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Sperm DNA damage and seminal antioxidant activity in subfertile men

Fernando Lopes[1] | Patrícia Pinto-Pinho[2,3,4,5] | Isabel Gaivão[6,7] | Ana Martins-Bessa[4,7] | Zélia Gomes[8] | Osvaldo Moutinho[8] | Maria Manuel Oliveira[9] | Francisco Peixoto[9] | Rosário Pinto-Leite[2]

[1]School of Health and Care Professions, University of Portsmouth, Portsmouth, UK

[2]Genetics/Andrology Laboratory, Centro Hospitalar de Trás-os-Montes e Alto Douro, E.P.E, Vila Real, Portugal

[3]CITAB, Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[4]Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[5]Laboratory of Signal Transduction, Department of Medical Sciences, Institute of Biomedicine – iBiMED, University of Aveiro, Aveiro, Portugal

[6]Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

[7]CECAV, Animal and Veterinary Research Centre, UTAD, Vila Real, Portugal

[8]Obstetrics Service, Centro Hospitalar de Trás-os-Montes e Alto Douro, E.P.E, Vila Real, Portugal

[9]CQVR, Centro de Química de Vila Real, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

Correspondence
Fernando Lopes, School of Health and Care Professions, University of Portsmouth, Block 2, St Andrews Court, St Michael's Road, PO1 2UP Portsmouth, UK.
Email: fernando.lopes@port.ac.uk

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Abstract

Supraphysiological ROS levels can lead to apoptosis, lipid peroxidation, and DNA and protein damage. This pilot study aimed to investigate the sperm oxidative damage in subfertile men, to describe the relationship between the antioxidant system and ROS. Sixty-four semen samples were categorised according to the evaluated routine parameters (WHO, *WHO laboratory manual for the examination and processing of human semen*, 2010). Results were cross-referenced with the DNA damage [Comet (n = 53) and TUNEL (n = 49) assays], antioxidant enzyme activity [SOD (n = 51), CAT (n = 48) and GST (n = 48)], and content of total thiols (n = 36), lipid hydroperoxides (n = 35) and MDA (n = 31). Compared to pathospermic samples, normozoospermic presented 40%–45% fewer spermatozoa with fragmented DNA, 19% fewer hydroperoxides, and slightly higher total thiols and MDA levels. Asthenozoospermic/asthenoteratozoospermic samples had the lowest GST activity. SOD and CAT showed a similar trend. Our results evidenced significant positive correlations between DNA damage and immotile spermatozoa; SOD and CAT, GST and total thiols; CAT and GST; total thiols and sperm concentration; and MDA levels and head/midpiece abnormalities and hydroperoxides. This work contributes to the existing body of knowledge by showing that the oxidative status correlates with the classic sperm analysis parameters. Oxidative stress and DNA damage evaluation might be a valuable diagnostic and prognostic tool in cases of idiopathic male subfertility.

KEYWORDS

antioxidant, DNA damage, infertility, oxidative status, sperm quality

1 | INTRODUCTION

Male infertility continues to be a clinical challenge with several contributing factors such as anatomy, hormones, genetics, epigenetics, biochemistry, immunology, infections, lifestyle or environmental exposure (Hayden et al., 2018; Jungwirth et al., 2012; Moghbelinejad et al., 2018). Its aetiology and pathogenic mechanisms remain unknown in about 30% of the cases, which is known as idiopathic infertility (Duca et al., 2019).

Evidence suggests that sperm damage mediated by reactive oxygen species (ROS) has a major effect on male idiopathic fertility (Bisht et al., 2017). ROS are free radicals or nonradicals as hydrogen peroxide, highly reactive, derived from the metabolism of oxygen and present in all aerobic organisms (Prieto-Bermejo et al., 2018). Depending on the concentration, location and time of exposure, ROS can have a beneficial or harmful effect on spermatozoa. At normal physiological levels, ROS are essential for motility, capacitation, hyperactivation, acrosome reaction and, therefore, fertilisation (Agarwal & Sengupta, 2020; Di Meo et al., 2016). Overproduction of free radicals exceeding the antioxidant capacity of both the spermatozoa and the seminal plasma, known as oxidative stress (OS), can lead to apoptosis, lipid peroxidation, low sperm quality, and protein and DNA damage (Aitken, 2017; Subramanian et al., 2018).

The antioxidant system includes enzymatic and nonenzymatic antioxidant pathways that may protect cells against the adverse effects of ROS (Micheli et al., 2016).

The sulfhydryl group (also called thiol group) can be considered one of the most reactive chemical groups present in biological systems, reacting with a wide range of ROS and electrophilic compounds. The maintenance of free protein sulfhydryl groups is essential for proper folding and protein activity (Champroux et al., 2016). Also, excessive protein oxidation can lead to enhanced susceptibility to sperm DNA injury. Thus, the nonoxidised protein content is a good indicator of the antioxidant system capacity (Champroux et al., 2016; Nowicka-bauer & Nixon, 2020). Another appropriate tool to evaluate OS is by studying lipid peroxidation on the spermatozoa. Hydroperoxide quantification is commonly used as a measure of early damage by ROS in lipids (Gay & Gebicki, 2003; Grintzalis et al., 2013; Rahmanto et al., 2010). Another option is to assess the levels of stable lipid peroxidation end products, like malondialdehyde (MDA), in spermatozoa and seminal plasma (Dutta et al., 2019).

This pilot study aimed to quantify biomolecular alterations and antioxidant enzymatic activity in the seminal plasma to investigate the relationship between the antioxidant system and ROS, using clinical and biochemical parameters of the spermatozoa of subfertile men. This study further aimed to elucidate the potential source of DNA fragmentation in human spermatozoa.

2 | MATERIAL AND METHODS

This pilot study comprised a final cohort of 64 males attending the Fertility Support Centre consultations at the Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD), E.P.E., Vila Real, Portugal, during a 6-month study period. Participants currently on any medication, tonics or antioxidant supplementation, and those suffering from any acute infection were excluded because of their well-known potential impact on ROS levels.

All the 64 anonymous semen samples were obtained by masturbation following 3–5 days of sexual activity abstinence and collected into sterile containers. Routine semen analysis (liquefaction time, volume, pH, viscosity, sperm count, motility and morphology) was carried out after liquefaction, in accordance with WHO guidelines (World Health Organization, 2010).

The different ejaculated volume meant that the number of techniques performed per sample varied. Therefore, based on the available sample volume and the sample volume required for each technique, the most comprehensive set of assays were performed (Figure 1).

Following routine sperm analysis, DNA damage was analysed by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling (TUNEL) assay technique ($n = 53$), and alkaline comet assay ($n = 49$). The activity of three antioxidant enzymes was also investigated: superoxide dismutase (SOD; $n = 51$), catalase (CAT; $n = 48$) and glutathione S-transferase (GST, $n = 48$). Finally, three oxidative stress markers were evaluated: total thiols ($n = 36$), hydroperoxides ($n = 35$) and malondialdehyde (MDA, $n = 31$).

This study was approved by the Ethics Committee of CHTMAD (Doc. nº 257/2020-P. C.A.). In accordance with the Declaration of Helsinki (as revised in Declaration of Helsinki, 2000—*WMA—The World Medical Association*, 2000), all participants were provided with information regarding the study and written consent was obtained prior to study enrolment.

2.1 | DNA damage

2.1.1 | TUNEL assay

TUNEL assay, as described by Muratori et al. (2000), was applied in 53 samples with the In situ *Cell Death Detection Kit* (Roche Diagnostics) to quantify DNA free 3′-OH ends.

Three sperm aliquots (200 μl) were washed 3 times (in 1× PBS, pH 7.2, centrifuged at 420 *g* for 10 min), then fixed in paraformaldehyde 4% and frozen at −20°C until use. After thawing, two washes with 200 μl of 1× PBS supplemented with 1% bovine serum albumin (BSA) were carried out, and spermatozoa were permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate (100 μl, for 2 min on ice). Sperm samples were washed twice again and split into 2 aliquots. One was incubated in 12.5 μl of the label solution with DNase I recombinant and the other was incubated with all reagents except terminal deoxynucleotidyl transferase enzyme (TdT; negative control). The labelling reaction was carried out for 1 hr in the dark at 37°C. After labelling, two subsequent washes were conducted to eliminate nonspecific fluorescence. Finally, spermatozoa were resuspended in 1× PBS. In some samples, positive controls were also prepared, but

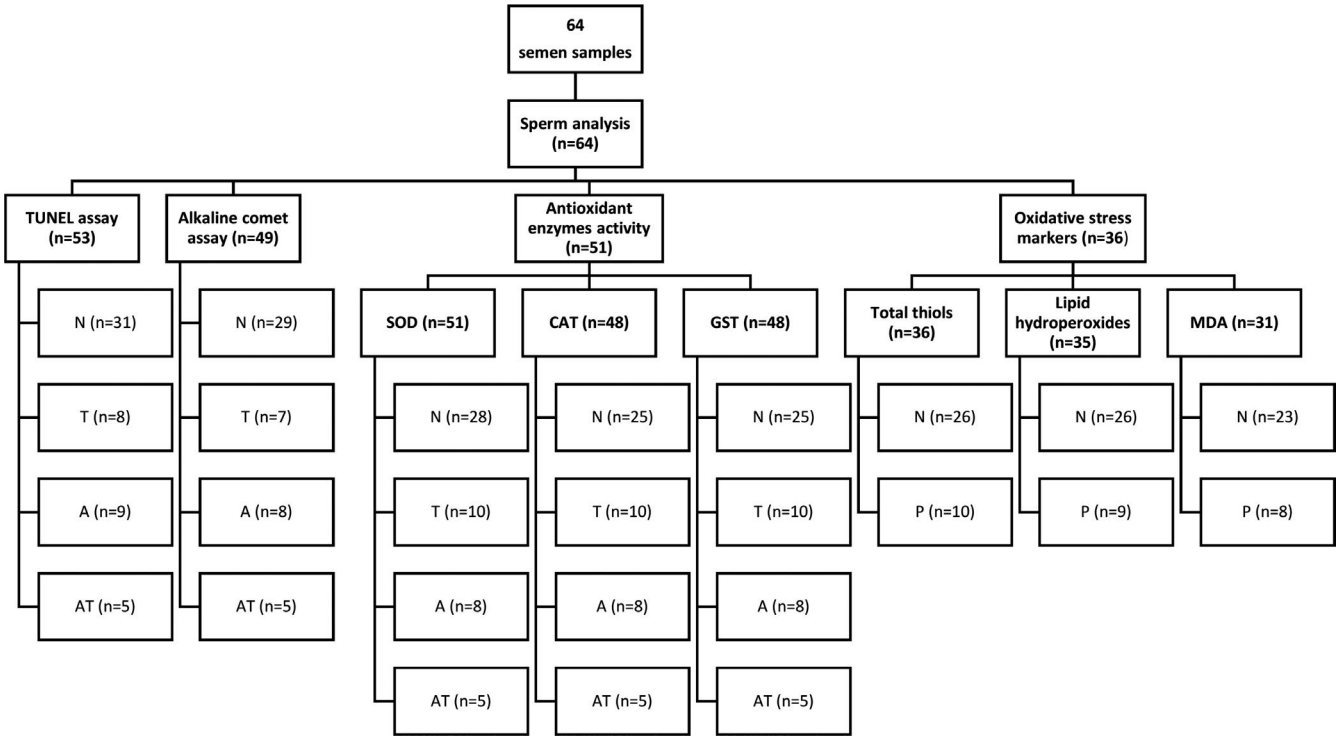

FIGURE 1 Distribution of the 64 semen samples by performed technique and groups [normozoospermic and oligozoospermic samples (N); pathospermic samples (P) composed by teratozoospermic and oligoteratozoospermic samples (T), asthenozoospermic and oligoasthenozoospermic samples (A), and asthenoteratozoospermic and oligoasthenoteratozoospermic samples (AT), according to WHO (2010)]

with an additional treatment with DNAse I (DNase I free of RNase 1 U/μl, 1,000 U; Fermentas, EU), 2 international units (IU) for 20 min at 37°C, before the labelling reaction. The number of spermatozoa undergoing apoptosis was observed in random fields in each slide and 200 spermatozoa were counted. The apoptotic index (%) was defined as follows: (number of apoptotic spermatozoa/total number of spermatozoa) × 100.

2.1.2 | Alkaline comet assay

The alkaline comet assay was performed in 49 samples, according to Sipinen et al. (2010). Three aliquots of 6×10^4 spermatozoa per sample were centrifuged (420 *g*, for 10 min at room temperature) and the pellet resuspended in cold PBS. They were kept on ice until mixed 1:1 with 2% low melting point agarose (w/v in PBS). Samples were deposited in duplicate on two regular glass slides pre-coated with 1% normal melting point agarose and covered by an 18 × 18 mm slide coverslip. Slides were placed for 5 min at 4°C. Then, the coverslips were removed, and cell lysis was achieved by washing the slides with two successive lysis solutions at 4°C, for 60 min each. The first lysis buffer contains 2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100 and 10 mM DTT (pH 10), and the second lysis buffer consists of the first buffer with 0.05 mg/ml proteinase K.

Before electrophoresis, the DNA unwinding was promoted by incubating the slides in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.2), for 30 min at 4°C. The electrophoresis tank (CSL-COM20, Cleaver Scientific Ltd; 31 × 34 × 9 cm) was always filled with 20 slides and 1.2 L of the buffer, the necessary amount to cover slides only with a thin layer of the buffer. Then, electrophoresis was conducted at 25 V, 0.8 V/cm on a platform for another 30 min, at 4°C.

Finally, slides were immersed and neutralised in 1× PBS (10 min, 4°C), followed by a 10-min distilled water immersion, at 4°C. The visualisation and scoring of the comets were performed using a Nikon Eclipse E400 fluorescent microscope (original magnification 200×). DAPI staining and visual image analyses of DNA damage were performed according to Collins et al. (2008). The DNA damage was quantified by visual classification, according to their tail length and intensity and length. The nucleoids were classified into 5 increasing DNA damage classes, where class 0 represent nucleoids with no tail and class 4 the nucleoids with almost all the DNA in the tail. The total score was expressed on a scale of 0–400 arbitrary units (AU) per 100 scored nucleoids. A total of 50 nucleoids on each gel were classified, always by the same technician, previously trained by a comet assay expert.

2.2 | Assessment of the oxidative stress status

The evaluation of the oxidative stress status was performed based on two different approaches, considering the antioxidant defence

system and oxidative stress markers (oxidant products). Three aliquots of liquified semen were centrifuged at 300 *g* for 10 min, and the sperm pellets were washed and resuspended in PBS. The seminal plasma (supernatant) was carefully removed and transferred to microfuge tubes. Aliquots of seminal plasma were frozen at −20°C until examination according to the following assays.

2.2.1 | Antioxidant enzymes activity

SOD

SOD was measured according to the method of McCord and Fridovich (1968), in 51 samples. This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with nitro blue tetrazolium chloride (NBT). The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD inhibits the rate of formazan dye formation by 50% under the conditions of the assay at 420 nm, and SOD activity was expressed as U min^{-1} mg $protein^{-1}$.

CAT

CAT activity was measured with a Clark-type oxygen electrode (Hansatech) as described by del Río et al. (1977), in 48 samples. The catalase activity was expressed as μmol H_2O_2 min^{-1} mg $protein^{-1}$.

GST

GST activity was measured spectrophotometrically at 340 nm, according to the method of Chikezie et al. (2009), in 48 samples. The GST activity was expressed as μM of CDNB min^{-1} mg $protein^{-1}$, and values were calculated using the extinction coefficient of the conjugated molecule (9.6×10^3 M^{-1} cm^{-1}).

2.2.2 | Oxidative stress markers evaluation

In this study, one marker for protein oxidation (total thiols) and two markers of lipid peroxidation (lipid hydroperoxides and MDA) were analysed.

Total thiols

Seminal plasma samples' free sulphydryl content was analysed according to the method of Suzuki et al. (1990), in 36 samples. In this assay, the free sulphydryl (-SH) groups in seminal plasma react with 5,5′dithiobis-2-nitrobenzoic acid (DTNB) to form a yellow dianion of 5-thio-2-nitrobenzoic acid (TNB) that can be measured spectrophotometrically at 412 nm. The TNB molar absorption coefficient is 13.6 × 103 M^{-1} cm^{-1} as published by Ellman (1959). Results were expressed as mM/mg.

Lipid hydroperoxides

The quantification of lipid hydroperoxides was performed according to a modified ferrous oxidation–xylenol orange (FOX) method, described by Devasagayam et al. (2003), in 35 samples. Values obtained by this method were expressed in H_2O_2 equivalents/mg protein, and results were normalised considering Eq. H_2O_2 10^{-7}cells.

MDA

The lipid peroxide levels in the seminal plasma were measured using a thiobarbituric acid reactive substances (TBARS) assay, which essentially monitors MDA production, based on the method of Ottolenghi (1959), in 31 samples. The amount of MDA was calculated using its extinction coefficient (1.56 × 105 M^{-1} cm^{-1}). The MDA content was measured at 530 nm and expressed as μM/MDA mg, and results were normalised considering μmol MDA 10^{-9} cells.

2.3 | Statistical analysis

All experiments were replicated at least three times on independent assays. Data were expressed as mean ± standard deviation. Data sets were analysed by ANOVA, Student's *t* test and Newman–Keuls multiple comparison test, using the GraphPad Prism ver. 8.0 (GraphPad Software). Spearman's correlation coefficients (ρ) were computed using IBM® SPSS® Statistics 25 software (IBM Corp.). A *p*-value (p) < .05 was regarded as a statistically significant difference.

3 | RESULTS

3.1 | Sperm analysis and samples distribution

After the routine sperm analysis (Table 1), the 64 ejaculates were classified into 4 groups according to their quality and the World Health Organization (2010) criteria, with the following distribution: N (61% with normozoospermia and oligozoospermia); T (16% with teratozoospermia and oligoteratozoospermia); A (14% with asthenozoospermia and oligoasthenozoospermia); and AT (9% with the asthenoteratozoospermia and oligoasthenoteratozoospermia).

For the determination of oxidised proteins and lipid peroxidation, due to the small number of ejaculates analysed by each technique, the 64 ejaculates were divided into only two study groups: N (61%), enclosing normozoospermic and oligozoospermic samples; and P (39%), comprising the pathospermic samples (teratozoospermia, oligoteratozoospermia, asthenozoospermia, oligoasthenozoospermia, asthenoteratozoospermia and oligoasthenoteratozoospermia).

3.2 | DNA damage

3.2.1 | TUNEL assay

Samples from groups T (n = 8), A (n = 9) and AT (n = 5) presented more DNA damaged cells (%) than group N (n = 31; T- 15.9 ± 4.8, A- 16.0 ± 8.3, and AT- 17.4 ± 7.4 vs. N- 9.6 ± 4.2; n = 53; p < .05;

TABLE 1 Semen parameters ($n = 64$) grouped in normozoospermic and oligozoospermic samples (N), teratozoospermic and oligoteratozoospermic samples (T), asthenozoospermic and oligoasthenozoospermic samples (A), and asthenoteratozoospermic and oligoasthenoteratozoospermic samples (AT), according to WHO (2010)

	N ($n = 39$)	T ($n = 10$)	A ($n = 9$)	AT ($n = 6$)
Age (years)	32.69 ± 0.98	27.50 ± 3.27	36.67 ± 2.74	28.67 ± 2.74
Semen volume (ml)	2.71 ± 0.26	3.06 ± 0.39	2.97 ± 0.25	3.61 ± 0.76
Abstinence time (days)	3.65 ± 0.22	4.75 ± 0.59	3.44 ± 0.24	3.00 ± 0.52
pH	8.08 ± 0.06	7.98 ± 0.09	8.10 ± 0.18	7.87 ± 0.19
Sperm concentration (10^6 SPZ/ml)	100.77 ± 11.78	59.75 ± 26.62	27.97 ± 9.69	182.09 ± 165.43
Progressive motility (%)	17.74 ± 2.72	12.00 ± 3.25	4.67 ± 1.40	4.17 ± 2.02
Immotile spermatozoa (%)	16.36 ± 1.47	23.60 ± 2.02	47.33 ± 3.80	50.33 ± 4.78
Vitality (%)	82.84 ± 1.31	78.30 ± 1.61	56.22 ± 5.23	55.83 ± 8.03
Hypoosmolarity (%)	82.28 ± 1.31	78.10 ± 1.59	51.44 ± 3.76	54.00 ± 8.47
Normal forms (%)	7.26 ± 0.52	2.80 ± 0.88	5.44 ± 0.58	2.67 ± 0.33
Head abnormalities (%)	89.51 ± 1.11	94.40 ± 1.18	91.67 ± 0.97	95.17 ± 0.98
Midpiece abnormalities (%)	36.72 ± 2.46	52.10 ± 5.36	53.56 ± 5.18	62.17 ± 3.11
Tail abnormalities (%)	11.44 ± 1.20	18.80 ± 2.39	22.89 ± 3.89	26.67 ± 5.60
Teratozoospermia index	1.49 ± 0.03	1.72 ± 0.07	1.57 ± 0.08	1.84 ± 0.03

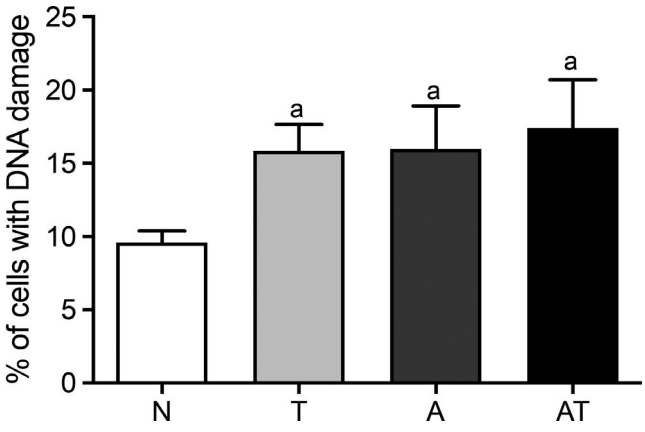

FIGURE 2 Percentage of sperm cells with fragmented DNA evaluated by TUNEL assay. N—normozoospermic and oligozoospermic ($n = 31$); T—teratozoospermic and oligoteratozoospermic ($n = 8$); A—asthenozoospermic and oligoasthenozoospermic ($n = 9$); AT—asthenoteratozoospermic and oligoasthenoteratozoospermic ($n = 5$). (a) Statistically significant relative to N

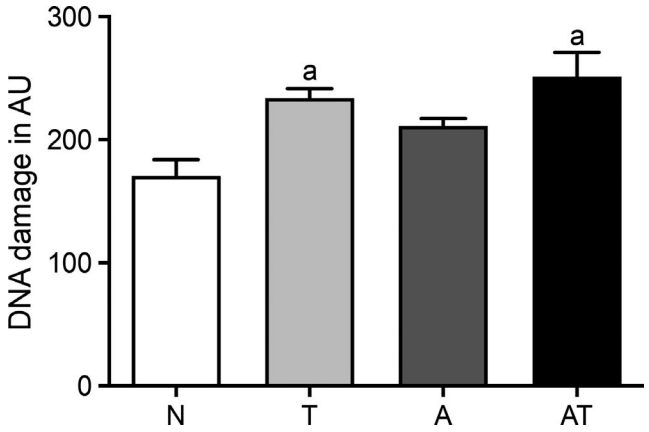

FIGURE 3 Sperm DNA damage evaluated by Comet assay and quantified in arbitrary units (AU). N—normozoospermic and oligozoospermic ($n = 29$); T—teratozoospermic and oligoteratozoospermic ($n = 7$); A—asthenozoospermic and oligoasthenozoospermic ($n = 8$); AT—asthenoteratozoospermic and oligoasthenoteratozoospermic ($n = 5$). (a) Statistically significant relative to N

Figure 2). However, no statistically significant differences were found among the groups T, A and AT (Figure 2).

3.2.2 | Comet assay

Samples from groups T ($n = 7$), A ($n = 8$) and AT ($n = 5$) presented numerically higher damage than group N ($n = 29$; T—234 ± 18.8 AU, A—211 ± 15.8 AU and AT—251 ± 44.0 AU vs. N—171 ± 57 AU). Statistical differences were found between groups N and T, and between groups N and AT ($p < .05$; Figure 3).

3.3 | Antioxidant enzymes activity

3.3.1 | SOD

SOD activity is illustrated in Figure 4a ($n = 51$). Differences between the groups were not statistically significant ($p = .663$). Nevertheless, groups with asthenozoospermia (A—0.3971 ± 0.1198 U/mg protein, $n = 8$; AT—0.4000 ± 0.1453 U/mg protein, $n = 5$) have a numerically lower activity when compared to groups N (0.4344 ± 0.1176 U/mg protein, $n = 28$) and T (0.4600 ± 0.1176 U/mg protein, $n = 10$; $p > .05$).

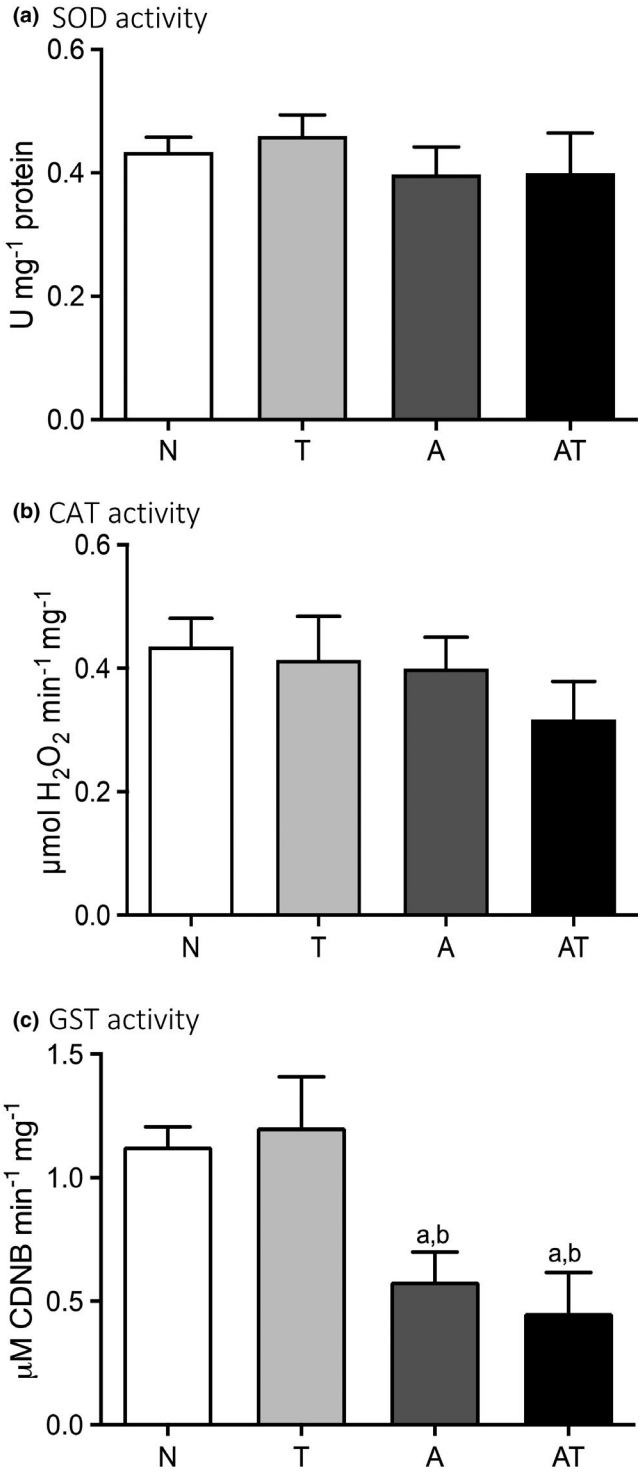

FIGURE 4 Activity of antioxidant enzymes in the sperm samples. (a) Activity of SOD (n = 51 samples) defined as the amount of SOD inhibiting 50% of NBT reduction (U/mg protein); (b) activity of CAT (n = 48 samples) defined in µmol H_2O_2 min^{-1} mg^{-1}; (c) activity of GST (n = 48 samples) defined in µM CDNB min^{-1} mg^{-1}. N—normozoospermic and oligozoospermic (SOD, n = 28; CAT and GST, n = 25); T—teratozoospermic and oligoteratozoospermic (n = 10); A—asthenozoospermic and oligoasthenozoospermic (n = 8); AT—asthenoteratozoospermic and oligoasthenoteratozoospermic (n = 5). (a) Statistically significant with respect to N. (b) Statistically significant with respect to T

3.3.2 | CAT

Values for CAT activity are shown in Figure 4b (n = 48). Groups A (0.4000 ± 0.1335 µmol H_2O_2 min^{-1} mg^{-1}, n = 8) and AT (0.3175 ± 0.1226 µmol H_2O_2 min^{-1} mg^{-1}, n = 5) had a numerically slightly lower activity when compared to groups N (0.4352 ± 0.2078 µmol H_2O_2 min^{-1} mg^{-1}, n = 25) and T (0.4140 ± 0.2219 µmol H_2O_2 min^{-1} mg^{-1}, n = 10). It corresponds to a reduction of 27% compared with N, and of 23.3% compared with T ($p > .05$).

3.3.3 | GST

Figure 4c shows GST activity (n = 48). Differences between group N (1.1254 ± 0.3304 µM CDNB min^{-1} mg^{-1}, n = 25) and group A (0.5796 ± 0.3170 µM CDNB min^{-1} mg^{-1}, n = 8; p = .0012) and AT (0.4519 ± 0.3299 µM CDNB min^{-1} mg^{-1}, n = 5; p = .0016) proved significant. Equally, a significant difference between group T (1.2034 ± 0.5786 µM CDNB min^{-1} mg^{-1}, n = 10) and groups A (p = .0251) and AT (p = .0389) was found.

3.4 | Oxidative stress markers evaluation

3.4.1 | Total thiols

No significant differences between the two groups were observed (p = .6073) although the P group demonstrated a slight increase (8.3%; N—6.67 ± 2.62 mM/mg, n = 26; P—7.22 ± 2.62 mM/mg, n = 10; Figure 5).

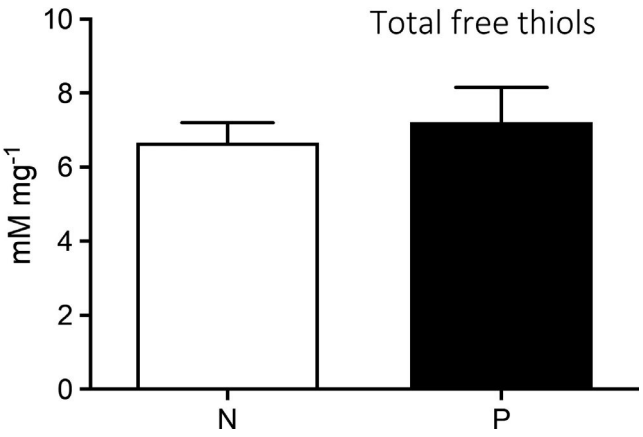

FIGURE 5 Determination of protein damage by total free thiols (mM/mg). N—normozoospermic and oligozoospermic (n = 26); P—teratozoospermic, oligoteratozoospermic, asthenozoospermic, oligoasthenozoospermic, asthenoteratozoospermic and oligoasthenoteratozoospermic (n = 10)

3.4.2 | Lipid hydroperoxides

A statistically significant difference was found between group N (6.51 ± 1.19 Eq. H_2O_2 10^{-7} cells, $n = 26$) and group P (7.99 ± 1.47 Eq. H_2O_2 10^{-7}cells, $n = 9$; $p = .0145$), being the latter approximately 22.7% higher (Figure 6).

3.4.3 | MDA

By contrast, the TBARS assay did not yield significant differences between the two groups. Nevertheless, group P contained a slightly higher MDA (12.2%; N- 16.6 ± 2.8 µmol MDA 10^{-9} cells, $n = 23$; P- 18.7 ± 3.4 µmol MDA 10^{-9} cells, $n = 8$; Figure 7).

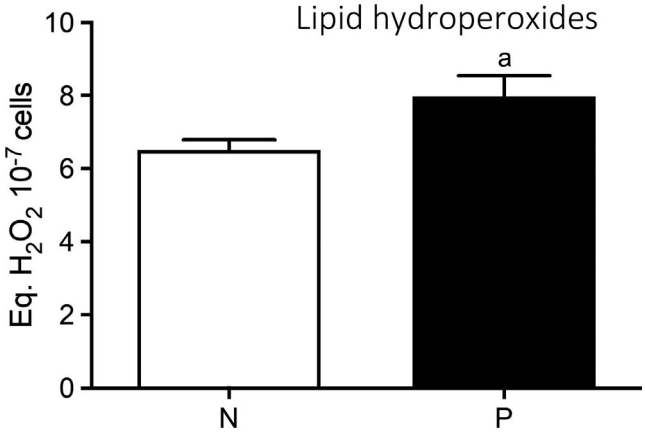

FIGURE 6 Lipid hydroperoxides (Eq. H_2O_2 10^{-7} cells). N—normozoospermic and oligozoospermic ($n = 26$); P—teratozoospermic, oligoteratozoospermic, asthenozoospermic, oligoasthenozoospermic, asthenoteratozoospermic and oligoasthenoteratozoospermic ($n = 9$). (a) Statistically significant with respect to N

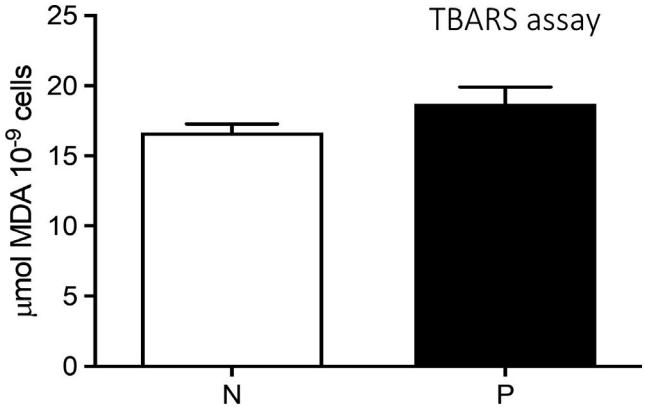

FIGURE 7 TBARS assay (µmol MDA 10^{-9} cells). N—normozoospermic and oligozoospermic ($n = 23$); P—teratozoospermic, oligoteratozoospermic, asthenozoospermic, oligoasthenozoospermic, asthenoteratozoospermic and oligoasthenoteratozoospermic ($n = 8$)

3.5 | Correlations

3.5.1 | Correlation analysis between semen parameters and DNA damage

Comet assay results were positively correlated with immotile spermatozoa ($\rho = .332$, $p < .05$; Figure 8a). TUNEL results found a significantly positive correlation with immotile spermatozoa ($\rho = .348$, $p < .01$; Figure 8b), while a negative correlation with sperm concentration ($\rho = -.322$, $p < .05$; Figure 8c) and vitality ($\rho = -.312$, $p < .05$; Figure 8d) was observed (Table 2).

3.5.2 | Correlation analysis between semen parameters and antioxidant enzymes, total thiols, hydroperoxides and MDA

SOD activity was positively correlated with ejaculate volume ($\rho = .259$, $p < .05$), CAT ($\rho = .796$, $p < .001$), GST ($\rho = .777$, $p < .001$) and total thiols ($\rho = .409$, $p < .05$), and negatively correlated with ejaculate pH ($\rho = -.319$, $p = .010$). With reference to GST, a significant positive correlation with ejaculate volume ($\rho = .363$, $p < .01$), abstinence time ($\rho = .367$, $p < .01$) and CAT ($\rho = .802$, $p < .001$) was also observed, while a negative correlation with ejaculate pH was found ($\rho = -.365$, $p < .01$; Table 3).

In addition to the correlation with SOD, total thiols were found to be positively correlated with sperm concentration ($\rho = .529$, $p < .001$; Table 4; Figure 9) and negatively correlated with the ejaculate volume ($\rho = -.340$, $p < .05$; Table 4).

TBARS assay results were positively correlated with hydroperoxides ($\rho = .339$, $p < .05$; Figure 10a), the percentage of head morphological abnormalities ($\rho = .440$, $p < .05$), percentage of midpiece abnormalities ($\rho = .602$, $p < .001$) and teratozoospermia index ($\rho = .552$, $p < .001$; Figure 10b). A negative correlation between TBARS results and the percentage of typical morphological spermatozoa was observed ($\rho = -.405$, $p < .05$; Table 4; Figure 10c).

4 | DISCUSSION

Three main topics were evaluated and correlated in our study, the spermatic parameters, the DNA damage and the oxidative stress status.

DNA damage in male germ cells has been linked to the aetiology of multiple diseases, namely infertility, miscarriage, dominant genetic disorders and diverse neurological disorders such as epilepsy, autism and schizophrenia (De Iuliis et al., 2009). To study the DNA damage in our work, we used two different techniques, the TUNEL and comet assays.

Concerning the TUNEL assay, the percentage of spermatozoa with fragmented DNA varied significantly between the analysed groups (Figure 2). Similar results are found in other works

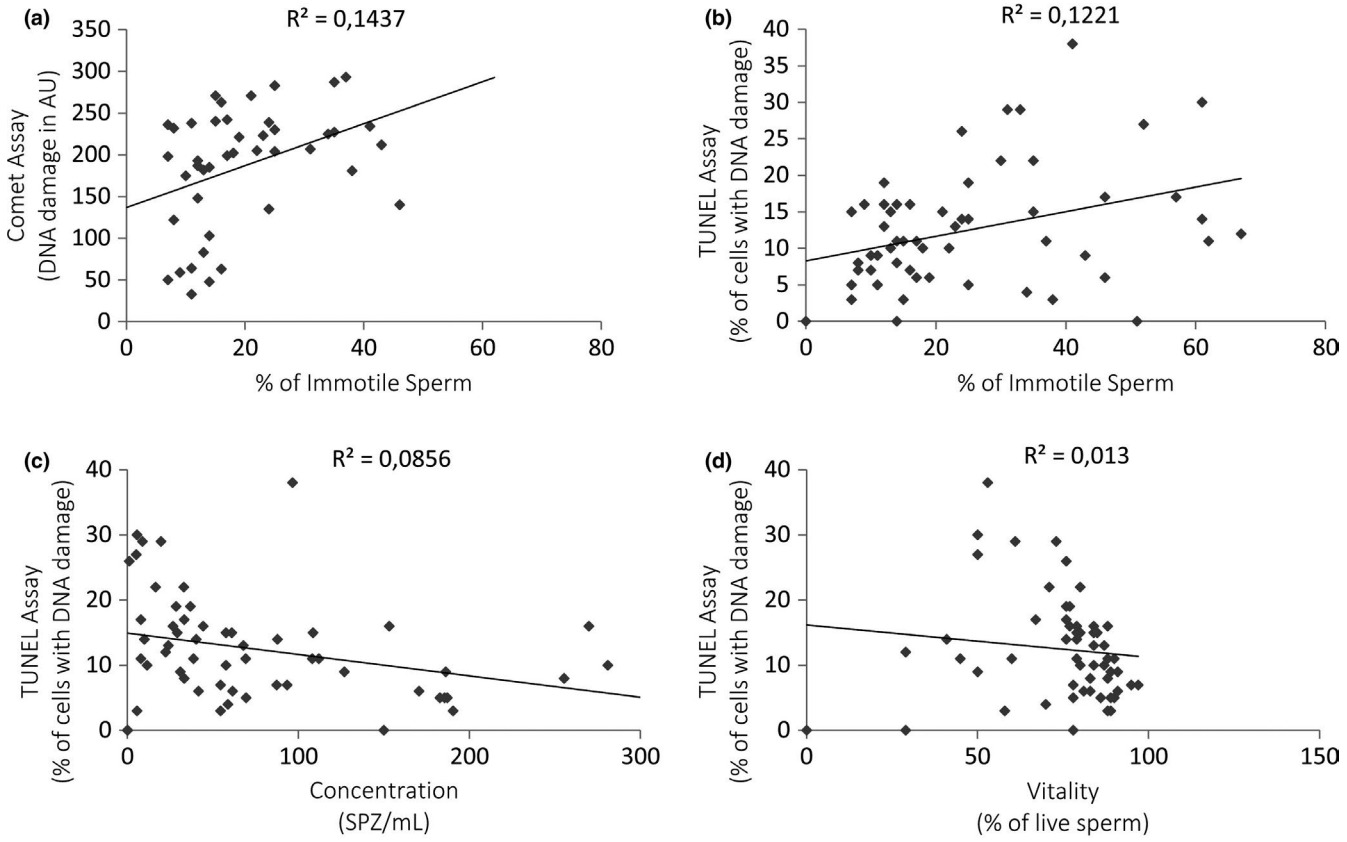

FIGURE 8 Scatter plots corresponding to the correlation between (a) comet assay and immotile sperm (%); (b) TUNEL assay and immotile sperm (%); (c) TUNEL assay and sperm concentration (10^6 sperm cells per ml); and (d) TUNEL assay and the vitality of sperm cells (%)

comparing normo- and teratozoospermia (Brahem et al., 2011; Mehdi et al., 2009; Sun et al., 1997), and normo- and asthenozoospermia (Muratori et al., 2000; Sun et al., 1997). There were also statistically significant positive correlations between the percentage of spermatozoa with fragmented DNA and the percentage of immotile spermatozoa and significant negative correlations with sperm concentration and vitality (Table 2). Similar results are described in the literature, with negative correlations between DNA damage and motility (Muratori et al., 2000; Sun et al., 1997), morphology (Brahem et al., 2011; Mehdi et al., 2009; Muratori et al., 2000; Sun et al., 1997), sperm concentration (Sun et al., 1997) and vitality (Mitchell et al., 2011). Regarding comet assay, group N presented samples with less DNA damage than groups T and AT (Figure 3), which is in accordance with other research works (Ahmad et al., 2007; Kumar et al., 2011; Shamsi et al., 2010; Sheikh et al., 2008). Besides, we found a significant positive correlation between DNA damage and the percentage of immotile spermatozoa (Table 2), as previously described in the literature (Lu et al., 2002; Morris et al., 2002; Sheikh et al., 2008; Silver et al., 2005).

ROS have a determinant role regarding the causes of abnormal sperm function. Excessive ROS production the seminal plasma seems to be associated with reduced sperm fertilising potential, impaired metabolism, motility and morphology (Agarwal & Sengupta, 2020). ROS-induced cell damage occurs due to an unbalanced cell redox, especially when the antioxidant systems cannot compensate for the increased ROS. In the seminal fluid, adequate levels of SOD and CAT play a crucial role in this balance (Abdallah et al., 2009; Khosrowbeygi & Zarghami, 2007; Kobayashi et al., 1991).

Regarding SOD activity, although the two groups that have asthenozoospermia presented slightly lower activity, similarly to other research, we did not find significant differences among the four studied groups (Figure 4a; Hsieh et al., 2002; Khosrowbeygi & Zarghami, 2007; Sanocka et al., 1996; Tavilani et al., 2008; Tkaczuk-Włach et al., 2002). Only Ben Abdallah et al. (2009) described an exception. They found an increased SOD activity in oligoasthenо- and asthenozoospermic samples comparing to normozoospermic samples. Our work evidenced that as SOD levels increase in sperm samples, the CAT, GST, thiol levels and ejaculate volume increase too, and the ejaculate pH decreases (Table 3). Shiva et al. (2011) also found positive correlations with CAT, GST and total thiols, and Tavilani et al. (2008) with CAT. A positive correlation between SOD and MDA concentration (Abdallah et al., 2009; Tavilani et al., 2008) and a negative correlation with sperm concentration and normal morphology (Abdallah et al., 2009) are described in the literature also.

Concerning CAT activity, our results follow the same trend observed for SOD activity (Figure 4b and Table 3) and are similar to what has been described previously (Abdallah et al., 2009; Khosrowbeygi & Zarghami, 2007; Shiva et al., 2011; Tavilani et al., 2008).

TABLE 2 Correlation between comet assay, TUNEL assay and the other parameters analysed

	TUNEL assay			Comet assay		
	n	*ρ*	*p*	*n*	*ρ*	*p*
Age	56	−.093	.496	48	−.062	.677
Ejaculate volume	56	−.156	.250	48	−.074	.616
Abstinence time	53	−.067	.635	47	−.042	.778
pH	56	.147	.280	48	−.075	.614
Concentration (SPZ/ml)	56	**−.322***	**.015*** (−)**	48	−.094	.525
Immotile spermatozoa	56	**.348****	**.009**** (+)**	48	**.332***	**.021*** (+)**
Progressive spermatozoa	56	−.061	.657	48	−.079	.593
Vitality	56	**−.312***	**.019*** (−)**	48	−.173	.241
Hypoosmolarity	56	−.240	.075	48	−.236	.106
Typical spermatozoa	56	−.033	.808	48	−.191	.192
Head abnormalities	56	.051	.710	48	−.103	.488
Midpiece abnormalities	56	.180	.184	48	.222	.130
Tail abnormalities	56	.143	.294	48	.111	.453
Teratozoospermia index	56	.176	.194	48	.171	.246
TUNEL assay	–	–	–	48	.219	.135
Comet assay	48	.219	.135	–	–	–
SOD	56	.050	.716	48	.205	.162
GST	56	.001	.993	48	.065	.658
CAT	56	−.006	.965	48	.117	.428
TBARS	23	.093	.674	21	.381	.089
Hydroperoxides	26	.164	.424	23	−.120	.587
Total thiols	26	.013	.948	20	.238	.274

* Significance higher than 95%.

** Significance higher than 99%; (+) positive correlation; (−) negative correlation; *ρ*. Spearman's rank correlation coefficient.

GST activity is very important for a normal cell function since sperm cells use GSH through the catalytic activity of GST to maintain functional competence (motility, vitality, mitochondrial state, oocyte binding capacity and fertilisation) during exposure to H_2O_2 or lipid peroxidation products (Andonian & Hermo, 2003; Hayes & Strange, 2000; Vorobets et al., 2018). A novel finding of this study was the significant difference in GST activity found between groups N/T and groups A and AT (Figure 4c). This analysis proved to be very interesting since these results suggest that GST is closely related to changes in motility. It is not possible to compare the present findings with other studies, because as far as we know, there are no published works on the subject so far. Only Hashemitabar et al. (2015), when looking at sperm gene expressions, found that GSTMu3 was down-regulated in asthenozoospermic samples when compared to normozoospermic samples. These results appear to be in line with evidence identifying GST as a relevant bioindicator for impaired fertility (Vani et al., 2010; Vorobets et al., 2018).

We must refer that, for both SOD and GST activities, there is a negative correlation with the ejaculate pH (Table 3), which is explained by their optimal pH activity of 7.0 and 7.8 respectively (Habig & Jakoby, 1981; Keele et al., 1971).

The changes observed concerning the activity of the antioxidant enzymes may reflect the aim of protecting spermatozoa against oxidative damage during spermiogenesis since mature spermatozoa are unable to synthesise proteins (Díez-Sánchez et al., 2003).

In this study, three oxidative stress markers were used, namely the content of total thiols, the formation of hydroperoxides and thiobarbituric acid reactive species (MDA).

It is already described that oxidative stress in seminiferous tubules during spermatogenesis can lead to an alteration of the spermatozoa's thiol concentration, potentially causing pathological alterations (Haidl et al., 2015; Homa et al., 2015). Our results show a lower thiol concentration in the normozoospermic samples than in the pathospermic samples (Figure 5), which are in line with previously published data (Ebisch et al., 2006; Králíková et al., 2017). Furthermore, total thiols show positive correlations with sperm concentration and SOD activity, and negatively with ejaculate volume. It also seems that total thiols may negatively affect sperm morphology, namely the sperm midpiece, and be potentially affected by GST activity (Table 4). Lewis et al. (1997) found statistically significant differences between asthenozoospermic and normozoospermic samples. Zini et al. (2001) described significant differences between

TABLE 3 Correlation between SOD, GST and CAT and the remaining results

	SOD			CAT			GST		
	n	*ρ*	*p*	*n*	*ρ*	*p*	*n*	*ρ*	*p*
Age	65	−.085	.498	65	−.083	.509	65	−.105	.404
Ejaculate volume	65	**.259***	**.037*** (+)	65	.235	.060	65	**.363****	**.003**** (+)
Abstinence time	61	.191	.139	61	.126	.334	61	**.367****	**.004**** (+)
pH	65	**−.319****	**.010**** (−)	65	−.180	.152	65	**−.365****	**.003**** (−)
Concentration (SPZ/ml)	65	−.034	.788	65	−.075	.550	65	.117	.335
Immotile spermatozoa	65	−.149	.237	65	−.127	.315	65	−.181	.149
Progressive spermatozoa	65	.112	.373	65	.110	.383	65	.144	.254
Vitality	65	.024	.847	65	.053	.676	65	.073	.563
Hypoosmolarity	65	−.032	.803	65	−.006	.961	65	.036	.777
Typical spermatozoa	65	−.038	.765	65	.023	.857	65	−.041	.743
Head abnormalities	65	−.041	.745	65	−.065	.607	65	−.018	.886
Midpiece abnormalities	65	−.090	.477	65	−.126	.318	65	−.223	.074
Tail abnormalities	65	−.038	.761	65	−.031	.804	65	−.073	.565
Teratozoospermia index	65	−.077	.544	65	−.098	.438	65	−.192	.126
TUNEL assay	56	.050	.716	56	−.006	.965	56	.001	.993
Comet assay	48	.205	.162	48	.117	.428	48	.065	.658
SOD	–	–	–	65	**.796****	**<.001**** (+)	65	**.777****	**<.001**** (+)
CAT	65	**.796****	**<.001**** (+)	–	–	–	65	**.802****	**<.001**** (+)
GST	65	**.777****	**<.001**** (+)	65	**.802****	**<.001**** (+)	–	–	–
TBARS	31	.075	.690	31	−.173	.353	31	−.162	.385
Hydroperoxides	35	−.056	.750	35	−.237	.170	35	−.127	.467
Total thiols	35	**.409***	**.015*** (+)	35	.214	.217	35	.296	.085

* Significance higher than 95%.

** Significance higher than 99%; (+) positive correlation; (−) negative correlation; *ρ*. Spearman's rank correlation coefficient.

the levels of thiol groups in the ejaculates from fertile and infertile men and even a positive correlation with the denaturation of DNA. Negative correlations between total thiols and sperm concentration, motility and morphology were also reported by several authors (Irvine et al., 2000; Sun et al., 1997; Zini et al., 2001). The spermatic DNA is highly compressed, owing to the substitution of protamine histones rich in cysteine residues. The thiol oxidation in these groups is physiological to stabilise the tail and, consequently, gather sperm motility and DNA stabilisation. Thus, an increase in this oxidation may lead to an increase in susceptibility to DNA damage. Despite this, and similarly to our results, Zini et al. (2001) found no differences in the DNA damage among men with different levels of thiols concentration. The strong positive correlations found with sperm concentration and SOD activity and the negative correlation with the ejaculate volume observed are similar to the results described by other authors (Lewis et al., 1997; Shiva et al., 2011; Zini et al., 2001).

The hydroperoxide levels are commonly used as an early indication of damage by free radicals, and other ROS formed in the lipid environment (Gay & Gebicki, 2003; Grintzalis et al., 2013; Rahmanto et al., 2010). Normozoospermic and pathospermic samples differed significantly on hydroperoxide concentration, which, in turn, showed a tendency to correlate negatively with sperm concentration (Figure 6; Table 4).

Regarding the MDA, the differences found in the study groups (Figure 7) also seem to agree with other studies that found higher amounts of MDA negatively correlated with sperm motility (Colagar et al., 2013; Mehrotra et al., 2013; Subramanian et al., 2018; Tomar et al., 2017), sperm morphology (Atig et al., 2012; Benedetti et al., 2012; Colagar et al., 2013; Subramanian et al., 2018) and sperm concentration (Colagar et al., 2013; Mehrotra et al., 2013; Subramanian et al., 2018). Moreover, we found significant positive correlations between MDA levels and abnormal head, abnormal midpiece and teratozoospermia index, and a negative correlation with the morphologically normal spermatozoa (Table 4). Peroxidation of phospholipids in mammalian sperm causes membrane damage, which is associated with loss of motility, impaired metabolism, impaired acrosome reaction reactivity and morphology changes (Ebisch et al., 2006; Engel et al., 1999). As reported by Calamera et al. (2003), spermatozoa from normozoospermic men had lower

TABLE 4 Correlation between the total thiols, lipid hydroperoxides, quantity of MDA and the remaining results

	Total thiols			Lipid hydroperoxides			TBARS		
	n	*ρ*	*p*	*n*	*ρ*	*p*	*n*	*ρ*	*p*
Age	35	.070	.691	35	.020	.910	31	.076	.684
Ejaculate volume	35	**−.340***	**.046*** **(−)**	35	−.003	.987	31	.175	.345
Abstinence time	33	.278	.117	33	−.261	.142	29	.088	.650
pH	35	−.266	.123	35	.274	.111	31	.087	.640
Concentration (SPZ/ml)	35	**.529****	**<.001**** **(+)**	35	−.295	.086	31	−.146	.433
Immotile spermatozoa	35	.030	.863	35	−.150	.388	31	.060	.747
Progressive spermatozoa	35	−.121	.488	35	−.008	.965	31	−.145	.437
Vitality	35	.065	.712	35	−.025	.887	31	.117	.532
Hypoosmolarity	35	.031	.861	35	.014	.936	31	.122	.514
Typical spermatozoa	35	.017	.924	35	−.199	.252	31	**−.405***	**.024*** **(−)**
Head abnormalities	35	.090	.606	35	.258	.134	31	**.440***	**.013*** **(+)**
Midpiece abnormalities	35	.316	.064	35	.136	.435	31	**.602****	**<.001**** **(+)**
Tail abnormalities	35	.088	.617	35	.128	.463	31	.004	.981
Teratozoospermia index	35	.295	.085	35	.113	.519	31	**.552****	**<.001**** **(+)**
TUNEL assay	26	.013	.948	26	.164	.424	23	.093	.674
Comet assay	23	.238	.274	23	−.120	.587	21	.381	.089
SOD	35	**.409***	**.015*** **(+)**	35	−.056	.750	31	−.075	.690
CAT	35	.214	.217	35	−.237	.170	31	−.173	.353
GST	35	.296	.085	35	−.127	.467	31	−.162	.385
TBARS	31	−.035	.850	31	**.399***	**.026*** **(+)**	–	–	–
Hydroperoxides	35	−.246	.155	–	–	–	31	**.399***	**.026*** **(+)**
Total thiols	–	–	–	35	−.246	.155	31	−.035	.850

* Significance higher than 95%.

** Significance higher than 99%; (+) positive correlation; (−) negative correlation; *ρ*. Spearman's rank correlation coefficient.

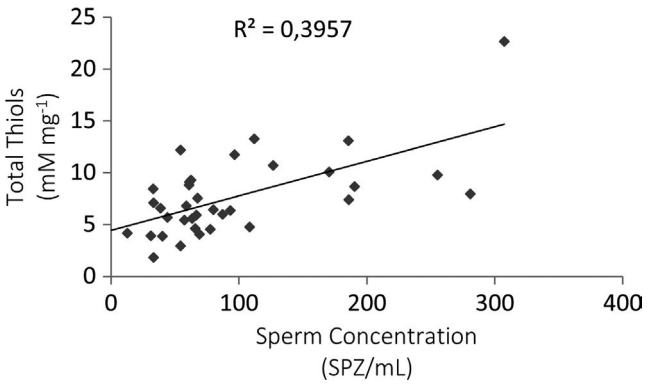

FIGURE 9 Scatter plot corresponding to the correlation between total thiols (mM/mg) and sperm concentration (10^6 sperm cells per ml)

unsaturated fatty acid content than spermatozoa from asthenozoospermic individuals. It suggests that they are less susceptible to ROS-induced peroxidative damage, which could explain our results. In our study, the concentration of MDA and hydroperoxides showed to be positively correlated (Table 4).

In sum, spermatozoa (as all aerobic cells) are prone to biomolecular alterations when under oxidative stress that promotes modification in their physiology and behaviour. Despite the limited size sample, we showed that MDA and hydroperoxides seem strongly correlated and could serve as a good indicator of sperm morphology and motility. It also appears that although there is an increased antioxidant enzymatic activity in group T, this was not enough to decrease the damage caused by ROS, potentially supporting the theory that spermatozoa with alterations in its morphology has increased production of ROS. Furthermore, decreased GST activity seems to be intimately associated with reduced motility and DNA damage with pathospermia. The TUNEL assay appears to be a reliable tool for assessing cell death. Our results suggest that the study of oxidative stress and DNA damage might be useful tools in idiopathic male subfertility to aid with diagnostics and prognosis.

This work has added to the existing body of knowledge by verifying that correlations between the oxidative status (including antioxidant enzymatic activity and ROS effect on proteins and lipids) and the classic sperm analysis indicators seem to exist. However, larger-size population studies are required to extend and verify our findings

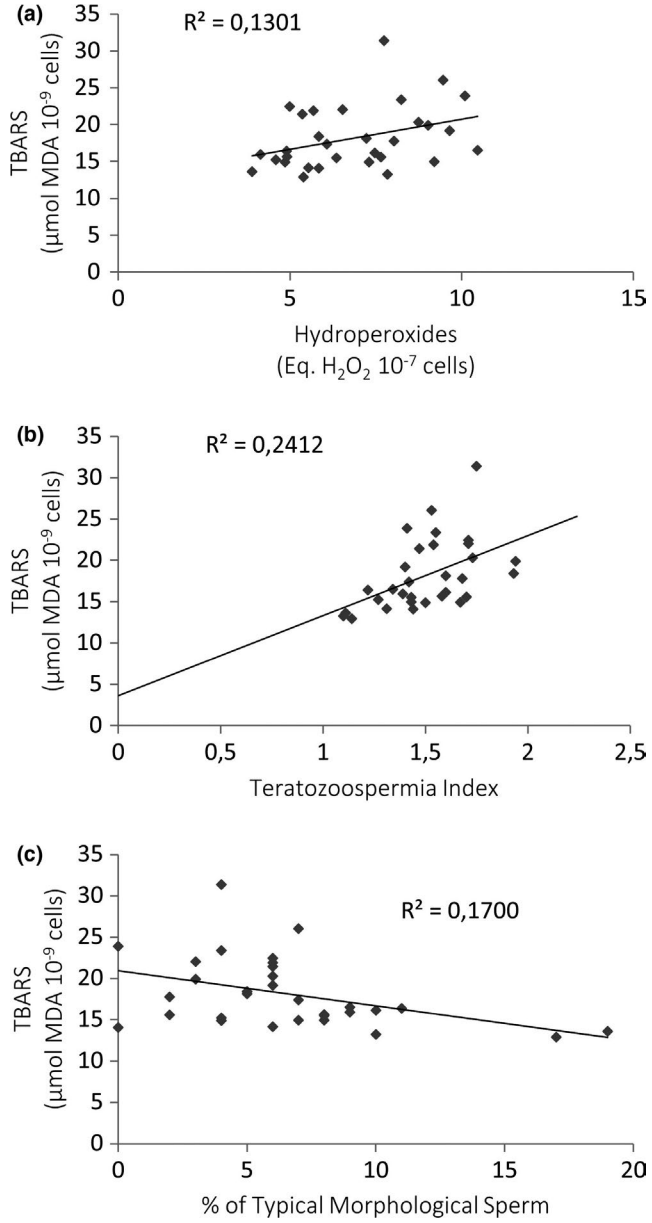

FIGURE 10 Scatter plots corresponding to the correlation between (a) TBARS (µmol MDA 10^{-9} cells) and hydroperoxides (Eq. H_2O_2 10^{-7} cells); (b) TBARS (µmol MDA 10^{-9} cells) and teratozoospermic index; and (c) TBARS (µmol MDA 10^{-9} cells) and percentage of typical sperm cells

to specific medical conditions affecting male fertility. While not providing definitive evidence, our work informs the potential design of a more comprehensive study and provides clear trends.

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CONFLICT OF INTEREST

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Fernando Lopes https://orcid.org/0000-0002-9080-877X
Rosário Pinto-Leite https://orcid.org/0000-0003-4128-0494

REFERENCES

Abdallah, F. B., Dammak, I., Attia, H., Hentati, B., & Ammar-Keskes, L. (2009). Lipid peroxidation and antioxidant enzyme activities in infertile men: Correlation with semen parameter. *Journal of Clinical Laboratory Analysis*, *23*(2), 99–104. https://doi.org/10.1002/jcla.20297

Agarwal, A., & Sengupta, P. (2020). Oxidative stress and its association with male infertility. In S. J. Parekattil, S. C. Esteves, & A. Agarwal (Eds.), *Male infertility* (pp. 57–68). Springer International Publishing. https://doi.org/10.1007/978-3-030-32300-4_6

Ahmad, L., Jalali, S., Shami, S. A., & Akram, Z. (2007). Sperm preparation: DNA damage by comet assay in normo-and teratozoospermics. *Archives of Andrology*, *53*(6), 325–338. https://doi.org/10.1080/01485010701730963

Aitken, R. J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*, *84*(10), 1039–1052. https://doi.org/10.1002/mrd.22871

Andonian, S., & Hermo, L. (2003). Immunolocalization of the Yb1 subunit of glutathione S-transferase in the adult rat epididymis following orchidectomy and efferent duct ligation. *Journal of Andrology*, *24*(4), 577–587.

Atig, F., Raffa, M., Ali, H. B., Abdelhamid, K., Saad, A., & Ajina, M. (2012). Altered antioxidant status and increased lipid per-oxidation in seminal plasma of Tunisian infertile men. *International Journal of Biological Sciences*, *8*(1), 139. https://doi.org/10.7150/ijbs.8.139

Benedetti, S., Tagliamonte, M. C., Catalani, S., Primiterra, M., Canestrari, F., Stefani, S. D., Palini, S., & Bulletti, C. (2012). Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. *Reproductive Biomedicine Online*, *25*(3), 300–306. https://doi.org/10.1016/j.rbmo.2012.05.011

Bisht, S., Faiq, M., Tolahunase, M., & Dada, R. (2017). Oxidative stress and male infertility. *Nature Reviews Urology*, *14*(8), 470–485. https://doi.org/10.1038/nrurol.2017.69

Brahem, S., Mehdi, M., Elghezal, H., & Saad, A. (2011). Detection of DNA fragmentation and meiotic segregation in human with isolated teratozoospermia. *Journal of Assisted Reproduction and Genetics*, *28*(1), 41–48. https://doi.org/10.1007/s10815-010-9482-8

Calamera, J., Buffone, M., Ollero, M., Alvarez, J., & Doncel, G. F. (2003). Superoxide dismutase content and fatty acid composition in subsets of human spermatozoa from normozoospermic, asthenozoospermic, and polyzoospermic semen samples. *Molecular Reproduction and Development: Incorporating Gamete Research*, *66*(4), 422–430. https://doi.org/10.1002/mrd.10368

Champroux, A., Torres-Carreira, J., Gharagozloo, P., Drevet, J. R., & Kocer, A. (2016). Mammalian sperm nuclear organization: Resiliencies and vulnerabilities. *Basic and Clinical Andrology*, *26*(1), 1–22. https://doi.org/10.1186/s12610-016-0044-5

Chikezie, P. C., Uwakwe, A. A., & Monago, C. C. (2009). Glutathione S-transferase activity of erythrocyte genotypes HbAA, HbAS and HbSS in male volunteers administered with fansidar and quinine. *African Journal of Biochemistry Research*, *3*(5), 210–214.

Colagar, A. H., Karimi, F., & Jorsaraei, S. G. A. (2013). Correlation of sperm parameters with semen lipid peroxidation and total antioxidants levels in astheno-and oligoasheno-teratospermic men. *Iranian Red Crescent Medical Journal*, *15*(9), 780.

Collins, A. R., Oscoz, A. A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Štětina, R. (2008). The comet assay: Topical issues. *Mutagenesis*, *23*(3), 143–151. https://doi.org/10.1093/mutage/gem051

Declaration of Helsinki 2000 – WMA – The World Medical Association. (2000). Retrieved September 23, 2020, from https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/doh-oct2000/

De Iuliis, G. N., Thomson, L. K., Mitchell, L. A., Finnie, J. M., Koppers, A. J., Hedges, A., & Aitken, R. J. (2009). DNA damage in human spermatozoa is highly correlated with the efficiency of ch. *Biology of Reproduction*, *81*(3), 517–524.

del Río, L., Ortega, M. G., Lopez, A. L., & Górge, J. L. (1977). A more sensitive modification of the catalase assay with the Clark oxygen electrode: Application to the kinetic study of the pea leaf enzyme. *Analytical Biochemistry*, *80*(2), 409–415. https://doi.org/10.1016/0003-2697(77)90662-5

Devasagayam, T. P. A., Boloor, K. K., & Ramasarma, T. (2003). *Methods for estimating lipid peroxidation: An analysis of merits and demerits*.

Díez-Sánchez, C., Ruiz-Pesini, E., Montoya, J., Pérez-Martos, A., Enríquez, J. A., & López-Pérez, M. J. (2003). Mitochondria from ejaculated human spermatozoa do not synthesize proteins. *FEBS Letters*, *553*(1–2), 205–208. https://doi.org/10.1016/S0014-5793(03)01013-5

Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. (2016). Harmful and beneficial role of ROS. *Oxidative Medicine and Cellular Longevity*, *2016*, 1–3. https://doi.org/10.1155/2016/7909186

Duca, Y., Calogero, A. E., Cannarella, R., Condorelli, R. A., & La Vignera, S. (2019). Current and emerging medical therapeutic agents for idiopathic male infertility. *Expert Opinion on Pharmacotherapy*, *20*(1), 55–67. https://doi.org/10.1080/14656566.2018.1543405

Dutta, S., Majzoub, A., & Agarwal, A. (2019). Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab Journal of Urology*, *17*(2), 87–97. https://doi.org/10.1080/2090598X.2019.1599624

Ebisch, I. M. W., Peters, W. H. M., Thomas, C. M. G., Wetzels, A. M. M., Peer, P. G. M., & Steegers-Theunissen, R. P. M. (2006). Homocysteine, glutathione and related thiols affect fertility parameters in the (sub) fertile couple. *Human Reproduction*, *21*(7), 1725–1733. https://doi.org/10.1093/humrep/del081

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, *82*(1), 70–77. https://doi.org/10.1016/0003-9861(59)90090-6

Engel, S., Schreiner, T., & Petzoldt, R. (1999). Lipid peroxidation in human spermatozoa and maintenance of progressive sperm motility. *Andrologia*, *31*(1), 17–22. https://doi.org/10.1111/j.1439-0272.1999.tb02837.x

Gay, C. A., & Gebicki, J. M. (2003). Measurement of protein and lipid hydroperoxides in biological systems by the ferric–xylenol orange method. *Analytical Biochemistry*, *315*(1), 29–35. https://doi.org/10.1016/S0003-2697(02)00606-1

Grintzalis, K., Zisimopoulos, D., Grune, T., Weber, D., & Georgiou, C. D. (2013). Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides. *Free Radical Biology and Medicine*, *59*, 27–35. https://doi.org/10.1016/j.freeradbiomed.2012.09.038

Habig, W. H., & Jakoby, W. B. (1981). [27] Glutathione S-transferases (rat and human). In W. Jakoby (Ed.), *Methods in enzymology* (Vol. *77*, pp. 218–231). Elsevier.

Haidl, F., Haidl, G., Oltermann, I., & Allam, J. P. (2015). Seminal parameters of chronic male genital inflammation are associated with disturbed sperm DNA integrity. *Andrologia*, *47*(4), 464–469. https://doi.org/10.1111/and.12408

Hashemitabar, M., Sabbagh, S., Orazizadeh, M., Ghadiri, A., & Bahmanzadeh, M. (2015). A proteomic analysis on human sperm tail: Comparison between normozoospermia and asthenozoospermia. *Journal of Assisted Reproduction and Genetics*, *32*(6), 853–863. https://doi.org/10.1007/s10815-015-0465-7

Hayden, R. P., Flannigan, R., & Schlegel, P. N. (2018). The role of lifestyle in male infertility: Diet, physical activity, and body habitus. *Current Urology Reports*, *19*(7). https://doi.org/10.1007/s11934-018-0805-0

Hayes, J. D., & Strange, R. C. (2000). Glutathione S-transferase polymorphisms and their biological consequences. *Pharmacology*, *61*(3), 154–166. https://doi.org/10.1159/000028396

Homa, S. T., Vessey, W., Perez-Miranda, A., Riyait, T., & Agarwal, A. (2015). Reactive oxygen species (ROS) in human semen: Determination of a reference range. *Journal of Assisted Reproduction and Genetics*, *32*(5), 757–764. https://doi.org/10.1007/s10815-015-0454-x

Hsieh, Y., Sun, Y., Chang, C., Lee, Y., Tsai, H., & Lin, C. (2002). Superoxide dismutase activities of spermatozoa and seminal plasma are not correlated with male infertility. *Journal of Clinical Laboratory Analysis*, *16*(3), 127–131. https://doi.org/10.1002/jcla.10029

Irvine, D. S., Twigg, J. P., Gordon, E. L., Fulton, N., Milne, P. A., & Aitken, R. J. (2000). DNA integrity in human spermatozoa: Relationships with semen quality. *Journal of Andrology*, *21*(1), 33–44.

Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., & Krausz, C. (2012). European Association of urology guidelines on male infertility: The 2012 update. *European Urology*, *62*(2), 324–332. https://doi.org/10.1016/j.eururo.2012.04.048

Keele, B. B., McCord, J. M., & Fridovich, I. (1971). Further characterization of bovine superoxide dismutase and its isolation from bovine heart. *Journal of Biological Chemistry*, *246*(9), 2875–2880. https://doi.org/10.1016/S0021-9258(18)62263-X

Khosrowbeygi, A., & Zarghami, N. (2007). Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. *BMC Clinical Pathology*, *7*(1), 1–6. https://doi.org/10.1186/1472-6890-7-6

Kobayashi, T., Miyazaki, T., Natori, M., & Nozawa, S. (1991). Protective role of superoxide dismutase in human sperm motifity: Superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Human Reproduction*, *6*(7), 987–991. https://doi.org/10.1093/oxfordjournals.humrep.a137474

Králíková, M., Crha, I., Huser, M., Melounová, J., Zakova, J., Matejovicova, M., & Ventruba, P. (2017). The intracellular concentration of homocysteine and related thiols is negatively correlated to sperm quality after highly effective method of sperm lysis. *Andrologia*, *49*(7), e12702. https://doi.org/10.1111/and.12702

Kumar, D., Kalthur, G., Mascarenhas, C., Kumar, P., & Adiga, S. K. (2011). Ejaculate fractions of asthenozoospermic and teratozoospermic patients have differences in the sperm DNA integrity. *Andrologia*, *43*(6), 416–421. https://doi.org/10.1111/j.1439-0272.2010.01105.x

Lewis, S. E. M., Sterling, E. S. L., Young, I. S., & Thompson, W. (1997). Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertility and Sterility*, *67*(1), 142–147. https://doi.org/10.1016/S0015-0282(97)81871-7

Lu, H. O., Zhang, L., Zhang, N., Tang, J., Ding, X. P., & Tang, Y. (2002). Detection of DNA damage of human sperm using single cell electrophoresis. *Zhonghua Nan Ke Xue = National Journal of Andrology*, *8*(6), 416–418.

McCord, J. M., & Fridovich, I. (1968). The reduction of cytochrome c by milk xanthine oxidase. *Journal of Biological Chemistry*, *243*(21), 5753–5760. https://doi.org/10.1016/S0021-9258(18)91929-0

Mehdi, M., Khantouche, L., Ajina, M., & Saad, A. (2009). Detection of DNA fragmentation in human spermatozoa: Correlation with

semen parameters. *Andrologia*, *41*(6), 383–386. https://doi.org/10.1111/j.1439-0272.2009.00953.x

Mehrotra, A., Katiyar, D. K., Agarwal, A., Das, V., & Pant, K. K. (2013). *Role of total antioxidant capacity and lipid peroxidation in fertile and infertile men*.

Micheli, L., Cerretani, D., Collodel, G., Menchiari, A., Moltoni, L., Fiaschi, A. I., & Moretti, E. (2016). Evaluation of enzymatic and non-enzymatic antioxidants in seminal plasma of men with genitourinary infections, varicocele and idiopathic infertility. *Andrology*, *4*(3), 456–464. https://doi.org/10.1111/andr.12181

Mitchell, L. A., De Iuliis, G. N., & Aitken, R. J. (2011). The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: Development of an improved methodology. *International Journal of Andrology*, *34*(1), 2–13. https://doi.org/10.1111/j.1365-2605.2009.01042.x

Moghbelinejad, S., Mozdarani, H., Ghoraeian, P., & Asadi, R. (2018). Basic and clinical genetic studies on male infertility in Iran during 2000–2016: A review. *International Journal of Reproductive BioMedicine*, *16*(3), 131. https://doi.org/10.29252/ijrm.16.3.131

Morris, I. D., Ilott, S., Dixon, L., & Brison, D. R. (2002). The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Human Reproduction*, *17*(4), 990–998. https://doi.org/10.1093/humrep/17.4.990

Muratori, M., Piomboni, P., Baldi, E., Filimberti, E., Pecchioli, P., Moretti, E., & Maggi, M. (2000). Functional and ultrastructural features of DNA-fragmented human sperm. *Journal of Andrology*, *21*(6), 903–912.

Nowicka-Bauer, K., & Nixon, B. (2020). Molecular changes induced by oxidative stress that impair human sperm motility. *Antioxidants*, *9*(2), 134. https://doi.org/10.3390/antiox9020134

Ottolenghi, A. (1959). Interaction of ascorbic acid and mitochondrial lipides. *Archives of Biochemistry and Biophysics*, *79*, 355–363. https://doi.org/10.1016/0003-9861(59)90414-X

Prieto-Bermejo, R., Romo-González, M., Pérez-Fernández, A., Ijurko, C., & Hernández-Hernández, Á. (2018). Reactive oxygen species in haematopoiesis: Leukaemic cells take a walk on the wild side. *Journal of Experimental & Clinical Cancer Research*, *37*(1), 125. https://doi.org/10.1186/s13046-018-0797-0

Rahmanto, A. S., Morgan, P. E., Hawkins, C. L., & Davies, M. J. (2010). Cellular effects of peptide and protein hydroperoxides. *Free Radical Biology and Medicine*, *48*(8), 1071–1078. https://doi.org/10.1016/j.freeradbiomed.2010.01.025

Sanocka, D., Miesel, R., Jedrzejczak, P., & Kurpisz, M. K. (1996). Oxidative stress and male infertility. *Journal of Andrology*, *17*(4), 449–454.

Shamsi, M. B., Venkatesh, S., Tanwar, M., Singh, G., Mukherjee, S., Malhotra, N., & Dada, R. (2010). Comet assay: A prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. *Indian Journal of Medical Research*, *131*(5), 675.

Sheikh, N., Amiri, I., Farimani, M., Najafi, R., & Hadeie, J. (2008). Correlation between sperm parameters and sperm DNA fragmentation in fertile and infertile men. *International Journal of Reproductive BioMedicine*, *6*(2), 10–13.

Shiva, M., Gautam, A. K., Verma, Y., Shivgotra, V., Doshi, H., & Kumar, S. (2011). Association between sperm quality, oxidative stress, and seminal antioxidant activity. *Clinical Biochemistry*, *44*(4), 319–324. https://doi.org/10.1016/j.clinbiochem.2010.11.009

Silver, E. W., Eskenazi, B., Evenson, D. P., Block, G., Young, S., & Wyrobek, A. J. (2005). Effect of antioxidant intake on sperm chromatin stability in healthy nonsmoking men. *Journal of Andrology*, *26*(4), 550–556. https://doi.org/10.2164/jandrol.04165

Sipinen, V., Laubenthal, J., Baumgartner, A., Cemeli, E., Linschooten, J. O., Godschalk, R. W. L., Van Schooten, F. J., Anderson, D., & Brunborg, G. (2010). In vitro evaluation of baseline and induced DNA damage in human sperm exposed to benzo[a]pyrene or its metabolite benzo[a]pyrene-7,8-diol-9,10-epoxide, using the comet assay. *Mutagenesis*, *25*(4), 417–425. https://doi.org/10.1093/mutage/geq024

Subramanian, V., Ravichandran, A., Thiagarajan, N., Govindarajan, M., Dhandayuthapani, S., & Suresh, S. (2018). Seminal reactive oxygen species and total antioxidant capacity: Correlations with sperm parameters and impact on male infertility. *Clinical and Experimental Reproductive Medicine*, *45*(2), 88–93. https://doi.org/10.5653/cerm.2018.45.2.88

Sun, J.-G., Jurisicova, A., & Casper, R. F. (1997). Detection of deoxyribonucleic acid fragmentation in human sperm: Correlation with fertilization in vitro. *Biology of Reproduction*, *56*(3), 602–607.

Suzuki, Y., Lyall, V., Biber, T. U. L., & Ford, G. D. (1990). A modified technique for the measurement of sulfhydryl groups oxidized by reactive oxygen intermediates. *Free Radical Biology and Medicine*, *9*(6), 479–484. https://doi.org/10.1016/0891-5849(90)90125-3

Tavilani, H., Goodarzi, M. T., Vaisi-Raygani, A., Salimi, S., & Hassanzadeh, T. (2008). Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa. *International Brazilian Journal of Urology*, *34*(4), 485–491. https://doi.org/10.1590/S1677-55382008000400011

Tkaczuk-Włach, J., Kankofer, M., & Jakiel, G. (2002). Activity of superoxide dismutase and glutathione peroxidase in human semen in normozoospermia and spermatopathy. *Annales Universitatis Mariae Curie-Sklodowska. Sectio D: Medicina*, *57*(2), 369–375.

Tomar, G., Joshi, T., Varghes, A., Sasidharan, S., & Kural, M. R. (2017). Relationship of antioxidant system and reactive oxygen species with clinical semen parameters in infertile men. *Journal of Family Medicine and Primary Care*, *6*(3), 574. https://doi.org/10.4103/2249-4863.222051

Vani, G. T., Mukesh, N., Siva Prasad, B., Rama Devi, P., Hema Prasad, M., Usha Rani, P., & Pardhanandana Reddy, P. (2010). Role of glutathione S-transferase Mu-1 (GSTM1) polymorphism in oligospermic infertile males. *Andrologia*, *42*(4), 213–217. https://doi.org/10.1111/j.1439-0272.2009.00971.x

Vorobets, M. Z., Fafula, R. V., Besedina, A. S., Onufrovych, O. K., & Vorobets, D. Z. (2018). Glutathione s-transferase as a marker of oxidative stress in human ejaculated spermatozoa from patients with pathospermia. *Regulatory Mechanisms in Biosystems*, *9*(2), 287–292. https://doi.org/10.15421/021842

World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen*.

Zini, A., Kamal, K. M., & Phang, D. (2001). Free thiols in human spermatozoa: Correlation with sperm DNA integrity. *Urology*, *58*(1), 80–84. https://doi.org/10.1016/S0090-4295(01)00997-9

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