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Effects of adverse semen parameters on total oxidation status and DNA/chromatin integrity

Maryam Imani^{1†}, Minoo Vahedi Raad^{2†}, Maliheh Afsari¹ and Farzaneh Fesahat^{1*} 

Abstract

Background Infertility is a major health problem that affects 7% of the men's population. Oxidative stress (OS) plays a significant role in the pathophysiology of male infertility. The purpose of this study comparatively evaluated the total anti-oxidation status and DNA/chromatin integrity in semen samples among different infertile men's groups compared with the normozoospermic men.

Methods This cross-sectional study contains four experimental groups, including teratozoospermia (Exp I), asthenoteratozoospermia (Exp II), oligoasthenoteratozoospermia (Exp III), and azoospermia (Exp IV), as well as the control group of normozoospermic men. The total antioxidant capacity (TAC) and total oxidant status (TOS) were assessed by applying the enzyme-linked immunosorbent assay. The chromatin/DNA damage was assessed in semen samples of all study groups by applying chromomycin A3 (CMA3) and toluidine blue (TB) staining methods.

Results The results showed significantly higher proportions of TB+ and CMA3 positive sperm in all experimental groups compared to controls ($P < 0.001$). TAC, TOS, and the ratio of TAC to TOS were significantly different in all experimental groups compared to the normozoospermic men ($P < 0.001$).

Conclusion Our study demonstrated that at least one sperm parameter abnormality, such as teratozoospermia could cause serious defects at the levels of DNA/chromatin as well as the antioxidants to oxidant balance of human spermatozoa in subfertile men with abnormal spermogram. Infertile men with sperm morphological abnormalities may benefit from simultaneous assessment of sperm DNA defects and OS.

Keywords Chromatin quality, DNA defects, Male infertility, Oxidative stress, Sperm

1 Background

Infertility is the main health concern that affects about 7% of men [1]. Male infertility can be caused by different reasons, such as failure of the testis, obstruction, cryptorchidism, low semen quality, sperm abnormalities and agglutination, unexplained infertility, varicocele,

ejaculatory defects, endocrine disruption, congenital disorders, environmental factors, and lifestyle [2–5]. Male infertility can be related to the over production of reactive oxygen species (ROS) in the semen. Specified physiological levels of ROS are essential for different natural mechanisms including sperm maturation, acrosome reaction, capacitation, and fertilization process [6]. The overabundance of ROS decreases the antioxidant defense of semen, which leads to oxidative stress (OS) [7]. Malondialdehyde (MDA), one of the main lipid peroxidation biomarkers in plasma membrane, causes a decrease in sperm motility, count, and viability and increases the morphological abnormalities via oxidative damage of lipids in biological membranes [8]. OS affects the integrity of the plasma membrane and induces premature capacitation, which causes spermatozoa less qualified

[†]Maryam Imani and Minoo Vahedi Raad are co-first author also contributed to this study equally.

*Correspondence:
Farzaneh Fesahat
farzaneh.fesahat@gmail.com

¹ Reproductive Immunology Research Center, Shahid Sadoughi University of Medical Sciences, Yazd 8916188635, Iran

² Department of Anatomical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

for fertilization and decreases sperm motility [9]. OS can affect the mitochondrial copy number, mitochondrial DNA deletion, DNA methylation, and other DNA damage in spermatozoa, which affects semen quality and can be used as a factor to determine male fertility potential [10, 11]. The oxidative stress index (OSI) is a rapid, facile, and inexpensive indicator to determine the oxidant/antioxidant ratio in seminal plasma and serum accurately. The OSI offers an invaluable and objective assessment of redox status [12].

Sperm DNA integrity is vital to the successful fertilization process and the transfer of the parental genetic content. Impaired chromatin packaging can cause the production of free radicals, which indirectly leads to DNA backbone break by decreasing protamine and forming disulfide bonds, as the oxidative attack seems to cause poor sperm morphology [13]. Men with abnormal sperm parameters show more degrees of DNA damage [14]. The decreased seminal antioxidants may be a main factor in the process of death associated with DNA damage in teratozoospermic men [15]. Men with teratozoospermia may have a higher risk of sperm DNA break [16]. Infertile men with isolated teratozoospermia (iTZS) had higher neutrophil-to-lymphocyte ratio (NLR) than normozoospermic men and increased sperm DNA fragmentation

(SDF) values than an infertile participant with isolated azoospermia, isolated oligozoospermia, or normal semen parameters [17]. Abnormal morphology can be caused by damage to sperm DNA, defects in chromatin density, and related unsuccessful pregnancy outcomes. The evidence highlighted the role of OS and SDF in the emergence of sperm with poor morphology [18]. The main aim of our study was to assess the differences in sperm parameters, total antioxidant capacity (TAC), total oxidant status (TOS), OSI, and chromatin quality in men with different sperm parameters and whether the presence of one sperm parameter abnormality such as teratozoospermia could affect both OS and SDF in subfertile men with variable sperm parameters.

2 Methods

2.1 Study population

In this cross-sectional study, patients were divided into five groups, which include four experimental groups and a control group. The experimental groups included 15 teratozoospermic men (Exp I, T), 13 asthenoteratozoospermic men (Exp II, AT), 13 oligoasthenoteratozoospermic men (Exp III, OAT), and 18 azoospermic men (Exp IV). Also, the control group included 22 fertile men with normal sperm parameters (Fig. 1). In this

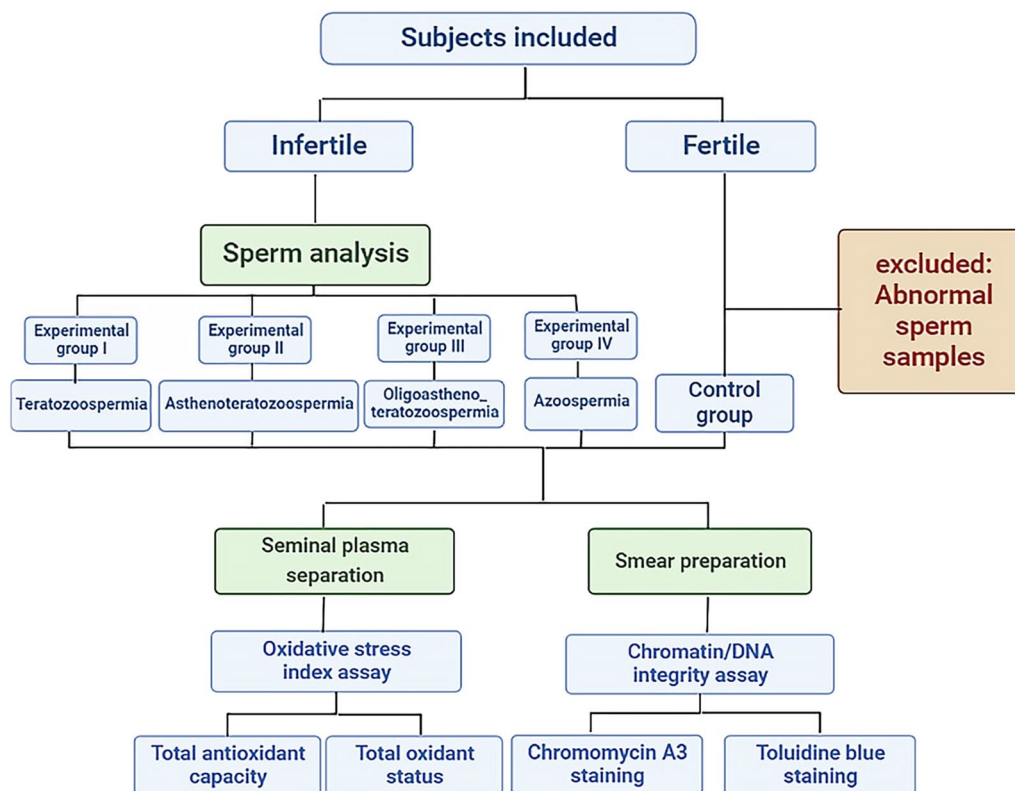


Fig. 1 The schematic view of study design

study, the semen samples were collected from 81 participants referred to Yazd reproductive sciences institute. The inclusion criteria of the control group were having normal sperm parameters and at least one child in two previous years, body mass index (BMI) ≤ 25 , no history of smoking, no varicocele disease and no drug consumption, and age < 40 . In the experimental groups, the inclusion criteria were the presence of BMI ≤ 25 , no varicocele and infection disease and diabetes, no history of smoking, no drug consumption, no alcohol consumption, and age < 40 . Exclusion criteria in all study groups included people with Pyospermia, fever, infectious diseases in the last three months, genetic problems, any infections or inflammatory diseases in the reproductive tract, sexually transmitted diseases, or erectile disorder. Our study was approved by the Ethics Committee of Shahid Sadoughi University, Yazd, Iran with approval code: IR.SSU.SPH.REC.1399.004. The consent form was signed by all participants before entering the study.

2.2 Semen analysis

The sample collection method was by masturbation with 2–5 days of sexual abstinence. In the liquefaction process, samples were incubated at 37 °C for 20 min. All assessments of sperm parameters were done based on the World Health Organization 2021 criteria. For evaluating sperm motility, a hundred spermatozoa were assessed by a phase-contrast Microscope with $\times 400$ magnifications. The percentages of progressive, non-progressive, and immotile spermatozoa were analyzed according to WHO [10]. The Diff-Quik staining kit (FaradidPardaz Pars Inc., Iran) was used to assess sperm morphology. The smears were stained according to the kit protocol. After that, 200 spermatozoa were evaluated by light microscopy at $\times 1000$ magnification. The normal sperm percentage was recorded based on WHO [10].

2.3 Assessments of oxidative stress index

To evaluate the concentrations of total oxidant level (TOS) and total antioxidant level (TAC), we separated the seminal plasma of the samples from sperm cells by centrifugation at 1800 g for 10 min. The supernatant was applied to evaluate the OSI in seminal plasma. The OSI is computed by dividing TOS by TAC. The commercial kits (Naxifer TM, Navand, Salamat Co., Urmia, Iran) were applied to quantify the TOS and TAC. For preparing the samples, 10^7 cells of seminal plasma were washed with PBS and then homogenized with 1 ml Lysing Buffer. After centrifugation at 10,000 rpm for 10 min, the supernatant was separated and used as a sample. The reagents were placed at room temperature 30 min before use. If crystals were observed, the solution was homogenized by vortexing. For the TAC assay, after the preparation, reagent 2

was mixed with reagent 3 (1:1) and vortexed. Then, five-fold the volume of the prepared solution, R1 solution was added. The final solution was used as a working solution. 5 μ l of the sample/standard were poured into the wells of the plate (all standards and samples were assayed in duplicate), followed by adding 250 μ l of the working solution. After 5 min, maximum absorption was recorded by a plate reader at 593 nm. For the TOS assay, after the preparation of the working and standard solution according to kit protocol, 30 μ l of the sample/standard were added to the wells. 200 μ l of reagent 1 and 10 μ l of reagent 2 was added to the wells and incubated for 20 min. After 20 min, maximum absorption was recorded by a plate reader at 530 nm. By using statistical software such as Excel, the standard curve graph of different concentrations was obtained and according to the line formula, the TAC and TOS were calculated.

2.4 Sperm DNA/chromatin integrity

After sperm analysis, each semen sample was smeared for toluidine blue (TB) and Chromomycin A3 (CMA3) staining. To assess sperm DNA/chromatin integrity, TB and CMA3 staining were performed to determine the chromatin damage and protamine deficiency of each sample.

2.4.1 Toluidine blue staining

In the first step, smears were prepared and dried. Then, they were fixed in 96% ethanol-acetone (1:1) at 4 °C for 30 min. 0.1 Na HCl was used to hydrolyze samples at 4 °C for 5 min. The samples were washed with distilled water and then stained with 0.05% TB for 10 min at room temperature. In the last step, 200 sperm cells were evaluated at $\times 1000$ magnification with light microscopy. Normal spermatozoa heads were stained pale blue, and abnormal spermatozoa heads were stained dark blue or purple. Abnormal spermatozoa percentage (TB⁺) was recorded [19].

2.4.2 Chromomycin A3

To indicate protamine deficiency, we performed Chromomycin A3 (CMA3) staining. After preparing and drying smears, Carnoy's solution (methanol/glacial acetic acid, 3:1) was applied to fix slides at 4 °C for 10 min. Staining of smears was performed by CMA3 solution (Sigma). Then, the slides were washed, and 200 spermatozoa were calculated by a fluorescent microscope (Olympus BX5) at $\times 1000$ magnification. The bright yellow and dull yellow spermatozoa were considered as CMA3⁺ and

CMA3⁻, respectively. Finally, the abnormal spermatozoa percentage (CMA3⁺) was recorded [14].

2.4.3 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 20 (IBM, California, United States) was used. To assess the normality of the data, Shapiro–Wilk test was performed. Data were expressed as mean \pm SEM. Variables were analyzed using the Kruskal–Wallis variance analysis test and the Mann–Whitney U test. In addition, Spearman's test was applied to calculate the correlation coefficient. $P < 0.05$ was considered statistically significant.

3 Results

Every single one of the subjects was national Iranian, with a total mean (\pm SD) age of 33.78 (\pm 5.29) years and a total mean (\pm SD) BMI of 24.29 (\pm 3.24). The age and BMI of all contributors were well-adjusted among all groups. Age and BMI did not show any significant differences ($P \geq 0.05$).

Semen concentration and sperm morphology were significantly higher in the normozoospermic group compared to the other four groups ($P < 0.001$). The sperm normal morphology significantly declined in the infertile men with Teratozoospermia compared to the Oligoasthenoteratozoospermia group ($P < 0.01$). As expected, the percentages of immotile and motile sperms were respectively higher and lower in the II and III experimental groups compared to the controls ($P < 0.001$). Semen volume analysis did not show any difference that is worthy of attention. The semen analysis data regarding azoospermic samples were excluded due to the lack of sperm (Table 1).

The chromatin integrity data demonstrated significantly higher proportions of TB⁺ and positive CMA3

spermatozoa in different sperm parameter variations of the experimental groups compared to the controls ($P < 0.001$).

There was a significant decrease in seminal plasma TAC levels in all experimental groups compared to the controls ($P < 0.001$). Moreover, TOS concentration significantly increased in the experimental groups compared to the normozoospermia group ($P < 0.05$). The OSI was almost in the balance range in the control group (OSI = 0.78 \pm 0.09), while it was significantly higher (OSI = 2.77 \pm 0.41) in all seminal plasma samples of the experimental groups ($P < 0.001$) (Table 2).

Sperm concentration, progressive motility, and normal morphology were negatively correlated with CMA3⁺, TB⁺, and TOS levels. There was a significant positive correlation between each evaluated sperm parameter and TAC levels. BMI and age of participants were not significantly correlated with any sperm parameters (Table 3).

4 Discussion

The aim of this study was to understand the pathology of nuclear defects leading to poor sperm morphology by analyzing important markers of the OS. In the current study, Sperm DNA/chromatin integrity, OS, and sperm parameters of different infertile men's groups were compared with the normozoospermic men and clarified using standard tests.

In brief, the findings of this study indicated a significant difference between the experimental groups and control group regarding sperm parameters. Sperm chromatin quality and DNA integrity, assessed by TB and CMA3 stains, showed a significant increase in teratozoospermic different groups compared to the control group, indicating defects in protamine and sperm chromatin in infertile men. Our data were in agreement with Ying Ma et al. research suggested that amorphous head familial teratozoospermia might be caused by abnormal

Table 1 Comparison of patients' characteristics, semen analysis results between the groups

Parameters	Experimental group I (n = 15)	Experimental group II (n = 13)	Experimental group III (n = 13)	Control group (n = 22)	P value
Age (year)	33.60 \pm 3.44	32.92 \pm 4.55	34.92 \pm 3.30	33.73 \pm 4.1	0.657
Body mass index (kg/m ²)	24.10 \pm 2.6	23.32 \pm 3.4	23.66 \pm 3.1	24.50 \pm 3.2	0.716
Semen volume (ml)	3.3 \pm 0.3	3.6 \pm 0.2	3.8 \pm 0.4	3.5 \pm 0.3	0.82
Semen concentration ($\times 10^6$ /ml)	38.0 \pm 4.0	38.9 \pm 4.4	7.8 \pm 0.9	61.4 \pm 6.0	<0.001
Progressive motile sperm (%)	37.6 \pm 1.9	18.3 \pm 3.5	21.6 \pm 2.3	38.0 \pm 0.8	<0.001
Non-progressive motile sperm (%)	12.4 \pm 0.7	9.6 \pm 1.4	9.3 \pm 1.0	11.7 \pm 0.6	0.04
Immotile sperm (%)	50.0 \pm 1.8	71.9 \pm 4.2	69.0 \pm 2.9	50.1 \pm 0.9	<0.001
Sperm morphology (%)	2.0 \pm 0.1	1.8 \pm 0.2	1.3 \pm 0.2	4.0 \pm 0.1	<0.001

Data were not normally distributed (nonparametric) according to the Shapiro–Wilk test. Data were presented as Mean \pm SD. Bold indicated that $P < 0.05$ was regarded as a significant value. The Kruskal–Wallis H test was used. Experimental group I: Teratozoospermia, Experimental group II: Astheno-teratozoospermia, Experimental group III: Oligoasthenoteratozoospermia, and the control group: Normozoospermia

Table 2 Comparison of sperm DNA/chromatin integrity and anti-/oxidation status among different groups

Parameters	Experimental group I ^a (n = 15)	Experimental group II ^b (n = 13)	Experimental group III ^c (n = 13)	Experimental group IV ^d (n = 18)	Control group (n = 22)	P-value
CMA3	36.0 ± 1.40	37.23 ± 1.72	44.46 ± 2.19	–	27.36 ± 0.73	< 0.001 < 0.001 ^{a,e, c,e b,e}
TB	77.07 ± 2.59	71.62 ± 3.65	71.92 ± 2.02	–	40.6 ± 2.63	< 0.001 < 0.001 ^{a,e, c,e b,e}
TAC	4.49 ± 0.37	4.25 ± 0.26	3.68 ± 0.36	3.47 ± 0.29	7.38 ± 0.78	< 0.001 < 0.001 ^{a,e, c,e b,e d,e}
TOS	10.6 ± 0.71	10.12 ± 1.27	9.74 ± 0.77	9.40 ± 0.82	4.60 ± 0.24	< 0.001 < 0.001 ^{a,e, c,e b,e d,e}
TAC/TOS (OSI)	2.62 ± 0.30	2.59 ± 0.40	3.09 ± 0.54	3.10 ± 0.40	0.78 ± 0.09	< 0.001 < 0.001 ^{a,e, c,e b,e d,e}

Data were not normally distributed (nonparametric) according to the Shapiro–Wilk test. Data were presented as Mean ± SEM. The Kruskal–Wallis H test was used to determine if there are statistically significant differences between all groups and Mann–Whitney test was used to compare between two groups. Bold indicated that $P < 0.05$ was regarded as a significant value

CMA3 Chromomycin A3; TB Toluidine blue; TAC Total antioxidant capacity; TOS Total oxidant status; OSI Oxidative stress index

^a Experimental group I: Teratozoospermia

^b Experimental group II: Astheno-teratozoospermia

^c Experimental group III: Oligoasthenoteratozoospermia

^d Experimental group IV: Azoospermia

^e The control group: Normozoospermia

Table 3 Correlations between sperm parameters and the indicators of chromatin integrity and anti-/oxidation status

Variable	CMA3	TB	TAC	TOS
Sperm concentration	–0.47** (< 0.001)	–0.31* (0.02)	0.46** (< 0.001)	–0.30* (0.02)
Progressive motility	–0.47** (< 0.001)	–0.30* (0.02)	0.46** (< 0.001)	–0.34** (0.01)
Normal morphology	–0.61** (< 0.001)	–0.62** (< 0.001)	0.53** (< 0.001)	–0.45** (< 0.001)

The Spearman correlation test was used. In each cell, the top value is r while the bottom data represents the P -value. The P -value < 0.05 indicated statistical significance

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

CMA3 Chromomycin A3; TB Toluidine blue; TAC Total antioxidant capacity; TOS Total oxidant status

chromatin condensation induced by disturbances in the process of histone protamine replacement process, and the elasticity of sperm nuclei could be a new index for assessing the sperm quality [20]. Moreover, these findings are in line with the results reported by Ammar et al. who used TUNEL, single-cell gel electrophoresis (comet test), toluidine blue, and acridine orange as four assays to assess the integrity of the nuclear sperm in men with isolated teratozoospermia. The aforementioned experiments demonstrated positive correlations between DNA damage and various morphological anomalies [21, 22]. In addition, the results of another study by Ammare et al. in 2020 provided clear evidence that apoptotic changes are

closely related to abnormal sperm morphology and DNA damage [15]. So, sperm protamine deficiency and DNA damage might be one of the pathways which can lead to defects in sperm morphology.

OS damage is one of the most important causes of altered sperm morphology and function and is considered an important factor in male infertility [23]. Some evidence indicates that the presence of abnormal sperm morphology and motility in semen samples could be due to the OS occurrence in infertile males [24]. Furthermore, seminal ROS overproduction may be the primary cause of teratozoospermia [25].

The imbalance between reactive oxygen species and antioxidants causes oxidative stress and may be regarded as an important contributor to infertility in men [26]. We measured the level of TOS and TAC in the seminal plasma samples. As expected, the findings of this study showed an increase in the TOS levels and a significant decrease in seminal plasma antioxidant levels in all experimental groups compared to the control group. We also detected a negative correlation between Sperm parameters (concentration, progressive motility, and normal morphology) and CMA3, TB, and TOS levels. There were significant positive correlations between each of the evaluated sperm parameters and TAC levels. In this regard, the results of a study that compared patients with teratozoospermia with fertile men found that the semen ROS production, hypocondensed chromatin, denatured DNA, and fragmented DNA were significantly higher than those for fertile men according to their ROS levels.

The semen ROS production, hypocondensed chromatin, denatured DNA, and fragmented DNA were significantly higher than those for fertile men based on their ROS level. The abnormal sperm morphology was correlated positively with all these parameters.

A correlation between ROS production and DNA integrity markers was also found. SDF is the main cause of sperm morphology defects and oxidative stress is the major cause of DNA fragmentation in spermatozoa [27]. Previous studies also suggested that damage to spermatozoa DNA caused by oxidative stress could have a critical effect on the etiology of infertility [28]. Due to oxidative damage to the DNA backbone, poor semen morphology appears to be caused most commonly by defective chromatin compaction, which may induce DNA breaks and free radicals. The DNA backbone may be broken indirectly by reducing protamination and disulfide bond formation due to these [21]. Colagar et al. reported that seminal TAC decreased in asthenoteratozoospermia and Oligoasthenoteratozoospermic men compared to healthy individuals, similar to our results [29]. In addition, Dehghanpour et al., aiming to compare semen parameters, protamine deficiency, and apoptosis between patients with teratozoospermia (tapered heads) and those with normozoospermia, suggested that apoptosis and abnormal chromatin packaging in tapered-head spermatozoa may contribute to the impaired fertility of teratozoospermic patients with this kind of abnormality, causing impaired fertility in these patients [30].

Treating the patient with oral antioxidant vitamins is often a standard procedure to reduce ROS formation and amend male fertility competency [31]. Evidence suggests indiscriminate consumption of antioxidants may damage sperm cells through a reductive-stress-induced state. Therefore, the “antioxidant paradox” must be carefully avoided and fully investigated. Because of these issues, oxidation–reduction potential (ORP) evolved as a valuable tool for assessing the overall balance between oxidants and antioxidants (reductants) in semen [26].

To avoid reductive stress, the oxidative status of the seminal plasma must also be evaluated pre-treatment to proceed with micronutrient supplementation safely. Supplements have the ability to improve male fertility potential and support sperm quality under these conditions [32].

According to recent reports as well as our findings, it seems that evaluating the oxidative status, antioxidant defense systems, and DNA damage, along with semen parameters might be a useful tool for the diagnosing and treating male infertility. More investigation is necessary to improve therapy options and our knowledge of these pathways.

5 Conclusion

In conclusion, our study demonstrated that DNA defects are much higher in abnormal semen samples than in normozoospermic ones. Also, the subfertile men with poor semen parameters represented significantly higher rates of OSI in comparison with semen samples with normal qualities. From this perspective, simultaneous assessment of sperm DNA defects and OS offers supplementary metrics for sperm quality analysis and may help in determining the most effective treatment method for teratozoospermic men.

Abbreviations

AT	Asthenoteratozoospermia
BMI	Body mass index
CMA3	Chromomycin A3
DNA	Deoxyribonucleic acid
Exp	Experimental group
iTZS	Isolated teratozoospermia
MDA	Malodialdehyde
NLR	Neutrophil-to-lymphocyte ratio
OAT	Oligoasthenoteratozoospermia
OS	Oxidative stress
OSI	Oxidative stress index
ROS	Reactive oxygen species
SD	Standard deviation
SDF	Sperm DNA fragmentation
SEM	Standard error of means
SPSS	The statistical package for the social sciences
T	Teratozoospermia
TAC	Total antioxidant capacity
TB	Toluidine blue
TOS	Total oxidant status
WHO	World health organization

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Author contributions

FF designed and directed the experiment. MA and MV contributed to sample preparation. MI carried out the experiment. MV performed the analytic calculations and contributed to the interpretation of the results. All authors helped shape the research and manuscript. We can confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Availability of data and materials

The data supporting the findings of this study are available from the corresponding author Dr. Farzaneh Fesahat on request.

Declarations

Ethics approval and consent to participate

Our study was approved by the Ethics Committee of Shahid Sadoughi University, Yazd, Iran with approval code: IR.SSU.SPH.REC.1399.004. The consent form was signed by all participants before entering the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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