



Analysis of the correlation between sperm DNA integrity and conventional semen parameters in infertile men

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ABSTRACT

Objective: A male factor is responsible in approximately 30-40% of couples receiving infertility treatment. Routinely, such couples undergo semen analysis including parameters such as sperm count, motility and morphology. Generally, the analysis of sperm DNA damage, shown to have a significant clinical importance by many studies, is recognized as an advanced test that is not included in routine infertility tests. Intracytoplasmic sperm injection method, commonly employed in the current infertility treatment protocols, lowers the fertilization rate, however, fertilization can occur even with a damaged DNA which is known to pose a risk in the subsequent pregnancy period. The relation between sperm morphology and the degree of sperm DNA damage has not yet been understood clearly. In this study, we aimed to investigate the association between routine semen analysis and sperm DNA integrity assay, another advanced but costly method.

Material and methods: The degree of DNA damage was compared with the results of semen analysis, based on the WHO criteria, in 399 male patients who received comet assay for sperm DNA integrity. The statistical correlation analyses were performed with Windows SPSS statistical package program.

Results: Accordingly, the sperm DNA damage was found to be correlated with all 3 parameters (sperm count, forward motility, and morphology) examined by the semen analysis ($p<0.001$). Total sperm DNA Damage Count was 226, 216, and 210 arbitrary units in patients with a sperm count <15 mil/mL, forward moving motility $<32\%$, and normal morphology $<4\%$, respectively. The difference with the normal individuals was statistically significant ($p<0.001$).

Conclusion: In light of the comet assay results, higher degree of sperm DNA damage is associated with significant impairment of all seminal parameters.

Keywords: Male infertility; morphology; motility; sperm DNA damage.

Introduction

In almost half of the infertile couples, a male factor is involved. These reproductive problems are routinely evaluated by conventional semen analysis. However, 15% of cases of male factor infertility have normal semen analysis which complicates a definitive diagnosis. There is a strong association between sperm genetic damage and infertility. The involvement of sperm DNA damage in infertility has been shown by many *in vitro* and *in vivo* studies. It has been found that spermatozoa with denatured and fragmented DNA have a significant relationship with impaired seminal parameters, being significantly higher in infertile males (25% and 27%, respectively) than

in fertile ones (10% and 13%, respectively) ($p=0.028$ and $p=0.016$).^[1] As the degree of spermatozoa with damaged DNA increases ($>30-40\%$), the likelihood of natural pregnancy drops.^[2,3] Sperm DNA of poor quality has been shown to impair fertilization by *in vitro* studies. In fact, when the degree of sperm with damaged DNA is above 4% in patients receiving *in vitro* fertilization (IVF), the fertilization rates drop from 58% to 38% ($p<0.05$).^[4] The importance of these data during intracytoplasmic sperm injection (ICSI) emerges from the fact that damaged DNA may not inhibit fertilization, thereby allowing the formation of embryos with this defective genetic material.^[5,6] Therefore, it is important to know the degree of spermatozoa with damaged DNA in

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the ejaculate in order to predict the fertilization rate and determine the risks that may affect the embryo.

Although high degrees of sperm DNA damage are correlated with impaired seminal parameters such as reduced sperm count and motility, men with normal seminal parameters can also exhibit high degrees of sperm DNA damage.

In this study, we compared the results of conventional semen analyses and sperm DNA damage assay in 399 patients diagnosed with male factor infertility in the infertility clinic.

Material and methods

This study was performed with patients who applied to Ankara University Infertility Research Center between 2010 and 2014. Men who failed to have a child despite 1 year of attempt were included in the study. Azoospermic men were excluded from the study. The sperm analyses were performed based on the WHO 2010 criteria. Seminal fluid samples were collected following a fasting period of 72 hours; a sperm count ≥ 15 million, forward motility $\geq 32\%$, and Diff-Quik morphology $\geq 4\%$ were recognized as normal.

Ankara University Faculty of Medicine's Institutional Ethical Committee approved the study and the written informed consent was obtained from the entire subject who participated in the study.

Analysis of total sperm DNA damage

Total Sperm DNA Damage Count (TDC), developed by Singh et al.^[7], was analyzed by alkaline assay with minor modifications. First, sperm cells were washed, followed by the mixing of 10 μ L of fresh sperm cell suspension (around 20,000 cells) with 80 μ L of 0.7% low-melting-point agarose (LMA) (Sigma) in PBS at 37°C. Subsequently, 80 μ L of this mixture was layered onto slides that had been previously coated with 1.0% hot (60°C) normal melting point agarose (NMA), covered with a coverslip at 4°C for at least 5 min to allow the agarose to solidify. After removing the coverslips, the slides were embedded in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA⁻²Na; 10 mM Tris-HCl, pH 10-10.5; 1% Triton X-100 and 10% DMSO added just before analysis) and 15 μ L proteinase K for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mmol/L NaOH and 1 mmol/L Na₂EDTA, pH >13) at 4°C for unwinding (40 min), and then subjected to electrophoresis (25 V/300 mA, 25 min). All of the above steps were conducted without applying direct light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide, covered with a coverslip, and analyzed using a fluorescence microscope (Olympus BX51). The images of 100 randomly chosen nuclei were analyzed visually from each subject, as described elsewhere. Each image was classified according to the intensity of

the fluorescence in the comet tail and was given a value of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4), so that the total scores ranged between 0 and 400 arbitrary units (AU) (Figure 1).^[8]

Statistical analysis

The study data analysis was performed using Statistical Package for the Social Sciences for Windows, version 15.0 (SPSS, Inc., Chicago, IL, USA). The statistical analyses between compared groups were performed by two-sample t-test. Each p value <0.05 was considered statistically significant. Pearson and Spearman correlation coefficients were calculated between seminal parameters and TDC analysis. Reliability of the scoring systems was tested with the correlation coefficient (*r*).

Results

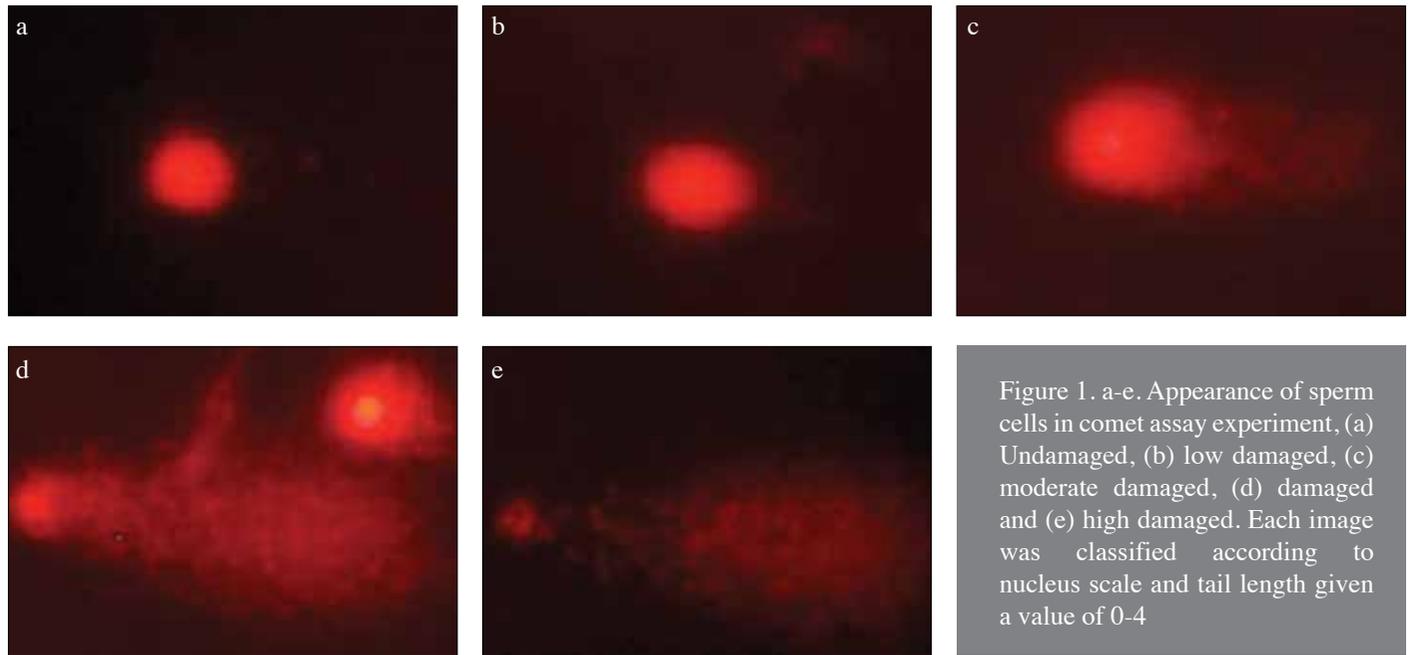
A total of 399 consecutive patients were included in the study. The mean age was 28 years (range: 23-42 years). Seminal parameters and TDC results were compared in these patients. Of the 399 infertile patients, mean sperm count was 45.7 10⁶/mL and forward motility was 37%. Of the 392 patients, mean Kruger morphology was 2.37% (Table 1).

In our study, seminal parameters were categorized based on the WHO 2010 criteria. Sperm count was classified as <15 million and ≥ 15 million groups. When sperm count and TDC were compared, 249 patients with normozoospermia, a sperm count ≥ 15 million, had a TDC of 191 AU, while 144 patients with oligozoospermia, <15 million, had a TDC value of 226 AU ($p < 0.001$) (Figure 2a).

The patients were categorized with regard to sperm morphology, as well: <4% and $\geq 4\%$ groups. A total of 294 patients with a morphology rate <4% had a TDC of 210 AU, while 97 patients with normal morphology ($\geq 4\%$) exhibited a TDC of 186 AU ($p < 0.001$) (Table 2).

In the present study, we observed the relation of TDC with sperm count, motility, and morphology. According to the correlation analysis, all three seminal parameters showed a negative correlation with TDC and the correlation coefficients were -0.301, -0.248 and -0.207, respectively (Table 2) (Figure 2b).

Forward motility rate was categorized in two groups: >32% and ≤ 32 . A total of 145 patients with a motility rate >32% had a TDC of 216 AU, whereas the patient group with a motility rate ≤ 32 showed a TDC of 194 AU ($p < 0.001$) (Figure 2c). Comparison of the TDC values was also done between normozoospermic infertile men and infertile men with Oligoasthenoteratozoospermia (OAT). TDC was found significantly higher in OAT group than those of the normozoospermic group; as 211 and 176 AU, respectively (Table 3) (Figure 2d).



Seminal parameters	n	Mean (min-max)
Sperm count x 10 ⁶ /mL	399	45.70 (0-280)
Forward motility (%)	377	37.00 (0-92)
Morphology (4%)	392	2.37 (0-21)

Discussion

Current evidences reveal that the most influential cause of sperm DNA damage is oxidative stress. Such events are accompanied by impaired spermiogenesis and increased ROS production due to spermatozoa. Impaired spermiogenesis is probably caused by oxidative stress leading to disrupted protein translation. Thus, spermatozoa differentiate into germ cells with certain deficiencies. Insufficient protamination also causes poor compaction of DNA which renders it vulnerable to oxidative stress. Finally, defective sperm cells may prematurely enter apoptosis, which is accompanied by the activation of mitochondrial ROS generation. As a consequence of this cascade, in addition to the disruption of sperm production, the spermatozoa lose their motility and exhibit oxidative DNA damage that leads to the creation of DNA fragmentation.^[9]

Our results indicate that DNA damage is significantly higher in infertile men with OAT when compared with those of the normozoospermic men. Many studies have been performed to investigate the relation between sperm DNA damage and conventional seminal parameters. Most of these studies have yielded results indicating an inverse correlation of DNA damage with seminal parameters including sperm count, motility, and morphology.^[10-12]

In our study, among patients with a mature sperm count $\geq 15 \times 10^6/\text{mL}$, the TDC was 191 AU, whereas those with a mature sperm count $< 15 \times 10^6/\text{mL}$ had a TDC of 226 AU (Figure 2a). The low sperm count was associated with impaired spermatogenesis; the oxidative stress generated by increasing the ROS level hinders the maturation of germ cells by inducing DNA damage in the germ cells and accelerating apoptosis via cytochrome C and caspase 9 and caspase 3.^[13]

Abortive apoptotic pathway has been proposed as an important etiology for the DNA breaks in the spermatozoa of infertile patients.^[14] In the testes, normally, apoptosis prevents the overproduction of germ cells and selectively destroys injured germ cells. However, Sertoli cells are only able to support a limited number of germ cells in the testis.^[15] There is an optimal germ cell to Sertoli cell ratio for the spermatogenesis to proceed normally. During this developmental process, excess immature germ cells are removed by apoptosis. If this process is impaired, the germ cell to Sertoli cell ratio is perturbed which can in turn interrupt the normal spermatogenic cascade by the result of an abortive apoptotic process. Indeed, men exhibiting deficiencies in their semen profile often possess a large number of Fas bearing spermatozoa which leads to cell death through apoptosis.^[16] This fact prompts the suggestion that these dysfunctional cells are the product of an incomplete apoptotic cascade. In case of exposure of human spermatozoa to hydrogen peroxide, apoptotic cascade has been shown to be triggered by the activation of caspase 3,^[17] which may explain the association of high DNA damage and low mature spermatozoa count in our series. It has also been demonstrated that the activation of apoptotic cascade results in the stimulation of mitochondrial free radical generation.^[14] Mitochondrial source of ROS can make an additional

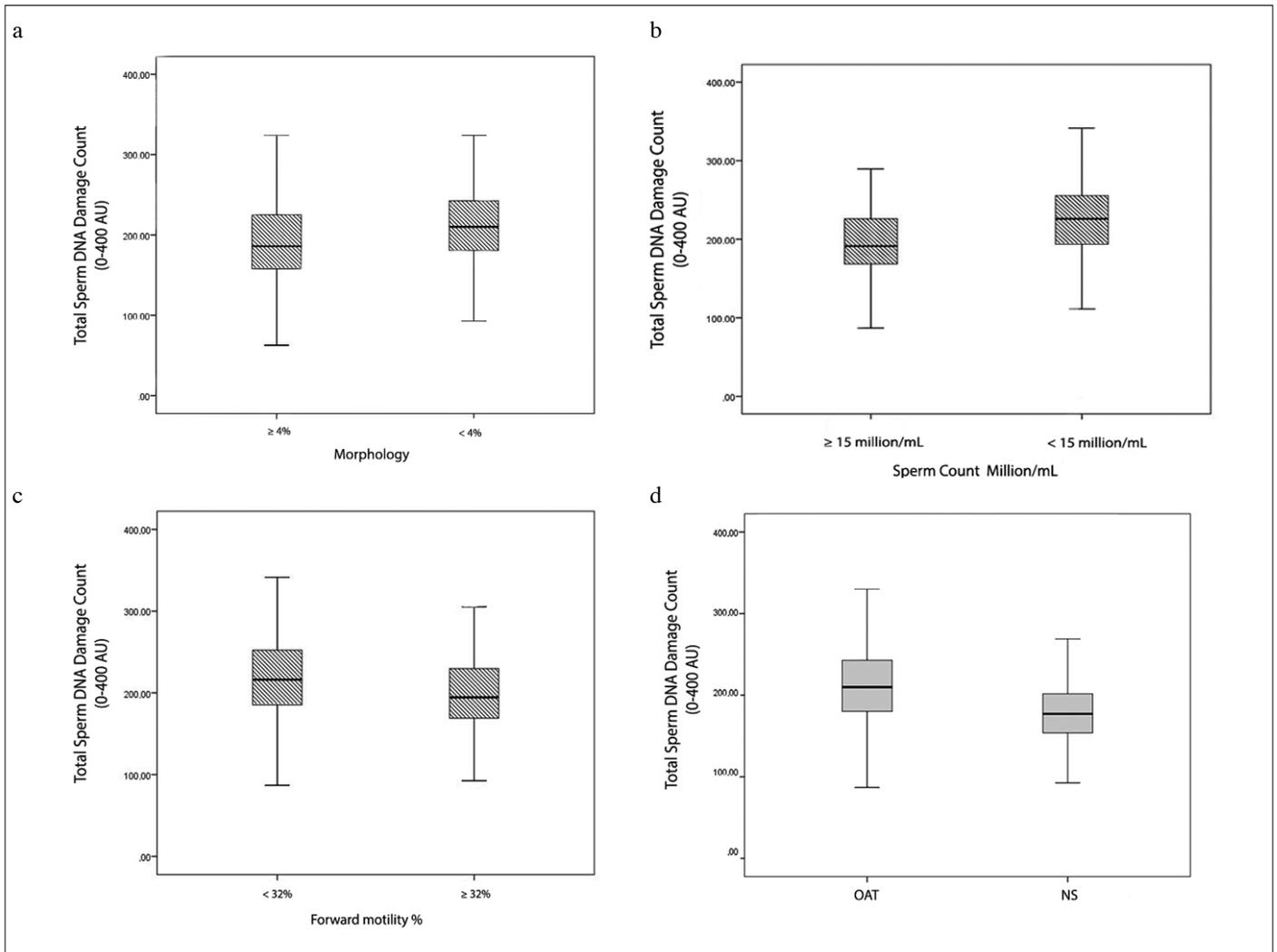


Figure 2. a-d. The graphics of comparison between total sperm DNA damage count and Kruger morphology (a) sperm count (b) forward motility (c) in infertile men. Comparison of the total sperm DNA damage values in NS infertile men (All seminal parameters are in normal ranges) and infertile men with OAT (d)

contribution to the induction of oxidative stress and DNA damage in spermatozoa.

In our study, sperm morphology and TDC values showed a significant correlation; patients with a morphology rate $<4\%$ had a TDC of 186 AU, while patients exhibiting a morphology rate $\geq 4\%$ showed a TDC of 210 AU (Figure 2b). In the literature, a significant association has been reported between normal sperm morphology rate and DNA fragmentation.^[18]

Particularly, the incidence of serious morphologic disorders such as abnormally small heads, megaloccephaly, severe tail abnormalities, or globozoospermia has a significant correlation with abnormal chromatin structure and DNA strand breaks.^[19] In such cases, there are also parallel increases in aneuploidy rates and unexplained recurrent pregnancy losses.^[20]

The shaping of the sperm head is probably related to the degree of chromatin condensation as well as developmental factors.^[21] It has been suggested that the shaping of sperm heads may depend on the specific patterns of assembly of DNA-protein complexes established during chromatin condensation in the nuclei.^[22] On the other hand, nuclear shape has been linked to vacuoles and heterogeneity in chromatin condensation.^[23] During spermiogenesis phase, last stage of spermatogenesis, significant rearrangements take place in the sperm nucleus during the transition from round spermatids to spermatozoa^[24] condensation of nuclear chromatin, formation of the flagellum apparatus, and development of the acrosomal cap. The most prevalent rearrangement is the reshaping of the nucleus. Within the nucleus, the chromatin granules undergo progressive condensation due to replacement of the transitional proteins by protamins. Protamins form a fine homogeneous material that uniformly fills the entire

Table 2. Correlation between total sperm DNA damage count and seminal parameters in infertile men

Seminal parameters		n (%)	Total sperm DNA damage count (0-400 AU)	Coefficient	p
Count 10 ⁶ /mL	<15	144 (36.64)	226±54.39	r= -0.301	<0.001
	≥15	249 (63.36)	191±48.20		
Forward motility, %	<32	145 (39.18)	216±55.13	r= -0.248	<0.001
	≥32	225 (60.82)	194±49.38		
Morphology, %	<4	294 (75.20)	210±50.12	r= -0.207	<0.001
	≥4	97 (24.8)	186±56.46		

Table 3. Comparison of the total sperm DNA damage values in normozoospermic infertile men and infertile men with OAT

Seminal parameters	n (%)	Total sperm DNA damage count (0-400 AU)	p
Normozoospermia*	67 (83)	176.7±44.4	<0.001
OAT**	332 (17)	211.8±53.4	<0.001

OAT: oligoasthenoatozoospermia
 *All seminal parameters are in normal ranges
 **Oligoasthenoatozoospermia; count <15x10⁶/mL, forward motility <32%, morphology <4%

sperm nucleus. In light of all those events, the positive correlation between sperm DNA damage and impaired sperm morphology may be associated with the fact that sperm DNA damage leads to impairment in the sperm chromatin structure.

In our study, the degree of DNA damage and sperm motility impairment showed a positive correlation. In cases with a motility rate <32%, TDC was 216 AU, whereas patients with a motility rate ≥32% showed a TDC of 194 AU (Figure 2c). It is a well known fact that oxidative stress, which causes sperm DNA damage, induces peroxidation in the lipid-containing sperm plasma membrane via ROS, thereby leading to structural and functional damage in the spermatozoa.^[25] At the same time, ROS can impair the sperm motility by damaging the axonemal structure or reducing the intracellular adenosine triphosphate (ATP).^[26] Therefore, increased oxidative stress is a very effective factor in the impairment of sperm motility and the development of asthenozoospermia. The most cytotoxic oxygen metabolite among ROS products has been shown to be hydrogen peroxide. Indeed, when Percoll-separated spermatozoa are treated with hydrogen peroxide, there is a progressive decrease, leading to a complete arrest in the sperm flagellar beat frequency. However, once demembrated, ROS-immobilized spermatozoa regain motility.^[27] ROS-related increases in the motility can be prevented with catalase.^[28] The impairment in sperm motility has also been explained by apoptosis. It has been suggested that oxidative stress

causes the generation of spermatozoa with poorly remodelled chromatin. These defective cells have a tendency to enter in an apoptotic pathway associated with motility loss.^[14]

In experimental studies, a dramatic increase in DNA fragmentation has been observed after exposure to ROS products such as xanthine and xanthine oxidase. The results demonstrate that oxidative stress impairs sperm motility, as well as DNA integrity, by altering intracellular signalling pathways through changes in tyrosine phosphorylation and acid phosphatase activity.^[29] In other studies, oxidative stress has been shown to promote a dose-dependent increase in tyrosine nitration and S-glutathionylation, while altering motility and the ability of spermatozoa to undergo capacitation.^[30]

Another cause of the impairment in sperm motility is the disruption in apoptotic balance due to oxidative stress. Apoptotic mediators such as caspases have been shown to occur at high levels in sperms with impaired motility.^[31,32] A significant positive correlation between caspase activity in the sperm midpiece and the DNA fragmentation has been shown in the low motility fractions of patients, suggesting that caspase-dependent apoptotic mechanisms can originate in the cytoplasmic droplet or within the mitochondria, and function in the nucleus.^[31]

The most frequently used tests to assess sperm DNA damage are the Sperm Chromatin Structure Assay (SCSA), the single-cell gel electrophoresis assay (COMET), the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and the sperm chromatin dispersion (SCD) test. Because of its high precision and sensitivity in the determination of cell death and DNA damage, we prefer comet assay. The comet assay is a single cell gel electrophoresis of immobilized sperm which involves their encapsulation in agarose, followed by lysis and electrophoresis.^[7] Although the technique is often tedious and may damage the cell membrane changing the distribution of the cell population of live, apoptotic or necrotic cells, it can provide more specific information about the extent and heterogeneity of DNA damage compared to TUNEL staining.^[33] The TUNEL assay is also im-

peded by the highly compacted nature of sperm chromatin and necessitate the introduction of a chromatin decondensation step, prior to commencement of the assay in order to avoid potential artefacts.^[34] On the other hand, both the TUNEL assay and the SCD test have been reported to require higher levels of DNA and chromatin damage before they can detect structural changes, indicating that they have lower sensitivity than the SCSA and the comet assay.^[35]

In conclusion, as far as the comet assay can detect, high degrees of sperm DNA damage are accompanied by significant impairment in all seminal parameters. However, further studies including larger samples, homogenous patient groups, and comparison of different methods are required to reach a definitive conclusion.

Ethics Committee Approval: Ethics committee approval was obtained (approval number: 154-4951).

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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