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Factors Affecting Sperm DNA Fragmentation in Men with Unexplained Infertility

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Abstract

Normal sperm DNA integrity is of paramount importance for normal fertilization, embryo development and normal pregnancy. Although semen parameters are normal, a significant proportion of men with unexplained infertility have high sperm DNA damage indicating that other biological or life- style factors as well as oxidative mechanisms may be involved. This case–control study sought to determine clinical, biochemical and lifestyle predictors associated with SDF in men with unexplained infertility. Seventy men were recruited and divided into two groups according to DNA fragmentation index: a normal-DNA group (n = 51) and an abnormal-DNA group (n = 19). The anthropometric measures, smoking, MDA and other oxidative-stress biomarkers—catalase (CAT) and superoxide dismutase (SOD)—and acute phase proteins—C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin—were determined. Comparison There was no age difference between groups (P = 0.312) while BMI showed borderline result (P=0.06). The only clinical factor related to increased DNA damage of sperm was smoking, p = 0.04. The individuals with anomalous quantified DNA profiles had notably higher oxidative stress showing significantly elevated MDA concentrations (p = 0.002), and significant decrease of CAT (p= 0.01) and SOD activities (p= 0.003). In addition, all evaluated positive acute phase proteins were significantly elevated in men with abnormal DNA fragmentation as compared to those without (CRP: p = 0.022, SAA: p = 0.014, haptoglobin: p = 0.031). History medicals variables, such as diabetes mellitus (p = 0.41) and varicocele (p = 0.08), did not show statistical significance. In conclusion, results in the present study suggest that smoking adjunct to elevated systemic inflammation and enhanced oxidative stress contribute significantly to DNA damage among men with unexplained infertility. These findings emphasise the clinical value to include oxidative and inflammatory profiling in standard of care assessment for infertility and make a case for interventions targeting systemic inflammation and oxidative load in order to help improve reproductive outcome.

Keywords: DNA Damage, MDA, CAT, SOD, CRP, SAA, Haptoglobin

Introduction

Infertility affects about 15% of couples worldwide, with male infertility responsible for nearly half (Agarwal et al., 2015). Traditional semen analysis— concentration, motility and morphology— has been the reference method for estimating male fertility potential. Nevertheless, a large proportion of men who cannot father children have normal semen parameters and are diagnosed as having “unexplained” or “idiopathic” infertility. This disparity reflects the shortcomings of conventional semen analysis and indicates the necessity for more sensitive biomarkers to provide an indication of functional and genomic integrity of sperm. In the last years, sperm DNA damage (frequently assessed as sperm

DNA fragmentation [SDF]) has arisen as an important parameter to define the male reproductive competence (Alahmar et al., 2022).

Sperm DNA integrity is vital for the success of fertilization, embryogenesis and normal fetal development. The accumulation of DNA strand breaks may also prevent the zygote from forming, block the embryo cleavage process and even decrease the rates of implantation. Increased SDF has been linked to impaired natural fertility, poor success with assisted reproductive techniques (ART), i.e., IVF and ICSI and is a risk factor for early pregnancy loss (Zeqiraj et al., 2018). A number of studies after 2015 invariably state that sperm DNA integrity offers prognosis over and above semen parameters, and may account for a failed reproduction in couples with otherwise normal evaluations (Sharma et al., 2016).

SDF is frequently the underlying but unrecognised pathogenesis in unexplained infertile men. Studies have shown that a significant percentage of such men present with high DNA fragmentation despite the presence of normal sperm levels, motility and morphology (Hwang et al., 2011). Furthermore, increased fragmentation has been associated with poor embryo quality and reduced fertilization and clinical pregnancy rates even in the ART setting, highlighting its clinical significance. These results corroborate an increasing awareness that evaluation of DNA damage ought to be considered as a part of routine semen analysis, since the conventional tests are unable to detect an obvious source in many instances (Bach & Schlegel, 2016).

There are a variety of biological and environmental factors that can produce sperm DNA fragmentation. Among this oxidative stress is the most common causative factor and over accumulation of ROS can cause not only single but also double-strand DNA breaks and affect sperm chromatin (Agarwal et al., 2021). Faulty chromatin remodelling, especially in protamination, during spermato-genesis can expose the genome to oxidative and enzymatic damage. Moreover, abortive apoptosis (when the process of programmed cell death is triggered, but not finalized) might lead to spermatozoa with irreversibly damaged DNA being passed on into the ejaculate (Muratori et al., 2015). These endogenous defects are frequently aggravated by exosomes as a result of smoking, obesity, excessive heat exposure, environmental pollutants and occupational risks documented to raise SDF levels (Barati et al., 2020).

Recent studies have also highlighted the importance of systemic metabolic and endocrine derangements, such as insulin resistance, dyslipidemia, obesity-associated inflammation in favor of inducing oxidative stress thus leading to DNA damage in sperm. It has been documented significant correlations between lifestyle, modifications of redox regulation and compromised genomic stability in the male germ line. Studies in more recent times have examined epigenetic changes and biochemical molecules such as iron-related ones and oxidative DNA adducts, providing a finer characterization of the molecular determinants of sperm DNA damage (Lira et al., 2024).

In view of the complex and multivariate characteristics of sperm DNA fragmentation, etiological factors are needed to enhance diagnostic precision and to advise therapeutic procedures. Current evidence suggests that SDF is a measure of the net outcome of testicular function, oxidative equilibrium and environmental and lifestyle influences. However, despite the volume of investigations, the relative contribution of each to complications—particularly in idiopathic male infertility—is not yet precisely defined (Cho et al., 2017). Thus, the current study aims to evaluate and identify the determinants of elevated sperm DNA fragmentation among men diagnosed with unexplained infertility in this study. Through the analysis of biochemical, lifestyle and clinical factors, this study aims to draw out common factor(s) that lead to DNA integrity compromise despite normal standard semen parameters.

Methods

Patients and data collection

This was a case-controlled study with 70 men with unexplained infertility (UI) who referred to the andrology outpatient clinic. All subjects were subjected to semen analysis, and evaluation of sperm DNA fragmentation, oxidative stress biomarkers, acute phase proteins and some clinical and life style determinants. Unexplained infertility was described as failure to conceive after ≥12 months of regular intercourse in couples without obvious cause of infertility despite a normally functioning ovulatory process and tubal patency, hormonal profile (surrogate measure for ovarian reserve) and normal clinical assessment of the female partner. Exclusion criteria included medical history of a chronic systemic disease (eg, diabetes mellitus, hypertension, liver and renal disease), known endocrine

imbalance or genital infection, prior chemotherapy- or radiotherapy treatment, and any current use of hormonal therapy/antioxidants /steroids drug during the latest 3 months. Other exclusion criteria comprised of azoospermia, gross urogenital malformations, active smokers who smoked more than two packs a day and unwillingness to give an informed consent. The study protocol was approved by the institutional ethics committee, and all procedures used conformed to the standards set forth in the Declaration of Helsinki. All participants gave written informed consent before entry. Semen was obtained by masturbation in sterile containers after 3–5 days of sexual abstinence. Thirty minutes after the thaw process at 37°C, DNA fragmentation was determined by TB staining. Sperms were air-dried, fixed in freshly prepared Carnoy's solution and stained with 0.05% Toluidine Blue in acetate buffer (pH 4.0) for 10 min. Spermatozoa with normal chromatin colored were light blue, fragmented DNA appeared as deep royal-blue to violet stained spermatozoa. A minimum of 200 spermatozoa per slide were scored and the percentage of DNA fragmentation determined. All dye reagents were purchased from Authentic Corp, China.

The oxidative stress markers measured were malondialdehyde (MDA), catalase (CAT) and superoxide dismutase (SOD). A volume of about 5 ml of venous blood was collected from all volunteers, allowed to clot at room temperature and centrifuged at 3000 rpm for 10 min. The serum was then aliquoted and frozen at −20°C for analysis. MDA levels were determined with a thiobarbituric acid reactive substances (TBARS) assay kit (China), according to spectrophotometric measurement of the MDA–TBA complex. Catalase (CAT) activity was analyzed by an inductance period colorimetric assay kit description of the dismolition speed of H-Gr--H2O2 (China), SOD measurement using a commercial inhibition-based colorimetric kit analysis (China). Each assay was done in duplicate with the intra-assay and inter-assay variation being less than 10%.

The levels of acute-phase proteins, such as C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin, were measured with enzyme-linked immunosorbent assayed (ELISA) kits purchased from Chinese certified matrices. Serum was thawed once before analysis, and all assays were performed according to manufacturer instructions. Quantities were stated in mg/L for CRP and SAA, g/L haptoglobin.

The clinical and lifestyle factors of the enrolled, including age, body mass index (BMI), smoking status, history of diabetes mellitus and clinically diagnosed varicocele was documented for each participant. BMI was estimated as the ratio "weight/height²" (kg/m²) and varicocele was verified by physical assessment, whenever necessary complemented with Doppler ultrasonography. Smoking status was further classified as current smoker and non-smoker.

The statistical analysis was carried out with SPSS (version XX). Continuous variables were presented as mean ± SD and categorical variables counts and percentages. Male patients with and without sperm DNA fragmentation were compared by independent-samples t-test for continuous variables and chi-square test for categorical variables. The Pearson correlation coefficients were calculated between the DNA fragmentation and biochemical or clinical variables. Statistical significance was defined by a $p< 0.05$.

The Results

Figure 1 The distribution of the results of TB staining in the study population is shown in Figure 1, representing the percentage of males with normal or abnormal chromatin integrity. Among 70 subjects, 51(72.86%) men exhibited normal TB staining, which show mostly intact sperm chromatin packaging. By comparison, abnormal staining patterns with altered chromatin structure and predicted increase in DNA instability were identified in 19 (27.14%) male subjects.

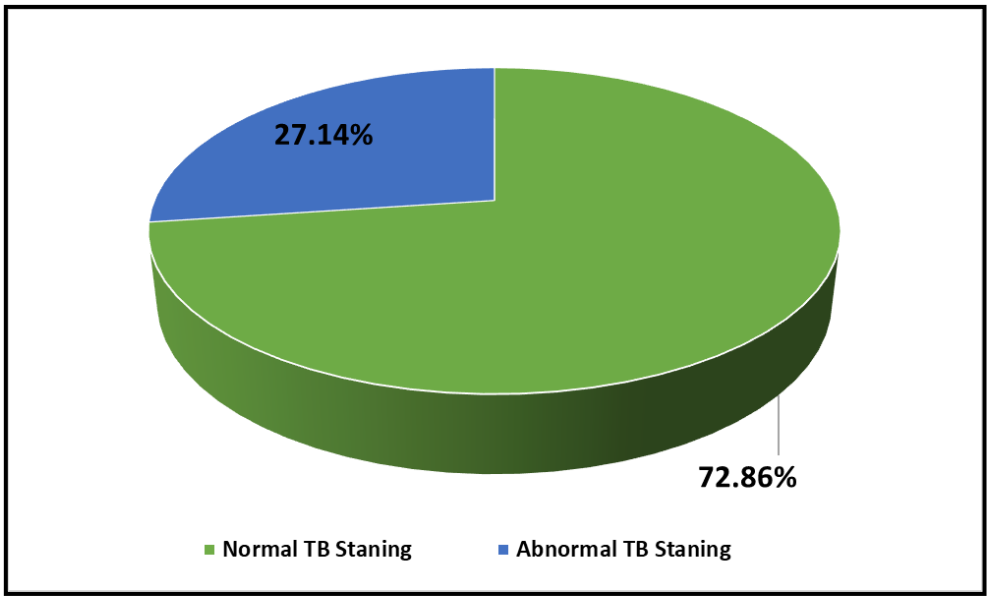

Figure 1. Percentage of patients according to response of sperm to toluidine blue staining (TB)

Statistical demographic and lifestyle characteristics of men with normal and abnormal sperm DNA integrity. Overall, DNA fragmentation levels did not correlate to age (p = 0.312), and the distribution between age groups was relatively equal suggesting that age alone may not be a critical factor determining sperm DNA stability in this population. A borderline association with abnormal sperm DNA was found to be associated with BMI (p = 0.06). An even greater proportion (47.37% vs. 23.53%) of the men in the abnormal group were obese; while no true statistical significance was attained, this may have at least a relatively strong influence on tests of DNAP considering the comparatively small sample size of the cohort. Only smoking was significantly associated with the abnormal sperm DNA (p = 0.04). More than half the men with abnormal DNA (52.63 per cent) were smokers compared with just 25.49 per cent of those with normal DNA integrity.

Table 1. Comparison of age, BMI and smoking habits between patients with normal and abnormal sperm DNA

Items		Normal =N)51(		Abnormal =N)19(		(P value)
		Freq.	%	Freq.	%	
Age	25-34	18	35.29	9	47.37	0.312 (NS)
	35-44	24	47.06	7	36.84	
	> 45	9	17.65	3	15.79	
BMI	Normal	11	21.57	2	10.53	0.06 (NS)
	Overweight	28	54.90	8	42.11	
	Obese	12	23.53	9	47.37	
Smoking	Yes	13	25.49	10	52.63	0.04 (N)
	No	38	74.51	9	47.37	

NS: Non- Significant at P value >0.05, S: Significant at P value <0.05

There were stark differences in oxidative stress profiles amongst men with intact or impaired sperm DNA integrity. Serum malondialdehyde, an indicator of lipid peroxidation, was markedly higher in subjects with DNA fragmentation. In contrast, activities of catalase and superoxide dismutase were significantly decreased, suggesting an impaired antioxidant defence system. P for all markers < 0.01) further supports oxidative stress as a key mediator of sperm DNA damage in unexplained infertility (Table 2).

Table 2. Comparison of oxidative stress markers between patients with normal and abnormal sperm DNA

Markers	Normal =N)51(		Abnormal =N)19(		(P value)
	Mean	SD	Mean	SD	
Malondialdehyde (MDA)	2.35	0.42	3.18	0.51	0.002 *
Catalase (CAT)	9.84	1.77	7.12	1.64	0.01 *
Superoxide Dismutase (SOD)	5.92	1.11	4.03	0.96	0.003 *

*** High Significant at P value <0.01**

When inflammatory markers were compared between normal sperm DNA and abnormal sperm DNA fragmentation, all three acute-phase reactants SAA, haptoglobin, CRP were statistically higher in the men with high levels of DNA breakage. In the abnormal group, the CRP level was significantly increased, suggesting a high system inflammatory state that possibly leads to oxidative injury in male reproductive tract. Serum amyloid A (SAA) levels were also higher in the abnormal DNA group, reinforcing that subclinical inflammation could affect sperm chromatin integrity. Haptoglobin levels were also increased in men with abnormal DNA fragmentation, indicating that activation of the acute-phase response occurred.
(table 3).

Table 3. Comparison of acute phase proteins between patients with normal and abnormal sperm DNA

Acute phase proteins	Normal =N)51(		Abnormal =N)19(		(P value)
	Mean	SD	Mean	SD	
CRP (mg/L)	2.15	0.88	3.42	1.21	0.022 *
SAA (mg/L)	5.73	2.06	8.14	2.57	0.014 *
Haptoglobin (g/L)	0.84	0.21	1.03	0.27	0.031 *

*** Significant at P value <0.05**

by There was no significant relationship between sperm DNA fragmentation with background of DM and varicocele, when medical history was compared in terms of both groups. The prevalence of diabetes was slightly higher among men with abnormal DNA (21.0%) than those with normal DNA integrity (11.8%), but the difference was not significant (p = 0.41). No firm conclusions can be drawn toward varicocele as it seemed more prevalent among the abnormal group (26.3%) versus the normal group (13.7%); again, this was a borderline nonsignificant pattern (p = 0.08) as shown in (table 4).

Table 4. Comparison medical history between patients with normal and abnormal sperm DNA

Items		Normal =N)51(		Abnormal =N)19(		(P value)
		Freq.	%	Freq.	%	
Diabetes Mellitus	Yes	6	11.80	4	21.00	0.41 (NS)
	No	45	88.20	15	79.00	
Varicocele	Yes	7	13.70	5	26.30	0.08 (NS)
	No	44	86.30	14	73.70	

NS: Non- Significant at P value >0.05

Discussion

In this study, we investigated factors associated with sperm DNA damage in men with unexplained infertility and showed that marker of oxidative stress and acute-phase inflammatory proteins were higher in men with abnormal sperm DNA fragmentation. Demographic and

lifestyle factors, including age and BMI, were not statistically significant predictors of DNA fragmentation but smoking did have a statistically significant association. These observations emphasize the multifactorial etiology of sperm DNA damage and are in accordance with recent evidence pointing to oxidative and inflammatory pathways as central pathways underlying male infertility (Agarwal et al., 2016; Agarwal et al., 2019).

The results of the current study come in accordance with the study of Al-Sultani et al. (2015) who observed that sperm chromatin staining test showed a significantly higher percentage of abnormalities in chromatin structure for infertile men than that for fertile controls. Even though some patients had semen parameters that fell within these cut-off values, there was a high incidence of chromatin alterations and augmented DNA instability which suggested the presence of potential hidden molecular abnormalities. These findings strongly suggest that DNA fragmentation and chromatin damage in sperm can be considered two independent predictors of male infertility and may be indeed a significant factor in those men with idiopathic infertility, where conventional semen analysis does not permit the diagnosis.

The fact that age did not play a significant role varied from some other articles showing an increase in DNA fragmentation due to the accumulation of oxidative stress and lowering repair capacity on advancing of paternal age (Basaria, 2013). On the other hand, previous evidence revealed a small or non-significant correlation between age and sperm DNA integrity in samples with relatively narrow limited ranges such as the present sample. Accordingly, the failure to find such an association in our study might not so much indicate biological lack of effect as demographic homogeneity (Petersen et al., 2018).

BMI appeared to be of borderline significance, reflecting the increasing but not always coherent evidence for an association between overweight or obesity and sperm DNA instability. Increased BMI is often correlated with higher scrotal temperature, systemic inflammation and altered hormonal milieu that could compromise spermatogenesis (Campbell et al., 2015). However, there are reports that the interpretation of BMI is limited in prediction of DNA fragmentation per se without considering metabolic syndrome or severe adiposity. The null pattern found here leaves open the possibility that an underlying effect is evident in larger samples of mice or with greater variation (or skew) in body weight (Santi et al., 2024).

Smoking was identified as an important risk factor related to sperm DNA damage. This result is consistent with numerous studies that have demonstrated the induction of genotoxic agents, increased ROS production and decreased antioxidant defence in testicular tissue following exposure to tobacco, resulting in an increase in DNA fragmentation (Sharma et al., 2016) Several meta-analyses have also shown that smokers are affected more for semen quality and fragmentation than non-smokers. The large effect in the current study further confirms that smoking is a proven, preventable risk factor for male infertility (Kunzle et al., 2003).

The levels of oxidative stress markers (MDA, CAT and SOD) were significantly different in patients with abnormal sperm DNA. Higher levels of MDA represent more lipid peroxidation, a reflection of oxidative damage in the plasma membrane associated with defective spermatozoa. The decreased expressions or abnormal activities of antioxidant enzymes, including CAT and SOD also proved oxidative imbalance. These results are in good agreement with recent studies showing oxidative stress is the primary pathway that leads to sperm DNA fragmentation, as ROS directly attack nucleoprotein structures and disrupt their packaging during spermatogenesis (Aitken, 2020). Moreover, more recent studies show that also low levels of ROS are capable of causing double strand breaks and apoptosis in germ cells. Thus, the biochemical profile of our sample would be consistent with oxidative stress as a driving pathogenic pathway (Takalani et al., 2023).

Acute-phase proteins, such as CRP, SAA, and haptoglobin were similarly elevated in the abnormal DNA group; implying a systemic disk-related inflammation. CRP is regarded as an established biomarker for low grade inflammation and has been implicated in reduced sperm quality in some recent studies (Azenabor et al., 2015). Elevated SAA has also been associated with chronic inflammatory conditions, which are reported to compromise testicular microcirculation and oxidative status. The increase of haptoglobin concentration reflects also activation inflammatory status and the possible increment in oxidative stress due to its capacity to bind free hemoglobin, thus modulating ROS generation Fe- dependent. Recent studies have shown that the idiopathic infertile man presents a higher level

of inflammatory mediators, indicating that inflammation and oxidative stress cooperate in causing sperm DNA damage. Thus, the current results are consistent with the existing and recent mechanistic models (Potiris et al., 2025).

With respect to medical history; neither diabetes mellitus nor varicocele were statistically significant in this cohort. Effects of diabetes Although diabetes is known to be linked with elevation in ROS, advanced glycation products and disrupted spermatogenesis, the impact may differ based on glycemic control, duration and the occurrence of complications (Condorelli et al., 2017) . The lack of significance observed may indicate well-managed condition or few diabetic individuals. Likewise, varicocele is well established to be associated with increased oxidative stress and sperm DNA damage (fragmentation) though the low P value, in this case borderline, indicates a potential under representation of cases of varicocele submitted for LC or mild grades of varicocele were not severe enough to influence fragmentation to any great extent. This is in accordance with the findings that varicocele-related DNA damage may also vary based on clinical grade, time of evolution and testicular impairment (Kantartzi et al., 2007).

Conclusion

Smoking, oxidative stress and inflammatory activation were identified as the major contributors of sperm DNA damage in men with unexplained infertility. These results support current guidelines suggesting lifestyle modification, checking antioxidant status, and evaluating systemic inflammation in idiopathic male infertility treatment. Larger studies with consideration of environmental exposures and antioxidant treatment responses will be necessary to have a clearer evaluation of the multifactorial aspects of sperm DNA integrity.

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