation. Consequently, in order to clarify our results, we have thenmeasuredseminal MDA level which was significantly increased in the patients group.

This significant rise of seminal lipid peroxidation in the teratzoospermic group can be explained by the significant decrease of their enzymatic antioxidant activity.

These observations were consistent with the finding of Atig et al. [32,33] and highlighted the fundamental scavenging activity of these antioxidant enzymes against lipid membrane oxidation [28]. In addition, elevated seminal MDA concentrations were closely associated with poor sperm morphology, particularly with abnormal head and tail forms. This may also support the hypothesis that the cytoplasmic membrane could be the primary structure involved in morphological abnormalities of the spermatozoa. At the same time, it is the main sperm structure attacked by the ROS [35]. In fact, it was clearly reported thathuman spermatozoa are particularly vulnerable to oxidation because they are well endowed with polyunsaturated fatty acids that are

Table 6
Inter-correlation between the different oxidative biomarkers.

	Oxidative biomarkers				
	GPx	Catalase	SOD	Iron	MDA
1	–	–	–	–	–
2	*NS*	–	–	–	–
3	*NS*	r = 0.319[b] $p<0.01$	–	–	–
4	r= -0.162[a] $p<0.05$	*NS*	*NS*	–	–
5	r= -0.224[b] $p<0.01$	*NS*	*NS*	r = 0.266[b] $p<0.01$	–

Note: Statistical analysis was performed using the Spearman Rank Order correlation test.
[a] Significant difference with control group ($P < 0.05$).
[b] Highly significant difference with control group ($P < 0.01$). *NS* Not significant.

susceptible to free radical attack at the alpha methylene carbons adjacent to the carbon-carbon double bonds [36,37].

The Catalase activity was assessed in the seminal plasma of our subjects. Since iron is an essential co-factor of this antioxidant enzyme, we decided to determine its seminal level and estimate its influence on semen quality. Our findings showed a significant increase of seminal Iron level in the study group compared to the controls. These findings are in agreement with several other studies [38,39]. They suggested that iron plays an essential role in spermatogenesis and male infertility. Consistently with Huang et al. [40] results, we found significant positive correlations between iron and MDA concentrations in semen of teratozoospermic men. Iron might be the mediator of the oxidative attacks by inducing lipid peroxidation. The mechanisms behind this theory were discussed in the studies of Aitken et al. [36,41] who has demonstrated that in the presence of Ferrous Ions promoter, the Malondialdehydegeneration represents the catalytic breakdown of the lipid hydroperoxides in the sperm plasma membrane and the subsequent propagation of a peroxidation chain reaction through the creation of alkoxyl and peroxyl radicals.

In this respect, we noted a significant correlation between decreased GPx activity as a protective factor and the high levels of iron, but to our knowledge, there is no data in the literature to compare our results with. Our findings sustain the possible link between oxidative stressinduced by iron and its deleterious effect on sperm morphology.

This theory can also be confirmed by the positive correlation observed between seminal iron levels with abnormal sperm morphology especially sperm head abnormalities observed in our investigations. Interestingly, the morphological and biochemical alterations were accompanied by a remarkable DNA damage, which was evaluated by of the COMET assay; a highly sensitive method for evaluating the single- and double-strand DNA breaks. Teratozoospermic men presented a remarkable increase in DNA single- and double-strand breaks, shown by the significant increase in tail DNA percentages: tail length, olive moment and Tail moment as COMET DNA markers. Thus, we found a significant association between sperm morphology and sperm DNA damage. The COMET DNA markers were particularly correlated with the incidence of sperm head abnormalities.

These correlations suggest that sperm head defects may be in part due to a reduction in sperm DNA integrity. This is in agreement with different other studies [14,17].We have also reported a significant correlation between DNA fragmentation and total abnormal tails. Using a TUNEL coupled flow cytometry, Muratori et al. [42] reported that the extent of sperm DNA fragmentation was positively related to poor morphology and associated with defects of the sperm tail. It's not easy to understand the mechanisms leading to DNA breaks due to the multiple intrinsic and extrinsic factors involved [28]. In this study, increased levels of sperm DNA fragmentation observed in teratozoospermic group may be caused by the unbalanced oxidative status found in its seminal plasma.

Generally, when a spermatozoon is subjected to oxidative stress, it is clear that both the plasma membrane and the DNA will come under attack [43]. In this respect, we clearly showed that decreased antioxidant enzymes and elevated seminal MDA and iron concentrations were strongly associated with increased sperm DNA damage. Our finding was consistent with the results of Atig et al. [33] who confirmed the important role of seminal antioxidant enzymes in counteracting the deleterious effects of oxidative attacks on lipid and nuclear compound of the spermatozoon. The link between seminal oxidative stress and sperm DNA damage may also be combined with spermiogenesis defects [44,45]. This may lead in turn, to ROS overproduction as characterized by the prevalence of DNA damage [14,46].

Conclusion

The association between impaired seminal antioxidant activity with increased LPO and DNA damage in semen samples containing a high proportion of sperm morphological abnormalities indicated that the lack of seminal antioxidant system can be a risk factor of sperm nuclear DNA damage leading to poor morphology. Increased iron content may be an important factor involved in the mechanism of oxidative stress-mediated DNA breaks in teratozoospermic semen. This data suggest that the measure of oxidative stress biomarkers could be recommended during the routine male infertility assessment. Nevertheless, the COMET assay can be a useful tool to detect the prevalence of sperm DNA damage and to identify potential male infertility. Accordingly, we suggest further comparative studies connecting the spermatozoa morphology and the DNA integrity with more oxidative stress markers, such as microelements balance and protein oxidation.

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