http://www.ijwhr.net
doi 10.15296/ijwhr.2019.75
Open Access Original Article
IJWHR
International Journal of Women's Health and Reproduction Sciences
Vol. 7, No. 4, October 2019, 451–460
ISSN 2330-4456

Paternal Smoking in Relation to Sperm Quality and intracytoplasmic Sperm Injection Outcomes

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Abstract

Objectives: The present study focused on tobacco smoke and its effect on semen parameters, sperm DNA quality (compaction and fragmentation) and clinical outcomes after intracytoplasmic sperm injection (ICSI) therapy

Materials and Methods: The semen samples were divided according to smoking status into the following 2 groups, 98 heavy-smokers (G1) and 43 non-smokers (G2). Semen was prepared and purified using the PureSperm gradients according to the WHO guidelines 2010. Protamine deficiency (CMA3 positivity) was assessed by chromomycin CMA3 staining and sperm DNA fragmentation (sDF) by TUNEL assay.

Results: The mean concentration and the total motility were significantly higher in G2 in comparison to G1 ($P=0.014$, and $P=0.026$ respectively) and the results were similar for the mean percent of the progressive motility and normal morphology ($P=0.0001$). CMA3+ and sDF in G2 were significantly lower in comparison to G1 (20.35 ± 13.34% vs. 33.30 ± 22.33%, $P=0.001$; 14.23 ± 13.07% vs. 26.68 ± 19.77%, $P=0.0001$). Meanwhile, there were no significant differences in the ICSI outcomes, except for the pregnancy rate, which was significantly higher in G2 than in G1 (0.60 ± 0.49% vs. 0.38 ± 0.48%; $P=0.013$). In G1, CMA3+ correlated negatively with sperm concentration (r = -0.233, $P=0.021$) but positively with sDF (r = 0.484, $P=0.0001$). In G2, sDF correlated negatively with progressive motility and morphologically normal spermatozoa (r = -0.304, p=0.047; r = -0.361, $P=0.017$ respectively).

Conclusions: The findings of this study revealed that tobacco smoking altered sperm parameters and later affected the pregnancy results in ICSI therapy. CMA3 and TUNEL tests are therefore useful as a supplementary test before any ART treatment to ensure a good prognosis.

Keywords: Infertility, Smoking, Sperm DNA quality, ICSI outcomes

Introduction

Infertility is a big problem that many couples worldwide have to face. In fact, it is now affecting nearly 15% of couples that, according to the World Health Organization (WHO), (1) fail to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.

A population based-study carried out by Agarwal et al to estimate male infertility distribution around the world, showed that the male factor distribution in infertility was between 20% and 70% and the proportion of infertile men was within the 2.5%-12% range (2).

The traditional way of diagnosing male infertility is by determining the classical semen parameters, as described in laboratory guidelines defined by the WHO (1).

After semen analysis, normal semen parameters provide no firm conclusions to clinicians either about the fertility status of a patient or the outcomes of any infertility treatment. Various studies have demonstrated that abnormalities during the sperm chromatin organization may lead to male infertility (3-5) and may later influence the fertilization, the embryo quality and its development (6,7). This means that sperm DNA analysis with the standard semen analysis may help to reveal any hidden sperm DNA abnormality in infertile men with idiopathic infertility. In the light of the increasing body of evidence for DNA integrity and its importance for the ART outcome, many methods have been developed, within the last decade, to reveal any changes, such as fragmentation and the protamination, in the sperm chromatin status and maturity. But the use of such techniques as complementary biomarkers beside semen analysis is still controversial.

Environmental and lifestyle factors, like nutrition, drinking alcohol, physical activity and tobacco smoke, play an important role in the aggravation of the idiopathic male infertility problem.

In fact, exposure to external toxicants leads to different alterations during the various phases (mitotic, meiotic and post-meiotic) of spermatogenesis (8). As a matter of fact, current debate about these issues seems to confirm that the male fertility impairment is increasing and that this might indeed be associated with environmental factors and life-styles. However, in this study, we have focused on tobacco smoke and its effect on semen parameters, sperm DNA quality and clinical outcomes after intracytoplasmic sperm injection (ICSI) therapy.

Received 11 June 2018, **Accepted** 23 December 2018, **Available online** 16 January 2019

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Materials and Methods

Study Design

Semen samples were collected randomly from male partners of couples undergoing ICSI treatment. This was carried out in the laboratory of Biochemistry and Molecular Biology of Reproductive Medicine within the Department of Obstetrics and Gynaecology, the Women's Hospital Saarland Clinic, Germany.

Smoking Evaluation

Ae participant who smokes more than one packet/day for 10 years or 2 packets/day for 5 years was considered to be a heavy-smoker, and the participant who did not smoke was considered to be a non-smoker. According to this definition, patients were divided into the following two groups: heavy-smokers (n = 98) and non-smokers (n = 43).

Sperm Processing

The samples were collected, by masturbation, after a minimum of 2 days and a maximum of 3 days of sexual abstinence.

The specimen container was kept on the heating stage or in the incubator (37°C) for 30-60 minutes for liquefaction. Then, macroscopic (ejaculate appearance, viscosity, pH, and volume) and microscopic (spermatozoa concentration, motility, vitality, aggregation, and morphology in semen) evaluations were done according to the WHO laboratory manual (1).

All semen samples were treated (the purification step) to remove cells other than spermatozoa by loading each sample onto 40%–80% discontinuous PureSperm gradients (Nidacon International, Sweden) and then centrifuged at 500 x g for 20 minutes at room temperature. The upper layer was aspirated until the ring without any touching of the pellet. Next, the pellet was re-suspended in 1 mL of G-IVF Plus medium (Vitrolife, Sweden) and then centrifuged (330 x g/10 min). The supernatant was eliminated and the pellet was suspended in 0.5 mL of G-IVF Plus medium (Vitrolife, Sweden) and kept in an incubator (6% CO_2, 37°C) for at least two hours before ICSI.

Sperm Chromatin Condensation Assay (Chromomycin A3 Assay)

For the sperm DNA condensation assessment, the Chromomycin A3 assay, as described by Hammadeh et al was used but with some modifications.

The first step was fixation, namely, putting the slides in methanol-glacial acetic acid (3:1) for 1 hour and then leaving them to air-dry at room temperature. To each slide, 25 µL of CMA3 stain solution was added and the slide was then incubated in the dark for 30 minutes at room temperature. After awash with PBS buffer, the slides were mounted and then kept overnight at 4°C in the dark. On each slide, 200 spermatozoa were evaluated using a fluorescence microscope (Olympus, Japan): bright green spermatozoon represents a low protamination state (CMA3 positive) and dull green spermatozoa are CMA3 negative.

Sperm DNA Fragmentation TUNEL

A Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used for the sperm DNA fragmentation assessment, as previously described by Borini et al.

The first step was the fixation of smears in slides with 4% paraformaldehyde (Sigma-Aldrich, Germany) for 2 hours at room temperature. Then in the permeabilization step, the smears were incubated with 0.1% Triton for 15 minutes at room temperature. To each slide, 25 µL of the TdT-labelled nucleotide mixture was added and then the slide was incubated overnight at 37°C in a humidified chamber. The slides were then washed with PBS. After that, 25 µL of DAPI (Sigma-Aldrich, Germany) was added to each slide as a counter stain. On each slide, 200 spermatozoa were evaluated using a fluorescence microscope (Olympus, Japan) via a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470: green-stained spermatozoa are TUNEL-positive whereas blue-stained spermatozoa are TUNEL-negative.

ICSI Procedure and Embryo Evaluation

After 3-4 hours of oocyte retrieval, a decoronation of the cumulus-corona oocyte cell complex was performed using hyaluronidase (SynVitro Hyadase, Origio, Denmark) and the intracytoplasmic sperm injection (ICSI) was given for metaphase II oocytes using a micromanipulation system (Narishige, Japan) and an inverted microscope (Zeiss, Germany) (Figure 1).

For embryo culture and assessment, the microdrop culture system and the Embryo Scope time-lapse incubator (Vitrolife, Sweden) were used. After injection, the oocytes were distributed in an Embryo Slide culture dish (Vitrolife, Sweden) that could hold 12 embryos. In each well of the dish, 25 µL of global total culture medium (Life Global, Canada) was added. The incubation conditions were: Temperature = 37°C, 5.5% Oxygen (O_2) and 5.5% carbon dioxide (CO_2).

The embryo quality grade was assessed on day 3 after

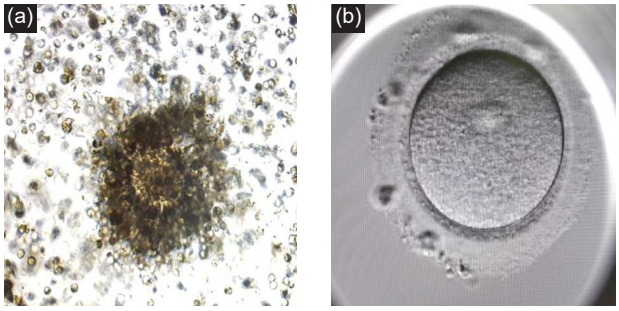

Figure 1. Intracytoplasmic sperm injection (ICSI) Day; (a) A cumulus-corona oocyte cell complex, (b) Denuded Metapahse II (MII) Oocyte.

the injection (the cleavage stage) according to the division symmetry, the cytoplasmic fragmentation proportion (Grade 1: 0-10% of cytoplasm fragmented, Grade 2: 11-20% of cytoplasm fragmented, grade 3: >20 of cytoplasm fragmented) (9) using the EmbryoViewer Software (Vitrolife, Sweden) (Figure 2).

The embryo quality was also assessed on day 4 (Morula stage) (Figure 3) and day 5 (Blastocyst stage), using Gardner's blastocyst grading scale and including expansion, inner cell mass (ICM) and trophectoderm epithelium (TE) (10) (Figure 4).

In 30% of the cases the embryo was transferred in the cleavage stage and in 70% in the blastocyst stage. The average of embryo transfer was 2 embryos/patient.

Statistical Analysis

Data were analyzed using the IBM SPSS for Windows software package version 24.0, USA. The descriptive statistics of the different studied parameters were expressed as mean ± standard deviation (M ± SD). The samples were not-normally distributed. The Mann-Whitney U test was therefore applied to compare the continuous variables and the Spearman correlation test was used to determine the correlation between the different studied parameters. The association degree was described by the correlation coefficient (r), the P value ≤ 0.05 was considered to be statistically significant and $P < 0.01$ was considered to be statistically highly significant.

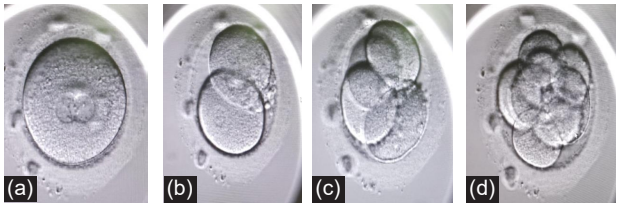

Figure 2. Embryo cleavage stages after ICSI; (a) Fertilized oocyte (Zygote) with 2 polar bodies and 2 central equal pronuclei (PNs) (18 h post-ICSI), (b): A 2-blastomeres human embryo (Day 1 post-ICSI), (c): A 4-blastomeres human embryo (Day 2 post-ICSI), (d): A 8-blastomeres human embryo (Day 3 post-ICSI).

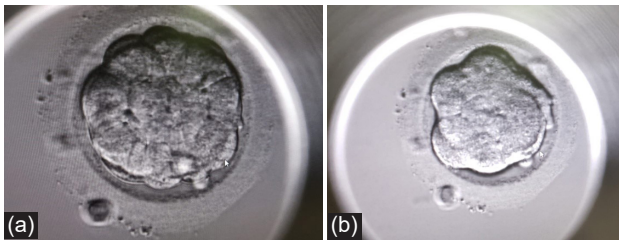

Figure 3. Progressive compaction of human embryo on day 4 post-ICSI leading to the formation of morula.

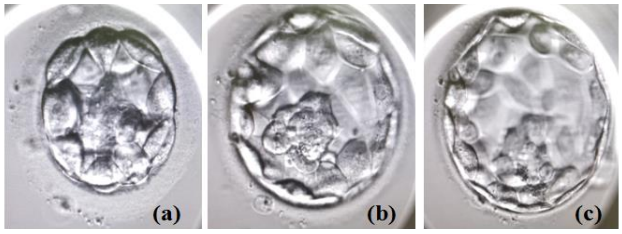

Figure 4. Blastocyst formation (Day 4-5 post-ICSI); (a) Human embryo with early cavitation, (b) Early blastocyst, (c) Expanded blastocyst.

Results

The Characteristics of the Study Population

Table 1 provides a summary of the statistical analysis of the sperm parameters, protamine deficiency, sperm DNA fragmentation, and ICSI outcomes. The means ± SD of the sperm volume, sperm concentration, total motility, progressive motility, and morphologically normal spermatozoa were (3.33 ± 1.57 (mL), 79.03 ± 59.68 (10^6/mL), 42.80 ± 20.99%, 20.55 ± 17.17%, 6.94 ± 8.40% respectively). The CMA3+ had a mean value of 29.35 ± 20.86% and the sperm DNA fragmentation (sDF) had a mean value of 22.89 ± 18.85%.

The mean percentage of the fertilization rate was 79.04 ± 19.85%. The mean values of the number of cleaved, grade 1 (G1), and grade 2 (G2) embryos were 6.65 ± 4.74, 2.83 ± 2.48, 1.87± 0.56. The embryo mean grade score was 1.87 ± 0.56 and the mean pregnancy rate was 0.45 ± 0.5%.

A Comparison of the Studied Parameters Between the Non-smokers and the Heavy-Smokers

The patients were later divided, according to their smoking status, into 2 groups, namely, non-smokers (n = 43) and heavy-smokers (n = 98).

By comparing the semen parameters between the 2 groups (Table 2), we found that the mean concentration and the total motility were significantly higher in the non-smoker group ($P = 0.014$, and $P = 0.026$ respectively) and found similar results for the mean percent of the

Table 1. Descriptive Statistics of Studied Parameters for All Patients (n = 141)

Parameters	Mean ± SD
Semen volume (mL)	3.33 ± 1.57
Sperm concentration (10^6 per mL)	79.03 ± 59.68
Total motility (%)	42.80 ± 20.999
Progressive motility (%)	20.55 ± 17.17
Morphologically normal spermatozoa (%)	6.94 ± 8.40
Protamine deficiency (CMA3+) (%)	29.35 ± 20.86
Sperm DNA fragmentation (sDF) (%)	22.89 ± 18.85
Fertilization rate (%)	79.04 ± 19.85
Number of cleaved embryos	6.65 ± 4.74
Number of grade 1embryos (G1)	2.34 ± 2.56
Number of grade 2 embryos (G2)	2.83 ± 2.48
Embryos' grade score	1.87 ± 0.56
Pregnancy rate (%)	0.45 ± 0.5

SD: standard deviation

Table 2. Comparison of the Semen Analysis Parameters Between Non-smokers and Heavy-Smokers

Parameter (Unit)	Non-smokers (n=43) Mean ± SD	Heavy-smokers (n=98) Mean ± SD	*P* Value
Semen volume (mL)	3.71 ± 1.76	3.17 ± 1.46	0.181
Sperm concentration (10^6 per mL)	98.56 ± 64.63	70.46 ± 55.59	0.014*
Total motility	48.42 ± 21.83	40.34 ± 20.25	0.026*
Progressive motility	31.42 ± 22.24	15.78 ± 11.66	0.0001**
Morphologically normal spermatozoa (%)	12.91 ± 12.76	4.32 ± 2.93	0.0001**
Protamine deficiency (CMA3+) (%)	20.35 ± 13.43	33.30 ± 22.33	0.001**
Sperm DNA fragmentation (sDF) (%)	14.23 ± 13.07	26.68 ± 19.77	0.0001**

SD: standard deviation.
**Correlation is highly significant at the 0.01 level ($P < 0.01$).
*Correlation is significant at the 0.05 level ($P < 0.05$).

progressive motility and normal morphology ($P = 0.0001$).

Furthermore, the mean percentage of protamine deficiency (CMA3+) in the group of non-smokers was significantly lower in comparison to that of the heavy-smokers (20.35 ± 13.34 vs. 33.30 ± 22.33, $P = 0.001$). The mean percentage of sDF showed a high difference between the non-smoker group and heavy-smoker one (14.23 ± 13.07 vs. 26.68 ± 19.77, $P = 0.0001$).

Moreover, except for the pregnancy rate, which was significantly higher in the group of non-smokers than in that of the heavy-smokers (0.60 ± 0.49% vs. 0.38 ± 0.48%; $P = 0.013$), there were no significant differences in the other clinically investigated parameters (fertilization rate, number of cleaved embryos, number of grade 1 (G1) and grade 2 (G2) embryos and the embryos' grade score) between the two groups (Table 3).

Correlation Between the Different Sperm Parameters and Protamine Deficiency

In the heavy-smokers group (Table 4), the mean percentage of the sperm concentration correlated positively with the mean percentages of total motility, progressive motility, and morphologically normal spermatozoa (r = 0.294, r = 0.515, r = 0.531; $P < 0.01$) but correlated negatively with the protamine deficiency (r = -0.233, $P = 0.021$). The total and progressive motility showed a high positive correlation with the mean of morphologically normal spermatozoa (r = 0.439, r = 0.583 respectively; $P = 0.0001$). In addition, a significant correlation (r = 0.484, $P = 0.0001$) between CMA3+ and sDF was shown (Table 4).

Table 5 illustrates the correlations between the mean percentage of the different sperm parameters, the protamine deficiency (CMA3+) and sDF in the non-smokers group. The semen volume correlated negatively with the concentration (r = -0.338, $P = 0.027$) but the mean percentage of total motility correlated positively with the mean percentage of morphologically normal spermatozoa (r = 0.663, $P = 0.0001$) and negatively with the mean percentage of sDF (r = -0.304, $P = 0.048$). In addition, the progressive motility correlated positively with morphologically normal spermatozoa (r = 0.830, $P = 0.0001$) but negatively with the mean percentage of sDF (r = -0.304, $P = 0.047$). In addition, the mean percentage of morphologically normal spermatozoa showed a significant negative correlation with the mean percentage of sDF (r = -0.361, $P = 0.017$).

Table 3. Comparison of the ICSI-Outcomes Between Non-smokers and Heavy-Smokers

Parameter (Unit)	Non-smokers (n=43) Mean ± SD	Heavy-smokers (n=98) Mean ± SD	*P* Value
Fertilization rate (%)	78.23 ± 19.48	79.40 ± 20.10	0.691
Number of cleaved embryos	6.70 ± 4.75	6.63 ± 4.76	0.923
Number of grade 1 embryos (G1)	2.67 ± 2.93	2.19 ± 2.39	0.567
Number of grade 2 embryos (G2)	2.49 ± 2.31	2.98 ± 2.55	0.195
Embryos grade score	1.99 ± 0.61	1.82 ± 0.54	0.229
Pregnancy rate (%)	0.60 ± 0.49	0.38 ± 0.48	0.013*

SD: standard deviation.
*Correlation is significant at the 0.05 level ($P \leq 0.05$).

Correlation Between Protamine Deficiency and ICSI Results

In the non-smokers group (Table 6), the mean percentage of the sperm DNA fragmentation correlated positively with the number of cleaved embryos (r = 0.394, $P = 0.009$), the number of grade 1 embryos (r = 0.341, $P = 0.025$) and the number of grade 2 embryos (r = 0.316, $P = 0.039$). However, the remaining parameters showed no significant difference.

Table 7 shows that in the heavy-smokers group, neither the mean percent of CMA3+ nor sDF correlate with the clinical parameters after ICSI.

Discussion

Several lifestyle behaviours, such as nutrition, sport, drinking alcohol and tobacco smoking, are reported to

Table 4. Correlation Between the Investigated Sperm Parameters and Protamine Deficiency (CMA3+) in the Heavy-Smokers Group (n=98)

		Semen volume (mL)	Sperm concentration (10^6 per mL)	Total motility (%)	Progressive motility (%)	Morphologic-ally normal spermatozoa (%)	CMA3+ (%)	sDF (%)
Semen volume (mL)	*r*	1.000	-0.042	0.004	-0.024	0.097	-0.117	-0.029
	P		0.681	0.965	0.812	0.343	0.251	0.776
Sperm concentration (10^6 per mL)	*r*	-0.042	1.000	**0.294****	**0.515****	**0.531****	**-0.233***	**-0.263****
	P	0.681		**0.003**	**0.0001**	**0.0001**	**0.021**	**0.009**
Total motility (%)	*r*	0.004	**0.294****	1.000	**0.677****	**0.439****	0.133	0.101
	P	0.965	**0.003**		**0.0001**	**0.0001**	0.193	0.321
Progressive motility (%)	*r*	-0.024	**0.515****	**0.677****	1.000	**0.583****	-0.097	-0.063
	P	0.812	**0.0001**	**0.0001**		**0.0001**	0.340	0.534
Morphological-ly normal spermatozoa (%)	*r*	0.097	**0.531****	**0.439****	**0.583****	1.000	-0.140	-0.177
	P	0.343	**0.0001**	**0.0001**	**0.0001**		0.169	0.081
CMA3+ (%)	*r*	-0.117	**-0.233***	0.133	-0.097	-0.140	1.000	**0.484****
	P	0.251	**0.021**	0.193	0.340	0.169		**0.0001**

**Correlation is highly significant at the 0.01 level ($P < 0.01$).
*Correlation is significant at the 0.05 level ($P \leq 0.05$).

Table 5. Correlation Between the Different Sperm Parameters and Protamine Deficiency (CMA3+) in the Non-smoker Group (n=43)

		Semen volume (mL)	Sperm concentration (10^6 per mL)	Total motility (%)	Progressive motility (%)	Morphologica-lly normal spermatozoa (%)	CMA3+ (%)	sDF (%)
Semen volume (mL)	*r*	1.000	**-0.338***	0.184	0.242	0.227	0.147	-0.113
	P		**0.027**	0.237	0.117	0.142	0.345	0.469
Sperm concentration (10^6 per mL)	*r*	**-0.338***	1.000	0.159	0.107	0.110	-0.240	-0.297
	P	**0.027**		0.309	0.494	0.481	0.121	0.053
Total motility (%)	*r*	0.184	0.159	1.000	**0.874****	**0.663****	0.037	**-0.304***
	P	0.237	0.309		**0.0001**	**0.0001**	0.815	**0.048**
Progressive motility (%)	*r*	0.242	0.107	**0.874****	1.000	**0.830****	0.018	**-0.304***
	P	0.117	0.494	**0.0001**		**0.0001**	0.907	**0.047**
Morphologic-ally normal spermatozoa (%)	*r*	0.227	0.110	**0.663****	**0.830****	1.000	-0.146	**-0.361***
	P	0.142	0.481	**0.0001**	**0.0001**		0.350	**0.017**
CMA3+ (%)	*r*	0.147	-0.240	0.037	0.018	-0.146	1.000	0.256
	P	0.345	0.121	0.815	0.907	0.350		0.098

**Correlation is highly significant at the 0.01 level ($P < 0.01$).
*Correlation is significant at the 0.05 level ($P \leq 0.05$).

have an influence on both male and female reproductive health, and, in a number of cases, they may even have an effect on epigenetic mechanism alterations, which may be associated with major common human maladies (11,12).

Around 46% of males of reproductive age between 20- and 39-years-old are cigarette smokers (13). Almost 75% of the men that are daily smokers live in countries that have a medium or high human development index (14).

For this reason, we focused, in this current study, on the influence of paternal tobacco smoke on the male reproductive function: sperm parameters, sperm DNA maturity and ICSI outcomes.

In this study, we found in our heavy-smokers group (n = 98) a significant decrease ($P<0.01$) in standard semen parameters, namely, sperm concentration, total motility, progressive motility, and sperm normal morphology, in comparison to our non-smokers group (70.46 ± 55.59 10^6/mL vs. 98.56 ± 64.63 10^6/mL, 40.34 ± 20.25 % vs. 48.42 ± 21.83%, 15.78 ± 11.66 % vs. 31.42 ± 22.24% and 4.32 ± 2.93% vs. 12.91 ± 12.76 % respectively) (Table 2).

These results are in accordance with various other studies that reported that the toxic elements in tobacco smoking had negative effects on semen quality (15-18). The Practice Committee of the American Society for Reproductive Medicine also reported in 2012 that the relationship between tobacco consumption and reduced sperm parameters is a dose-response relationship (19). In a meta-analysis, Sharma et al concluded that smoking

Table 6. Correlation Between Protamine Deficiency (CMA3 positivity), Sperm DNA Fragmentation and ICSI Results in Non-smokers Group (n=43)

		Fertilization Rate (%)	Number of Cleaved Embryos	Number of Grade 1 Embryos	Number of Grade 2 Embryos	Embryo Grade Score
Protamine deficiency (CMA3+) (%)	*r*	0.039	-0.037	0.186	-0.235	-0.107
	P	0.805	0.813	0.232	0.130	0.496
Sperm DNA fragmentation (sDF) (%)	*r*	0.077	**0.394****	**0.341***	**0.316***	-0.045
	P	0.624	**0.009**	**0.025**	**0.039**	0.773

**Correlation is highly significant at the 0.01 level ($P < 0.01$).
*Correlation is significant at the 0.05 level ($P \leq 0.05$).

Table 7. Correlation Between Protamine Deficiency (CMA3 positivity), Sperm DNA Fragmentation and ICSI Results in Heavy-Smokers Group (n=43)

		Fertilization Rate (%)	Number of Cleaved Embryos	Number of Grade 1 Embryos	Number of Grade 2 Embryos	Embryo Grade Score
Protamine deficiency (CMA3+) (%)	*r*	0.152	0.082	0.009	0.061	0.145
	P	0.135	0.421	0.929	0.550	0.153
Sperm DNA fragmentation (sDF) (%)	*r*	0.050	0.117	0.086	-0.007	0.034
	P	0.625	0.252	0.402	0.946	0.736

**Correlation is highly significant at the 0.01 level ($P < 0.01$).
*Correlation is significant at the 0.05 level ($P \leq 0.05$).

had a generally negative influence on standard semen parameters and that this was generally more obvious in infertile male patients than in the common population because their spermatozoa are probably more sensitive to the inhaled toxic chemicals (20).

Nevertheless, other studies reported that smoking had no meaningful effect on conventional sperm parameters (21-24). Further studies at the molecular level are therefore needed to find out how tobacco smoking affects sperm function and to shed more light on the clinical condition.

The main causes of DNA alteration were reported to be the defective repair of double or single-stranded DNA breaks caused by topoisomerase II during chromatin remodelling (25), abortive apoptosis (26), aberrant protamination (27-29), the abnormal expression of transition proteins (30-32), interaction between toxic chemicals and/or heavy metals with protamines (33), and oxidative stress (34).

In the last decade, more studies have been focused on the mechanisms by means of which environmental and lifestyle factors, especially smoking, have an influence on the sperm genome and epigenome (35) and have a potential effect on the developing embryo (8, 36).

Tobacco smoking is in fact associated with high levels of seminal reactive oxygen species (ROS) causing oxidative DNA damage (37-40). It has also been reported that tobacco smoke contents are correlated with DNA adduct formation leading to DNA damage (41,42).

The following two techniques were used in the current study to evaluate the sperm DNA integrity: TUNEL for sDF assessment, and CMA3 staining for protamine deficiency (CMA3+) assessment. In the heavy-smokers group, the mean percentage of sDF and CMA3+ were significantly higher ($P < 0.01$) than that of the non-smokers group ($26.86 \pm 19.77\%$ vs.$14.23 \pm 13.07\%$ and $33.30 \pm 23.33\%$ vs. $20.35 \pm 13.43\%$ respectively) (Table 2).

These results are in line with a number of studies that used different techniques for the determination of sperm DNA fragmentation. Most of the human researches using a TUNEL assay demonstrated that the levels of DNA fragmentation in smokers was higher than in non-smokers (24,37,43). Similar results were found in other studies using different techniques to evaluate DNA fragmentation (15,44-46). Contradictory studies, however, have concluded that there is no correlation between smoking and DNA damage (47,48).

On the other hand, a significant positive correlation has been found between protamine deficiency (CMA3+) and DNA fragmentation (sDF) ($r = 0.484$, $P = 0.0001$) in the group of heavy-smokers (Table 4), but there was no correlation ($r = 0.256$, $P = 0.098$) between these 2 parameters in the non-smokers group (Table 5). Similar results have been demonstrated by other groups who reported that abnormal protamination leads to abnormal chromatin condensation and raises the sensitivity of sperm DNA to external stress causing an oxidative attack (37,49-51).

In the heavy-smokers group (Table 4), the mean percentage of the sperm concentration correlated negatively with the protamine deficiency CMA3+ ($r = -0.233$, $P = 0.021$) and the sDF ($r = -0.263$, $P = 0.009$). A similar situation was observed in the non-smokers group (Table 5), with the sperm DNA fragmentation sDF being correlated negatively with the mean percentage of total motility ($r = -0.304$, $P = 0.048$), the mean of progressive motility ($r = -0.304$, $P = 0.047$) and the mean percentage

of morphologically normal spermatozoa (r = -0.361, $P = 0.017$). These results support the claim that sperm DNA damage has a negative influence on sperm quality, thereby confirming earlier studies (52-54).

However, a number of studies have not succeeded in demonstrating a relationship between traditional seminal parameters and sperm DNA damage (24,37,55).

Moreover, the use of different techniques to measure alterations in sperm DNA has been a controversial issue in the field of assisted reproduction field. Bungum et al demonstrated that the pregnancy rate after intrauterine insemination (IUI) decreased when the DNA fragmentation index (DFI) was higher than 20% (56). In other studies, it was concluded that couples who did not become pregnant after IVF treatment had a DNA fragmentation higher than 25% and their embryo quality correlated negatively with sDF (57). According to a study conducted by Oleszczuk et al, DFI above 40% is correlated with a danger of early miscarriage (58).

In the present study, by comparing the clinical investigated parameters between the heavy-smokers group and the non-smokers group, the pregnancy rate was seen to be significantly higher in the group of non-smokers than in the heavy-smokers group ($0.60 \pm 0.49\%$ vs. $0.38 \pm 0.48\%$; $P = 0.013$) but other parameters showed no significant differences (Table 3). This is similar to the results of a study done on couples that had a normal conception but the male partner was a smoker; this study demonstrated that smoking has negative effects on fecundity and the time-to-pregnancy in a dose-dependent manner (59).

Low fecundity in smokers has also been reported to be related to acrosin activity (60). The correlation between smoking and the sperm's ability to fertilize the oocyte was also studied by Sofikitis and colleagues, who demonstrated that smoking correlates negatively with the sperm's potential to fertilize the oocyte (61).

Furthermore, toxic elements resulting from tobacco combustion have been proved to decrease the mitochondrial activity and cause impairment in chromatin organization and, as a result, alter the fertilization capability (12,62).

In the present study, in the heavy-smoker group, the fertilization rate, the number of cleaved embryos, the number of grade 1 embryos, the number of grade 2 embryos, and the embryos' grade score had a correlation neither with the mean percentage of protamine deficiency (CMA3+) nor with sperm DNA fragmentation (sDF) (Table 7).

These results are in agreement with other studies, which reported no significant correlation between the fertilization rate, the quality of the embryo and pregnancy rates after IVF or ICSI (63-66).

Henkel et al showed that there was no correlation between DNA fragmentation (TUNEL test) and the fertilization rate. Similar results had been found earlier by Benchaieb et al, who found no correlation between DNA fragmentation (TUNEL assay) and embryo quality (67). In both groups, however, it was shown that sperm DNA damage has a negative impact on pregnancy, and, more recently, Amiri-Yekta et al came to the same conclusion (68).

In contrast, in the non-smoker group, the mean percentage of the sDF correlated positively with the number of cleaved embryos (r = 0.394, $P = 0.009$), the number of grade 1 embryos (r = 0.341, $P = 0.025$), and the number of grade 2 embryos (r = 0.316, $P = 0.039$) (Table 6). This coincides with the results from a study, conducted by Payne and colleagues, in which they demonstrated that the higher the sperm DNA fragmentation is, the higher the pregnancy result is (69). This contradiction can be explained by the fact that, after fertilization, a good quality oocyte is capable of repairing the sperm DNA damage (70) but, if not, this can have a negative impact on the embryo development (71,72).

In conclusion, the current study strongly suggests that CMA3 staining and TUNEL measuring of the sperm's DNA alterations (compaction and fragmentation respectively) caused by various factors, such as tobacco smoking, may be useful as supplementary tests before any ART treatment to ensure a good prognosis especially in cases of idiopathic infertility and repetitive miscarriage.

Ethical Issues

This study was approved by the Institutional Ethics Committee of Saarland University (under the code of ethics PHRC/HC/13/14). All the subjects provided written consent before participation in this study.

Conflict of Interests

The authors declare that they have no conflict of interests.

Financial support

The Department of Obstetrics and Gynaecology, Saarland University, Germany

Acknowledgments

We thank the Department of Obstetrics, Gynaecology and Reproductive Medicine, University Hospital of Saarland, for their financial support and we are also grateful to the patients included in this study.

Special thanks to Prof. Dr. Charles Goodall who done required language amendments and linguistic revision for this article

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