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Tobacco smoking and its impact on the expression level of sperm nuclear protein genes: *H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2*

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Abstract

The aim of this current study was to investigate the influence of tobacco smoke on sperm quality determined by standard parameters, on sperm DNA maturity tested by chromomycin A3 (CMA3) staining, on sperm DNA fragmentation tested by TUNEL assay and on the transcript level of sperm nuclear proteins *H2BFWT*, *PRM1*, *PRM2*, *TNP1* and *TNP2* genes quantified by RT-PCR. One hundred forty-one (141) sperm samples (43 nonsmokers (G.1) and 98 heavy smokers (G.2)) of couples undergoing ICSI were enrolled in this study. In G2, a significant decrease in standard semen parameters in comparison with nonsmokers was shown ($p < .01$). In contrast, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) were significantly higher in G2 than in G1 ($p < .01$). Furthermore, the studied genes were differentially expressed ($p < .01$), down-regulated in the spermatozoa of G.2 compared to that of G.1 (fold change <0.5) and were significantly correlated between each other ($p < .01$). Moreover, in comparison with G1, the protamine mRNA ratio in G2 was significantly higher ($p < .01$). It can therefore be concluded that smoking alters mRNA expression levels of *H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2* genes and the protamine mRNA ratio and consequently alters normal sperm function.

KEYWORDS
male infertility, sperm DNA, sperm nuclear proteins, tobacco, transcript level

1 | INTRODUCTION

Spermatogenesis is an epigenetically elevated controlled process and any interruption at any phase might be the cause of male infertility (Das et al., 2017). Progressive protamination of the sperm genome during spermiogenesis leads to the elimination of histone-carrying epigenetic signals. Protamination thus plays a part in the epigenetic regulation of the spermatozoa and any environmental factor affecting protamination may be considered as an epigenetic signal, such as DNA methylation and histone modification, influencing the transcription regulation after fertilisation (Donkin & Barrès, 2018).

In addition, human spermatozoa carry a different kind of mRNA molecules (>5,000 types) containing at least 100 miRNAs (Castillo et al., 2015; Güneş & Kulaç, 2013). Until now, the exact function of these mRNA molecules is still not clear because the protein synthesis is disabled in spermatozoa (Savadi-Shiraz et al., 2015). A number of studies have demonstrated that the sperm transcript accompanies the paternal genome throughout fertilisation and consequently affects the early embryo development (Jodar et al., 2013; Sendler et al., 2013).

The H2B histone family member W, testis-specific (*H2BFWT*) is one of two H2B variants that are present in the male gamete. The only information known about *H2BFWT* is that this histone variant's main function is the facilitation of the transition histone–protamine and the epigenetic control of gene transcription and its association with telomeres, which suggests it plays a putative role in fertilisation during early chromatin remodelling (Churikov, Siino, et al., 2004).

Moreover, the transition nuclear proteins also play an important role as intermediaries in histone–protamine replacement in sperm chromatin. The transition protein 1 gene (*TNP1)* is localised on chromosomes 2, and the code for transition protein TP1, which is important in DNA repair mechanisms, and in the reduction of the interaction DNA-nucleosome core, in addition to transition protein 2 (TP2), are all needed for the complete editing of protamine P2 (Meistrich et al., 2003). In contrast to *TNP1*, the transition protein 2 gene (*TNP2)* expression has been demonstrated to be different between mammals. TP2 is a DNA-compacting protein important for the initiation of the binding of protamine with sperm DNA (Boissonneault, 2002).

The protamine 1 gene (*PRM1)* and protamine 2 gene (*PRM2)* are the protamine genes which are located beside *TNP2*, the transition protein 2 gene, on chromosome 16: 16p13.3 forming a multigenic cluster (Oliva, 2006). This cluster is bordered by the matrix attachment regions (MARs) containing repetitive alanine elements that present at sites of methylation. These MARs are important for the appropriate regulation of protamine gene expression, independent of the methylation state (Schmid et al., 2001).

Gene silencing is mediated by methylation and its activation is mediated by hypomethylation, which permits binding between the nuclear matrix and chromatin, thereby retaining a targeted opening of the chromatin domains, known as potentiation (Schmid et al., 2001). It has been reported that in late pachytene spermatocytes, the protamine cluster is potentiated and then transcribed later in round spermatids (Martins & Krawetz, 2007b). *PRM1-PRM2-TNP2* gene loci include a TATA-box, which is important for the transcription initiation by facilitating the bind between the transcription factors and the promoter. The transcription is also regulated by binding between cAMP response elements (CRE) and a number of CRE proteins (Oliva, 2006).

Once transcribed, protamine transcripts are saved as ribonucleoproteins (RNPs), which are translationally repressed and translated later in elongated spermatids (Castillo et al., 2015).

We have, therefore, focused on the relative quantification of *H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2* gene expression and investigated the influence of tobacco smoke on the transcript level of these sperm nuclear proteins. Moreover, we have also assessed its influence on sperm DNA quality and maturity.

2 | METHODS AND MATERIALS

2.1 | Sample handling

This study was approved by the locally appointed Ethics Committee [Institutional Review Board (Number: 195/11)]. Informed consent was obtained from all patients involved in this study.

Semen (n = 141) was collected from male partners of couples undergoing intracyptoplasmic sperm injection treatment (ICSI) in the assisted reproduction laboratory (Arryan Hospital, Riyadh, Saudi Arabia).

The individuals were of reproductive age (25–49 years). Each patient had a physical examination and was interviewed following their completion of a set questionnaire (about Medical History, Job, Lifestyle and tobacco Smoke). Heavy smokers were males who smoked at least one pack a day for 10 years or 2 packs a day for 5 years at least, and nonsmokers were males who did not smoke.

The sample was kept at the heating stage for 30 min, resulting in liquefaction at 37°C. After that, the sperm samples were evaluated according to the WHO laboratory manual (WHO, 2010).

Patients who had genetic disorders, such as Y-chromosome microdeletion or Klinefelter's syndrome, cryptorchidism, hypogonadism (a hormonal disorder), present or past cancer treatment, varicocele, drug abuse, and female partners with any history of subfertility were excluded from this study.

2.2 | Evaluation of sperm DNA quality

Semen smears were prepared for a sperm DNA integrity assessment. Then, Chromomycin A3 (CMA3) staining was used to evaluate the condensation of the sperm DNA, as described by Manicardi et al. (1995).

The slides were fixed with acetic acid-methanol (1:3) for 1 hr. 25 μl of CMA3 stain solution was added to each slide and kept for 30 min at room temperature (RT) in the dark. After washing with phosphate-buffered saline (PBS), the slides were mounted and then kept overnight at 4°C in the dark. From each slide, 500 spermatozoa were evaluated, using a fluorescence microscope (Olympus, Japan). Spermatozoa were classified as follows: bright green spermatozoon (CMA3 positive) had low protamination, and dull green spermatozoa (CMA3 negative) had normal protamination (Amor et al., 2019).

The terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) test was used for the sperm DNA fragmentation assessment (Borini et al., 2006). The different steps in this assay were performed according to the manufacturing company's instructions of the in situ cell death detection kit fluorescein (Roche Diagnostics GmbH, Germany). 500 spermatozoa were evaluated from each slide using a fluorescence microscope (Olympus, Japan). Spermatozoa were classified as follows: green stained spermatozoa (TUNEL-positive), and blue stained spermatozoa (TUNEL-negative; Amor et al., 2019).

2.3 | RNA isolation from human spermatozoa

Before RNA isolation, each sample was treated by discontinuous Puresperm gradients (40%–80%) (Nidacon International, Sweden). Then, to guarantee the elimination of somatic cells, the samples were washed with a lysis buffer (0.1% SDS, 0.5% Triton X-100 in double-distilled water).

The total RNA was isolated from the purified semen samples, according to a modified protocol of the Isolate II RNA/DNA/Protein Kit (Phenol-free) (Bioline, UK). Then, the purity and the quantity of the isolated RNA were checked by determining the spectrophotometric ratio of absorbance at 260/280 nm using the NanoDrop spectrophotometer ND-2000c (Thermo Scientific, USA). The integrity of the isolated RNA was checked on an RNA Nano 6,000 chip via an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.4 | Reverse transcription and quantitative PCR

A real-time quantitative PCR (RT-qPCR) technique was used for the quantification of the expression level of the five studied genes, namely the H2B histone family member W, the testis-specific (H2BFWT), the transition protein 1 (TNP1), the transition protein 2 (TNP2), the protamine 1 (PRM1), the protamine 2 (PRM2) and the reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The total RNA was converted into cDNA in a 30 μl reaction volume using a miScript reverse transcription kit (Qiagen, Germany); all the procedures were carried out according to manufacturers' recommendations.

In brief, 300 ng of the isolated RNA was mixed with a miScript HiFlex Buffer (5×), a miScript Reverse transcriptase mix, a miScript nucleic mix and RNase free water. The mix was incubated in a thermocycler for 60 min at 37°C, then for 5 min at 95°C to inactivate the transcriptase mix.

Quantitative PCR (qPCR) was performed using a StepOnePlus™ System (7500 Fast Applied Biosystems, USA).

The cDNA produced was used as the template for qPCR analysis, which was performed using a SYBR Green and QuantiTect primer assay (Qiagen, Germany), according to the manufacturers' recommendations. In addition, a no template control (NTC) and a no reverse transcriptase control (NRT) were included in each run. All qPCR experiments were performed in triplicate and the resulting Ct values were normalised to GAPDH.

2.5 | Statistical analysis

IBM SPSS for the Windows software package version 24.0 (SPSS Inc., USA) was used to analyse the data obtained in the current study. After application of the skewness test, the Kurtosis test, and the *Z*-value and Shapiro test, it was obvious that the samples were not normally distributed. Thus, for the comparison of the quantitative variables between the heavy-smoker and nonsmoker groups, the Mann–Whitney *U* test was used, and for the determination of the correlation between the different studied parameters, the Spearman test was applied.

The relative RNA quantity in the samples of this study was calculated separately by using the comparative ΔCt method. The threshold cycle (Ct) reflects the cycle number at which the fluorescence curve is generated within the reaction across the threshold for qPCR. The ΔCt was calculated by subtracting the Ct values of GAPDH from the Ct values of the RNA of interest, where ΔCt = [(Ct RNA of interest) − (Ct RNA of GAPDH)]. The ΔΔCt was then calculated by subtracting the mean ΔCt of the control groups from the ΔCt of the case groups, where ΔΔCt = [(ΔCt cases − ΔCt controls)]. The fold change cut-off for the mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ equation (Livak & Schmittgen, 2001). The protamine *PRM1*: *PRM2* mRNA ratio was calculated, as described previously by Steger et al., 2003.

3 | RESULTS

On comparing the studied parameters between the heavy smokers and nonsmokers (Table 1), we found that total motility and concentration were significantly higher in the group of nonsmokers ($p = .026$ and $p = .014$ respectively) and similar findings were noted for normal morphology and progressive motility ($p \leq .0001$; Table 1).

Furthermore, in the group of nonsmokers, the mean percentage of CMA3 positivity in the heavy smokers group (33.30 ± 22.33) was significantly higher than that of CMA3 positivity in nonsmokers group (20.35 ± 13.34; $p \leq .001$). Similarly, the sDF was significantly higher in the heavy smokers group in comparison with the difference in the nonsmokers group (26.68 ± 19.77 versus 14.23 ± 13.07; $p \leq .0001$ respectively; Table 1).

On the other hand, the relative amounts of the investigated genes mRNA (mean delta ct) (*H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2*) were differentially expressed among the compared groups. This difference between the group of nonsmokers and the group of heavy smokers was highly significant ($p < .01$; Table 1). Furthermore, the protamine mRNA ratio was also significantly elevated in the case group in comparison with that in the control group (0.60 ± 1.08 versus 0.11 ± 0.84; $p = .001$; Table 1).

Since the correlation between delta Ct (ΔCt) and the gene expression level was contradictory, the higher delta Ct values indicated that the gene expression was decreased. This is shown in Table 2 where the *H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2* were down-regulated (fold change <0.5).

3.1 | Correlation between the expression levels of the different studied genes and sperm parameters

Sperm parameters of the nonsmoker group showed no correlation with the relative expression levels (ΔCt) of the *PRM1*, *PRM2*, *TNP1*, *TNP2* and *H2BFWT* genes (Table 3).

However, in the heavy-smoker group, the expression levels of *PRM1* and *TNP1* genes correlated positively with the sperm concentration ($r = .335$, $p = .001$ and $r = .391$, $p \leq .0001$ respectively), total motility ($r = .203$, $p = .045$ and $r = .238$, $p = .018$ respectively), progressive motility ($r = .337$, $p = .001$ and $r = .361$, $p \leq .0001$ respectively) and morphologically normal spermatozoa ($r = .214$, $p = .035$ and $r = .294$, $p = .003$ respectively), while the expression level of *PRM2*

TABLE 1 Comparison of the studied parameters between heavy smokers and non-smokers groups

Parameters	Heavy smokers ($n = 98$)	Nonsmokers ($n = 43$)	p-Value
Semen volume (ml)	3.00 (0.70–9.0)	3.00 (1.20–8.30)	.181
Sperm concentration (10^6 per ml)	58.00 (2.00–209.0)	95.00 (9.00–286.0)	.014*
Total motility (PR + NP %)	39.00 (3.00–86.0)	49.00 (2.00–91.0)	.026*
Progressive motility (PR %)	15.00 (0–50)	31.00 (0–80.0)	.0001**
Morphologically normal spermatozoa (%)	4.00 (0–13)	7.00 (0.00–53.0)	.0001**
CMA3 positivity (%)	29.00 (0–98)	16.00 (0–63)	.001**
Sperm DNA fragmentation (sDF) (%)	22.50 (0–97)	10.00 (2–60)	.0001**
ΔCt H2BFWT	14.27 (2.77–22.69)	9.83 (1.55–23.5)	.001**
ΔCt TNP1	1.88 (−4.17–4.35)	0.06 (−4.87–3.0)	.001**
ΔCt TNP2	9.53 (2.03 ± 20.0)	5.75 (−32.09 −15.7)	.0001**
ΔCt PRM1	1.11 (−5.39–7.39)	−0.69 (−6.38–2.9)	.002**
ΔCt PRM2	1.50 (−4.97– 9.9)	−0.85 (−5.58–4.9)	.001**
Protamine (P1–P2) mRNA ratio	0.65 (−2.76– 4.8)	0.16 (−2.88–2.2)	.001**

Note: Results are expressed as median values and interquartile range (25th–75th percentile).

Abbreviation: Spz, spermatozoa.

**p-Value is statistically high significant at the .01 level.

*p-Value is statistically significant at the .05 level.

TABLE 2 Mean expression levels (delta Ct) of H2BFWT, TNP1, TNP2, PRM1 and PRM2 genes from spermatozoa in the case group (heavy smokers) compared to the control group (non-smokers)

Genes	Mean delta Ct Nonsmokers	Mean delta Ct Heavy smokers	Fold change (FC)	Log_2 fold change	Regulation
H2BFWT	10.75	13.35	0.16	−2.6	Down
TNP1	−0.04	1.17	0.43	−1.21	Down
TNP2	5.61	9.52	0.06	−3.91	Down
PRM1	−0.64	0.66	0.40	−1.3	Down
PRM2	−0.53	1.25	0.29	−1.78	Down

TABLE 3 Correlation between the mRNA relative amount of the studied genes and sperm parameters in the control group (non-smokers, $n = 43$)

Expression level		Semen volume (ml)	Sperm concentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologically normal spermatozoa (%)
ΔCt PRM1	*r*	−.142	−.035	−.062	−.146	−.143
	p	.363	.821	.693	.352	.362
ΔCt PRM2	*r*	−.197	−.036	−.072	−.164	−.166
	p	.205	.817	.645	.293	.288
ΔCt TNP1	*r*	−.184	.079	−.044	−.175	−.241
	p	.237	.615	.778	.262	.119
ΔCt TNP2	*r*	−.176	.064	.013	−.077	−.120
	p	.290	.704	.937	.645	.473
ΔCt H2BFWT	*r*	−.177	−.075	.179	.251	.150
	p	.274	.644	.270	.119	.356

correlated as being significantly positive to the sperm count ($r = .329$, $p = .001$) and the progressive motility ($r = .338$, $p = .001$; Table 4).

Moreover, the relative expression level of *TNP2* showed a high-positive correlation with the sperm count ($r = .369$, $p = .001$), the progressive motility ($r = .359$, $p = .001$) and the morphologically normal sperm form ($r = .303$, $p = .004$; Table 4).

In addition, a significant positive correlation was shown between the relative amount of *H2BFWT* mRNA and the mean percentage of sperm with progressive motility ($r = .230$, $p = .027$; Table 4).

3.2 | Correlation between the expression levels of the investigated genes and protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF)

By examining the correlation between the mRNA relative amount of the studied genes (*TNP1*, *TNP2*, *PRM1*, *PRM2* and *H2BFWT*), and protamine deficiency (CMA3 positivity) as well as sperm DNA fragmentation (sDF), no correlation was shown either in nonsmokers (Table 5) or in heavy smokers (Table 6). However, in the heavy smokers' group (Table 6), the protamine mRNA ratio showed a high-positive correlation with both the CMA3 positivity ($r = .413$, $p \leq .0001$) and the sDF ($r = .302$, $p = .003$).

3.3 | Correlation between the relative expression levels of the studied genes: *H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2*

As shown in Table 7, the relative expression level of the *H2BFWT* showed a positive correlation with the relative amount of *TNP2* mRNA ($r = .487$, $p = .003$) in the nonsmokers' group.

Moreover, the relative expression level of *TNP1* showed a highly positive correlation with the expression levels of *TNP2*, *PRM1* and *PRM2* ($r = .737$, $r = .920$, $r = .887$; $p \leq .001$).

The *TNP2* relative expression level correlated positively with the relative amount of *PRM1* and *PRM2* ($r = .731$; $r = .709$ respectively, $p \leq .0001$).

The correlation between the *PRM1* and *PRM2* expression levels was highly positive and significant ($r = .961$, $p \leq .0001$).

In addition, the protamine mRNA ratio correlated positively with the expression levels of *TNP2* ($r = .349$, $p = .032$) and *PRM2* ($r = .488$, $p = .001$).

In the heavy smokers' group (Table 8), the *H2BFWT* expression level showed a significant positive correlation with the transition proteins 1 (*TNP1*) and 2 (*TNP2*) relative expression levels ($r = .357$, $p \leq .0001$ and $r = .354$, $p = .001$ respectively).

Moreover, a positive correlation with protamines 1 (*PRM1*) and 2 (*PRM2*) and an mRNA relative amount was found ($r = .254$, $p = .014$ and $r = .398$, $p \leq .0001$ respectively).

The relative amount of TNP1 gene mRNA demonstrates a highly significant positive correlation ($p < .001$) with the following mRNA relative amounts of *TNP2* ($r = .814$), *PRM1* ($r = .859$) and *PRM2* ($r = .822$).

Similarly, the *TNP2* gene mRNA's relative amount showed a highly positive correlation ($p \leq .0001$) with the expression level of *PRM1* ($r = .903$) and *PRM2* ($r = .887$).

The *PRM1* and *PRM2* expression levels also correlated positively significant with each other ($r = .926$, $p \leq .0001$).

In contrast to the group of nonsmokers, in the heavy smokers' group, the protamine mRNA ratio correlated as significantly positive to *TNP2* mRNA relative amount ($r = .307$, $p = .004$), *PRM2* mRNA relative amount ($r = .445$, $p \leq .0001$) and to *H2BFWT* mRNA relative amount ($r = .342$, $p = .001$; Table 8).

4 | DISCUSSION

4.1 | Smoking and sperm DNA damage

In the last 10 years, various research group were focused on the mechanisms by means of which different lifestyles and

TABLE 4 Correlation between the mRNA relative amount of the studied genes and sperm parameters in the case group (heavy smokers, $n = 98$)

Expression level		Semen volume (ml)	Sperm concentration (10^6 per ml)	Total motility (PR + NP. %)	Progressive motility (PR. %)	Morphologically normal spermatozoa (%)
ΔCt PRM1	*r*	−.008	**.335****	**.203***	**.337****	**.214***
	p	.940	**.001**	**.045**	**.001**	**.035**
ΔCt PRM2	*r*	−.028	**.329****	.187	**.338****	.166
	p	.782	**.001**	.066	**.001**	.103
ΔCt TNP1	*r*	−.076	**.391****	**.238***	**.361****	**.294****
	p	.458	**.0001**	**.018**	**.0001**	**.003**
ΔCt TNP2	*r*	−.027	**.369****	.197	**.359****	**.303****
	p	.805	**.0001**	.067	**.001**	**.004**
ΔCt H2BFWT	*r*	−.135	.058	.064	**.230***	.084
	p	.199	.581	.543	**.027**	.421

**Correlation is significant at the .01 level.

*Correlation is significant at the .05 level.

TABLE 5 Correlation between the mRNA relative amount of the studied genes, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the control group (non-smokers, $n = 43$)

Expression level		CMA3 positivity (%)	sDF (%)
ΔCt PRM1	*r*	−.135	.222
	p	.387	.153
ΔCt PRM2	*r*	−.134	.230
	p	.390	.138
ΔCt TNP1	*r*	−.179	.133
	p	.250	.397
ΔCt TNP2	*r*	−.171	.039
	p	.305	.814
ΔCt H2BFWT	*r*	−.038	−.023
	p	.814	.887
Protamine (P1–P2) mRNA ratio	*r*	−.091	.100
	p	.560	.524

TABLE 6 Correlation between the mRNA relative amount of the studied genes, protamine deficiency (CMA3 positivity) and sperm DNA fragmentation (sDF) in the case group (heavy smokers, $n = 98$)

Expression level		CMA3 positivity (%)	Sperm DNA fragmentation (sDF) (%)
ΔCt PRM1	*r*	.036	−.055
	p	.722	.587
ΔCt PRM2	*r*	.165	.069
	p	.104	.499
ΔCt TNP1	*r*	.002	−.008
	p	.982	.935
ΔCt TNP2	*r*	.029	−.039
	p	.789	.721
ΔCt H2BFWT	*r*	.076	−.002
	p	.469	.983
Protamine (P1–P2) mRNA ratio	*r*	**.413****	**.302****
	p	**.0001**	**.003**

**Correlation is significant at the .01 level.

environmental factors, such as tobacco smoking, influence the sperm epigenome and genome (Harlev et al., 2015) and also have a potential effect on the developing embryo (Beal et al., 2017; Donkin & Barrès, 2018).

In fact, cigarette smoke has been associated with high levels of seminal reactive oxygen species (ROS), leading to oxidative sperm DNA damage (Hammadeh et al., 2010; Kumar et al., 2015; La Maestra et al., 2015; Opuwari & Henkel, 2016). The contents of the cigarette have been reported to have a correlation with the formation of the DNA adduct, causing sperm DNA damage (Perrin et al., 2011; Phillips & Venitt, 2012).

The CMA3 positivity and the sDF were higher in the heavy smokers' group ($p \leq .001$; Table 1).

These results are in accordance with various studies that determined sperm DNA fragmentation, using different techniques. The majority of study groups using a TUNEL assay concluded that the DNA fragmentation levels in nonsmokers were lower than those in heavy smokers (Aydin et al., 2013; Hammadeh et al., 2010). The same conclusions were reached by others using various assays to evaluate the sperm DNA fragmentation (Cui et al., 2016; El-Melegy & Ali, 2011; Mitra et al., 2012; Taha et al., 2014). However, some contradictory studies demonstrated that there was no correlation between smoking and DNA damage (Bojar et al., 2013; Bounartzi et al., 2016).

4.2 | The relative expression level of the studied genes

To our knowledge, this is in fact the first study to examine the relative amount of the *H2BFWT* gene and its correlation with the relative amount of the transition proteins and protamine genes.

The findings of the present study are in agreement with other studies which reported that *H2BFWT* is synthesised in the human testes and combined to form sperm nuclei during spermatogenesis (Churikov, Siino, et al., 2004; Wu et al., 2015) and to be associated with chromatin compaction during spermiogenesis (Gineitis et al., 2000).

The *H2BFWT* and *TNP2* gene expression levels correlation (Table 7) indicate an association between these two proteins and the probability that this testis-specific histone may be replaced by TP2 during chromatin condensation, but further studies are still needed to fully understand this synchronisation.

The correlation between the expression levels of *TNP1*, *TNP2*, *PRM1* and *PRM2* (Table 7) is in accordance with the results of other studies indicating that during chromatin remodelling the transition proteins TP1 and TP2 are supplanted by the protamines P1 and P2 (Bao & Bedford, 2016; Rathke et al., 2014).

In fact, transition protein and protamine mRNA are similarly expressed in high quantities in round spermatids (Balhorn, 2007; Oliva, 2006) and their protein has a significant presence in the nuclei of elongating spermatid (Meistrich et al., 2003; Wu et al., 2000). Moreover, a deletion of *TNP1* or *TNP2* leads to an alteration in *PRM2* and consequently to a defect in chromatin condensation (Yu et al., 2000; Zhao et al., 2001).

The relative proportion of protamine quantities P1 and P2 has been proposed in many studies as a biomarker of the maturity and integrity of the sperm chromatin (Amor et al., 2019; Hammadeh et al., 2010).

The protamine mRNA ratio, in the nonsmokers' group, correlated positively with the expression levels of *TNP2* ($p = .032$) and *PRM2* ($p = .001$; Table 7). (P1/P2) deregulation is correlated with P2, more than P1 deregulation is (Barrachina et al., 2018; Hammadeh et al., 2010).

TABLE 7 Correlation between the mRNA relative amount of the studied genes in the control group (non-smokers, $n = 43$)

Expression level		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
ΔCt PRM1	*r*	1.000	**.961****	**.920****	**.731****	.257	.299
	p		**.0001**	**.0001**	**.0001**	.110	.052
ΔCt PRM2	*r*	**.961****	1.000	**.887****	**.709****	.237	**.488****
	p	**.0001**		**.0001**	**.0001**	.141	**.001**
ΔCt TNP1	*r*	**.920****	**.887****	1.000	**.737****	.236	.294
	p	**.0001**	**.0001**		**.0001**	.142	.055
ΔCt TNP2	*r*	**.731****	**.709****	**.737****	1.000	**.487****	**.349***
	p	**.0001**	**.0001**	**.0001**		**.003**	**.032**
ΔCt H2BFWT	*r*	.257	.237	.236	**.487****	1.000	.229
	p	.110	.141	.142	**.003**		.155

**Correlation is significant at the .01 level.

*Correlation is significant at the .05 level.

The correlation between the protamine mRNA ratio and *TNP2* can be clarified by the fact that the *TNP2* gene and *PRM2* gene is simultaneously regulated and expressed because they are in the same locus (Oliva & Castillo, 2011).

H2BFWT, *TNP1*, *TNP2*, *PRM1* and *PRM2* genes were down-regulated in the spermatozoa of heavy smokers compared to that of nonsmokers (fold change <0.5; Table 2).

Moreover, the protamine mRNA ratio, in the current study, was significantly higher in the heavy smokers in comparison with the nonsmokers ($p = .001$; Table 1). Moreover, the protamine mRNA ratio in heavy smokers correlated with the CMA3 positivity ($p \leq .0001$) and sDF ($p = .003$; Table 6).

Our results confirm the previous studies in our laboratory carried out by Hammadeh et al. (2010), who disclosed that the protamine 2 (P2) was under-expressed in the group of smokers in comparison with the nonsmokers' group and that the protamine ratio (P1/P2) was significantly elevated in the smokers' group.

Furthermore, they found that the levels of 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde (MDA), reactive oxygen species (ROS) and cotinine correlated significantly and were significantly lower in the group of nonsmokers in comparison with the smokers. Protamine P2 concentration and 8-OHdG correlated negatively and the protamine ratio (P1/P2) correlated positively with 8-OHdG, thereby confirming the association between DNA oxidative damage caused by smoking and protamination abnormalities in sperm chromatin (Hammadeh et al., 2010). Hamad et al. (2017) also proved that smoking has negative effects on protamine *PRM1* and that the *PRM2* gene expression and protamine transcript ratio were also significantly higher in the smokers' group (Hamad et al., 2017).

Other studies have demonstrated that *PRM1* and *PRM2* transcripts levels were low in asthenozoospermic, oligozoospermic (Aoki et al., 2005; Kempisty et al., 2007) and teratozoospermic cases (Savadi-Shiraz et al., 2015) and also generally in infertile men (Jodar et al., 2013) in comparison with normozoospermic men. Moreover, others are of the opinion that the protamine transcript ratio can be adapted to discriminate between fertile and infertile men (Depa-Martynow et al., 2012; Rogenhofer et al., 2013) and

TABLE 8 Correlation between the mRNA relative amount of the studied genes in the case group (heavy smokers, $n = 98$)

Expression level		ΔCt PRM1	ΔCt PRM2	ΔCt TNP1	ΔCt TNP2	ΔCt H2BFWT	Protamine (P1–P2) mRNA ratio
ΔCt PRM1	*r*	1.000	**.926****	**.859****	**.903****	**.254***	.150
	p		**.0001**	**.0001**	**.0001**	**.014**	.139
ΔCt PRM2	*r*	**.926****	1.000	**.822****	**.887****	**.398****	**.445****
	p	**.0001**		**.0001**	**.0001**	**.0001**	**.0001**
ΔCt TNP1	*r*	**.859****	**.822****	1.000	**.814****	**.357****	.186
	p	**.0001**	**.0001**		**.0001**	**.0001**	.066
ΔCt TNP2	*r*	**.903****	**.887****	**.814****	1.000	**.354****	**.307****
	p	**.0001**	**.0001**	**.0001**		**.001**	**.004**
ΔCt H2BFWT	*r*	**.254***	**.398****	**.357****	**.354****	1.000	**.342****
	p	**.014**	**.0001**	**.0001**	**.001**		**.001**

**Correlation is significant at the .01 level.

*Correlation is significant at the .05 level.

the ratio (P1/P2) was high in patients having fertility problems (Ni et al., 2016) and correlated with high sperm DNA fragmentation (Amor et al., 2018; Hammadeh et al., 2010; Ribas-Maynou et al., 2015).

This study is unique as it is the first, to our knowledge, to investigate the influence of smoking on the histone variant *H2BFWT* and the expression of the transition proteins *TNP1* and *TNP2*. A number of studies have examined the correlation between these genes' expression level and male infertility. They have found that *TNP1*, *TNP2* and the spermatid-specific linker histone H1-like protein (HILS1) mRNA levels are lower in the spermatozoa of asthenozoospermic patients compared to normozoospermic ones (Jedrzejczak et al., 2007), but another study, by Savadi-Shiraz et al. (2015), found that the *TNP2* transcript level is higher in teratozoospermic patients compared to normal ones. Moreover, the down-regulation of *H2BFWT* was found to be related to altered spermatogenesis (Churikov, Siino, et al., 2004).

Hamad et al. (2014) concluded that male smoking is related to a high histone-(H2B)-to-protamine ratio, causing an alteration in sperm DNA. Yu et al. (2014) confirmed this previous study and added that the histone–protamine transition and the transcription of protamine genes can indeed be affected by tobacco smoking. An earlier study reported that the testis-specific histone 2B (TSH2B) was expressed differently in infertile patients (Van Roijen et al., 1998). Zhang et al. (2006) showed a high ratio of histone levels to total nuclear protein in infertile patients.

Selit et al. (2013) reported that sperm DNA and RNA are negatively affected by smoking. Moreover, tobacco smoking is responsible for the down-regulation of microRNA mir-469, mir-466, mir450-b, mir-421 and mir-34b (Dashwood & Ho, 2007; Izzotti et al., 2011; Wei et al., 2015).

Cui et al. (2016) pointed out that the smoking habit is associated with an alteration of DNA integrity and inhibits DNA repair. They found that smoking was associated with sperm DNA fragmentation and reduced amounts of checkpoint kinase 1 (Chk1) transcripts ($p < .05$), which are important for the repair of sperm DNA damage.

Nevertheless, tobacco smoke is generally one of the stronger factors affecting DNA methylation (Lee & Pausova, 2013). It has been reported that nicotine adheres to nicotine acetylcholine receptors and raises the intracellular calcium, causing the downstream activation of the cAMP response element-binding protein, the main transcription factor of a great number of genes, (Shen & Yakel, 2009) including the genes investigated in this study (*H2BFWT*, *TNP1*, TNP2, *PRM1* and *PRM2*).

Satta et al. (2008) reported that nicotine downregulates the expression of a number of DNA methyltransferase (DNMT1) and other proteins of mouse neurons.

In addition, smoking is a major source of ROS, which leads to oxidative stress and the cysteine and thiol groups (2SH) of protamine are an easy target for oxidative stress constituents. Cotinine, on the other hand, has a negative effect on intra- and intermolecular disulphide bond formation, leading to less chromatin compaction in sperm and a high percentage of DNA fragmentation (Hammadeh et al., 2008).

Benzopyrene and vinyl chloride, other components of cigarette smoking, increase the linking of DNA adducts, which then participate in improper DNA replication and inaccurate protein synthesis (Ménézo et al., 2010).

Other studies have demonstrated that an aberrant protamine ratio (P1/P2) is correlated with male infertility and this is caused by a decrease in or an absence of protamine 2 (P2) expressions (Hamad et al., 2014; Hammadeh et al., 2010; Moghbelinejad et al., 2015). In the present study, we have found that an aberrant protamine ratio is correlated with a decrease in protamine 2, transition protein 2 and the testis-specific histone *H2BFWT* (Table 8).

However, an analysis of a larger sample size and experimental functional studies will be needed and these would allow a better understanding of the role of histone, transition proteins and protamines in sperm maturation and sperm function.

5 | CONCLUSION

To conclude, tobacco smoking negatively affects sperm DNA integrity, the expression of the five investigated genes (*H2BFWT*, *TNP1*, *TNP2*, *PRM1* and *PRM2*) and the protamine mRNA ratio. These variations consequently lead to impairment of the sperm quality.

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DISCLOSURE

Nothing to declare.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues data sets with DOIs.

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