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Semen Quality of Male Idiopathic Infertile Smokers and Nonsmokers: An Ultrastructural Study

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ABSTRACT: This retrospective study was aimed at evaluating the effects of cigarette consumption on semen parameters in a group of men with idiopathic infertility. The semen quality of 2 groups of men with idiopathic infertility, smokers (n = 118) and nonsmokers (n = 153), were compared. Conventional semen analysis was performed and sperm morphology was assessed by transmission electron microscopy (TEM). TEM data were elaborated by means of a mathematical formula based on a Bayesian technique able to furnish a fertility index (FI), and the percentages of sperm apoptosis, necrosis, and immaturity. Values of normality recommended by World Health Organization guidelines were used as a control for conventional semen analysis, and values from sperm of 25 men of proven fertility were used for TEM indices. Infertile smoker and nonsmoker patients showed similar sperm parameters, although sperm motility and TEM analysis values in both groups were significantly impaired compared with controls. Smoker patients were then classified as mild (≥1 and ≤10 cigarettes/d), moderate (>10 and <20 cigarettes/day), or heavy smokers (≥20 cigarettes/d). Sperm concentration and FI were significantly ($P < .05$) different among the 3 considered smoker classes. Comparing the pairs of smoker classes, sperm concentration and FI in heavy smokers were significantly lower ($P < .05$) than that observed in mild smoker and nonsmoker groups. Although semen quality in males with idiopathic infertility seems not to be dramatically affected by cigarette consumption, heavy smokers show significantly lower sperm concentration and FI: another strong reason to stop smoking.

Key words: Cigarette smoking, male idiopathic infertility, TEM.
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Cigarette smoking is a widely recognized health hazard, yet despite worldwide antismoking campaigns, some people continue to consume cigarettes on a regular basis, and the highest prevalence of smoking is observed in young adult males during their reproductive period (Langgassner, 1999).

Soares and Melo (2008), reviewing the literature concerning the relationship between cigarette smoking and reproductive function, highlighted a strong body of evidence indicating the negative effect of cigarette smoking on male and female fertility.

A consistent number of studies have claimed that cigarette smoking is correlated with alterations in sperm quality such as semen volume, sperm concentration, motility, and morphology (Vine et al, 1994; Zavos et al, 1998a,b; Ozgur et al, 2005; Guo et al, 2006; Pasqualotto et al, 2006, 2008; Ramlau-Hansen, 2007a), concomitant with a reduced concentration mainly of citrate and also of fructose (Kunzle et al, 2003). On the other hand, other studies did not find any alterations in conventional semen parameters (Hoidas et al, 1985; Trummer et al, 2002; Sepaniak et al, 2006). In infertile smoking men, the antioxidant level, particularly the superoxide dismutase level, was recently found to be correlated with sperm concentration and negatively correlated with leukocytospermia (Pasqualotto et al, 2008).

It has been suggested that cigarette smoking increases the percentage of morphologically altered spermatozoa (Evans et al, 1981; Elshal et al, 2008), especially in men who are heavy smokers or who have smoked for many years (Gaur et al, 2007); however, the extent and the localization of the morphological damage is still being debated (Mak et al, 2000; Guo et al, 2006). In the vast majority of the studies, sperm morphological evaluation has been performed by light microscope, although transmission electron microscope (TEM) is probably the only instrument able to analyze deeply the inner structure of organelles. In this regard, severe alterations in the flagellar ultrastructure of sperm from smokers was reported by Zavos et al (1998b), and recently our group, using a method of quantitative sperm analysis based on TEM, demonstrated that cigarette consumption in the presence of varicocele could represent a

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further risk in impairing human semen quality (Collodel et al, 2009).

This study was aimed at investigating whether the habit of smoking could negatively influence semen quality in a group of men with idiopathic infertility.

Materials and Methods

Patients

In this retrospective study, we selected 271 (aged 21–46) smoking or nonsmoking individuals with idiopathic infertility among the men examined at the Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena, between January 1998 and December 2007 (1256 patients). The median duration of infertility was 2.65 years (range 2–4 years) of unprotected sexual intercourse without conception. All patients were interviewed about their clinical and family history and their information was recorded in a database. Their clinical history encompassed the most common risk factors for male infertility, including diabetes, hypertension, allergy, cancer, gastritis, gastric ulcer, and so on.

Lymphocyte karyotype analysis, evaluated with the conventional cytogenetic method, hormonal profile, and microbiological investigations were performed. In patients with a sperm concentration of less than 10 million/mL, polymerase chain reaction (PCR) analysis on DNA extracted from blood lymphocytes was carried out to exclude the presence of Y chromosome microdeletions.

To exclude many coexisting factors, we used stringent patient selection criteria. Patients who were ex-smokers or men with a history of recreational drug use or alcohol consumption, occupational exposure to chemicals or excessive heat, varicocele or other anatomical injury, leukocytospermia or chronic genitourinary infections diagnosed by spermiocultures, altered hormonal levels, or pathologies related to infertility were excluded from this study.

Moreover, we did not include azoospermic men, individuals with an altered karyotype, carriers of chromosome Y microdeletions, or those with sperm defects of supposed genetic origin. Any febrile episode during the 2 months before analysis was reported.

Information on smoking habits included the number of cigarettes smoked per day and the number of years of smoking. Patients had begun to smoke between the ages of 12 and 24 years; we considered as ''smokers'' the patients who had smoked cigarettes for more than 10 years, and ''nonsmokers'' were men who had never smoked.

We first categorized infertile males as smokers (n = 118; age 31.9 ± 4.7 years; median, 30; range, 27–45) and nonsmokers (n = 153; age 31.3 ± 4.8 years; median, 31; range, 25–46), then we divided the smokers into mild (n = 42, 1–10 cigarettes/d, age 31.1 ± 4.2 years), moderate (n = 25, 11–20 cigarettes/d, age 32 ± 4.3 years), and heavy smokers (n = 51, >20 cigarettes/d, age 32.8 ± 5.8 years). The time of first smoking for mild smokers was 15.5 ± 4.41, for moderate smokers 16.8 ± 3.90, and for heavy smokers 16.05 ± 6.74.

All patients signed a declaration of informed consent to participate in the research. Approval by an institutional review board was not necessary because all analyses were part of the routine infertility investigation.

Semen samples from 25 fertile men (age 28.3 ± 3.8 years; median, 28.5; range, 22–35) without anatomical problems or infections and with normal karyotype were used as controls for TEM indices (Collodel and Moretti, 2008).

Reference values used for spermiogram data (semen volume, pH, sperm concentration, and motility) were those suggested by World Health Organization guidelines (WHO, 1999).

Semen Analysis

Light and Electron Microscopy—Semen samples from 271 patients were collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration, and motility were evaluated according to WHO guidelines (1999). As general rule, we evaluate only 1 sample for each patient, particularly for electron microscopy analysis.

For TEM, sperm samples were fixed in cold Karnovsky fixative and maintained at 4°C for 2 hours. Fixed semen was washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 hours, postfixed in 1% buffered osmium tetroxide for 1 hour at 4°C, dehydrated, and embedded in Epon Araldite. Ultrathin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate, and observed and photographed with a Philips CM10 transmission electron microscope (Philips Scientifics, Eindhoven, The Netherlands).

Three hundred ultrathin sperm sections were analyzed for each patient. Major submicroscopic characteristics were recorded by trained examiners who were blind to the experiment. TEM data were evaluated according to the mathematical formula (Baccetti et al, 1995) that considers 16 selected submicroscopic characteristics of sperm organelles, each of which can be present either in a normal state or in 1 or more different abnormal states, or defects. These characteristics, in a normal or abnormal state, were recorded during TEM examination and subsequently elaborated by means of a Bayesian technique (probability calculation) that is able to quantify the data obtained by TEM analysis by calculating the number of spermatozoa free of structural defects in a semen sample (the fertility index, FI). The lowest FI assuring normal fertility was observed to be slightly less than 2 million (Collodel and Moretti, 2008).

Moreover, this method also allows for obtaining the percentages of 3 main sperm pathologies; immaturity, necrosis, and apoptosis (Collodel and Moretti, 2008). Sperm pathologies are defined by typical ultrastructural characteristics. Altered acrosomes; misshapen, round, or elliptical nuclei with uncondensed chromatin; and the presence of cytoplasmic droplets were the characteristic features of immaturity. Marginated chromatin, cytoplasm with translucent vacuoles, and swollen and badly assembled mitochondria were the typical ultrastructural markers of apoptosis, whereas spermatozoa with broken plasma membrane, reacted or absent acrosome, misshaped nuclei with disrupted chromatin, and

Table 1. *Comparison of semen variables (optical microscopy) compared with World Health Organization (WHO, 1999) values, and sperm TEM variables compared with values of 25 men of proven fertility (TEM controls) in smoker and nonsmoker patients with idiopathic infertility. The significance is indicated as a mean or median on the basis of normal or nonnormal distribution of values. Values in bold are significantly different*

		Variables							
Groups	Measurement	Volume	pH	Concentration, $\times 10^6$	Motility, %	Apoptosis, %	Immaturity, %	Necrosis, %	Fertility Index, $\times 10^6$
118 Smoker patients	$\bar{x}$	3.78	7.6	61.27	**24.1**[a]	11.8	**70.8**[b]	**43.02**[b]	0.312
	Median	3.05	7.6	37	22	**9.01**[b]	71.81	41.9	**0.0807**[b]
	SD	2.83	0.25	70.3	13.15	8.52	12.52	17.36	0.481
	95% CI for mean	3.26–4.29	7.55–7.64	48.4–74.08	21.7–26.5	6.38–16.69	68.5–73.05	39.85–46.19	0.00663–0.4
153 Nonsmoker patients	$\bar{x}$	3.19	7.6	76.16	**23.84**[a]	10.28	**70.69**[b]	**43.42**[b]	0.32
	Median	3	7.6	43.5	24	**9.01**[b]	72.02	42.76	**0.105**[b]
	SD	1.45	0.26	102.92	12.04	7.48	13.25	16.55	0.461
	95% CI for mean	2.96–3.42	7.53–7.61	59.72–92.60	21.92–25.77	7.77–10.47	68.58–72.81	40.77–46.06	0.07–0.18
Controls	$\bar{x}$[c]	2–6[d]	7–8[d]	>20[d]	>50[d]	4.06[c]	48.83[c]	32.13[c]	7.386[c]
	Median[c]					4.06	47.29	34.63	3.807
	SD[c]					2.05	13.93	10.58	10.464

[a] $P < .0001$ for both groups of infertile males compared with controls (WHO values).
[b] $P < .001$ for both groups of infertile males compared with controls (TEM controls; Collodel and Moretti, 2008).
[c] TEM controls, n = 25.
[d] WHO values.

poor axonemal and periaxonemal cytoskeletal structures were affected by necrosis.

FI, immaturity, necrosis, and apoptosis values are able to express the sperm quality of each examined ejaculate in numbers. These scores are closely interconnected because an increase in these pathologies is concomitant with a decrease in the FI.

Statistical Analysis—Sperm characteristics from 118 smoker and 153 nonsmoker patients with idiopathic infertility were statistically analyzed with SPSS v.13.0 software (SPSS Inc, Chicago, Illinois). The Kolmogorov-Smirnov test was used to verify a normal or nonnormal distribution of values. A *t* test, in the case of normal distribution, or the Mann-Whitney test was used to compare the considered variables between smoker and nonsmoker groups.

In a second analysis, the data of smoker patients were categorized into 3 groups: mild, moderate, or heavy smokers. The variables among groups were analyzed by analysis of variance or by the Kruskal-Wallis test, and when statistically significant, the Tukey or the Dunn post hoc test was used to compare pairs of groups. Variables from mild, moderate, and heavy smoker groups were compared with a group of 153 nonsmoking men using the Dunnet or the Dunn post hoc test. All variables are expressed in the tables as means or medians. $P < .05$ was considered statistically significant.

Results

In this study, the semen quality of 271 selected idiopathic infertile patients was considered. Patients were divided into smokers (n = 118) and nonsmokers (n = 153). The values of the considered variables in the 2 groups regarding pH, semen volume, sperm concentration, progressive sperm motility (grade a + grade b), TEM indices such as the fertility index (FI), and the percentage of sperm immaturity, necrosis, and apoptosis are reported in Table 1.

In both groups of infertile males, pH, semen volume, and sperm concentration were normal, whereas progressive motility was significantly reduced compared with the values suggested for normality by WHO guidelines (1999). FI was significantly decreased, whereas apoptosis, necrosis, and immaturity were increased compared with the quantitatively elaborated values from men of proven fertility used as controls for TEM analysis. It is noteworthy that FI was 0.0807×10^6 for smoker patients and 0.105×10^6 for nonsmoker patients, both values lower than 2×10^6, the minimum number of sperm devoid of ultrastructural defects needed for natural fertility, as established by the mathematical method (Table 1).

Comparing the values of all considered variables between smoker and nonsmoker groups, nonsignificant differences were observed (Table 1).

Smoker patients were then divided, according to the number of cigarettes smoked daily, into groups of mild (n = 42, from 1 to 10 cigarettes daily), moderate (n = 25, from >10 to 20 cigarettes daily), and heavy (n = 51, >20 cigarettes daily) smokers, and the values of the considered variables are reported in Table 2. Sperm concentration and FI were significantly ($P < .05$)

Table 2. *Comparison of variables of different groups of smokers and the group of nonsmoking patients. Values in bold are significantly different*

No. of Cigarettes, d^{-1}	No. of Patients	pH	Volume	Motility (a + b), %	Concentration	Fertility Index, $\times 10^6$	Apoptosis, %	Necrosis, %	Immaturity, %
Nonsmokers	153	7.6	3	23.84	43.50	0.105	10.27	43.41	70.69
Mild (≥1 and ≤10)	42	7.6	3.5	24.07	42.88	0.11	11.86	41.87	69.94
Moderate (>10 and <20)	25	7.6	2.5	28.24	57	0.095	10.57	39.57	70.49
Heavy (≥20)	51	7.6	3	22.09	**21**[a,b]	**0.026**[a,b]	12.26	45.67	73.43

[a] $P < .05$ (heavy compared with mild smokers).
[b] $P < .05$ (heavy smokers compared with control group).

different among the 3 considered smoker groups. Comparing the pairs of smoker groups, sperm concentration and FI in heavy smokers were significantly lower ($P < .05$) than observed in mild smoker and nonsmoker groups.

Discussion

A causal relationship between cigarette smoking and impaired reproductive function is highly suspected in the literature (Dorfman, 2008). Many studies have examined the effects of cigarette smoking on male fertility, and the results have suggested a substantial negative effect on sperm production, motility, and morphology (Weber et al, 2002; Kunzle et al, 2003; Ramlau-Hansen, 2007a). A review of 27 studies concerning cigarette smoking and semen quality was carried out by Vine (1996) highlighting in smokers a mean reduction of 13% in sperm concentration, 10% in sperm motility, and 3% in morphologically normal sperm.

In this study, a comparison of variables from infertile smoker patients and infertile nonsmoker patients did not reveal any significant difference.

To better understand whether the effect of the number of cigarettes smoked daily might play a negative role in semen quality, we divided the smoker subjects into 3 groups (mild, moderate, and heavy smokers), and we compared them with the nonsmoker group. Only sperm concentration and FI were significantly reduced in heavy smokers compared with mild smokers and nonsmokers. It should be noted that the group of moderate smokers, despite a lower FI compared with that of mild and nonsmokers, showed higher motility and concentrations. This discrepancy might be explained by the fact that the fertility index is influenced by sperm concentration and motility, but also by morphological evaluation of the single organelles by TEM. In addition, the small size of the group of moderate smokers (only 25 patients) should be taken into account. In this study, only 1 semen sample from each patient was examined, but in such a large population, we supposed that it was sufficient to evaluate the relation between smoking habit in the case of idiopathic infertility and sperm quality.

Our data agree with those from other authors reporting a lower sperm quality in smoker patients (Kunzle et al, 2003; Pasqualotto et al, 2008). However, Ramlau-Hansen et al (2007a) demonstrated, in a large study of this field, an inverse dose-response relation between smoking and semen volume, total sperm count, and the percentage of motile sperm, concluding that current smoking in adult life moderately impairs semen quality. Reduction in sperm parameters was also observed by Kunzle et al (2003), analyzing semen samples from 655 smokers and 1131 nonsmokers. Our results are partially in accord with those of Ramlau-Hansen and Kunzle, although we found only reduced sperm concentration and FI in heavy smokers. In particular, we did not find a relation between smoking and sperm motility, and this could be partly due to the small size of our sample group and to the severe criteria used to select the population of men with idiopathic infertility used in this study. We also cannot exclude that our patients, particularly nonsmoker patients, might have been exposed to secondhand smoke, which can cause immediate harm (Dorfman, 2008) and, in particular, to prenatal exposure to tobacco smoke, which has been demonstrated to have an adverse effect on semen quality (Ramlau-Hansen et al, 2007b).

Several studies have reported that the mutagenic components of cigarette smoke adversely affect rapidly dividing cells, including germ cells in the testis (Sorahan et al, 1997).

In rats exposed to cigarette smoke, a concurrent reduction in the number of germ cells, Leydig cells, and Sertoli cells was observed and this could be because of the toxic substances in cigarettes or histological reactions from hypoxemia induced by smoke (Ahmadnia et al, 2007). Deficiency in sperm maturations (Yamamoto et al, 1998) and a secretory deficit of Leydig and Sertoli cells (Kapawa et al, 2004) in rats exposed to cigarette smoke have already been reported. All these observations could, in part, explain the diminished sperm concentration and FI detected in the analyzed group of smoker men with idiopathic infertility.

An adverse effect of reactive oxygen species (ROS) on sperm quality is a possibility that could justify the observed sperm damage, since elevated levels of ROS have been found in infertile smoking men (Saleh et al, 2002), who showed concomitantly decreasing levels of antioxidants in seminal plasma (Pasqualotto et al, 2008).

The mechanisms by which cigarette smoking might affect semen quality could not exclude the direct involvement of toxic substances in cigarette smoke, such as nicotine, carbon monoxide, and recognized carcinogens and mutagens, such as radioactive polonium, cadmium, benzo(*a*)pyrene, and others. Most of these are known to affect male and female gametes and embryos (Zenzes, 2000; Jurasovic et al, 2004; Kumosani et al, 2008; Thompson and Bannigan, 2008).

Although the semen quality of males with idiopathic infertility seems not to be dramatically affected by cigarette consumption, heavy smokers show significantly lower sperm concentration and FI. Because much of the reduced fecundity associated with smoking could be reversed within a year of cessation (Dorfman, 2008), the clinical significance of the present finding should be to develop effective interventions aimed at helping patients stop smoking for the benefits to general health and for their fertility.

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