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Cigarette Smoking Disrupts Sperm Nuclear Maturation, Epigenetic Regulation, and CatSper-Mediated Calcium Signaling

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ABSTRACT

OBJECTIVE: The CatSper (cation channel of sperm) channel is the principal pH-sensitive and low-voltage-activated cation channel that regulates Ca^{2+} influx required for sperm hyperactivation. Exposure to cigarette smoke is known to affect semen parameters and sperm DNA integrity adversely. This study aimed to investigate the effects of cigarette smoking on semen analysis results, sperm DNA fragmentation, chromatin condensation, global DNA methylation, and CatSper4 immunoreactivity.

STUDY DESIGN: In this case-control study, 50 men who presented for infertility evaluation between November 2019 and November 2021 were divided into two groups: non-smokers (n=25) and those who smoked approximately one pack of cigarettes per day (n=25). Twenty of these cases were normospermic and 30 oligospermic. Semen analysis was performed according to the World Health Organization 2010 criteria. Sperm DNA fragmentation was assessed by acridine orange staining, chromatin condensation by aniline blue staining, global DNA methylation by 5-methylcytosine (5-mC) ELISA assay, and CatSper4 expression by immunofluorescence staining.

RESULTS: In normospermic men, no statistically significant smoking-related differences were observed in conventional semen parameters, DNA fragmentation, chromatin condensation, or CatSper4 positivity. In oligospermic men, smoking was associated with significantly higher sperm DNA fragmentation and significantly lower chromatin condensation and CatSper4 positivity. In contrast, sperm concentration, motility, and normal morphology did not differ significantly between smokers and non-smokers. Global DNA methylation levels were significantly higher in smokers than in non-smokers (1.81±0.52 vs. 1.11±0.35, respectively; p=0.0012).

CONCLUSION: Cigarette smoking may impair male fertility potential primarily through alterations in sperm DNA integrity, chromatin maturation, global DNA methylation, and CatSper4-related calcium signaling, particularly in oligospermic men. Larger-scale studies using individual-level datasets are required to confirm these findings and clarify their clinical significance.

Keywords: CatSper4; Chromatin condensation; Cigarette smoking; Global DNA methylation; Semen analysis; Sperm DNA fragmentation

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Introduction

Infertility is a major public health concern worldwide, affecting both women and men (1). Male fertility depends on multiple interrelated processes, including spermatogenesis, sperm nuclear integrity, and molecular pathways that regulate sperm function (2). In addition to genetic factors, accumulating evidence indicates that environmental exposures and lifestyle choices play a critical role in male reproductive health.

Tobacco use remains highly prevalent worldwide. According to recent estimates by the World Health

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Organization, approximately 1.25 billion adults worldwide use tobacco products. Although the prevalence of tobacco use has declined since 2000, it continues to affect nearly one-fifth of the adult population (3). Cigarette smoke contains more than 7,000 chemical compounds, many of which possess toxic and carcinogenic properties (4).

Oxidative agents and toxic constituents in cigarette smoke have been shown to increase oxidative stress in seminal plasma, often accompanied by leukocytosis, ultimately disrupting sperm membrane integrity and causing DNA damage (5,6). A comprehensive meta-analysis demonstrated that cigarette smoking adversely affects semen volume, sperm concentration, motility, and morphology (7). Furthermore, smoking has been associated with increased leukocyte counts and reduced semen quality in infertile men (8).

Semen analysis is the cornerstone of male infertility assessment and remains the most widely used diagnostic tool for evaluating the male factor; however, its ability to accurately predict fertility potential is limited (9). Consequently, interest has grown in complementary biomarkers such as sperm DNA integrity, chromatin maturity, and epigenetic regulators to provide a more comprehensive evaluation of male reproductive potential.

Epigenetic modifications, including DNA methylation, histone modifications, and chromatin remodeling, play a fundamental role in regulating gene expression across cell divisions (10,11). DNA methylation predominantly occurs through the formation of 5-methylcytosine (5-mC) at CpG dinucleotides and is catalyzed by DNA methyltransferases (12). Previous studies have reported that cigarette smoking can alter sperm DNA methylation profiles and that global DNA methylation levels may be associated with semen quality parameters (13,14).

The CatSper (cation channel of sperm) channel is a pH-sensitive, calcium-selective ion channel that mediates Ca^{2+} influx into sperm cells and plays a pivotal role in sperm hyperactivation, capacitation, and fertilization by regulating characteristic motility patterns (15-17). Furthermore, its 4 subunits have been demonstrated in human sperm. Our previous unpublished thesis studies showed that CatSper 4 may be important for human sperm.

Despite extensive evidence demonstrating the detrimental effects of cigarette smoking on conventional semen parameters, the underlying molecular and epigenetic mechanisms contributing to smoking-associated male infertility remain incompletely understood. In particular, limited data are available regarding the combined effects of smoking on sperm nuclear integrity, global DNA methylation, and calcium signaling pathways mediated by the CatSper channel, especially in men with oligospermia. Moreover, most previous studies have focused on isolated biomarkers rather than adopting an integrative approach encompassing both functional and epigenetic sperm parameters.

Therefore, the present study was designed to comprehensively evaluate the impact of cigarette smoking on semen quality, sperm DNA fragmentation, chromatin condensation, global DNA methylation, and CatSper4 immunoreactivity in normospermic and oligospermic men. We hypothesized that cigarette smoking would be associated with increased sperm DNA damage, altered epigenetic regulation, and reduced CatSper4 channel expression, with more pronounced effects in oligospermic individuals. By integrating conventional semen analysis with advanced molecular and epigenetic assessments, this study aims to provide deeper insight into the mechanisms by which smoking impairs male reproductive potential.

Material and Method

Study Population and Sample Collection: This case-control study was conducted through a collaboration between the Andrology Research Laboratory of Biruni University, the Life Sciences Laboratory of Yeni Yuzyil University, and Biruni University BirTech Laboratories. The relevant institutional ethics committee approved the study, and all participants provided written informed consent before enrollment. The Biruni University Scientific Research Projects Unit (BAP) supported the study under project number BIRUNI-BAP-2019-02-21.

The study included 50 men who presented to the urology clinic for infertility evaluation between November 2019 and November 2021. Participants were classified into two main groups based on semen analysis results: a normospermic control group (n=20) and an oligospermic patient group (n=30). In addition, participants were categorized according to smoking status as non-smokers (Group I, n=25) or smokers consuming approximately one pack of cigarettes per day (Group II, n=25). Comparative analyses were performed within normospermic and oligospermic subgroups according to smoking status.

Exclusion criteria included body mass index ≥30 kg/m², presence of systemic disease, azoospermia, and age over 45 years. Semen samples were collected after 2-7 days of sexual abstinence and analyzed according to the World Health Organization (WHO) 2010 laboratory guidelines (18).

This study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki. Ethical approval was granted by the Non-Interventional Clinical Research Ethics Committee of Biruni University (Approval No: 2018/18-04, Date: 20.07.2018). We obtained written informed consent from all participants before enrollment. All procedures involving human participants were performed in accordance with institutional regulations and internationally accepted ethical standards.

Semen analysis: Conventional semen parameters, including

sperm concentration, motility, and morphology, were evaluated according to WHO 2010 criteria. Samples were allowed to liquefy at room temperature before analysis, and experienced laboratory personnel performed all assessments under standardized conditions. Sperm morphology was evaluated according to WHO 2010 strict criteria, including detailed assessment of the acrosome, head, midpiece, and tail regions (18).

Sperm preparation for all analyses: Sperm samples were processed using SpermGrad™ density-gradient layers. Following separation through the 45% and 90% gradients, spermatozoa were washed with standard HTF solution and collected as pellets. The resulting pellets were used to prepare sperm smears and samples for methylation analysis.

Assessment of Sperm DNA Fragmentation (Acridine Orange Staining): Sperm smears were prepared on glass slides and fixed in Carnoy's fixative at room temperature for 30 minutes, followed by washing with phosphate-buffered saline (PBS). Slides were then incubated with acridine orange staining solution for 5 minutes in the dark and rinsed with PBS subsequently. Preparations were examined under a fluorescence microscope using a 100× oil immersion objective (excitation wavelength: 450-490 nm).

We evaluated at least 100 spermatozoa per slide. Acridine orange emits green fluorescence when bound to intact double-stranded DNA and red-orange fluorescence when bound to denatured or fragmented single-stranded DNA. The percentage of spermatozoa exhibiting DNA fragmentation was calculated accordingly.

Evaluation of Chromatin Condensation (Aniline Blue Staining): Sperm smears were prepared by placing 20 µL of semen on glass slides. Samples were fixed in 2% glutaraldehyde for 45 minutes and subsequently stained with aniline blue for 15 minutes. After rinsing with distilled water, slides were examined under a light microscope using a 100× oil immersion objective.

A minimum of 100 spermatozoa were evaluated per slide. Dark blue-stained spermatozoa were considered positive for histone retention, unstained spermatozoa were considered negative, and lightly stained or halo-like spermatozoa were classified as partially positive. The percentage of negatively stained spermatozoa was calculated as an indicator of chromatin condensation and nuclear maturity (19,20).

Immunofluorescent Detection of CatSper4 Protein: Immunocytochemical analysis of CatSper4 (Thermofisher Sci., USA). Samples were fixed with PFA and permeabilized in 0.1% Triton X-100, then blocked in 10% serum for 45 minutes at 25˚C. Samples were incubated with CatSper4 polyclonal antibody (Product No: PA5-103717) and mouse anti-beta-tubulin antibody at 37˚C for 1 hour. AlexaFluor594-conjugated goat anti-rabbit IgG (red) and AlexaFluor488-conjugated goat anti-mouse IgG (green) were used as secondary antibodies. DAPI was used as the core stain (blue).

After washing, slides were mounted with a Hoechst-containing mounting medium. Preparations were examined under a fluorescence microscope at 100× magnification, and CatSper4 positivity was calculated by counting at least 100 spermatozoa in randomly selected fields.

Global DNA Methylation Analysis: Global DNA methylation levels were measured using a 5-methylcytosine (5-mC) ELISA kit (EpigenTek, USA) following the manufacturer's instructions. Methylation analysis was performed on 22 cases: 11 normospermic and 11 oligospermic. We ensured that the number of smokers in each group was similar.

DNA Extraction: Gradient-separated sperm cells underwent DNA extraction with the ISOLATE II Genomic DNA Kit (Bioline, Germany). The extracted DNA was stored at –20˚C until analysis. DNA purity was assessed spectrophotometrically at 260 and 280 nm.

Briefly, 100 ng of genomic DNA from each sample was used per reaction. Absorbance was measured at 450 nm using a microplate reader. The amount of 5-mC (ng) was calculated based on the slope of the standard curve, and global DNA methylation was expressed as a percentage using the following formula:

5-mC% = [5-mC amount (ng)/input DNA amount (ng)]×100

This method provides a quantitative assessment of global DNA methylation levels (13,14).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 9.1.1). Continuous variables were expressed as mean ± standard deviation (SD). Data normality was assessed using the Shapiro-Wilk test. Comparisons between two independent groups (smokers vs. non-smokers) were conducted using an independent-samples t-test when normality assumptions were met. For all analyses, a p-value of less than 0.05 was considered statistically significant. Exact p-values were reported in the tables whenever applicable. For very small p-values, values were presented as $p<0.001$.

Results

Data in Table I are presented as mean±standard deviation. Comparisons between smokers and nonsmokers within each semen status group were made using independent-sample t-tests. Exact p-values are shown. We considered $p<0.05$ statistically significant.

There were no differences between the groups in sperm concentration, sperm motility, or normal morphology. However, smoking was associated with higher sperm DNA fragmentation in oligospermic individuals ($p=0.008$).

Table I: *Comparison of semen parameters, DNA integrity, chromatin condensation, and CatSper4 positivity between smokers and non-smokers within normospermic and oligospermic groups.*

Parameter	Normospermic Smokers (n=10)	Normospermic Non-Smokers (n=10)	p	Oligospermic Smokers (n=15)	Oligospermic Non-Smokers (n=15)	p
Sperm concentration	59.06±25.19	65.16±30.27	0.630	11.79±8.27	13.65±9.75	0.578
Sperm motility	33.25±11.34	36.67±13.25	0.543	20.19±16.23	27.11±9.69	0.167
Normal morphology	4.12±2.23	6.15±2.33	0.062	2.24±1.23	3.23±1.89	0.100
DNA fragmentation	36.20±16.94	30.80±13.00	0.434	44.73±12.36	28.00±18.86	0.008
CatSper4 positivity	46.20±23.71	52.70±23.17	0.543	22.26±12.36	45.86±18.30	**<0.001***
Chromatin condensation	51.27±22.34	68.36±19.45	0.085	13.34±9.76	29.89±8.69	**<0.001***

CatSper4 membrane channel protein positivity was lower in the oligospermic smoker group ($p<0.001$). The chromatin condensation value (aniline blue negative) was lower in oligospermic individuals and was statistically significant in the smoker group ($p<0.001$).

Global DNA methylation levels were assessed by measuring the percentage of 5-methylcytosine (5-mC%) in sperm DNA samples. Group means were calculated from individual values, and the results are presented in Figure 1. Smokers showed a statistically significant increase in global DNA methylation compared with nonsmokers (1.81±0.52 vs. 1.11±0.35; $p=0.0012$). This suggests that smoking may be associated with increased DNA methylation levels.

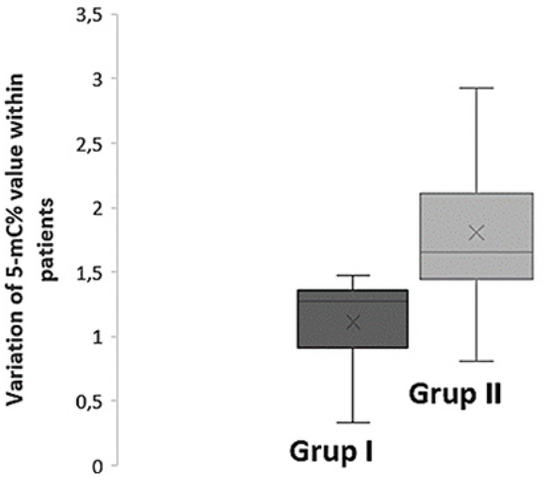

Figure 1: *Changes in global DNA methylation associated with cigarette smoking.*
Global DNA methylation levels (5-mC%) in smokers (Group II) and non-smokers (Group I). Data are presented as mean±SD, and individual data points are shown where available. Smokers showed a statistically significant increase compared with non-smokers (p= 0.0012)

Discussion

Reactive oxidants and toxic constituents in cigarette smoke have been shown to increase oxidative stress in seminal plasma, leading to disruption of sperm membrane integrity and DNA damage (5,6). Consistent with this mechanism, numerous studies and meta-analyses have demonstrated a strong association between cigarette smoking and deterioration of conventional semen parameters (7).

In the present study, smoking-related differences were most evident in oligospermic men, particularly for sperm DNA fragmentation, chromatin condensation, and CatSper4 positivity. In contrast, conventional semen parameters showed numerical but statistically non-significant differences between smokers and non-smokers in the subgroup analyses. These findings are in agreement with previous reports indicating that smoking negatively affects sperm nuclear integrity and maturation processes (8,21). The observed increase in DNA fragmentation and chromatin abnormalities suggests that smoking-induced oxidative stress may interfere with proper chromatin remodeling during spermiogenesis, thereby compromising sperm genomic stability.

In addition to structural alterations, our results demonstrated a significant increase in global DNA methylation levels (5-mC%) in smokers compared to non-smokers. DNA methylation is a key epigenetic mechanism regulating gene expression, and alterations in global methylation levels have been associated with impaired sperm function and reduced fertility potential (13,14). Therefore, the observed elevation in 5-mC% levels in smokers may reflect smoking-associated epigenetic dysregulation in sperm cells.

Although the precise mechanisms underlying these changes remain unclear, oxidative stress induced by cigarette smoke may play a central role. Reactive oxygen species can interfere with DNA methyltransferase activity and genomic stability, potentially leading to aberrant DNA methylation patterns. In this context, our findings suggest that cigarette smoking may influence not only sperm DNA integrity but also epigenetic regulation.

In the context of assisted reproductive technologies (ART), accumulating evidence suggests that epigenetic alterations in parental gametes may influence embryonic development, implantation, and long-term health outcomes in offspring (22-24). Therefore, identifying modifiable lifestyle factors, such as cigarette smoking, that impact the sperm epige-

netic profile is of considerable clinical importance. A more detailed characterization of smoking-induced epigenetic changes at gene- and locus-specific levels may provide valuable insights into potential transgenerational effects.

The CatSper channel is a key regulator of Ca^{2+} influx required for sperm hyperactivation and successful fertilization (15-17). CatSper4 encodes a sperm-specific CatSper calcium channel subunit that supports calcium influx, which is essential for sperm hyperactivation and fertilization. It is part of the CatSper complex and contributes to the mechanism enabling flagellated sperm motility. Consistent with this role, it is highly expressed in the male reproductive system and functions in reproductive physiology (25).

CatSper4 is localized to the plasma membrane, the sperm main segment, and the acrosomal vesicle, and is found in key regions for sperm signaling and motility. Channel activity is indirectly activated by progesterone via ABHD2-dependent hydrolysis of the endocannabinoid inhibitor 2-arachidonylglycerol (2AG), and voltage sensitivity is enhanced by intracellular alkalization and sperm capacitation. It is hypothesized to enable voltage-gated calcium channel activity and to participate in intracellular sodium ion transport and localization (26).

Pathogenic variants in CatSper4 have been reported in male infertility associated with impaired sperm function. This gene is also associated with impaired and overactive sperm capacitation, and its reproductive importance is supported by its role within the sperm duct complex (25). In the present study, we observed a significant reduction in CatSper4 positivity in oligospermic smokers. This finding suggests that in individuals with already compromised semen parameters, cigarette smoking may further impair Ca^{2+}-dependent signaling pathways that regulate sperm motility and fertilization capacity. Previous studies reporting associations between CatSper4 expression and sperm motility and morphology support this interpretation (27,28).

Limitations and Strengths: This study has several limitations. First, the sample size was relatively small, which may limit the generalizability of the findings. Second, smoking exposure was not quantified in terms of pack-years, preventing dose–response evaluation. Third, CatSper4 expression was assessed using immunoreactivity rather than functional assays.

Despite these limitations, this study has important strengths. It integrates conventional semen analysis with molecular and epigenetic markers, including sperm DNA fragmentation, chromatin condensation, global DNA methylation, and CatSper4 expression. This comprehensive approach provides a more holistic understanding of the mechanisms by which cigarette smoking affects male fertility.

Future studies involving larger cohorts, dose-response analyses, and comprehensive epigenetic profiling at the gene or locus level are warranted to validate and extend the present findings.

Conclusion

In conclusion, the present study suggests that cigarette smoking is associated with alterations in sperm nuclear integrity, chromatin maturation, CatSper4 immunoreactivity, and global DNA methylation, particularly in oligospermic men. Although conventional semen parameters such as sperm concentration, motility, and normal morphology showed numerical differences between smokers and non-smokers, these differences did not consistently reach statistical significance in subgroup analyses. Therefore, the findings should not be interpreted as evidence of a uniform smoking-related decline in all conventional semen parameters. The most prominent smoking-associated alterations were observed in sperm DNA fragmentation, chromatin condensation, and CatSper4 positivity in the oligospermic group, suggesting that cigarette smoking may primarily affect molecular and functional aspects of sperm quality rather than producing consistent changes in all routine semen parameters. In addition, the significant increase in global DNA methylation levels among smokers indicates that smoking may be associated with epigenetic dysregulation in sperm cells. Taken together, these findings support the view that cigarette smoking may impair male fertility potential through combined effects on sperm DNA integrity, chromatin organization, epigenetic regulation, and calcium-dependent signaling pathways. However, given the relatively small sample size and the lack of individual-level validation for some analyses, further large-scale studies including dose–response assessment, functional CatSper assays, and detailed epigenetic profiling are required to confirm these findings and clarify their clinical implications in male infertility.

Declarations

Acknowledgment: The authors thank all participants for their contribution to this study.

Ethics Approval and Consent to Participate: The Non-Interventional Clinical Research Ethics Committee of Biruni University approved the study (Approval No: 2018/18-04, Date: 20.07.2018). We obtained written informed consent from all participants. All procedures were conducted in accordance with the Declaration of Helsinki.

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Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' Contribution EC: Conceptualization, Methodology, Writing - original draft, Supervision. TE: Methodology, Investigation. ES: Investigation, Data curation. GD: Formal analysis, Visualization.TI: Supervision, Writing - review and editing.

All authors read and approved the final manuscript.
Conflict of Interest: The authors declare no conflict of interest.

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