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Fragmentation and Immunohistochemical Evaluation of Sperm Cells of Smoking Normospermic Infertility Individuals

Fragmentación del ADN y Evaluación Inmunohistoquímica de Espermatozoides de Individuos Fumadores con Infertilidad Normospérmica

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SUMMARY: Smoking is a significant environmental factor contributing to male infertility. This study aims to evaluate the effects of smoking on reproductive health by analyzing DNA fragmentation levels and immunohistochemical changes in sperm cells of normospermic infertile males who smoke. A total of 40 participants were included in the study. The study group consisted of normospermic infertile men who smoked, while the control group included non-smokers. Semen samples were analyzed for sperm count, motility, morphology, and vitality. DNA fragmentation levels were measured using acridine orange staining, and Caspase-3 levels were assessed through immunohistochemical analyses. The control group exhibited normal sperm parameters. In contrast, the DNA fragmentation rates in the smoking group were significantly higher, indicating compromised genetic integrity of sperm cells. Negative effects on sperm motility and morphology were also observed. Smoking significantly disrupts sperm DNA integrity, contributing to male infertility. These findings confirm the detrimental impact of smoking on male reproductive health and emphasize the importance of smoking cessation as a critical component of infertility treatment. Health policies should focus on strategies to reduce smoking prevalence.

KEY WORDS: DNA fragmentation; Sperm; Immunohistochemistry; Cigarettes.

INTRODUCTION

Approximately 20 % of infertility cases are solely attributed to male factors. When considering cases involving both male and female factors, this rate increases to 30–40 %. Male fertility evaluation is typically performed through semen analysis. However, approximately 15 % of infertile men present with normal semen analysis results, yet a definitive diagnosis cannot be established. This highlights the need for novel methods that can more accurately assess fertility and predict pregnancy outcomes. Recently, sperm DNA integrity has emerged as a prominent focus in this regard (Liu *et al*., 2024).

Studies conducted in recent years emphasize the significance of sperm nuclear DNA integrity in male infertility. These studies suggest that sperm DNA integrity may serve as a stronger biomarker for evaluating and predicting male infertility compared to routine semen analysis. Moreover, sperm apoptosis (programmed cell death) and DNA damage in sperm are considered potentially valuable biological markers for male infertility (Gil Juliá *et al*., 2021; Bisconti *et al*., 2021; Kaneko & Okada, 2024).
Smoking is one of the most prevalent social habits worldwide, carrying significant health risks. It is known to be more common among men than women. Cigarette smoke contains numerous toxic chemicals, mutagens, and carcinogens, adversely affecting both active smokers and passive smokers. These effects result in severe outcomes such as cancer, cardiovascular diseases, chronic respiratory diseases, and reproductive health problems. Additionally, the adverse effects of smoking on male reproductive health are evident through decreased sperm quality, DNA damage, and an increased risk of infertility (Pavuluri *et al*., 2024).

The negative impacts of smoking and tobacco products on the male reproductive system, particularly in terms of infertility, are a major concern. Various studies have demonstrated significant associations between smoking habits, including chewing tobacco and cigarette smoking,

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and semen parameters. These studies indicate that smoking can negatively affect sperm quality. For instance, a study on infertile Turkish men revealed a significant increase in sperm tail defects among individuals who smoked 20 or more cigarettes per day. Interestingly, higher progressive sperm motility was observed in men smoking more than 20 cigarettes daily compared to light smokers (Ozgur *et al*., 2005). This finding suggests that the effects of smoking on sperm function may be complex and influenced not only by the amount of consumption but also by individual differences and other environmental factors.

Prospective studies and larger sample sizes are needed to better understand the effects of smoking on male reproductive health. Such research could illuminate the mechanisms of damage caused by smoking and contribute to the development of strategies to prevent these effects. Studies have shown that leukocytes play a significant role in the male reproductive system, but the exact functions of seminal leukocytes remain unclear. Current research suggests that spermatozoon apoptosis is not always directly correlated with semen quality. In the absence of infections in the urogenital tract, one primary function of seminal leukocytes might be to eliminate apoptotic spermatozoa (Kaneko & Okada, 2024). This finding points to the potential regulatory role of seminal leukocytes in the reproductive system and underscores the need for further research to better understand these mechanisms.

Paternal smoking habits have been associated with a significant increase in the proportion of spermatozoa with DNA damage, which is linked to an elevated risk of childhood cancers and congenital anomalies. However, a limited number of studies have reported no clear relationship between sperm nuclear DNA damage and sperm function or quality (Albeitawi *et al*., 2024; Pavuluri *et al*., 2024). These conflicting findings suggest that the effects of smoking on sperm may be linked to complex mechanisms influenced by environmental, genetic, and individual factors. Further research is needed to clarify the precise impact of smoking on reproductive health and to fill knowledge gaps in this area.

Although the effects of smoking on male infertility are not yet fully established, smoking is considered a plausible risk factor for infertility. Evidence demonstrating the adverse effects of smoking on semen parameters supports this relationship. It is particularly important for both partners to quit smoking in cases where borderline or abnormal semen parameters are detected, as well as in couples with a history of recurrent pregnancy loss or infertility. Smoking cessation represents a simple yet effective intervention with the potential to improve reproductive health, and it is essential for healthcare professionals to provide counseling on this matter (Colagar *et al*., 2009).

Active smoking has been positively correlated with sperm DNA fragmentation, axonemal damage, and reduced sperm count. Smokers have been found to exhibit higher susceptibility to acid-induced DNA denaturation compared to non-smokers. This indicates higher levels of DNA strand breaks in the sperm of smokers (Mottola *et al*., 2024). These findings emphasize the detrimental effects of smoking on sperm structure and genetic integrity, highlighting the importance of further research to better understand the adverse effects of smoking on reproductive health.

High levels of reactive oxygen species (ROS) in seminal plasma have been associated with poor sperm morphology, reduced motility, and decreased sperm count (Elnashar *et al*., 2025). This finding suggests that ROS may contribute to infertility by adversely affecting sperm quality and underscores the potential harms of elevated ROS levels on reproductive health. DNA damage caused by ROS accelerates apoptosis processes in cells, directly correlating with a decrease in the number of viable spermatozoa and negatively impacting reproductive health (Pavuluri *et al*., 2024). ROS-induced DNA damage in sperm disrupts sperm function, reducing fertilization capacity.

In this study, we aim to examine sperm samples from infertile normospermic individuals who smoke using DNA fragmentation and immunohistochemical techniques. By investigating the potential effects of smoking on sperm DNA integrity and assessing changes in sperm quality, this research seeks to contribute to our understanding of the long-term impacts of smoking on reproductive health. The findings may aid in developing new strategies for infertility treatment.

MATERIAL AND METHOD

Patients. This study was conducted on semen samples obtained from a total of 40 infertile men who applied to the Urology Clinic at Dicle University Faculty of Medicine Hospital. Ethical approval for the study was obtained from the Non-Invasive Clinical Research Ethics Committee of Dicle University Faculty of Medicine (approval number 2024/257, dated September 25, 2024). Participants were divided into two groups: the first group consisted of 20 non-smokers, while the second group included 20 individuals who smoked at least five cigarettes per day. The individuals included in the study were selected based on the infertility criteria defined by the World Health Organization and had no history of chronic diseases such as diabetes, hypertension, or rheumatic diseases. Additionally, individuals with a history of urological surgery, genital infections, or cryptorchidism

were excluded. Semen samples from individuals diagnosed with azoospermia after microscopic analysis were excluded from the study. These selection criteria aimed to provide a more reliable assessment of the effects of smoking on semen parameters.

Semen collection and analysis. Semen samples were collected from patients who visited the clinic for spermiogram testing after 2–7 days of sexual abstinence. Samples were collected in sterile plastic containers labeled with the participants' names, surnames, and ages, using the masturbation method. The date and time of sample collection were recorded, and the samples were allowed to liquefy at room temperature for approximately 30 minutes.

For each sample, the duration of sexual abstinence, viscosity, volume, color, and liquefaction time were determined and recorded. Sperm count was evaluated using a Makler counting chamber. A small drop of the semen sample was placed on the chamber, and the total sperm count, progressively motile, immotile sperm count, and motility were assessed under a light microscope at 20x magnification.

Sperm staining and morphological evaluation. During morphological analysis, 100 spermatozoa per slide were evaluated, and all observed anomalies were recorded. This method enabled detailed analysis of spermatozoa morphology.

A drop of the semen sample was placed on a slide, and the amount was adjusted according to spermatozoa concentration. The sample was spread evenly using another slide and air-dried. Preparations were stained using the Spermac staining method (Ferti Pro NV, Industriepark Noord, Belgium). Slides were fixed in a fixative solution for 10 minutes, rinsed with water, and the excess liquid was carefully removed. Staining was performed using solutions A and B for 1.5 min each, followed by solution C for 30 s. The prepared slides were air-dried and examined under a light microscope at 100x magnification using immersion oil.

Swim-up method. Normozoospermic semen samples were evaluated using a Makler counting chamber and then transferred to 15 mL round-bottom Falcon tubes. Two milliliters of sperm preparation medium (G-IVF™ PLUS) were added to each tube. The tubes were incubated at 37 °C in a Heraeus Heracell 240 incubator with 5 % CO_2 and 95 % humidity at a 4° angle for 30–60 min.

After incubation, the medium at the top of the tubes was carefully aspirated without disturbing the lower semen layer. Five milliliters of sperm wash medium were added to the tubes, mixed thoroughly, and centrifuged at 300–600 g. The supernatant was carefully removed after centrifugation, ensuring optimal separation of cells during the sperm preparation process.

Eosin Y application protocol. The Eosin Y staining method was used for viability assessment. A 5 g/L Eosin Y solution was prepared in a 9 g/L aqueous sodium chloride solution. A drop of fresh semen sample was mixed with a drop of Eosin Y solution on a slide. The mixture was incubated for 30 s and examined under a light microscope at 40x magnification.

During evaluation, live cells remained unstained, while dead cells appeared red. For analysis, 100 spermatozoa were assessed for each sample, and the viability rate was determined. This method enabled accurate and reliable assessment of spermatozoa viability.

Evaluation of sperm count and motility. Standard manual techniques described in the World Health Organization's 2010 Laboratory Manual (Jellad *et al*., 2021) were applied to evaluate sperm count and motility. For analysis, 10 µL of semen sample was placed on a Makler counting chamber and examined under a light microscope with a 20x magnification.

During assessment, spermatozoa were categorized into three groups based on motility: rapidly progressive, slowly progressive, and immotile. Sperm concentration, motility, and morphology were determined using standard manual techniques. A minimum of 100 spermatozoa were counted for each semen sample, and analyses were conducted under 20x magnification in compliance with WHO criteria.

Acridine orange staining. Acridine orange is a fluorescent dye that binds to denatured regions of DNA under acidic conditions. It emits green fluorescence when bound to normal DNA and orange-red fluorescence when bound to denatured DNA.

Smear preparations were prepared by washing semen samples with special solutions to remove plasma, dead sperm, and cellular debris. Twenty microliters of the sample were spread on slides, fixed in Carnoy solution for approximately one hour, air-dried, and stained with acridine orange in a dark environment for 5 minutes. Slides were washed with distilled water and examined under a fluorescence microscope at 450–490 nm.

Spermatozoa emitting green fluorescence were considered normal, while those emitting orange-red fluorescence were considered to have DNA damage. An average of 100 spermatozoa were examined, and the percentage of DNA fragmentation was calculated (Bibi *et al*., 2022).

Caspase-3 immunostaining. Immunohistochemical staining was used to determine protein localization and density at the cellular level. Slides prepared from samples in Nunc 4-well culture chambers were rehydrated by washing with phosphate-buffered saline (PBS). Non-specific binding was blocked using a histostain kit containing non-immune serum for 20 min.

A primary antibody specific to Caspase-3 (catalog no: sc-7272, Santa Cruz Biotechnology, US) was applied to the slides, which were then sealed with stretch film and incubated overnight at 4 °C. The slides were washed with PBS three times for 5 min each to remove unbound antibodies.

Biotin-conjugated secondary antibodies were applied for 20 minutes, followed by horseradish peroxidase (HRP)-conjugated streptavidin. Diaminobenzidine (DAB) chromogen was used for 25 min in a dark environment, and slides were counterstained with hematoxylin. Prepared slides were washed, cleaned with alcohol, labeled with the date and group information, and mounted with a cover slip. Positive reactions were observed under a light microscope, and the proportion of positive sperm cells was calculated. Preparations were photographed to visualize protein expression.

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics software (version 25). Data distribution was assessed using the Shapiro-Wilk test. Statistical significance between two groups was determined using the Mann-Whitney U test. A p-value of <0.005 was considered statistically significant.

RESULTS

Demographic age findings of patients. The mean age ± standard deviation of patients presenting with infertility complaints is shown in Table I. The mean age of non-smoking patients was 29.3 ± 5.14 years, while it was 29.6 ± 5.83 years in the smoking group. No significant effect of smoking on age was observed (P = 0.9304) (Table I).

Duration of infertility. The mean duration of infertility ± standard deviation of patients presenting with infertility complaints is shown in Table II. The duration of infertility was 2.3 ± 0.72 years in the non-smoking group and 3.3 ± 1.3 years in the smoking group. A statistically significant difference was observed between the two groups in terms of infertility duration (P = 0.0080). Smoking was found to increase the duration of infertility. (Table II).

Sperm parameters findings

Semen volume. The mean semen volumes ± standard deviation of patients presenting with infertility complaints are shown in Table III. Semen volumes were found to be lower in the smoking group compared to the non-smoking group (1.7 ± 0.67 mL vs. 2.9 ± 0.81 mL). This difference between the non-smoking and smoking groups was statistically significant, indicating that smoking reduces semen volume (P < 0.0001).

Concentration findings. Sperm concentrations (million/mL) in the non-smoking group were higher compared to the smoking group (72.84 ± 48.9 vs. 69.08 ± 43.2). However, statistical analysis revealed no significant difference in sperm concentration between the two groups (P > 0.05) (Table IV). This result suggests that the impact of smoking on reduced sperm concentration is not pronounced.

Table I. Average age of patients.

Parameter	*Non-smoker (n= 20)*	*Smoker (n= 20)*	*P value**
Age	*29,3±5,14*	*29,6±5,83*	*0.9304*

* Mann Whitney U test.

Table II. Average infertility duration of patients.

Parameter	*Non-smoker (n= 20)*	*Smoker (n= 20)*	*P value**
Duration of infertility (years)	*2,3±0,72*	*3,3±1,3*	*0.0080*

* Mann Whitney U test.

Table III. Average semen volumes of patients.

Parameter	*Non-smoker (n=20)*	*Smoker (n=20)*	*P value**
Semen volume (mL)	*2,9±0,81*	*1,7±0,67*	*<0.0001*

* Mann Whitney U test.

Table IV. Mean semen concentrations of patients.

Parameter	*Non-smoker (n=20)*	*Smoker (n=20)*	*P value**
Sperm concentration (million/mL)	*72±3,8*	*68±5,1*	*P= 0.0064*

* Mann Whitney U test.

Sperm motility results. The motility rates were 84 % (±5.1) in the non-smoking group and 74 % (±3.4) in the smoking group (Table V). The difference in sperm motility between the groups was statistically significant, indicating that smoking reduces sperm motility (P < 0.0001).

Sperm (Kruger) morphology results. The mean ± standard deviation of sperm (Kruger) morphology in patients presenting with infertility complaints is provided in Table VI. The mean morphology was 14 % ± 3.8 in the non-smoking group and 12 % ± 2.9 in the smoking group. The negative effect of smoking on sperm morphology was found to be statistically significant (P = 0.0057).

Table V. Average sperm motility of patients.

Parameter	*Non-smoker (n=20)*	*Smoker (n=20)*	*P value**
Sperm motility rates (%)	*84±5,1*	*74±3,4*	*<0.0001*

* Mann Whitney U test.

Table VI. Mean sperm Kruger morphology of patients.

Parameter	*Non-smoker (n=20)*	*Smoker (n=20)*	*P value**
Sperm morphology (%)	*14±3,8*	*12±2,9*	*0.0057*

* Mann Whitney U test.

Histopathological staining findings

Non-smoking group. Figure 1 illustrates the results of sperm staining conducted on samples from non-smoking individuals. According to the staining findings, sperm cells exhibit more uniform morphology and less damage. Since individuals in this group are healthy normospermic patients, sperm defects are observed at minimal levels in our findings.

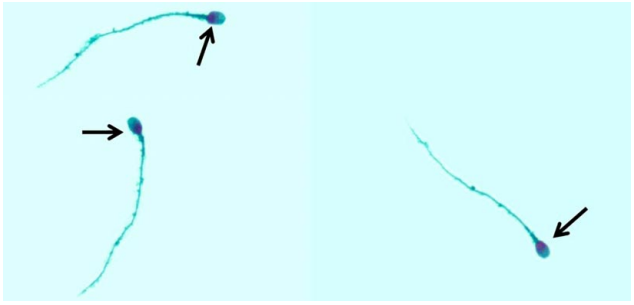

Fig. 1. Sperm morphology of non-smoking individuals. Arrow: sperm with normal morphology, Spermac Staining, Bar: 10 µm

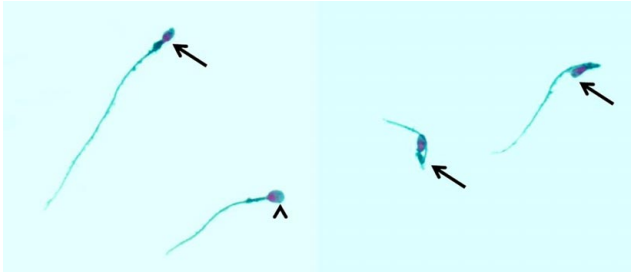

Fig. 2. Sperm morphology of the smoking group. Arrow: pinhead sperm with neck defect, arrowhead: globozoospermia, Spermac Staining, Bar: 10 µm

Smoking group. The sperm morphology of smoking individuals after morphological staining is shown in Figure 2. In sperm samples from smokers, abnormalities in sperm head shape, tail deformities, and abnormal acrosome structures are distinctly observed. The anomalies in the head, tail, and acrosome of the sperm indicate the adverse effects of smoking on sperm quality (Fig. 2).

Vitality findings

Non-smoking group. The staining showing the sperm vitality of non-smoking infertile individuals is presented in Figure 3. It was observed that most of the sperm were regular and viable, with only a few sperm being dead. The findings are consistent with those of a healthy normospermic patient.

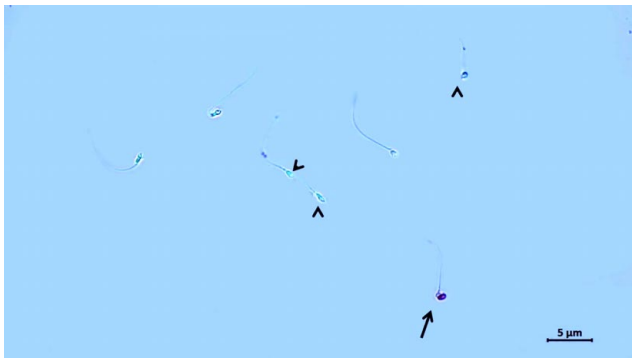

Fig. 3. Image showing sperm sample of non-smoking individuals. Arrow: dead sperm (red), arrowhead: live sperm (transparent), Eosin Y staining, phase contrast microscope, Bar: 5 µm

Another section showing the sperm vitality of non-smoking infertile individuals is shown in Figure 4. It was noted that most of the sperms were transparent and regular in the cros sectional area, while a few sperms were red and non-living. Our findings were parallel to the findings of the normospermic healthy person.

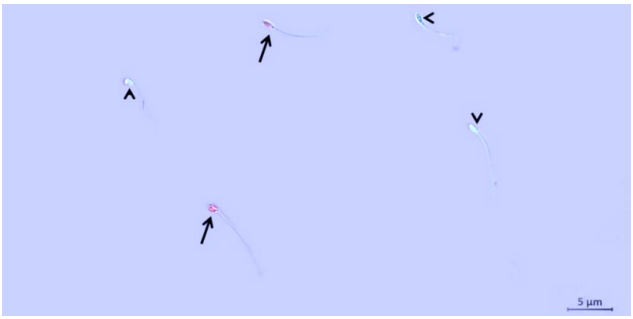

Fig. 4. Image showing sperm sample of non-smoking individuals. Arrow: dead sperm (red), arrowhead: live sperm (transparent), Eosin Y staining, phase contrast microscope, Bar: 5 µm

Smoking group. The staining showing the sperm vitality of infertile individuals who smoke is shown in Figure 5. It was noted that most of the sperm were red and dead. A few live sperm were found. According to our findings, smoking decreased sperm vitality.

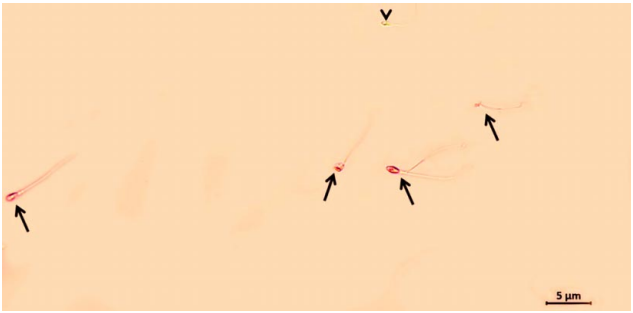

Fig. 5. Image showing the sperm sample of individuals who smoke. Arrow: dead sperm (red), arrowhead: live sperm (transparent), Eosin Y staining, phase contrast microscope, Bar: 5 µm

Another section showing the sperm vitality of infertile individuals who smoke is shown in Figure 6. It was noted that most of the sperm in the cross-sectional area were dead, and the live sperms with a transparent appearance were morphologically defective. According to our findings, it was observed that smoking reduces sperm vitality.

Acridine orange findings

Non-smoking group. In the findings of non-smoking infertile patients, it was observed that most of the sperms did not have DNA damage in terms of DNA fragmentation (Fig. 7). In the sperm samples examined, it was observed that the sperms in the area were alive and their DNA preserved its integrity.

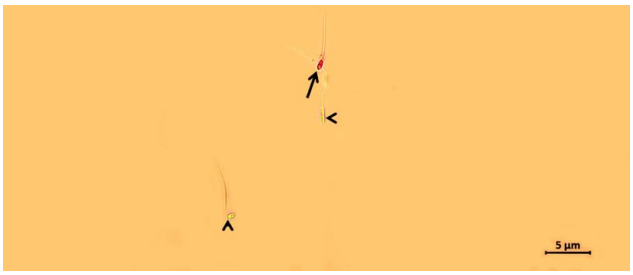

Fig. 6. Image showing sperm sample of non-smoking individuals. Arrow: dead sperm (red), arrowhead: live sperm (transparent), Eosin Y staining, phase contrast microscope, Bar: 5 µm

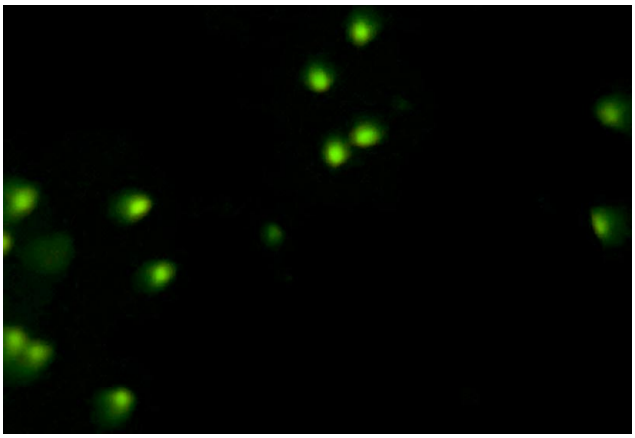

Fig. 7. Sperm DNA fragmentation staining of non-smoking patients. green color: DNA intact sperm, Acridine orange staining, Fluorescence microscope, Bar: 5 µm

Smoking group. In the findings of infertile patients who smoked, DNA damage was observed in most of the sperm in terms of DNA fragmentation (Fig. 8). In the sperm samples examined, it was noted that there was a loss of integrity in the DNA of the sperm in the area and the density of sperm with DNA defects. According to our findings, we observed that smoking increased DNA fragmentation.

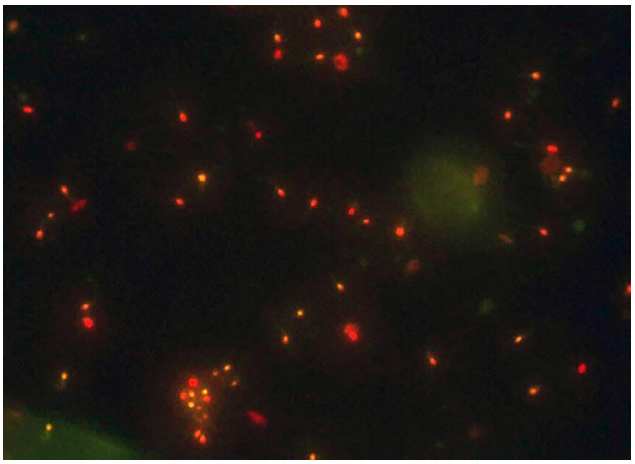

Fig. 8. Sperm DNA fragmentation staining of patients who smoke. Red color: sperm with damaged DNA, green color: sperm with intact DNA, Acridine orange staining, Fluorescence microscope, Bar: 5 µm

Caspase-3 immunostaining findings

Non-smoking group. When the sections belonging to non-smoking infertile patients were examined, it was observed that the sperms were weak in terms of Caspase-3 expression and did not undergo the apoptotic process (Fig. 9). Negative Caspase-3 expression was quite evident in the head, body and tail sections of the sperms. Immunohistochemically, it was observed that the sperms did not undergo DNA fragmentation.

Fig. 9. Caspase-3 immunostaining of sperm from smoking patients. Arrow: negative reaction; Caspase-3 immunostaining, Light microscope, Bar: 10 μm

Smoking group. In the findings of infertile patients who smoked, it was observed that Caspase-3 expression was intense in most of the sperm in terms of DNA fragmentation (Fig. 10). It can be said that Caspase-3 activity was especially high in the head region of the sperm and apoptotic processes were intense. According to our findings, it was observed that smoking induced apoptosis in normospermic patients and this situation was evident with Caspase-3 expression positivity.

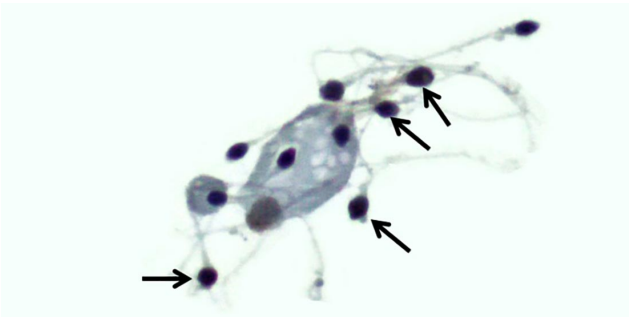

Fig. 10. Sperm Caspase-3 immunostaining of smoking patients. Arrow: positive reaction, Caspase-3 immunostaining, Light microscope, Bar: 10 μm

DISCUSSION

The findings of this study provide critical insights into the detrimental effects of smoking on sperm DNA integrity and the expression of key proteins within sperm cells of normospermic infertile individuals. Despite normal semen parameters, the elevated levels of DNA fragmentation observed in smokers highlight a potential mechanism underlying their infertility, emphasizing the inadequacy of conventional semen analysis in capturing subtle but significant molecular abnormalities. Immunohistochemical evaluation further revealed alterations in protein expression profiles associated with oxidative stress and apoptotic pathways, suggesting that smoking-induced oxidative damage may play a pivotal role in impairing sperm functionality. These results underscore the importance of incorporating advanced molecular and immunohistochemical assessments in the evaluation of idiopathic infertility cases, particularly among smokers.

In infertile couples, cases where male factor alone is the primary cause are observed in approximately 20 % of the cases, while situations where both male and female factors are involved increase this percentage to 30-40 %. A review of the literature reveals ongoing debates regarding whether cigarette smoking can establish a causal relationship with impaired reproductive function (Costa *et al*., 2023). However, a consensus has not yet been reached regarding the effects of tobacco on male infertility. Although mechanisms such as impaired spermatogenesis, the emergence of ultrastructural abnormalities, and apoptosis are suggested to be associated with smoking, the exact mechanisms and their significance are still being investigated (Verón *et al*., 2018).

In a large-scale study conducted on a total of 1104 infertile men, including 478 smokers, no significant difference was found between smokers and non-smokers regarding traditional semen parameters (sperm concentration, motility, and morphology) (Aryanpur *et al*., 2011). In another study, although lower semen parameters (morphology, motility, and concentration) were observed in smokers, it was noted that there was no statistically significant difference between the groups (Gruhl *et al*., 2023). Similarly, in our study, no significant difference was found between smokers and non-smokers in terms of sperm concentration. However, negative effects such as reduced sperm motility, decreased volume, and impaired morphology were observed in smokers. These findings reveal the harmful effects of smoking on various sperm quality parameters.

In a study by Collodel *et al*. (2010), smokers were divided into three groups based on the number of cigarettes smoked per day (light, moderate, and heavy smokers), and the effects on semen quality were evaluated. When compared to non-smokers, light smokers, and heavy smokers, only sperm concentration and fertility index (FI) were reported to have significantly decreased (Costa *et al*., 2023). In another study, no significant difference was found between smokers and non-smokers regarding semen volume (Chua *et al*., 2023).

Other studies have also reported no significant differences in sperm parameters between smokers and non-

smokers (Verón *et al*., 2018; Khan *et al*., 2023; Henriques *et al*., 2023; Albeitawi *et al*., 2024; Williamson *et al*., 2024). In our study, no statistically significant difference was found between smokers and non-smokers in terms of sperm concentration. However, negative effects such as reduced sperm motility, decreased volume, and impaired morphology were observed in smokers. Future studies with larger sample sizes are expected to better clarify the effects of smoking on semen parameters.

Some other studies have shown that smoking adversely affects sperm parameters. It has been reported that impairments in basic semen parameters, such as sperm concentration, motility, and morphology, occur. However, there is no consensus on the biological mechanisms through which these negative effects occur. Smoking has been suggested to impact sperm function through various biological processes, such as oxidative stress, DNA damage, and cellular-level changes, but the exact pathways and mechanisms are still under investigation (Colagar *et al*., 2009; Chua *et al*., 2023). This uncertainty underscores the need for more comprehensive studies to better understand the biological mechanisms behind smoking's effects on reproductive health.

In a study by Colagar *et al*. (2009), it was reported that smoking had a negative effect on sperm count, motility, and morphology. One of these negative effects was the generation of reactive oxygen species (ROS) caused by seminal oxidative stress, which has a destructive impact on sperm quality and function. Smokers were found to have higher oxidative stress in their semen compared to non-smokers. Several studies have shown that smoking triggers a series of mechanisms that increase seminal oxidative stress (Aydos *et al*., 2021).

Similarly, in another study, it was reported that smoking and alcohol consumption led to significant changes in sperm morphology, causing harmful effects on semen volume and sperm concentration (Zhang *et al*., 2024). In a study by Ramlau-Hansen *et al*. (2007), which involved 2542 healthy men between 1987 and 2004, it was reported that the amount of smoking showed a significant and dose-dependent relationship with semen characteristics. In smokers, significant reductions in semen volume, sperm concentration, total sperm count, and motile sperm percentage were observed. Especially in men who smoked more than twenty cigarettes a day, sperm concentration was found to be 19 % lower and total sperm count was 29 % lower than non-smokers, considering factors such as age, sexual abstinence period, and other factors affecting semen quality. In our study, no statistically significant difference was found between smokers and non-smokers in terms of sperm concentration ($p>0.05$). However, the literature suggests that the effects of smoking on sperm parameters may become more evident based on the amount of cigarettes smoked daily. Various studies have shown that smoking affects semen parameters to varying degrees, and these effects increase proportionally with the amount of smoking. Therefore, the insufficient classification of smoking quantity in our study may have prevented the exact manifestation of these effects. The literature emphasizes the need for more detailed grouping to better understand the effects of smoking on sperm quality. Specifically, groupings based on the number of cigarettes smoked per day make changes in semen parameters more evident. This situation highlights the importance of classifying the sample group in more detail based on the amount of smoking to more accurately evaluate the effects of smoking on sperm parameters. Such detailed classifications may help us better understand the effects of smoking on sperm parameters and improve the accuracy of research in this area.

Many studies have reported that smoking leads to poor sperm quality by creating negative effects on sperm concentration, motility, and morphology (Aziz *et al*., 2007; Fan *et al*., 2024; Wang *et al*., 2024). Other studies have found significant decreases in semen parameters, higher DNA fragmentation percentages, and cotinine levels in infertile smokers. These findings showed significant differences between light smokers and moderate or heavy smokers, and a negative relationship was also identified between DNA fragmentation percentage and semen parameters. Smoking is known to affect the hypothalamic-pituitary-gonadal axis by disrupting testicular microcirculation (Bisconti *et al*., 2021; Pavuluri *et al*., 2024). Additionally, the toxic effects of various chemical components in cigarettes are more pronounced on the germinative epithelium in smokers. It has been suggested that oxidants in cigarettes cause oxidative DNA damage in sperm, leading to higher levels of oxidative DNA damage in the sperm of smokers (Sergerie *et al*., 2005). Similar findings were obtained in our study. In infertile smokers, significant DNA damage was observed in most sperm with respect to DNA fragmentation (Fig. 8). In the examined samples, it was found that sperm lost their DNA integrity and that the density of sperm with DNA defects increased. Our findings suggest that smoking increases DNA fragmentation in sperm. These results support the idea that smoking leads to oxidative DNA damage in sperm. Furthermore, they confirm the literature findings that smoking exposure causes a decrease in semen parameters and has toxic effects on the germinative epithelium.

In a study by Aziz *et al*. (2007), it was found that individuals carrying spermatozoa with DNA fragmentation had significantly lower normal sperm

morphology and spermatozoa deformity index (SDI) values compared to the population without DNA fragmentation. While there is no consensus on the importance of DNA fragmentation in male infertility, it has been emphasized that the mechanisms behind its occurrence should be investigated in more detail (Aziz *et al*., 2007). In another study, a negative relationship between DNA damage and semen quality was reported. Schmid *et al*. (2007) in their study of 80 healthy non-smokers, found no statistically significant difference between DNA damage and traditional semen parameters. These findings suggest that the effects of smoking on sperm quality and DNA integrity have a complex relationship and require further investigation. Among the factors influencing infertility, smoking is emerging as an important toxic agent. However, since most of the studies have been conducted on individuals diagnosed with infertility, this limits the ability to clearly separate the effects of smoking. To more deeply explore the potential effects of smoking on semen, long-term studies on the general population are needed. Such studies could provide clearer data regarding secondary infertility or potential changes in semen parameters due to smoking.

Tesarik *et al*. (2002) study examined the critical role of paternal genetic and epigenetic effects on preimplantation development in embryos fertilized by ICSI. It has been shown that sperm DNA damage and quality issues adversely affect embryo development and implantation success. The study emphasizes the importance of paternal factors in early embryo development (Tesarik *et al*., 2002). In other studies, no clinically significant difference was observed between groups consuming alcohol and tobacco and those not consuming alcohol and tobacco regarding age and other semen parameters (Schmid *et al*., 2007). However, current scientific evidence reveals a significant decrease in sperm volume, normal sperm percentage, and motility with increasing age. Degenerative changes caused by aging in the germinal epithelium can adversely affect spermatogenesis and cause impairments in sperm morphology. While a significant change in sperm concentration is not observed with age, age-related changes can affect spermatogenesis. With increasing age, narrowing in the tubular lumen, degeneration of germ cells, reduced spermatogenic activity, and a decrease in the number of Leydig cells can be observed. However, sperm concentration may increase with age, which can be explained by the acceleration of spermatogenesis. Endocrinological disruptions may be one of the reasons for this increase. Age can affect not only sperm quality but also sperm DNA integrity (Lahimer *et al*., 2023).

CONCLUSION

This study has shown that sperm DNA integrity is impaired in normospermic infertile individuals who smoke and its negative effects on male reproductive health. Smoking has impaired genetic integrity by increasing sperm DNA fragmentation and has negative effects on sperm motility and morphology. Clinically, increases in DNA fragmentation and deteriorations in sperm parameters are important biological markers that should be considered in the diagnosis and treatment of male infertility. DNA fragmentation testing can be guiding in the evaluation of individuals with unexplained infertility, recurrent pregnancy losses and assisted reproductive technology failures. Since our study found that cigarette consumption has a significant effect on DNA damage, it is recommended that smoking history be questioned in detail in men receiving infertility treatment.

These findings contribute to our understanding of the complex effects of smoking on male infertility and enable the development of preventive and therapeutic strategies to improve reproductive health.

KARABAT, M.U.; AKKUS, M. & TUNCER, M.C. Fragmentación del ADN y evaluación inmunohistoquímica de espermatozoides de individuos fumadores con infertilidad normospérmica. *Int. J. Morphol., 43(3)*:722-731, 2025.

RESUMEN: El tabaquismo es un factor ambiental significativo que contribuye a la infertilidad masculina. Este estudio tiene como objetivo evaluar los efectos del tabaquismo en la salud reproductiva mediante el análisis de los niveles de fragmentación del ADN y los cambios inmunohistoquímicos en espermatozoides de hombres fumadores con infertilidad normospérmica. Se incluyó un total de 40 participantes. El grupo de estudio consistió en hombres fumadores con infertilidad normospérmica, mientras que el grupo control incluyó a no fumadores. Se analizaron muestras de semen para determinar el recuento, la motilidad, la morfología y la vitalidad de los espermatozoides. Los niveles de fragmentación del ADN se midieron mediante tinción con naranja de acridina y los niveles de caspasa-3 se evaluaron mediante análisis inmunohistoquímicos. El grupo control presentó parámetros espermáticos normales. Por el contrario, las tasas de fragmentación del ADN en el grupo fumador fueron significativamente mayores, lo que indica una integridad genética comprometida de los espermatozoides. También se observaron efectos negativos sobre la motilidad y la morfología de los espermatozoides. El tabaquismo altera significativamente la integridad del ADN espermático, lo que contribuye a la infertilidad masculina. Estos hallazgos confirman el impacto perjudicial del tabaquismo en la salud reproductiva masculina y enfatizan la importancia de dejar de fumar como componente crítico del tratamiento de la infertilidad. Las políticas sanitarias deben centrarse en estrategias para reducir la prevalencia del tabaquismo.

PALABRAS CLAVE: Fragmentación del ADN; Espermatozoides; Inmunohistoquímica; Cigarrillos.

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