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Potential effect of tobacco cigarettes smoking on global DNA methylation status and protamines transcripts in human spermatozoa

Mohammed M. Laqqan[1*] and Maged M. Yassin[2]

Abstract

Background: Epigenetics refers to an alteration in gene expression without alteration in the sequence of DNA and this process may be affected by environmental factors and lifestyle like cigarette smoking. This study was designed to evaluate the potential effect of cigarette smoking on the global DNA methylation status and the transcription level of protamine 1 and protamine 2 in human spermatozoa. A total of 188 semen samples were collected from men with a mean age of 34.9 ± 5.8 years old (98 heavy smokers and 90 non-smokers). The DNA and RNA were isolated from purified spermatozoa, then the status of global DNA methylation and the transcription level of protamine 1 and protamine 2 were evaluated using ELISA and qPCR, respectively. The chromatin non-condensation and DNA fragmentation in human spermatozoa were evaluated using chromomycin A3 staining and TUNEL assay, respectively.

Results: A significant increase has been found in the status of global DNA methylation in spermatozoa of heavy smokers compared to non-smokers (7.69 ± 0.69 ng/μl vs. 4.90 ± 0.40 ng/μl, $P < 0.001$). Additionally, a significant reduction has been found in transcription level of protamine 1 (25.49 ± 0.31 vs. 23.94 ± 0.40, $P < 0.001$) and protamine 2 (28.27 ± 0.39 vs. 23.45 ± 0.30, $P < 0.001$) in heavy smokers. A downregulation has been found in the transcription level of protamine 1 and protamine 2 with a fold change of 0.497 and 0.047, respectively. A significant increase has been shown in the level of DNA fragmentation and chromatin non-condensation in heavy smokers compared to non-smokers ($P < 0.001$). On the other hand, a significant positive correlation has been found between sperm chromatin non-condensation, sperm DNA fragmentation, transcription level of protamine 1, transcription level of protamine 2, and global DNA methylation status ($r = 0.304$, $P < 0.001$; $r = 0.399$, $P < 0.001$; $r = 0.216$, $P = 0.003$; $r = 0.494$, $P < 0.001$, respectively).

Conclusion: Tobacco cigarette smoking has a potential influence on the global DNA methylation and the transcription level of protamine genes in human spermatozoa, and consequently, affect negatively on the semen parameters.

Keywords: Global methylation, Heavy smoking, Human spermatozoa, Protamines

* Correspondence: m.laqqan@gmail.com
[1]Faculty of Health Sciences, Department of Medical Laboratory Sciences, Islamic University, Gaza, Palestinian Territories
Full list of author information is available at the end of the article

Background

Epigenetics is defined as an alteration in gene expression without alteration in the sequence of DNA [1]. Epigenetics includes modifications to histone proteins, DNA methylation, and non-coding RNA action [2]. DNA methylation is a major epigenetic modification involving the addition of a methyl (CH_3) group to the fifth position of cytosine nucleotide [3, 4] by DNA methyltransferase (DNMTs) to form 5-methylcytosine (5-mC) [5, 6]. The process of DNA methylation plays a critical role in various cellular processes for example X-chromosome inactivation, genomic imprinting, silencing of transposons, and regulation of gene expression [7]. Several previous studies have reported that gene silencing is associated with an increase in DNA methylation level [8, 9]. Epigenetics may be impacted by lifestyle and environmental factors [10], where cigarette smoking was classified as the most lifestyle that impact the epigenetic and the transcription level of genes [11, 12]. Cigarette smoke can alter DNA methylation through different mechanisms. First, DNA damage and subsequent recruitment of DNMTs [13]. Second, the influence of nicotine on the gene expression level at spermatozoa [14]. Third, it may alter the DNA methylation through the modulation of gene expression and the activity of DNA-binding factor indirectly [15]. On the other hand, the oxidative stress resulting from cigarette smoking can contribute to the loss of DNA methylation by the hydroxylation of 5-methylcytosine to form 5-hydroxymethylcytosine [16]. In addition, reactive oxygen species (ROS) can influence DNA methylation by acting on the activity and expression of DNMTs [17]. Several previous studies have evaluated whether there is an association between the alterations of DNA methylation, the transcription level of protamine in human spermatozoa, and cigarette smoking; one of these studies reported that an increase in the level of reactive oxygen species found in cigarette smoke leads to variation in the transcription level of protamines [18, 19]. Moreover, previous studies have been found a strong association between the reduction in the semen parameters, a decline in the sperm DNA integrity, elevation in the spermatozoa DNA damage, a change in the DNA methylation patterns, and cigarette smoking [20–24]. Another previous study found that the status of DNA methylation and the expression level of genes may be changed by smoking or exposure to cigarette smoke [25]. Nevertheless, people still consume cigarettes on a regular and continuous basis. Finally, the effect of cigarette smoking on the status of global DNA methylation, the transcription level of protamines, and the semen parameters remains a highly controversial issue [26–28].

This study was performed to (I) evaluate the potential effect of cigarette smoking on the status of global DNA methylation in human spermatozoa by comparison between the global DNA methylation level in heavy smokers and non-smokers men, (II) determine whether the transcription level of the protamine 1 (*PRM1*) and protamine 2 (*PRM2*) is different in heavy smokers compared to non-smokers, and to (III) study the correlation between the change in the status of global DNA methylation and the other parameters that were investigated.

Methods

Study population

A total of 188 semen samples were collected between October 2014 and December 2015 from males with a mean age of 34.9 ± 5.8 years. The men were classified into two groups according to the status of cigarette smoking: (I) heavy smokers group (n = 98), those who smoke ≥ 25 cigarettes/day, smoke duration at least 10 years and they still smoking, and (II) non-smokers group, who have never smoked cigarettes in any form during his life (n = 90). The sample size calculations were based on the formula for case-control studies. EPI-INFO statistical package version 3.5.1 was used with 95% CI, 80% power, and 50% proportion as conservative and OR > 2. The exclusion criteria for participation in this study were as follows: diabetes mellitus, alcohol intake, Varicocele, Y chromosome microdeletions, abnormal body mass index, abnormality in hormonal levels, and occupational exposures to known reproductive toxins or excessive heat. In contrast, the inclusion criteria were as follows: males from the same ethnicity and nationality, males have the same food supplementation or a good nutritional status, and non-smokers who have had one child at least.

Sample collection and preparation

The semen samples were collected by masturbation after 3 days of abstinence from sexual intercourse. The samples were allowed to liquefy for 30 min at 37 °C. Then, the Makler Chamber was used to evaluate the sperm count (Sefi-Medica, Haifa, Israel). The semen parameters were analyzed according to the World Health Organization guidelines [29]. All of the semen samples underwent the protocol of Somatic Cell Lysis Buffer (SCLB) to remove the somatic cells before the DNA extraction step from spermatozoa. Briefly, the liquefied semen samples were loaded onto 40% over 90% discontinuous Puresperm gradients (Nidacon International AB, Sweden) and then centrifuged at 500×*g* for 25 min at room temperature. After that, the pure spermatozoa were incubated with SCLB on ice for 30 min and washed three times with phosphate-buffered saline (PBS), and then centrifuged at 500×*g* for 10 min [30, 31]. Finally, the microscopic examination was used to prove the purity of the semen samples from somatic cells and other debris.

Evaluation of sperm chromatin condensation

The chromomycin A3 (CMA3) staining was used to evaluate the chromatin non-condensation at human spermatozoa. Briefly, four semen smears were prepared from each sample and all smears were fixed by using a fixative solution (methanol-glacial acetic acid, 3:1 respectively) at 4 °C for 20 min. The semen smears were air-dried at room temperature. After that, each smear was covered by 50 μl of staining solution (Sigma-Aldrich, St. Louis, MO, USA) and then incubated in a dark place at room temperature for 20 min. The phosphate-buffered saline (PBS) was used to wash all the slides, then the slides were mounted with 1:1 (v/v) glycerol/PBS incubated overnight at 4 °C. To estimate the results of CMA3 staining, the fluorescence microscope (Zeiss Photomicroscope III, Germany) was used to analyze 200 spermatozoa on each smear. Finally, the CMA3 staining was evaluated by differentiating the spermatozoa that stained with bright yellow (positive, bad spermatozoa) from spermatozoa that stained with a dull yellow (negative, good spermatozoa) [32].

Evaluation of DNA fragmentation of human spermatozoa

The DNA fragmentation of spermatozoa (sperm apoptosis) was assessed using the terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay [33]. The TUNEL assay was performed by using the in situ cell death detection kit following the guidelines of the manufacturer company (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, Smears were prepared using 10 μl of sperm suspension on microscope slides and allowed to air-dry and then fixed with 4% paraformaldehyde phosphate-buffered saline, pH 7.4 for 2 h at room temperature, then rinsed with PBS. Smears were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0 for 15 min at room temperature; 50 μl of the TdT-labeled nucleotide mixture (50 μl of enzyme solution and 450 μl of label solution) was added to each slide and incubated in a humidified chamber at 37 °C overnight in the dark. Negative controls without TdT enzyme were run in each replicate. Then, slides were rinsed two times in PBS and left to dry in the air followed by adding 25 μl of 5 μg/ml DAPI stain solution to each slide as a counterstain and then covered by coverslips. For evaluation, a total of 500 spermatozoa were analyzed on each slide, by distinguishing spermatozoa stained bright green (TUNEL positive, fragmented DNA) from those stained dull green (TUNEL negative, with intact DNA). A Zeiss Photomicroscope III was used for the fluorochrome evaluation (Zeiss Photomicroscope III, Germany) [34].

Nucleic acid isolation from human spermatozoa

The DNA and RNA were isolated from the human spermatozoa by using Isolate II DNA/RNA/Protein Kit and all steps were performed according to the guidelines of the manufacturer company (Bioline, UK). The Nanodrop spectrophotometer-2000c (Thermo Scientific, USA) was used to evaluate the purity and the quantity of isolated nucleic acid in order to ensure that the isolated nucleic acid is sufficient and suitable for global sperm DNA methylation and qPCR.

Global DNA methylation

The MethylFlash™ Methylated DNA Quantification ELISA Kit was used to evaluate the level of global DNA methylation (5-methylcytosine) in the human spermatozoa, and all the steps were performed according to the manufacturer's guidelines (Epigentek Group Inc., USA). Briefly, 100 ng of extracted DNA was incubated with the DNA-binding buffer solution at 37 °C for 90 min (the blank, a positive and negative control have been used in triplicate during this assay). After washing the microwell three times, the methylated DNA capture solution was added to each well and incubated for 1 h at room temperature. After that, the detection antibodies were added to each well and incubated at room temperature for 30 min. After washing three times, the developing solution was added to each well and incubated at room temperature in the dark place for 10 min, and at the end of the 10 min, the stop solution was added. The microplate ELISA reader was used to assess the absorbance at 450 nm. The global DNA methylation status (ng) was calculated using the equation: 5-mC(ng) = [(sample OD − blank OD)/100].

Reverse transcription and qPCR

The extracted RNA from human spermatozoa was converted to complementary DNA (cDNA) in a 30-μl reaction volume by using the miScript reverse transcription kit and all procured following the manufacturer's guidelines (Qiagen, Germany). The transcription level of protamine 1 (*PRM1*), protamine 2 (*PRM2*), and the housekeeping gene as a reference gene (*GAPDH*) were evaluated by using the qPCR instrument (7500 Fast applied Bio-systems, USA). The cDNA was used as a template, and all the primers included in this study (QuantiTect Primer) were used according to the guidelines of the manufacturer company (Qiagen, Germany). The reverse transcriptase control (NRT) and template control (NTC) were not involved in runs. All samples were analyzed in triplicate, and all the values of C_t were normalized to *GAPDH*.

Data analysis

The data of this study were analyzed using SPSS version 24.0 (SPSS Inc., USA). All samples included in this study were non-normally distributed (nonparametric) and that depending on the results of the skewness test, the

Kurtosis test, Z value, and Shapiro test. Consequently, the independent-sample t test (Mann–Whitney U test) was used to compare the means of quantitative variables between the study groups. Spearman's test was used to assess the association between the global DNA methylation, the transcription level of protamine, and semen parameters. The comparative ΔC_t method was used separately to calculate the relative RNA quantity in all samples. The ΔC_t was calculated by subtracting the C_t values of *GAPDH* from the C_t values of the target RNA, where ΔC_t = ([C_t RNA of protamine] − [C_t RNA of *GAPDH*]). Then, the $\Delta\Delta C_t$ was calculated by subtracting the mean ΔC_t of the control group from the ΔC_t of the cases group ($\Delta\Delta C_t$ = ΔC_t of heavy smokers − ΔC_t of non-smokers). The fold change of transcription level was calculated by using the following equation: $2^{-\Delta\Delta Ct}$ equation [35]. All the results of the above-mentioned tests were considered statistically significant when $p < 0.05$.

Results

The age of the males included in the study population was between 25.0 and 45.0 years, with a mean age of 34.9 ± 5.8 years. Table 1 illustrates the descriptive characteristics of the study population. As shown in Table 2, a significant decrease has been found in sperm count ($P = 0.003$), percentage of total sperm motility ($P < 0.001$), progressive motility ($P < 0.001$), normal form ($P < 0.001$), and semen volume ($P < 0.001$) in heavy smokers compared to non-smokers males. Conversely, a significant increase has been observed in percentage of sperm non-progressive motility ($P < 0.001$), non-motile ($P < 0.001$), and abnormal form ($P < 0.001$) in heavy smokers compared to non-smoker groups. A significant increase was found in the level of chromatin non-condensation (33.30 ± 2.26 vs. 19.32 ± 1.13; $P < 0.001$) and the level of DNA fragmentation (26.68 ± 2.00 vs. 11.66 ± 1.02; $P < 0.001$) in spermatozoa of heavy smokers compared to non-smokers (Fig. 1).

Global DNA methylation and protamines transcription

As illustrated in Fig. 1, a significant increase has been found in the status of global DNA methylation at spermatozoa of heavy smokers compared to non-smokers (7.69 ± 0.69 ng/μl vs. 4.90 ± 0.40 ng/μl, $P < 0.001$). A significant reduction has been found in heavy smokers compared to non-smokers in the transcription level of protamine 1 (25.49 ± 0.31 vs. 23.94 ± 0.40, $P < 0.001$) and transcription level of protamine 2 (28.27 ± 0.39 vs. 23.45 ± 0.30, $P < 0.001$) (Fig. 2). These results showed a downregulation in the transcription level of protamine 1 and protamine 2 with a fold change of 0.497 and 0.047, respectively, in the heavy smokers compared to the non-smoker (Table 3).

Correlation between the global DNA methylation and semen parameters

Table 4 showed a negative significant correlation between sperm concentration ($r = -0.189$; $P = 0.009$), the percentage of total sperm motility ($r = -0.303$; $P < 0.001$), progressive motility ($r = -0.514$; $P < 0.001$), normal form ($r = -0.498$; $P < 0.001$), and the status of global DNA methylation in human spermatozoa. In contrast, a positive significant correlation has been found between the percentage of sperm non-progressive motility ($r = 0.314$; $P < 0.001$), non-motile sperm ($r = 0.303$; $P < 0.001$), sperm abnormal form ($r = 0.498$; $P < 0.001$), level of chromatin non-condensation ($r = 0.304$; $P < 0.001$), DNA fragmentation level ($r = 0.399$; $P < 0.001$), and the global DNA methylation status.

Correlation between protamines transcription level and semen parameters

As indicated in Table 5, a significant positive correlation has been found between the transcription level of protamine

Table 1 Descriptive characteristics of the study population ($n = 188$)

Variables	Mean	Median	SD	Range
Semen volume (ml)	3.74	3.50	2.06	11.30
Sperm concentration (Mill/ml)	84.17	67.00	63.54	284.00
Total sperm motility (%)	47.51	48.00	21.19	89.00
Sperm progressive motility (%)	27.75	24.00	20.75	80.00
Sperm non-progressive motility (%)	19.76	16.00	13.33	72.00
Non-motile sperm (%)	52.49	52.00	21.19	89.00
Sperm normal form (%)	10.84	6.00	11.48	53.00
Sperm abnormal form (%)	89.16	94.00	11.48	53.00
Sperm chromatin non-condensation (CMA3-positive)	26.61	23.00	19.03	98.00
Sperm DNA fragmentation (TUNEL-positive)	19.49	12.50	17.43	97.00
Global DNA methylation level (ng/μl)	6.36	6.60	1.51	5.10

SD standard deviation

Table 2 Descriptive characteristics of heavy smokers compared to non-smokers (n = 188)

Variables	Heavy smoker (n = 98)	Non-smoker (n = 90)	P value
Semen volume (ml)	3.17 ± 0.15	4.35 ± 0.25	< 0.001
Sperm concentration (Mill/ml)	70.46 ± 5.62	99.10 ± 7.21	0.003
Total sperm motility (%)	40.34 ± 2.05	55.32 ± 2.05	< 0.001
Sperm progressive motility (%)	15.78 ± 1.18	40.79 ± 2.18	< 0.001
Sperm non-progressive motility (%)	24.56 ± 1.48	14.53 ± 0.97	< 0.001
Non-motile sperm (%)	59.66 ± 2.05	44.68 ± 2.05	< 0.001
Sperm normal form (%)	4.32 ± 0.30	17.93 ± 1.37	< 0.001
Sperm abnormal form (%)	95.68 ± 0.30	82.07 ± 1.37	< 0.001

All values presented as mean ± standard error; $P > 0.05$ not significant, $P < 0.05$ significant

1 and protamine 2 with the level of global DNA methylation (r = 0.216, P = 0.003; r = 0.494, P < 0.001, respectively). Moreover, protamine 1 and protamine 2 showed significant positive correlations with percentage of sperm non-progressive motility (r = 0.175, P = 0.016 and r = 0.245, P < 0.001, respectively); non-motile sperm (r = 0.290, P < 0.001 and r = 0.289, P < 0.001); sperm abnormal form (r = 0.464, P < 0.001 and r = 0.502, P < 0.001); level of chromatin non-condensation (r = 0.195, P = 0.007 and r = 0.261, P < 0.001), and level of DNA fragmentation (r = 0.153, P = 0.037 and r = 0.311, P < 0.001, respectively).

In contrast, a significant negative correlation has been observed between the transcription level of protamine 1 and protamine 2 with percentage of total sperm motility (r = − 0.290, P < 0.001 and r = − 0.289, P < 0.001, respectively); progressive motility (r = − 0.425, P < 0.001 and r = − 0.459, P < 0.001); and normal form (r = − 0.464, P < 0.001 and r = -− 0.502, P < 0.001, respectively). However, only

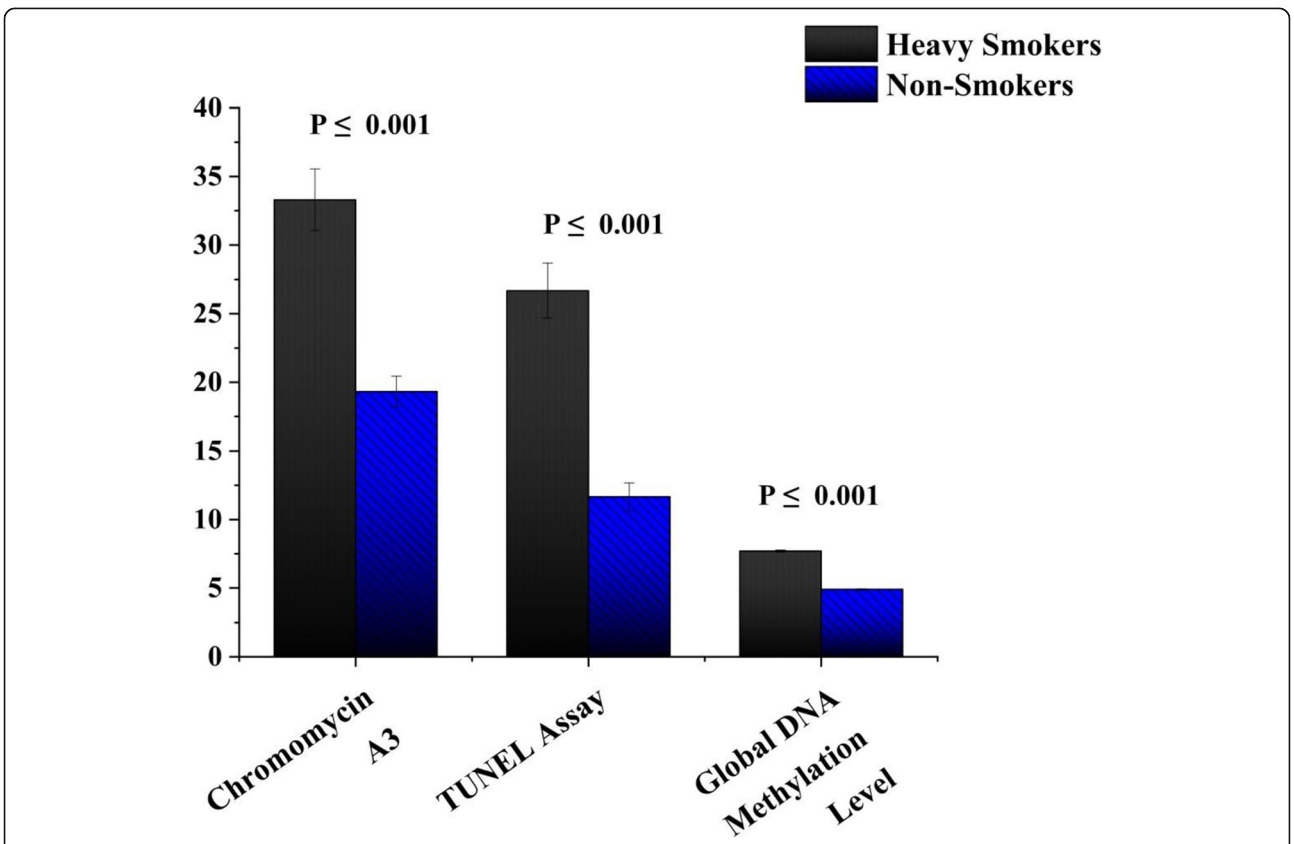

Fig. 1 Global DNA methylation, chromomycin A3, and TUNEL assay in heavy smokers compared to non-smokers. $P > 0.05$: not significant; $P < 0.05$: significant. Chromomycin A3: sperm chromatin non-condensation; TUNEL assay: sperm DNA fragmentation

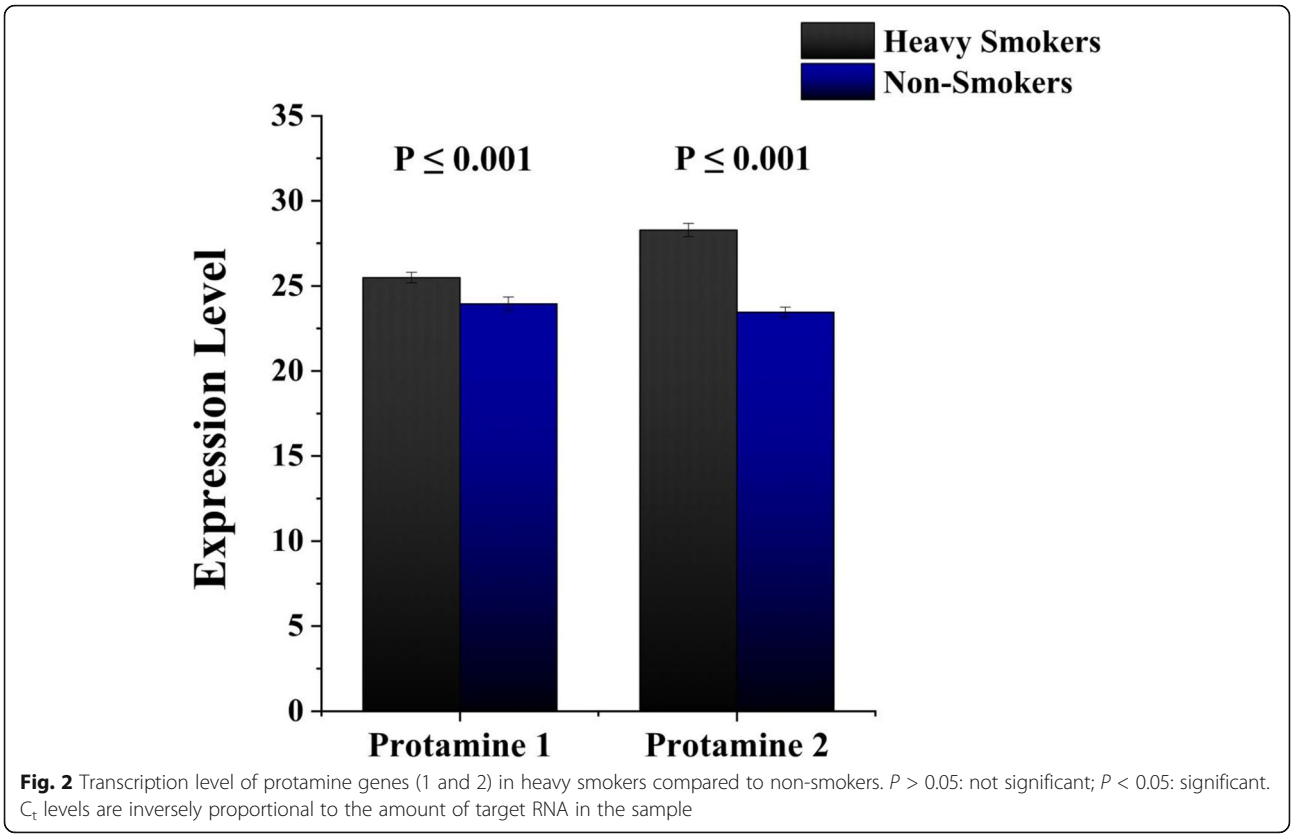

Fig. 2 Transcription level of protamine genes (1 and 2) in heavy smokers compared to non-smokers. $P > 0.05$: not significant; $P < 0.05$: significant. C_t levels are inversely proportional to the amount of target RNA in the sample

protamine 2 transcription level displayed a negative significant correlation with sperm count ($r = -0.215$ and $P = 0.003$).

Discussion

This study was performed to evaluate the potential impact of cigarette smoking on the status of global DNA methylation and transcription level of protamine1 and 2 in human spermatozoa. In humans, many histone variants are expressed during spermatogenesis and modulate the chromatin structure to facilitate the histone-to-protamine replacement to provide a full-function spermatozoon [36]. Consequently, any defect in the process of protamination leads to incorrect packaging of the sperm chromatin and makes the sperm more sensitive to DNA fragmentation, and that occurs through the high levels of ROS which might be coming from cigarette smoking [37]. The results of this study are consistent with previous studies that have found a significant elevation in the level of global DNA methylation in heavy smokers spermatozoa compared to non-smokers spermatozoa [24, 38]. Additionally, another study was shown a significant difference in the methylation level of CpGs that related to the *MAPK8IP* and *TKR* genes in the current smokers compared to the never smokers [39]. On contrary, a previous study pointed out no significant variation in the global DNA methylation level between smokers and non-smokers groups [40]. On the other hand, the present study found a significant variation in the transcription level of protamine genes in heavy smokers compared to non-smokers, and these results are consistent with the study performed by Hamad and his colleagues who observed that the transcription level of protamine genes (*PRM* 1 and *PRM* 2) in smoker males was significantly lower than the non-smokers [41]. Besides, another study has shown an alteration in the level of protamine gene transcription and DNA methylation in males who smoke tobacco [25]. The results of this study have supported the hypothesis proposed by Afanas'ev who pointed that the enzymes that regulate DNA methylation (DNMT) can be activated by tobacco smoking [42]. In the present study, a significant increase was observed in heavy smokers compared to non-

Table 3 Transcription levels of *PRM1* and *PRM2* genes in spermatozoa of heavy smokers compared to non-smokers ($n = 188$)

Variables	*PRM1* (ΔC_t)	*PRM2* (ΔC_t)
Heavy smoker	1.17	3.95
Non-smokers	0.16	− 0.47
ΔΔ C_t	1.01	4.42
Fold change	0.497	0.047
Regulation	Down	Down

ΔC_t delta cycle threshold

Table 4 Correlation between the status of global DNA methylation and semen parameters

Variables	Global DNA methylation level (ng/μl)	
	r	*P* value
Sperm concentration (Mill/ml)	− 0.189	0.009
Total sperm motility (%)	− 0.303	< 0.001
Sperm progressive motility (%)	− 0.514	< 0.001
Sperm non-progressive motility (%)	0.314	< 0.001
Non-motile sperm (%)	0.303	< 0.001
Sperm normal form (%)	− 0.498	< 0.001
Sperm abnormal form (%)	0.498	< 0.001
Sperm chromatin non-condensation (CMA3-positive)	0.304	< 0.001
Sperm DNA fragmentation (TUNEL-positive)	0.399	< 0.001

Spearman's test, *r* correlation coefficient; *P > 0.05* not significant, *P < 0.05* significant

smokers in each of the following parameters: chromatin non-condensation, DNA fragmentation, non-progressive motile, non-motile sperm, and sperm abnormal form, and these findings are matching with the results of previous studies [18, 43, 44]. Additionally, another study found an increase in the rate of cigarette smoking associated with a decline in sperm count, sperm motile, and semen volume [45]. Several studies showed that the substances in the cigarettes may cause insufficiency in spermatogenesis, an increase in the DNA fragmentation in sperm, a reduction in the chromatin condensation, and a decline in sperm count and motility [46, 47]. However, the results of the present study did not match with the previous studies that showed a non-significant increase in the level of DNA fragmentation in smoker males [48, 49]. Conversely, the results revealed a significant decline in heavy smokers compared to non-smokers in the following parameters: sperm count, total motility, progressive motility, and sperm normal form. This agrees with previous studies showing a significant reduction in the semen parameters in smokers males compared to non-smokers [42, 50].

A positive significant association was shown between the global DNA methylation status and semen parameters (sperm non-motility, non-progressive motility, and abnormal form); these results are matching with other studies that observed a positive significant correlation between the variation in DNA methylation level and the semen parameters abnormality [51, 52]. A significant positive correlation has been observed between the chromatin non-condensation, DNA fragmentation, and global DNA methylation level and these results coincide with a study that pointed out that, the increase in the methylation level at spermatozoa may lead to an increase the spermatozoa chromatin instability [53]. In contrast, previous studies observed a negative correlation between chromatin abnormalities and the change in sperm DNA methylation [54, 55]. One of the possible explanations for these findings is that the alterations in the global DNA methylation level under the effect of cigarette smoke lead to less chromatin compaction in spermatozoa. Consequently, the rate of sperm DNA fragmentation and sperm DNA exposure to damage will be increased.

On the other hand, a significant positive correlation has been found between protamines transcription and the status of global DNA methylation and these findings are in agreement with previous studies that found a significant relationship between the level of DNA methylation and genes transcription level at human spermatozoa [56, 57]. Conversely, other studies did not support this type of association [58, 59]. A significant correlation has

Table 5 Correlation between the transcription level of protamines and semen parameters

Variables	*PRM1* transcription level		*PRM2* transcription level	
	r	*P* value	r	*P* value
Sperm concentration (Mill/ml)	− 0.139	0.057	− 0.215	0.003
Total sperm motility (%)	− 0.290	< 0.001	− 0.289	< 0.001
Sperm progressive motility (%)	− 0.425	< 0.001	− 0.459	< 0.001
Sperm non-progressive motility (%)	0.175	< 0.016	0.245	< 0.001
Non-motile sperm (%)	0.290	< 0.001	0.289	< 0.001
Sperm normal form (%)	− 0.464	< 0.001	− 0.502	< 0.001
Sperm abnormal form (%)	0.464	< 0.001	0.502	< 0.001
Sperm chromatin non-condensation (CMA3-positive)	0.195	0.007	0.261	< 0.001
Sperm DNA fragmentation (TUNEL-positive)	0.153	0.037	0.311	< 0.001
Global DNA methylation level (ng/μl)	0.216	0.003	0.494	< 0.001

Spearman's test; *r* correlation coefficient, *P > 0.05* not significant, *P < 0.05* significant

been found between *PRM* 1, *PRM* 2 transcription levels, and the semen parameters (sperm count, total sperm motility, progressive motility, and sperm normal form), and these results are in the line with the previous studies that showed a negative significant association between the *PRM* 1/*PRM* 2 mRNA ratios and the same semen parameters [19, 60]. Therefore, all of these significant associations between the global DNA methylation, the transcription level of *PRM* 1, *PRM* 2, and semen parameters in smoker men support the hypothesis proposing that tobacco smoking has a negative influence on the semen parameters and might cause alterations in global DNA methylation and in the transcription levels of protamines which can affect the functions and capacities of spermatozoa.

Conclusion

The results of this study offer proof that tobacco cigarette smoking has a potential influence on the global DNA methylation and the transcription level of protamine genes in human spermatozoa, and consequently, impact negatively on the semen parameters.

Abbreviations

ROS: Reactive oxygen species; DNMTs: DNA methyltransferase; TNP: Transition nuclear proteins; *PRM* 1: Protamine 1; *PRM* 2: Protamine 2; SCLB: Somatic cell lysis buffer; PBS: Phosphate-buffered saline; CMA3: Chromomycin A3 staining; NRT: No reverse transcriptase control; NTC: No template control

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Authors' contributions

M. M. L collected and processed the samples and data analysis, and he was a major contributor in writing the manuscript. M. M. Y performed a review for data analysis, discussion preparation, and the writing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Palestinian Health Research Council (Reference No. PHRC/HC/13/14) and consent was provided according to the Declaration of Helsinki Committee. Besides, all participants signed an informed approval form to participate in this study. The samples were analyzed according to the guidelines and standard procedures of Al Basma Fertility Center.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Faculty of Health Sciences, Department of Medical Laboratory Sciences, Islamic University, Gaza, Palestinian Territories. [2]Faculty of Medicine, Department of Physiology, Islamic University, Gaza, Palestinian Territories.

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