Original Article — *Özgün Araştırma*
DOI: 10.24938/kutfd.385884 — Kırıkkale Üniversitesi Tıp Fakültesi Dergisi 2018;20(2):177-183

SPERM DNA DAMAGE ANALYSIS AMONG INFERTILE MALES WITH NORMAL SEMEN PARAMETERS AND A HISTORY OF ASSISTED REPRODUCTIVE TECHNIQUE FAILURE

Normal Sperm Parametrelerine Sahip, Yardımcı Üreme Yöntemi Başarısızlığı Öyküsü Bulunan İnfertil Erkeklerde Sperm DNA Hasar Analizi

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ABSTRACT

Objective: This study aims to document the sperm DNA damage results in infertile male patients with normal-standard semen parameters retrospectively.

Material and Methods: The study included samples from 500 male patients with a referred diagnosis of idiopathic infertility and a medical history of at least one assisted reproductive technique (ART) failure. All the patients included in this study were otherwise healthy individuals and had normal-standard semen parameters. We performed retrospective analysis, from the results obtained from samples. Tunel methodology was used, and fluorescence microscopy analysis was performed. From a morphological point of view, apparently normal mature sperms were analyzed.

Results: Increased sperm DNA damage rates were detected in our tested population. Advanced age did not correlate with a significant increase in sperm DNA damage ratios in our cohort.

Conclusion: The importance of sperm DNA damage should be considered as an etiological factor when evaluating infertile couples. Because the ratio of DNA damage might correlate with various indicators of fertility such as the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate and live birth rate, it is important to know the ratio of spermatozoa with damaged DNA in the ejaculate in order to predict the fertilization rate and determine the risks that may affect the embryo.

Keywords: *Sperm DNA damage, male infertility, sperm*

ÖZ

Amaç: Bu çalışmada retrospektif olarak, normal semen parametrelerine sahip infertil erkeklerde sperm DNA hasarı analizi sonuçlarının dokümantasyonu amaçlanmaktadır.

Gereç ve Yöntemler: Bu çalışmaya idiopatik infertilite olarak ele alınan ve takip edilen, ek olarak en az bir yardımcı üreme yöntemi başarısızlığı öyküsü bulunan 500 erkek hasta dahil edilmiştir. Tüm olgular, diğer açılardan sağlıklı, normal spermiyogram sonuçlarına sahip hastalar arasından seçilmiştir. Çalışmada Tunel yöntemi ile teste alınıp, floresan mikroskopi ile incelenen hasta örneklerindeki normal görünümlü spermlerde elde edilen sonuçlar dokumante edilmiştir.

Bulgular: Test edilen hasta grubunda artmış sperm DNA hasarı oranları gözlenmiştir. Bulguların yaştan bağımsız dağılım gösterdiği tespit edilmiştir.

Sonuç: İnfertil çift değerlendirmelerinde artmış sperm DNA hasarı da önemli bir parametre olarak göz önünde bulundurulmalıdır. Sperm DNA hasarının varlığı ve oranı, fertilizasyon düzeyi, embriyo yarıklanma oranı, implantasyon başarısı, gebelik ve canlı doğum oranı ile ilişkili olabilir. Bu nedenle sperm DNA hasarı analizi, bu çiftlerde prognoz tayini ve embriyonun sağkalımı ile riskleri konusunda fikir vermesi açısından önem arz etmektedir.

Anahtar Kelimeler: *Sperm DNA hasarı, erkek infertilitesi, sperm*

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Received / Geliş Tarihi: 29.01.2018 **Accepted /** Kabul Tarihi: 15.07.2018

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DOI: 10.24938/kutfd.385884

INTRODUCTION

Infertility is one of the most common health problems, affecting about one in five couples, and the male factor contributes to a considerable proportion of this condition. Mathews Duncan was the first to consider the epidemiology of infertility (1). The epidemiology of infertility is now very widely investigated in the literature, and the male factor makes the predominant etiologic contribution.

Male factor infertility assessment usually involves three steps: History, physical examination, and laboratory investigations. Laboratory analysis can begin with a spermiogram. Ejaculate consists of seminal plasma (99%), which includes the secretion of accessory glands, the prostatic gland, vesicula seminalis, and cellular population. In the clinical laboratory setting, the initial macroscopic examination of the ejaculate is followed by a series of evaluations. Liquefaction time, appearance, volume, viscosity, pH, sperm concentration, motility, morphology, and non-functional non-sperm elements should be carefully evaluated. Among these tests, only semen analysis can provide a real prognostic prediction of fertile, infertile, or in between. However, most male factor infertility cases have normal-standard semen analysis results, which includes the idiopathic group (2-4).

During normal spermatogenesis, immature germ cells are removed by apoptosis. If this process is impaired, the Sertoli cell ratio may become dominant, leading to disruption of the spermatogenic process. It has been found that the ratio of the spermatozoa with denatured and fragmented DNA is significantly higher in infertile males than in fertile ones. The likelihood of achieving natural pregnancy is inversely correlated with the ratio of spermatozoa to damaged DNA (5-7).

However, most male factor infertility cases have normal-standard semen analysis results, which reserves the idiopathic group and deserves further examination. Sperm DNA damage is an independent factor that cannot be readily examined during routine semen analysis. The involvement of sperm DNA damage in male factor infertility has already been shown in various studies.

In this study we are presenting the data belonging to 500 male factor infertility cases, which emphasizes the need for further evaluation of patients with male factor infertility that are routinely tested for infertility.

MATERIALS AND METHODS

Patients and samples

The study included samples from 500 patients with a referred diagnosis of idiopathic infertility. Patient characteristics are given in Table 1 considering the inclusion criteria of this study. All the patients were otherwise healthy individuals and had standard semen parameters. They were evaluated and diagnosed with their partners as having idiopathic infertility by their clinicians, and all were referred for sperm DNA damage testing. All the participants failed to have any successful pregnancy even after at least one assisted reproductive technique was performed. Semen analysis of the patients were performed once again by our laboratory following WHO criteria. All of the evaluated samples had a volume of more than 1.5 ml, containing more than 35% progressive motile sperm, with normal morphology, and semen containing acceptable leukocyte contamination.

We performed retrospective analysis from the results obtained from samples. Written informed consent was obtained from all subjects, and the study was approved by the ethics committee of Acibadem University, Istanbul (2017-13/52).

Sample Collection and Analysis Method

An ejaculate sample of 0.1 to 5 ml was collected from each participant. Patients were analyzed after 2–5 days of sexual abstinence. Only fresh semen was used, and immediate methanol fixed slides were prepared. A

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liquefaction procedure was performed at 37 ^{0}C for 15–20 minutes. For the procedure, 0.5–1 ml semen was mixed with 2–5 ml PBS in order to fill a centrifuge tube. After ten minutes of centrifugation at 1200 rpm, the pellet was resuspended in 2–5 ml PBS. Another centrifugation with the same conditions was followed by fixative addition to 1ml pellet. Ten minutes of –20^{0}C incubation was performed in a fixative solution (methanol). For each slide 30–40 µl pellet was spread.

The terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) methodology was used to analyse the samples. TUNEL is a direct quantification method of single and double-stranded DNA breaks. In this method, dUTP is incorporated at single-stranded and double-stranded DNA breaks in a reaction catalyzed by the enzyme TdT. The DNA breaks based on the incorporated dUTP are then labeled and can be visualized by bright field or fluorescent microscopy as well as flow cytometry. The first step of the method is fixation and permeabilization of the apoptotic cells as described above. The cells are then incubated with the TUNEL reaction mixture, which contains TdT and fluorescein-dUTP. During this incubation period, TdT catalyzes the addition of fluorescein-dUTP at free 3'-OH groups in single and double-stranded DNA. After washing steps to remove the unincorporated stain, the label at the damaged sites of the DNA can be visualized by fluorescence microscopy (Figure 1).

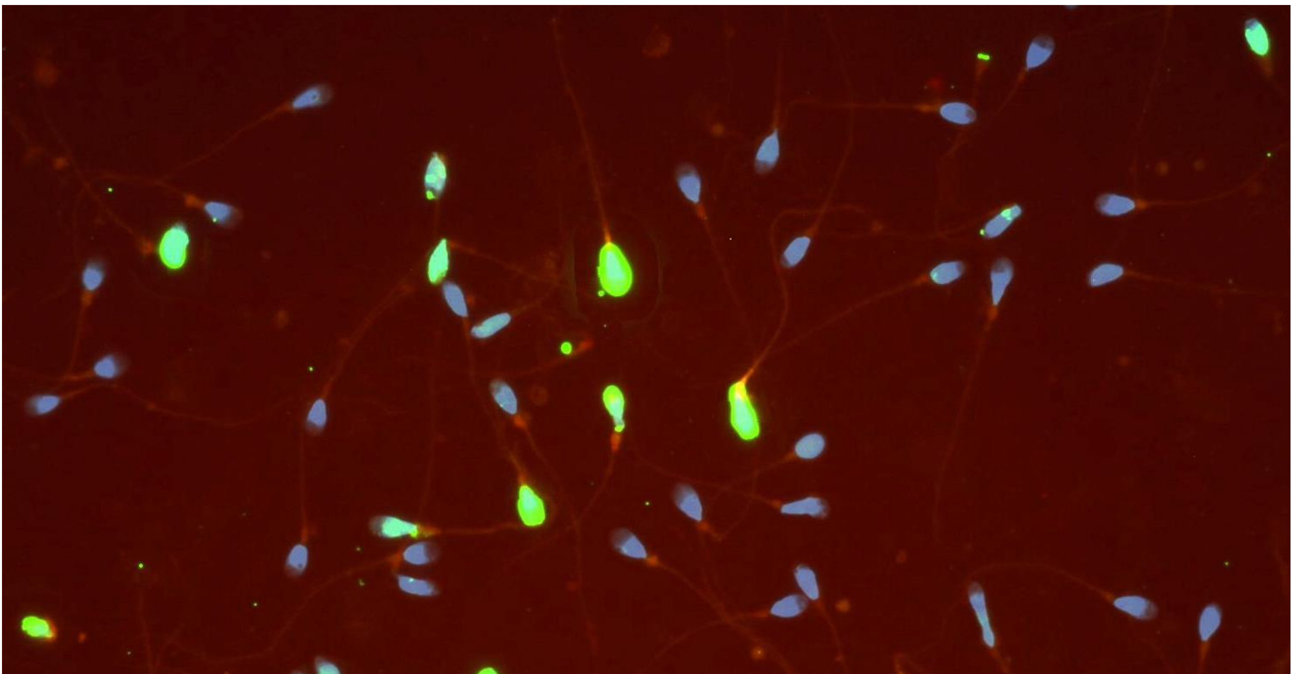

Figure 1. TUNEL assay. Blue sperms are TUNEL negative, green sperms are TUNEL positive, supporting finding of DNA fragmentation.

We used the In Situ Cell Death Detection Kit-Fluorescein (Roche Inc.) for TUNEL analysis according to the instructions of the manufacturer. Briefly, the slides from the above-mentioned fixation procedure were incubated in a freshly prepared 96ml PBS-4ml formaldehyde solution for one hour. Slides were agitated in PBS solution and then treated on ice with Triton X (1%) for two minutes. After this short treatment, they were washed with PBS and air dried twice. The procedure was performed in a dark room

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after this point. Five to ten µl of the enzyme buffer mix provided with the kit was dropped on each slide. One hour of incubation at 37^0C was performed after the slides were coverslipped. After this incubation, the coverslips were removed, and the washing procedure was performed three times using PBS solution. After air drying, 4-6 µl DAPI was added for counterstaining.

Sperms were then classified as TUNEL positive or negative according to fluorescence microscopy analysis. Approximately 1,000 apparently normal cells from each sample were analyzed, and TUNEL positive sperms were noted among the total analyzed population and expressed as a percentage of the total sperm in the population (Figure 1).

Validation and Statistical analysis

Validation procedure and cut off determination was performed using 100 healthy and young volunteer fathers. Cut off values were determined as 20% for the low value, and 20–30% was considered as a grey zone for the sperms analyzed. All the procedures were evaluated by the ISO 15189 accreditation process.

We have used mean, median, standard deviation, minimum and maximum values as descriptive statistics for numeric data. Frequency and percentages were used for categorical data. We investigated the difference in DNA damage between the three groups with one-way analysis of variance (ANOVA). Multiple comparisons were made with Tukey's test. The correlation between age and DNA was analyzed with linear regression and Pearson's correlation coefficient. Differences at a probability value (P) of 0.05 or less were considered to be statistically significant.

The significance of the difference between means was compared by Fisher's protected Least Significant Difference test, ANOVA sampling.

The relationships between the total number of spermatozoa and the DNA-fragmented nucleus index were examined by a single linear regression analysis, and the correlation coefficients were determined.

RESULTS

Five-hundred consecutive patients were included in the study. The mean age was 33.5 years (range: 23-58 years). Most of the patients were between 30 and 40 years of age (355 patients: 71%). Ninety-seven (19.4%) were between 20 and 30, and 48 (9.6%) were older than 40.

The TUNEL analysis was categorized based on the morphological criteria. Apparently normal sperms were selected as the target population. The ratio of spermatozoa to damaged DNA was classified as "low" for ratios under 20%, "grey zone" for ratios between 20–30%, and positive for ratios >30. Among 97 patients in the 20–30 age population, 67 (69%) had increased sperm DNA damage. This rate was 81.25% (39/48) among the older population (>40 years), and 76.3% (271/355) among the population of 30–40 years of age.

The difference between these three age groups was statistically significant. The second group's mean age was relatively lower than the other groups' (p = 0.01). But DNA analysis results among these groups were not different (p = 0.818). We have investigated the relationship between age and DNA, and the linear regression model was not significant. Their correlation was 3.3% (p = 0.316, R = 3.3%) (Table 2a-b,).

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Table 1. Patient characteristics and inclusion criteria to the study

Feature	Characteristic	Inclusion criteria
Age	23-58	<60
Female Factor	No	No female factor
Chromosome Analysis	Normal	Normal
Spermiogram	Normal morphology and count	Normal
ART procedure	Yes	At least 1 ART failure
Hormonal abnormality	No	No (hypergonadotrophic/hypogonadotrophic) hypogonadism, no abnormal Inhibin B or other abnormal results

ART: Assisted reproductive technique

Table 2a-b. Descriptive statistics

Age Distribution		
	Frequency	Percent
20-30	97	19.4
30-40	355	71
40-	48	9.6
Total	500	100,0

TUNEL Analysis Results Among Populations		
Age	Increased Sperm DNA Damage	Percent
20-30	67/97	69
30-40	271/355	76.3
40-	39/48	81.25
Overall	377/500	75.4

DISCUSSION

In this study, we have evaluated the sperm DNA damage in a very large series of male factor idiopathic infertility patients with the TUNEL method. To the best of our knowledge, this method has not been used sperm DNA damage in such a large cohort in previously published studies.

The hallmark of apoptosis is DNA degradation, which in the early stages, is selective to the internucleosomal DNA linker regions. The DNA cleavage may yield double-stranded and single-stranded DNA breaks (nicks). Both types of breaks can be detected by labelling the free 3'-OH termini with modified nucleotides (e.g., biotin-dUTP, DIG-dUTP, fluorescein-dUTP) in an enzymatic reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the template-independent polymerization of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA. This method has also been termed TUNEL (TdT-mediated dUTP-X nick end labeling). Alternatively, free 3'-OH groups may be labeled using DNA polymerases by the template-dependent mechanism called nick translation.

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However, the TUNEL method is considered to be more sensitive and faster. Widely used tests to assess sperm DNA damage include 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. The TUNEL assay was the method of choice because it allows qualitative detection of apoptosis at the single-cell level by fluorescence microscopy. It is sensitive and direct labeling procedure using fluorescein-dUTP reduces background labeling. It is fast and convenient for routine methods and allows for analysis of the samples directly after the TUNEL reaction. Also, no secondary detection system is required. The TUNEL method is also accurate because of its ability to identify apoptosis at a molecular level (DNA-strand breaks) and identify cells at the very early stages of apoptosis.

Various studies were reported recently that tested sperm DNA damage ratios in infertile men (5-9). Most of them hypothesized a negative correlation between fertility rates and sperm DNA damage rates. Aydos et al. as well as Evgeni et al. reported that a higher ratio of sperm DNA damage is associated with significant impairment of all seminal parameters (8,9). Wyrobek et al. focused on advanced age (10). In the center of these studies, oxidative stress was demonstrated as a causative agent of sperm DNA damage (11-13).

Independent from the etiological factor, the ratio of DNA damage might correlate with various problems of fertility such as the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth rate of the offspring. As the ratio of spermatozoa with damaged DNA increases (>30–40%), the likelihood of natural pregnancy drops (5-7).

Sperm DNA of poor quality have been shown to impair fertilization by in vitro studies. There is an observation that when the ratio of sperm with damaged DNA is high in patients receiving in vitro fertilization (IVF), the fertilization rates drop from 58% to 38% ($p<0.05$) (14). 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 (15,16).

The importance of sperm DNA damage should be considered as an etiological factor when evaluating infertile couples. The ratio of DNA damage might correlate with varying ratios of fertility failure rates resulting from the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth rate of the offspring. As a result, there should be a high association between sperm genetic damage and infertility. Therefore, it is essential to know the ratio of spermatozoa to damaged DNA in the ejaculate to predict the fertilization rate and determine the risks that may affect the embryo. Since early pregnancy loss may occur with an increase in the ratio of sperm DNA damage, couples being evaluated for recurrent pregnancy loss should undergo sperm DNA damage testing. Sperm DNA damage might have an adverse effect on achieving successful pregnancies following assisted reproduction treatment, so this test might be added to checklists for couples to consider before starting infertility therapy, and for those suffering from recurrent implantation failure.

Acknowledgement

I would like to thank to Duzen Laboratory Istanbul Kadikoy, Istanbul Mecidiyekoy, and Ankara Tunus staff for all their kind help and friendship.

Conflict of Interest

The author declares no conflict of interest.

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