# Possible Harmful Effects of Smoking Hookah on Sperm DNA Fragmentation Index and Protamine Genes Expression in Normozoospermic Men

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**ABSTRACT**

**OBJECTIVES:** In recent years, smoking water pipes or hookah has increased among adolescents in most countries. Although there is evidence in support of the negative effects of this type of smoking on human health, such as the increased risk of lung disease, little is known about the potential effects of hookah smoking on the male reproductive system, especially on the molecular aspects of sperm.

**PATIENTS AND METHODS:** This cross-sectional study examined sperm DNA fragmentation index, *protamine 1 and 2 (PRM1* and *PRM2*) genes expression, and oxidant status in normozoospermic hookah smokers in comparison with non-smoker controls.

**RESULTS:** Our results showed significantly higher rates of DNA fragmentation, protamine deficiency, and abnormal chromatin condensation in the spermatozoa of hookah smokers ($P < .0001$). Also, protamine gene expression showed a remarkable decrease in hookah smokers ($1.55 \pm 2.54$ and $0.33 \pm 0.54$) compared to the controls ($3.49 \pm 5.41$ and $1.22 \pm 1.96$), although the reduction was not statistically significant ($P = .155$ and $P = .066$, respectively). Moreover, a significantly higher level of semen MDA was observed in the case group compared to the controls ($0.39 \pm 1.04$ vs $0.15 \pm 0.21$; $P = .013$).

**CONCLUSION:** According to our study, although hookah smoking does not have a significant effect on sperm parameters, it may have deleterious effects on DNA integrity, oxidative status, and nuclear protein levels of spermatozoa.

**KEYWORDS:** Hookah, *Protamine 1*, *Protamine 2*, sperm, DNA fragmentation, chromatin

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## Introduction

Hookah or water pipe smoking (WPS) is a traditional method of tobacco consumption that emerged in India in the 16th century. WPS later became customary in the Middle East and the appearance of the instrument changed over time.[1] During 2009 to 2010 and 2013 to 2014, the prevalence of *past-30-days* WPS among adolescents (aged 18-24 years) increased from 7.8% to 18.2% and lifetime hookah use increased from 28.6% to 44.4%.[2,3] According to a systematic review of 76 articles on hookah smoking in Iran, 28.78% of participants reported total lifetime hookah use, 20.84% reported hookah use in the last year, and 16.36% in the last month.[4] Despite the large body of evidence on the negative effects of WPS on human health, including a higher risk of lung diseases,[5] little is known about the potential adverse effects of hookah use on the male reproductive tract. Albeitawi et al[6] investigated the effect of hookah smoking on the sperm parameters of 104 patients referred for in-vitro fertilization but did not find any significant changes in the sperm parameters of hookah users compared to those in the control group.

Sperm DNA integrity is required for the precise transmission of genetic information to the embryo. Research findings

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have revealed that sperm chromatin abnormalities or DNA damage are probable causes of male infertility due to cigarette smoking.[7] Smoking and oxidative stress have been identified as important extrinsic factors involved in the pathogenesis of DNA fragmentation in human spermatozoa. The high levels of free radicals present in cigarette smoke promote the production of Reactive Oxygen Species (ROS) in the seminal plasma, leading to oxidative stress (OS).[8] It seems that it is necessary to pay attention to the man's lifestyle[9] (such as smoking Hookah) and examine the DNA structure of the sperm in the treatment of couples' infertility with a normal sperm analysis.

*Protamine 1* (*PRM1*) and *protamine 2* (*PRM2*) are considered the most important genes in the production of nucleoproteins during the maturation of spermatozoa.[10] In the process of spermatogenesis, histones are first replaced by Transition Nuclear Proteins (TNPs), and subsequently, the TNPs are replaced by PRM1 and PRM2 in elongated spermatids.[11] Evidence indicates a correlation between *PRM1* and *PRM2* expression levels, sperm DNA fragmentation (SDF) levels, and male infertility issues.[12] To our knowledge, although there are limited studies with conflicting results about the effect of Hookah on sperm structure[6,13,14] and the possible adverse effects of WPS on the expression profile of these genes as well as DNA damage are not yet investigated specifically in men partner of infertile couples with normal sperm parameters. This cross-sectional study was therefore conducted to examine the possible harmful effects of WPS on sperm DNA fragmentation, *PRM1* and *PRM2* gene expression, and oxidant status in 2 groups—smokers and non-smokers.

## Patients and Methods

### Data collection

This study was approved by the ethics committee of Yazd Reproductive Sciences Institute with code IR.SSU.MEDICINE.REC.1398.297. Informed consent was obtained from the participants before beginning the study. Sixty-two eligible male partners of infertile couples with female factor infertility referred to Yazd Reproductive Sciences Research Institute for diagnosis and treatment from April 2020 until November 2021, were included in this cross-sectional study. They divided into 2 groups of men, including hookah smokers as the case group (n = 32) and non-smokers men as the control group (N = 30).[15] According to the pilot carried out to check the data dispersion in the case and control, the *S1* = 6.02, the *S2* = 8.11, and *d* = 5.51 was obtained. Considering the type 1 error was equal to 5% and the power of 80%, the sample size was set at 28.36.

$$n = \frac{\left(Z_{1-\alpha/2} + Z_{1-\beta}\right)^2 (S_1^2 + S_2^2)}{d^2}$$

The inclusion criteria for the case group consisted of normal sperm parameters, *using a hooker minimum of 3 times* per week for at least 30 to 45 minutes each time, and age 25 to 40 years. The inclusion criteria for the control group consisted of age under 40 years, normal sperm parameters, and no history of hookah smoking.[16] The exclusion criteria for both groups *were patients having* a history of chronic diseases (eg, hypertension, diabetes, and varicocele), history of urogenital infections, heavy cigarette smoking, alcohol consumption, *using a* specific medication, drug addiction, and abnormal sperm parameters based on the WHO reference criteria in 2010 and age over *40 years* (*study* design of this study was semantically shown in Figure 1).

*Specimen collection.* First, all the specimens were taken by masturbation after 2 to 7 days of abstinence. Following semen liquefaction, at 37°C, the analysis of each sample was carried out based on the WHO guidelines (2010) for basic sperm parameters.[17] The parameters were considered normal when semen volume was ⩾1.5 mL, sperm morphology ⩾4%, sperm concentration ⩾15 million/mL, and total sperm motility ⩾40%. After sperm analysis, the semen samples were smeared for aniline blue, toluidine blue (TB), Chromomycin A3 (CMA3), and sperm chromatin dispersion (SCD) tests. The remaining semen samples were used for further evaluation. Each sperm sample was separated from seminal plasma by centrifugation at 1800*g* for 10 minutes. The supernatant was applied for the measurement of Malondialdehyde (MDA) in seminal plasma. The spermatozoa pellets were re-suspended in a phosphate-buffered saline (PBS) due to remove the residual seminal plasma for molecular assessments.

### Sperm chromatin/DNA assessments

*Aniline blue staining.* Aniline blue staining was applied to distinguish the immature sperm cells with abnormal chromatin condensation from the mature ones. Briefly, each sperm smear was dried at Room Temperature (RT), then fixed in 4% formalin[18,19] for 5 minutes at RT, and was then rinsed in water and stained by 5% aniline blue (Merk, Germany) in 4% acetic acid solution for 10 minutes. After final rinsing and air drying of the slide, 200 spermatozoa were counted in several fields under light microscopy at 1000× magnification. Immature sperm was identified by dark blue color, while mature sperm remained unstained. The ratio of dark-blue spermatozoa to the total number of counted sperm was taken as immature sperm with poor chromatin condensation.

*Toluidine blue staining.* To evaluate the sperm chromatin structure, TB staining was performed.[20] Air-dried smears were fixed in fresh 96% ethanol-acetone (1:1) at 4°C for 1 hour. Thereafter, they were hydrolyzed in 0.1 N HCl at 4°C for 5 minutes following the air-drying stage. Afterward, the slides were washed 3 times in distilled water for 2 minutes and lastly stained with 0.05% TB in 50% McIlvaine citrate phosphate buffer with a pH of 3.5 for 5 minutes at RT. The slides were shortly rinsed in distilled water. Using optical microscopy with 1000× magnification, 200 spermatozoa

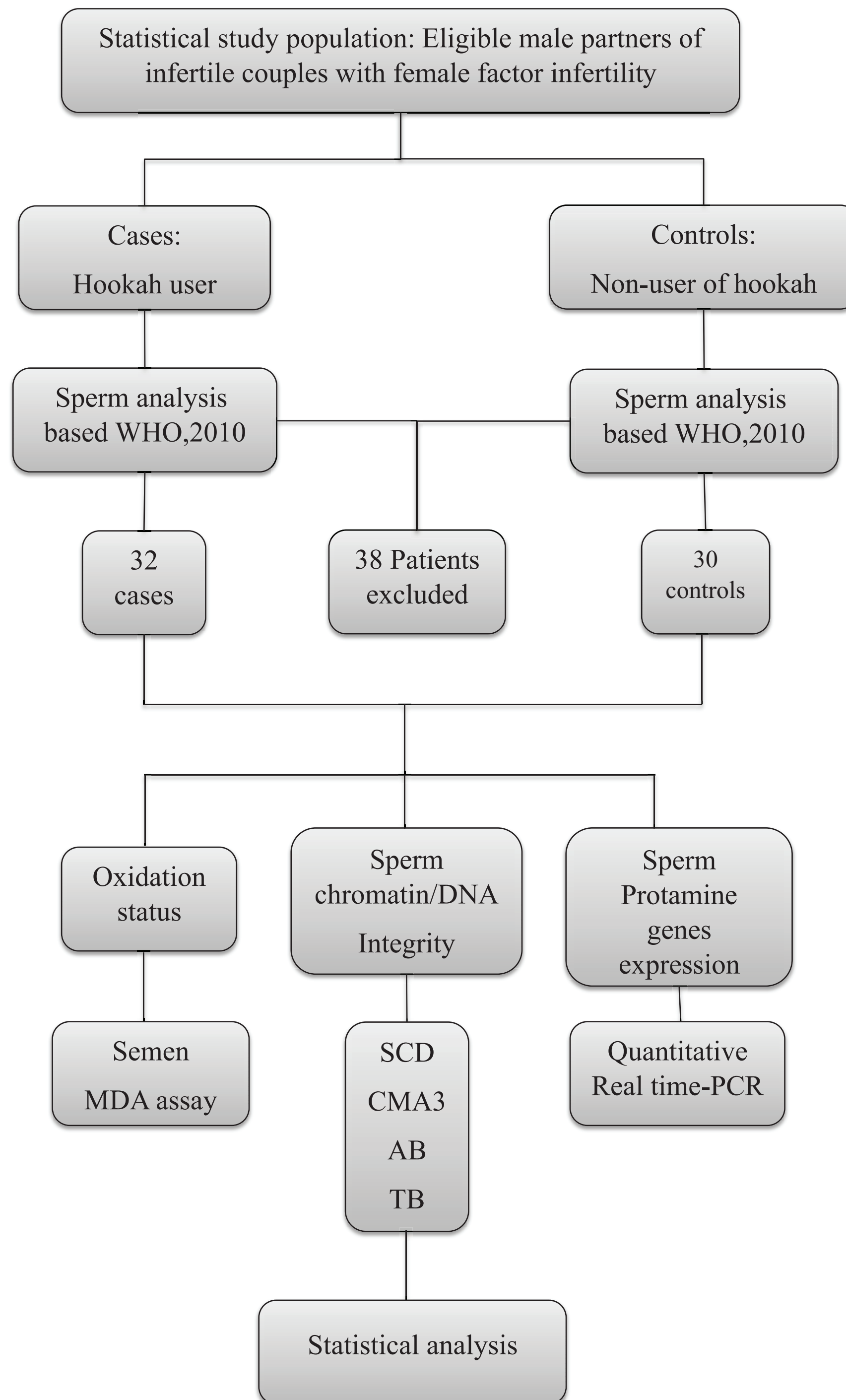


**Figure 1.** Schematic diagram of study design and methodology.

were counted in various fields of view for each slide. Spermatozoa with intact chromatin were light blue, while those with poor chromatin integrity could be seen in dark blue (or purple). The percentage of sperm cells with abnormal chromatin structure was determined in each sample.

*Chromomycin A3 staining.* The fluorescent dye chromomycin A3 (CMA3) competes with the protamine molecule to bind to the minor grooves of DNA. As such, it indicates the level of protamine deficiency in the chromatin structure indirectly.[21] The sperms with defective protamination were stained by CMA3, appearing as bright yellow following evaluation by fluorescent microscopy. Briefly, the dried smear of the sperm cell slides was fixed in Carnoy's fixative (methanol and glacial acid at a ratio of 1: 3) and stored in the refrigerator for 10 minutes. Then, 100 µL of CMA3 solution (Sigma, USA) was added to each fixed slide and kept in a dark room for 10 minutes. The slides were washed twice with McIlvaine buffer. The slides were mounted with DPX and examined with a fluorescence microscope with a 460-460-nm filter at 1000×

**Table 1.** Primer sequences.

| GENE | PRIMER SEQUENCE (5″-3″) | SEQUENCE AMPLIFIED | PRODUCT SIZE |
|---|---|---|---|
| *PRM1* | Forward—AGAGCCGGAGCAGATATTACC<br>Reverse—TCTACATCGCGGTCTGTACCT | NM_002761.2 | 119 bp |
| *PRM2* | Forward—ATCCACAGGCGGCAGCATC<br>Reverse—TTCCAGCTGGGGGTGAGGGG | NM_002762.3 | 144 bp |
| *B2M* | F-AGATGAGTATGCCTGCCGTG<br>R-TGCGGCATCTTCAAACCTC | NM_004048.2 | 106 bp |

Abbreviations: B2M, beta-2-microglobulin; PRDX2, Protamine 2; PRM1, Protamine 1.

magnification (Olympus BX5, Japan). By counting 200 sperms, the percentage of bright yellow sperms (CMA3+) and non-luminous sperms (CMA3−) was determined in each sample.

*Sperm chromatin dispersion test.* To investigate the level of DNA fragmentation of spermatozoa, the SCD test was administered by a commercial kit obtained from the Ideh Varzan Farda Company. Briefly, 50 µL of sperm suspension was mixed with low-melting agarose. Then, 20 µL of the mixture was placed on a pre-coated glass slide, and the droplet was spread with a coverslip. Each slide was kept at 4°C for 5 minutes. After removing the coverslip, staining was performed according to the manufacturer's instructions. Then, 200 spermatozoa were analyzed at a magnification of ×1000 by light microscopy. Based on the size of halo dispersion, SDF was taken as intact sperm DNA if a big halo was present, moderate DNA fragmentation when a medium halo was seen, and high DNA fragmentation when no halo was seen.

### *Measurement of the oxidant capacity of seminal plasma*

The oxidant status of seminal plasma was determined separately using a commercial MDA kit (Teb Pazhouhan Razi Co.) based on enzyme-linked immunosorbent assay (ELISA). The mean concentration of spermatozoa used in this study was $10 \times 10^6$/0.2 mL. The lipid peroxidation seminal plasma was evaluated using the reaction of Thiobarbituric Acid (TBA) with MDA as suggested by Yagi.[14] The MDA concentration was assessed using an Epoch microplate spectrophotometer (excitation 535 nm, emission 553 nm). The MDA fluorescence intensity of seminal plasma was assessed by various concentrations of Tetraethoxypropane as the standard. The data were presented as nmol MDA/mL of seminal plasma.

### *Gene expression assessment*

To synthesize complementary DNA (cDNA) and extract total RNA from all the sperm samples, special kits were used based on the manufacturers' instructions. The purity and concentration of the extracted RNAs were calculated by a spectrophotometer and the ratio of absorbance values obtained were 260/280 and 260 nm (PhotoBiometer, Eppendorf, Germany). The cDNA was synthesized from 1 µg of RNA utilizing RevertAid First Strand cDNA Synthesis Kit (Pars Tous Biotechnology, Iran) and the product was kept at −20°C. The relative gene expression levels were evaluated by a quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) (Applied Biosystems, CA, USA) and SYBR Green PCR Master Mix. cDNA (2 µL), forward primer (1 µL), reverse primer (1 µL), the master mix (10 µL), and 6 µL nuclease-free water was utilized for each reaction with the final volume adjusted to a total of 20 µL. All the reactions were duplicated. The qRT-PCR protocol was as follows: 10 minutes at 95°C, followed by 40 cycles of amplification at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. A melting curve stage was run after the cycling stage. Serum β-2 Microglobulin (B2M*)* was utilized as the reference gene. Table 1 summarizes the oligonucleotide primers used for all the genes. To analyze the relative expressions of each gene, the $2-\Delta\Delta Ct$ technique was employed. The mean CT value of the reference gene was calculated for the gene expression analysis as well.

### *Statistical analysis*

Statistical analyses were done using SPSS version 20 (Chicago, IL, USA). All numerical values were shown as mean ± SD. Statistical significance was assessed using an independent student *t*-test for the normally-distributed variables. Mann-Whitney test was used for the variables without normal distribution. The normality distribution was tested using the Kolmogorov-Smirnov test. The categorical variables were analyzed using the Chi-square test. The sample size was set according to the aforementioned formula and based on similar studies.[15,22] $P \leqslant .05$ was taken as the level of statistical significance.

## Results

Out of 100 couples referred with female factor infertility within the timeframe of sample collection, 62 participants were finally chosen and they were categorized into the cases

**Table 2.** Frequency distribution of education level and occupation in the study groups.

| VARIABLES | CASE (N = 32) | CONTROL (N = 30) | *P*-VALUE |
|---|---|---|---|
| Education | | | |
| Academic degree | 5 (15.63) | 10 (33.3) | .10 |
| Non-academic degree | 27 (84.37) | 20 (66.7) | |
| Occupation | | | |
| Driver | 4 (12.5) | 2 (6.67) | .71 |
| Employee | 11 (34.37) | 10 (33.3) | |
| Employer | 17 (53.13) | 18 (60.03) | |

**Table 3.** Comparison of patients' characteristics and semen analysis between 2 groups.

| VARIABLES | CASE | CONTROL | *P*-VALUE |
|---|---|---|---|
| Age (year) | 31.53 ± 4.85 | 33.57 ± 4.32 | .09 |
| BMI (kg/m²) | 25.71 ± 4.75 | 26.05 ± 4.01 | .77 |
| Volume (mL) | 2.81 ± 1.11 | 3.32 ± 1.83 | .19 |
| Sperm concentration (×10⁶/mL) | 40.5 ± 22.64 | 45.2 ± 36.77 | .54 |
| Total sperm number | 117.37 ± 84.91 | 132.40 ± 107.08 | .54 |
| Progressive motility (%) | 35 ± 17.20 | 36.6 ± 11.19 | .67 |
| Non-progressive motility (%) | 11.97 ± 3.27 | 11.5 ± 3.24 | .57 |
| Immotile sperm (%) | 54.34 ± 15.37 | 51.67 ± 9.86 | .42 |
| Total motility (%) | 46.96 ± 17.73 | 48.1 ± 9.90 | .76 |
| Normal morphology (%) | 4.88 ± 1.21 | 5.3 ± 1.48 | .22 |

Abbreviation: BMI, body mass index.
Data were presented as Mean ± SD as well as a percentage (%) by using a student *t*-test. The *P*-value <.05 was considered to indicate statistical significance.

(N = 32) and the controls (N = 30) based on the eligibility criteria, and the other participants were excluded due to lack of inclusion criteria. The mean age and BMI in the 2 groups did not differ significantly from each other. The participants were homogenous in terms of occupation and education (Table 2). Based on our results, the majority of the participants did not have an academic degree in the control and case groups (84.4% vs 66.7%, respectively). As expected, the sperm parameters including concentration, volume, total motility, and normal morphology were not different between groups (Table 3). Despite the slightly lower sperm concentration, volume, total motility, and normal morphology in the hookah smokers compared to the controls, these differences between the groups were not statistically significant ($P > .05$). As shown in Table 4, higher rates of spermatozoa with SDF and chromatin abnormalities were seen based on the CMA3, AB, TB, and SCD staining methods ($P < .0001$). Furthermore, although the *PRM1* expression and ratio of *PRM1* to *PRM2* showed a remarkable down-regulation in the spermatozoa of hookah smokers (1.55 ± 2.54 and 0.33 ± 0.54) compared to the controls (3.49 ± 5.41 and 1.22 ± 1.96), this reduction was not statistically significant ($P = .25$) (Figure 2). Finally, as can be

**Table 4.** Comparison of sperm DNA fragmentation status between the study groups.

| VARIABLES | CASE | CONTROL | *P*-VALUE |
|---|---|---|---|
| CMA3(+) | 70.22 ± 9.76 | 25.37 ± 10.51 | <.0001 |
| SCD(+) | 38.75 ± 9.66 | 28.07 ± 5.25 | <.0001 |
| AB(+) | 42.10 ± 9.43 | 30.67 ± 6.86 | <.0001 |
| TB(+) | 49.61 ± 5.23 | 41.47 ± 5.30 | <.0001 |

Abbreviations: AB, aniline blue; CMA3, Chromomycin A3; SCD, sperm chromatin dispersion; TB, toluidine blue.
Data were presented as Mean ± SD by using student *t*-test and Mann-Whitney test. The *P*-value <.05 was considered to indicate statistical significance.

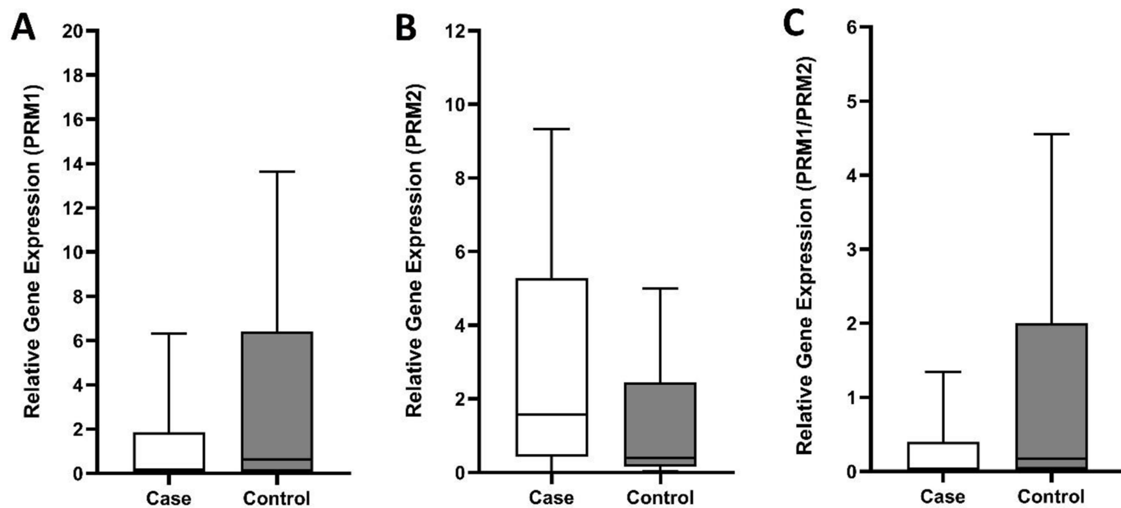


**Figure 2.** Comparison of gene expression profile between 2 study groups. The relative gene expression levels PRM1 (a), PRM2 (a) and PRM1/PRM2 (c) of each target gene were presented as Mean ± SD. $P < .05$ was considered a significant value. Mann–Whitney test was applied to compare differences between groups. There was no significant change between case and control groups regarding *PRM1*, and *PRM2* ($P > .05$). *PRM1: Protamine 1, PRM2: Protamine 2*, Case: normozoospermic hookah smokers, Control: non-smoker normozoospermic men.

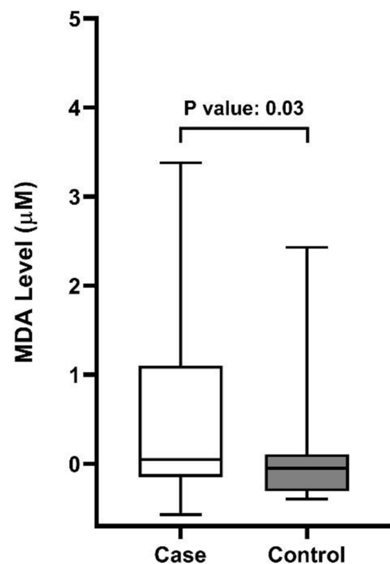


**Figure 3.** Comparison of oxidant status between 2 study groups. Data were presented as Mean ± SD by using Mann–Whitney test. MDA: malondialdehyde. The *P*-value <.05 was considered to indicate statistical significance.

observed in Figure 3, a significantly higher rate of semen MDA level was seen in the case group compared to the controls (0.39 ± 1.04 vs 0.15 ± 0.21, $P = .031$).

## Discussion

Recently, water pipe smoking (WPS) has become one of the most important health threats in people of reproductive ages, especially in Iran, Turkey, and Arab countries with high WPS prevalence[23,24] and has been demonstrated that WPS has more devastating effects than cigarettes due to longer duration of puffing (30-90 minutes/session), greater inhaled smoke, presence of flavors, and the social setting of the vaping session.[25,26] However, most hookah users also smoke cigarettes.

The findings have shown that tobacco and water pipe users are exposed to high toxicant loads because many of them are unaware of the associated toxicant load and consider WPS a safe alternative to cigarette smoking.[27] Meanwhile, in a single hookah smoking session, which typically lasts 45 minutes, hookah users get exposed to 3 to 9 times more carbon monoxide (CO) and 1.7 times more nicotine than cigarette smokers.[28] Therefore, to homogenize the samples and minimize any biases, we enrolled only individuals with a history of hookah use who smoked hookah at least 3 times a week, each session lasting 30 to 45 minutes.

In a previous study, the impact of tobacco smoking on semen quality has been systematically studied through the comparison of semen parameters between smokers and non-smokers. Concerning clinical outcomes, the study concluded that tobacco smoking leads to a lower sperm concentration and an increase in the number of morphological defects of spermatozoa. Nevertheless, the pH and motility of spermatozoa as well as the production of hormones involved in reproduction were not influenced in the infertile men studied.[29] Despite a large number of studies on the negative effects of cigarette smoking,[29-31] there is little research evaluating the influence of hookah smoking on semen quality. In a recent study published in 2020, Albeitawi et al investigated the effect of WPS on sperm parameters in 54 hookah smokers and 50 non-hookah smokers. They demonstrated no statistically significant difference between the 2 groups regarding sperm parameters,[6] which is following the present findings; however, it should be noted that factors such as age, amount and duration of hookah use, and genetic heredity may alter the results.

The production of small amounts of ROS in semen is a normal physiological process but an imbalance between ROS generation and scavenging antioxidants is detrimental to the sperm, which is also associated with male infertility.[32,33] A

recent literature review suggests that WPS can cause oxidative stress accompanied by several pathological conditions.[34] The present study showed a significantly higher oxidant by-product (MDA+) in the seminal plasma of the case group than that in the controls. Based on research literature, it can be concluded that WPS increases ROS generation and exposes smokers to high levels of potentially toxic free radicals.[34,35] The data of recent studies indicate that long-term hookah use not only increases MDA levels and causes SDF and sperm parameter abnormalities,[36] but it also has mutagenic effects.[37]

The comparison of SDF between our study groups demonstrated that WPS has a significant detrimental effect on chromatin/DNA integrity concerning DNA breakage, protamine content, and chromatin condensation (Table 3). Evaluation of sperm DNA damage is known as one of the most important sperm function tests for the detection of paternal genome integrity. Sperm DNA precision is required for a successful pregnancy and lives birth either during natural or assisted reproduction.[38] Accordingly, reducing the risk factors of sperm DNA damage can be practical as one of the major options for managing male infertility.[39] The findings of a newly published study on the impact of cigarette, hookah, and mixed smoking on sperm DNA fragmentation based on urinary samples among 60 men from Saudi Arabia showed a significantly elevated DNA fragmentation rate in all cigarette, hookah, and mixed smokers compared to the controls.[40] Despite the differences in the type of samples and study design, the results of our study were in line with the aforementioned study, suggesting the destructive role of any type of tobacco smoking on DNA apart from its tissue sources in the human body. It should be noted, however, that we used the SCD test in our study to evaluate SDF. The SCD assay is considered a reliable and inexpensive test and there is also a positive relationship between its results and the TUNEL assay as a standard laboratory test for detecting SDF abnormalities.[41] The main step in sperm nuclear chromatin packaging is the process of histone/protamine replacement, and it has been reported that any protamine alterations may lead to idiopathic male infertility.[42] Smoking can cause abnormalities in histone-to-protamine transition and may alter P1/P2 expression in human spermatozoa. Additionally, in comparison with non-smokers, the smokers had an elevated percentage of spermatozoa with a higher histone (H2B) to protamine ratio, causing alterations in the sperm chromatin structure during the process of spermiogenesis, and subsequently reducing fertility.[43] The possible mechanism of hookah consumption on sperm DNA according to the results of this study and aforementioned studies[38-43] may be supported by affecting histone replacement with protamine in the process of spermatogenesis due to the protamine expression alternations, resulting in uncompleted sperm chromatin packaging and chromatin instability, and increasing the sperm DNA damage. Moreover, the increase in ROS production, such as MDA by-product, due to the use of hookah could induce the apoptosis pathway during spermatogenesis and may hurt the male fertility potential.

To the best of our knowledge, no research was available showing the influence of WPS on transcript levels of sperm nuclear proteins, such as protamine. Nonetheless, the findings of a newly published study on the sperm samples of 43 non-smokers and 98 heavy cigarette smokers undergoing ICSI showed a significant negative effect of tobacco smoking on the gene expression of *H2BFWT*, *TNP1*, *TNP2*, *PRM1*, and *PRM2*, and the P1/P2 mRNA ratio. These variations subsequently led to sperm quality impairment.[44] In our study, mRNA levels of *PRMs* were different between the samples obtained from the cases and controls, but the expression ratio of *PRM1* to *PRM2* did not follow the direct pattern. Although the difference was not statistically significant, it might have been significant if our sample size was larger. It should be noted that, in our study, the selection of participants and samples who could meet all the eligibility criteria was rendered difficult by the COVID-19 pandemic, given the limited access to humans during this time. Also, our current sample size has been determined based on some other studies on this subject.[15] A larger sample size is therefore required to reach more reliable and reproducible diagnostic results in further studies.

## Conclusion

According to our study, hookah smoking may have destructive effects on the molecular markers of sperm cells rather than altering sperm parameters. It seems that WPS could have deleterious effects on chromatin/DNA integrity by increasing SDF and levels of oxidant markers as the most important factors involved in oxidative stress-induced male infertility. In addition, hookah smoking may cause different expression patterns of protamine genes, leading to an imbalance in PRM1/PRM2 ratio as the main causative factor in chromatin/DNA damage. To achieve efficient urological diagnosis/treatment, specifically in the male partner of infertile couples with normal sperm parameters, it is necessary to pay attention to their lifestyle and DNA structure and function of sperm, resulting in ART success.

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## Author Contributions

MTN, MAK, FF, and ART; Contributed to conception and design. MTN, MHS, MI, AN, and, SGE; Contributed to all experimental work, data and statistical analysis, and interpretation of data. ART, MHS, and MAK were responsible for overall supervision. MTN and FF; Drafted the manuscript, which was revised by ART and MAK. All authors read and approved the final manuscript.

## REFERENCES


1. Kadhum M, Sweidan A, Jaffery AE, Al-Saadi A, Madden B. A review of the health effects of smoking shisha. *Clin Med*. 2015;15:263-266.
2. Salloum RG, Thrasher JF, Kates FR, Maziak W. Water pipe tobacco smoking in the United States: findings from the National Adult Tobacco Survey. *Prev Med*. 2015;71:88-93.
3. Kasza KA, Ambrose BK, Conway KP, et al. Tobacco-product use by adults and youths in the United States in 2013 and 2014. *N Engl J Med*. 2017;376: 342-353.
4. Afrashteh S, Ansarifar A, Vali M, et al. Geographic distribution and time trends of water-pipe use among Iranian youth and teenage students: a meta-analysis and systematic review. *J Ethn Subst Abuse*. Published online September 6, 2021. doi:10.1080/15332640.2021.1943097
5. Rojewski AM, Zuromski KL, Toll BA. Strategies for smoking cessation among high risk populations to prevent lung cancer. *Expert Rev Respir Med*. 2017;11:85-87.
6. Albeitawi S, Hamadneh J, Al-Shatanawi TN, Al Mehaisen L, Al-Zubi M. Effect of hookah (water pipe) smoking on semen parameters. *Andrologia*. 2020;52:e13723.
7. Niu Z-H, Liu J-B, Shi T-Y, Yuan Y, Shi H-J. [Impact of cigarette smoking on human sperm DNA integrity]. *Zhonghua nan ke xue = Natl J Androl*. 2010;16: 300-304.
8. Henkel R, Kierspel E, Stalf T, et al. Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertil Steril*. 2005;83:635-642.
9. Yang H, Chen Q, Zhou N, et al. Lifestyles associated with human semen quality: results from MARHCS cohort study in Chongqing, China. *Medicine*. 2015;94:e1166.
10. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update*. 2007;13:313-327.
11. Rousseaux S, Caron C, Govin J, Lestrat C, Faure A-K, Khochbin S. Establishment of male-specific epigenetic information. *Gene*. 2005;345:139-153.
12. Imani M, Talebi AR, Fesahat F, Rahiminia T, Seifati SM, Dehghanpour F. Sperm parameters, DNA integrity, and protamine expression in patients with type II diabetes mellitus. *J Obstet Gynaecol*. 2021;41:439-446.
13. Yu B, Qi Y, Liu D, et al. Cigarette smoking is associated with abnormal histone-to-protamine transition in human sperm. *Fertil Steril*. 2014;101:51-57.e1.
14. Fawzy IA, Kamal NN, Abdulla AM. Reproductive toxicity of tobacco shisha smoking on semen parameters and hormones levels among adult Egyptian men. *Res J Environ Toxicol*. 2011;5:282-292.
15. Vandekerckhove FW, De Croo I, Gerris J, Vanden Abbeel E, De Sutter P. Sperm chromatin dispersion test before sperm preparation is predictive of clinical pregnancy in cases of unexplained infertility treated with intrauterine insemination and induction with clomiphene citrate. *Front Med*. 2016;3:63.
16. Panner Selvam MK, Agarwal A, Pushparaj PN, Baskaran S, Bendou H. Sperm proteome analysis and identification of fertility-associated biomarkers in unexplained male infertility. *Genes*. 2019;10:522.
17. Gottardo F, Kliesch S. [Semen analysis: spermiogram according to WHO 2010 criteria]. *Urologe A*. 2011;50:101-108.
18. Pourmasumi S, Khoradmehr A, Rahiminia T, Sabeti P, Talebi AR, Ghasemzadeh J. Evaluation of sperm chromatin integrity using aniline blue and toluidine blue staining in infertile and normozoospermic men. *J Reprod Infertil*. 2019;20:95-101.
19. Mostafa RM, Nasrallah YS, Hassan MM, Farrag AF, Majzoub A, Agarwal A. The effect of cigarette smoking on human seminal parameters, sperm chromatin structure and condensation. *Andrologia*. 2018;50:e12910.
20. Kim H-S, Kang MJ, Kim SA, et al. The utility of sperm DNA damage assay using toluidine blue and aniline blue staining in routine semen analysis. *Clin Exp Reprod Med*. 2013;40:23-28.
21. Dehghanpour F, Tabibnejad N, Fesahat F, Yazdinejad F, Talebi AR. Evaluation of sperm protamine deficiency and apoptosis in infertile men with idiopathic teratozoospermia. *Clin Exp Reprod Med*. 2017;44:73-78.
22. Koubaa A, Triki M, Trabelsi H, Baati H, Sahnoun Z, Hakim A. The effect of a 12-week moderate intensity interval training program on the antioxidant defense capability and lipid profile in men smoking cigarettes or hookah: a cohort study. *Sci World J*. 2015;2015:639369.
23. Mosharraf S, Allahdadin M, Reyhani M. Comparison of adverse pregnancy outcomes between hookah and non-smoking women. *J Midwif Reprod Health*. 2019;7:1499-1505.
24. Ansari-Moghaddam A, Rakhshani F, Shahraki-Sanavi F, Mohammadi M, Miri-Bonjar M, Bakhshani N-M. Prevalence and patterns of tobacco, alcohol, and drug use among Iranian adolescents: a meta-analysis of 58 studies. *Child Youth Serv Rev*. 2016;60:68-79.
25. Kim H, Brinkman MC, Sharma E, Gordon SM, Clark PI. Variability in puff topography and exhaled CO in waterpipe tobacco smoking. *Tob Regul Sci*. 2016;2:301-308.
26. Cobb CO, Blank MD, Morlett A, et al. Comparison of puff topography, toxicant exposure, and subjective effects in low- and high-frequency waterpipe users: a double-blind, placebo-control study. *Nicotine Tob Res*. 2015;17:667-674.
27. Primack BA, Carroll MV, Weiss PM, et al. Systematic review and meta-analysis of inhaled toxicants from waterpipe and cigarette smoking. *Public Health Rep*. 2016;131:76-85.
28. Brinkman MC, Kim H, Gordon SM, et al. Design and validation of a research-grade waterpipe equipped with puff topography analyzer. *Nicotine Tob Res*. 2016;18:785-793.
29. Bundhun PK, Janoo G, Bhurtu A, et al. Tobacco smoking and semen quality in infertile males: a systematic review and meta-analysis. *BMC Public Health*. 2019;19:36.
30. Zhang ZH, Zhu HB, Li LL, Yu Y, Zhang HG, Liu RZ. Decline of semen quality and increase of leukocytes with cigarette smoking in infertile men. *Iran J Reprod Med*. 2013;11:589-596.
31. Collodel G, Capitani S, Pammolli A, Giannerini V, Geminiani M, Moretti E. Semen quality of male idiopathic infertile smokers and nonsmokers: an ultra-structural study. *J Androl*. 2010;31:108-113.
32. Agarwal A, Virk G, Ong C, du Plessis SS. Effect of oxidative stress on male reproduction. *World J Mens Health*. 2014;32:1-17.
33. Afsari M, Fesahat F, Talebi AR, et al. ANXA2, SP17, SERPINA5, PRDX2 genes, and sperm DNA fragmentation differentially represented in male partners of infertile couples with normal and abnormal sperm parameters. *Andrologia*. 2022;54:e14556.
34. Badran M, Laher I. Waterpipe (shisha, hookah) smoking, oxidative stress and hidden disease potential. *Redox Biol*. 2020;34:101455.
35. Taati B, Arazi H, Suzuki K. Oxidative stress and inflammation induced by waterpipe tobacco smoking despite possible protective effects of exercise training: a Review of the literature. *Antioxidants*. 2020;9:777.
36. Suleiman I, Mohammed A, Kawu M, Tanko Y, Akor-Dewu M. Maleficent effects of chronic tobacco shisha smoke exposure on sperm DNA fragmentation, count, motility and morphology in adult male wistar rats. *J Afr Assoc Physiol Sci*. 2021;9:48-57.
37. Omolaoye TS, El Shahawy O, Skosana BT, Boillat T, Loney T, du Plessis SS. The mutagenic effect of tobacco smoke on male fertility. *Environ Sci Pollut Res*. 2022;29:62055-62066.
38. Esteves SC, Santi D, Simoni M. An update on clinical and surgical interventions to reduce sperm DNA fragmentation in infertile men. *Andrology*. 2020;8:53-81.
39. Panner Selvam MK, Ambar RF, Agarwal A, Henkel R. Etiologies of sperm DNA damage and its impact on male infertility. *Andrologia*. 2021;53:e13706.
40. Ahmed AS, Aldubayan MA, Ahmed HA, et al. Impact of smoking on heavy metal contamination and DNA fragmentation. *Environ Sci Pollut Res*. 2021;28: 13931-13941.
41. Grèze C, Guttmann A, Pons-Rejraji H, et al. Can the SCD test and terminal uridine nick-end labeling by flow cytometry technique (TUNEL/FCM) be used interchangeably to measure sperm DNA damage in routine laboratory practice? *Basic Clin Androl*. 2019;29:17.
42. Akmal M, Aulanni'am A, Widodo MA, Sumitro SB, Purnomo BB. The important role of protamine in spermatogenesis and quality of sperm: a mini review. *Asian Pac J Reprod*. 2016;5:357-360.
43. Hamad MF, Shelko N, Kartarius S, Montenarh M, Hammadeh ME. Impact of cigarette smoking on histone (H2B) to protamine ratio in human spermatozoa and its relation to sperm parameters. *Andrology*. 2014;2:666-677.
44. Amor H, Zeyad A, Hammadeh ME. Tobacco smoking and its impact on the expression level of sperm nuclear protein genes: H2BFWT, TNP1, TNP2, PRM1 and PRM2. *Andrologia*. 2021;53:e13964.