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An electron microscope study of the axonemal ultrastructure in human spermatozoa from male smokers and nonsmokers

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Objective: To investigate possible abnormalities or deterioration of the sperm axonemal ultrastructure in men who have smoked a large quantity of cigarettes (>20 per day) for a prolonged period.

Design: Semen specimens were collected by patients via masturbation; qualitative characteristics of the sperm were assessed and ultrastructural analysis of the sperm axoneme was performed using standard operating procedures for electron transmission microscopy.

Setting: The Andrology Institute of Lexington, Lexington, Kentucky, and the Department of Histology and Embryology, University of Salonika, Greece (collaborative effort).

Patient(s): Twenty-nine men (mean age ± SD, 30.7 ± 2.1 years) who smoked a mean (±SD) of 30.7 ± 2.1 cigarettes per day for 10.7 ± 0.7 years and 15 men who never smoked (mean age ± SD, 30.4 ± 2.2 years) participated in this study.

Main Outcome Measure(s): Ultrastructural organization of the sperm axoneme in male smokers and nonsmokers.

Result(s): Changes in the number and the arrangement of axonemal microtubules were noted in the smoker group when compared to the nonsmoker group. The incidence of axonemal abnormalities was higher in spermatozoa from smokers compared with that in spermatozoa from nonsmokers.

Conclusion(s): Smoking a large quantity of cigarettes per day, under the conditions of the current study, severely affected the ultrastructure of the flagellum and, more specifically, it affected the axoneme of the human spermatozoon. (Fertil Steril® 1998;69:430–4. ©1998 by American Society for Reproductive Medicine.)

Key Words: Cigarette smoking, spermatozoa, axoneme ultrastructure, electron microscopy

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Cigarette smoke contains a large number of substances, including nicotine, carbon monoxide, and recognized carcinogens and mutagens such as radioactive polonium, benzo(a)pyrene, dimethylbenz(a)anthracene, dimethylnitrosamine, naphthalene, and methnapthalene (1–5). However, the toxicity of many of these constituents in cigarettes and cigarette smoke have not been evaluated for their effect on human spermatozoa at the ultrastructural level.

Inhalation of cigarette smoke, whether through active or passive smoking, leads to absorption of these substances through the pulmonary vasculature and blood-borne circulation throughout the body (4). It is also possible that those same substances could end up in the seminal plasma of smokers through various modes of diffusion and active transport (5). Because other toxins affect reproduction, it is believed that the reproductive capacity in men may be impaired by exposure to a small but increasing number of environmental or occupational toxic agents (6).

Chemical agents or mutagens may affect male reproduction by direct effect on the testes and their ability to produce sperm through the process of spermatogenesis (3, 5, 7). Those mechanisms may involve the hormonal control of spermatogenesis or directly affect the germ and Sertoli cells within the seminiferous tubules (5, 8–11). Furthermore, fluctuations in hormones responsible for the regulation of spermatogenesis and the

TABLE 1

Clinical data for 29 smokers and 15 nonsmokers.

Patient group	No. of patients	Age of husband	Age of wife	No. of years married	No. of years that husband smoked	Smoking intensity*
Smoker	29	33.3 ± 1.2	28.3 ± 1.2	4.9 ± 0.6	10.9 ± 0.7	30.7 ± 2.1
Nonsmoker	15	30.4 ± 2.9	26.0 ± 2.0	4.0 ± 2.0	0	0

Note: All values are means ± SD unless otherwise indicated.

* No. of cigarettes smoked per day by husband.

sex drive have been documented in male smokers (5, 8). Various investigators have shown that smoking is associated with detrimental effects on sperm concentration, sperm motility, and the percentage of morphologically normal spermatozoa (4, 5, 11–21).

The effect of smoke metabolites on human Leydig cell function is controversial despite the reported adverse effects of those metabolites on Leydig cell function in animals (5, 11). A review of the literature suggests that the influence of smoking on the ability of men to reproduce may be caused by impaired spermatogenesis secondary to various hormonal alterations (4, 5, 8). It has been shown that cigarette smoking is associated with a higher incidence of gross sperm abnormalities and other quantitative and qualitative characteristics (5, 11–14, 16–18, 21, 22). However, data obtained from morphologic analysis of superficial sperm compartments using light or electron microscopy, without assessing the ultrastructure of those compartments, remains inconclusive (5, 13, 16, 21, 22).

The sperm tail provides the spermatozoon with a means of motility and is composed of the neck and the middle, principal, and end pieces. The entire length of the sperm tail or axoneme is composed of nine pairs (doublets) of filaments or microtubules that are arranged radially around two central filaments (9 + 2 arrangement). This arrangement of filaments is surrounded by nine coarse outer fibers. The coarse outer fibers appear to be associated with the nine pairs of filaments. Furthermore, the entire middle piece is covered by mitochondria that generate the energy needed for sperm motility. Sperm axonemal deficiencies are often the cause of lowered progressive motility and fertility, as has been observed in asthenozoospermic patients (23–26).

The objective of this study was to evaluate any possible abnormalities or deterioration of the sperm axonemal ultrastructure in men who had smoked a large quantity of cigarettes per day for a long period and in men who had never smoked.

MATERIALS AND METHODS

Semen Collection and Assessment

Forty-four patients (29 smokers and 15 nonsmokers) attending our infertility clinic were randomly selected and participated in this study. An attempt was made to choose men of similar clinical characteristics (Table 1). Patients who had smoked more than 20 cigarettes per day for at least 10 years were considered for the smoker group. Patients who never smoked were considered for the nonsmoking group. Patients were instructed to produce a semen specimen via masturbation after 3–4 days of sexual abstinence.

FIGURE 1

Normal ultrastructural arrangement of the sperm tail axoneme (cross-section) in nonsmoking patients. The sperm axoneme is composed of nine pairs (doublets) of filaments, which are arranged radially around two central filaments (9 + 2 arrangement).

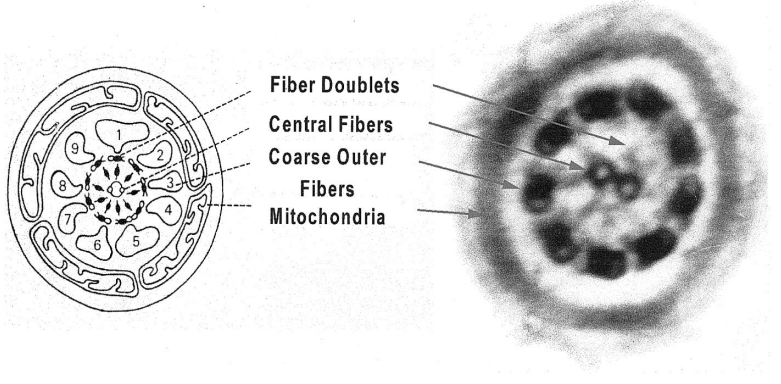
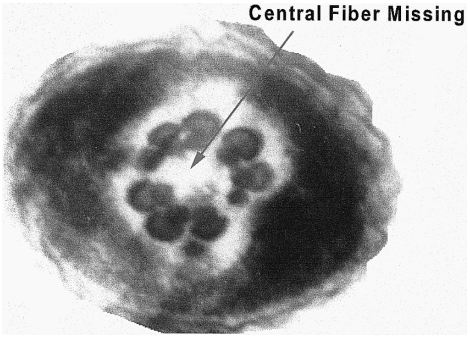


FIGURE 2

Loss of central fiber components from the sperm axoneme in spermatozoa from smokers.



The semen samples were evaluated for volume (mL), count ($\times 10^6$), motility (%), and morphological characteristics (%) after complete liquefaction (30 minutes). Morphologic features (not ultrastructural) assessed included those of the sperm head, middle piece, and tail. A total of 200 spermatozoa per specimen were assessed according to the World Health Organization guidelines (27). The total functional sperm fraction (TFSF; $\times 10^6$) parameter (inclusive term) was also calculated as the product of sperm count by motility by normal morphology (28, 29). The measurements were performed under blinded conditions by the same technician.

Semen Preparation and Ultrastructural Assessment of the Axoneme

Semen specimens were washed twice by centrifugation (800 *g* for 10 minutes) and resuspended using phosphate-buffered saline medium. The resulting sperm pellet was fixed for 1–15 hours by immersion in a 0.1 M phosphate-buffered solution (pH 7.4) containing 3.0% glutaraldehyde, followed by further fixation in buffered 1.5% osmium tetroxide (1 hour); the pellet was then embedded in Epon Araldite (Ernes Fullman, Inc., Schenectady, NY). Tissue sections were obtained using a Reichert OM U2 ultramicrotome (C. Reichert AG, Vienna, Austria) and stained with 1.0% toluidine blue in borax. Thin sections that displayed pale gold to silver interference colors were obtained with glass knives, double stained with uranyl acetate and lead citrate, and examined using a Siemmens Elmiskop 1A electron microscope (Siemmens, West Berlin, Germany).

The various components of the flagellar axoneme were assessed directly from electron micrographs (Figs. 1–3). Ultrastructual evaluation of the sperm tail cross-sections was performed by assessing 100 axonemes per patient. The number of central filaments, filament doublets, and coarse outer fibers were counted and compared between the two patient populations.

Statistical Analysis

The data obtained were analyzed with General Linear Model procedures (30). Student's *t*-tests were used to determine the degree of significance for the various mean variables obtained. The variables evaluated by the statistical model included smoking status, years of smoking, and the various sperm characteristics assessed.

RESULTS

Results obtained in this study are summarized in Tables 1–4. The ultrastructural arrangement of the normal axonemal region in spermatozoa obtained from a nonsmoker is depicted in Figure 1. Abnormalities in the ultrastructural arrangement typical of spermatozoa obtained from smokers are depicted in Figures 2 and 3. Clinical profiles for smokers and nonsmokers were similar with regard to the age of the patients, the age of their wives, and the number of years married (Table 1).

Semen characteristics from nonsmoking patients, except semen volume, showed significantly higher values ($P < 0.05$) than those of patients that smoked (Table 2). Sperm characteristics such as count, motility, normal morphology, and TFSF values were 26%, 26%, 40%, and 118.0% higher in sperm specimens from nonsmokers than

FIGURE 3

Loss of fiber doublet and/or coarse outer fibers components from the sperm axoneme in spermatozoa from smoker subjects.

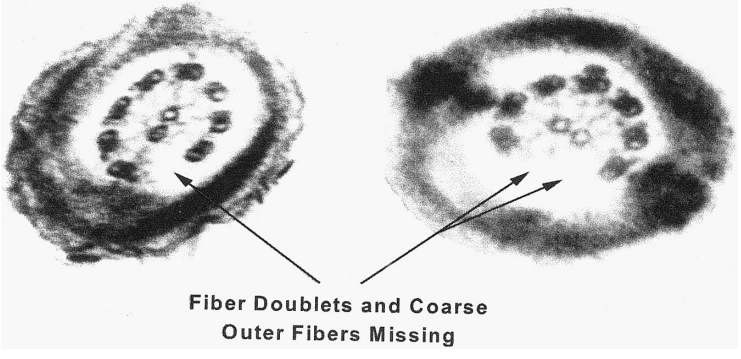


TABLE 2

Characteristics of semen specimens from smokers and nonsmokers.

Patient group	Volume (mL)	Count ($\times 10^6$)	Motility (%)	Grade (0–4)	Morphology (%)	Total functional sperm fraction ($\times 10^6$)*
Smoker	3.8 ± 0.2	187.4 ± 4.0	56.4 ± 3.0	2.5 ± 0.3	59.2 ± 2.3	62.6 ± 4.1
Nonsmoker	3.8 ± 0.5	235.5 ± 36.2†	71.0 ± 5.6†	3.6 ± 0.2†	81.6 ± 2.5†	136.4 ± 13.4†

Note: All values are means ± SD unless otherwise indicated.

* Calculated as the product of sperm count by motility by morphology.

† $P<0.05$ (sperm characteristics noticed in the specimens obtained from nonsmokers vs. smokers).

from smokers, respectively. The TFSF in sperm specimens from smokers and nonsmokers accounted for 33% and 58% of the total number of sperm per ejaculate, respectively. The percentage distribution of morphologic abnormalities in the head, middle piece, and tail regions was similar between sperm specimens obtained from smokers and nonsmokers (Table 3).

Statistically significant differences were noted in the total number of spermatozoa showing a specific abnormality (head, middle piece, and tail) between sperm specimens from smokers and nonsmokers ($P<0.05$; Table 3). The results obtained from the ultrastructural assessment of the axoneme showed that approximately 1% of the smokers and 73.4% of the nonsmoker axonemal components were intact and that 99% and 26% of the spermatozoa in those respective specimens showed some sort of ultrastructural abnormality (Table 4). In the smoker group, the most severe ultrastructural abnormality was the disappearance of fiber doublets, which accounted for 94% of the ultrastructural abnormalities.

DISCUSSION

In this study, the effect of smoking on semen characteristics and specifically on the axoneme of the sperm tail at the level of the principal piece was evaluated. The clinical profiles for all patients participating in this study were similar except for smoking habits (Table 1). Patients selected for the smoking group had a long history of smoking a large number of cigarettes per day. From the clinical point of view, spermatozoa from smokers showed decreased sperm qualitative and quantitative characteristics (Table 2). Although semen volume was not reduced in smokers, the spermatozoa count decreased 26%. Spermatozoa from smokers exhibited lower motility and progressive motility (grade) characteristics.

Lower motility and progressive motility problems have been associated with abnormalities noted within the ultrastructure of the flagellum and the axonemal structures of the sperm tail (5, 11, 17, 23). The distribution (percentage) of gross abnormalities was similar when compared between the

TABLE 3

Distribution of morphologic abnormalities in spermatozoa from smokers and nonsmokers.

	Spermatozoa region		
Patient group	Head	Middle piece	Tail
Smoker	43.9 ± 12.1 (57.5)	3.2 ± 1.9 (4.2)	29.3 ± 2.6 (38.3)
Nonsmoker	26.1 ± 4.0 (60.3)*	1.2 ± 0.5 (2.7)*	16.0 ± 1.0 (37.0)*

Note: All values are means ± SD (%). Morphologic analysis included gross abnormalities of the sperm head, middle piece, and tail.

* $P<0.05$ (specimens obtained from nonsmokers vs. smokers).

TABLE 4

Ultrastructural assessment and distribution of axonemal abnormalities in spermatozoa from smokers and nonsmokers.

	Patient group	
Type of axonemal fiber*	Smoker	Nonsmoker
Central fibers		
Two central fibers intact (%)	53	88
One central fiber missing (%)	7	2
Two central fibers missing (%)	33	1
Indiscriminate central fibers missing (%)†	7	9
Fiber doublets		
Nine fiber doublets intact (%)	6	92
One fiber doublet missing (%)	2	2
Two fiber doublets missing (%)	9	2
Two fiber doublets missing (%)	12	0
>Three fiber doublets missing (%)	41	0
Indiscriminate fiber doublets missing (%)†	30	4
Coarse outer fibers		
Nine coarse outer fibers intact (%)	33	92
One coarse outer fiber missing (%)	23	0
Two coarse outer fibers intact (%)	16	0
Three coarse outer fibers intact (%)	12	0
>Three coarse outer fibers intact (%)	16	0
Indiscriminate coarse outer fibers missing (%)†	0	8

* The axoneme of a normal spermatozoon consists of two intact central fibers, nine fiber doublets, and nine coarse outer fibers.

† Axonemal components that were missing but could not be differentiated were considered as indiscriminate.

two groups. However, spermatozoa from smokers exhibited a higher incidence of abnormalities (total numbers) in the head, middle piece, and tail regions ($P<0.05$; Table 3).

Reductions in the quantity and quality of spermatozoa from smokers was reflected by calculation of the TFSF (Table 3). The TFSF for nonsmoker specimens was approximately 2.2 times higher than the TFSF for smoker specimens. Because there were such large semen quality differences between smoker and nonsmoker specimens, tissue sections were prepared to verify the type of abnormalities that existed within the ultrastructure of the flagellum and the axonemal structures of the sperm tail.

In general, the cross-sections of spermatozoa from nonsmokers had normal axonemal arrangements, which consisted of two central fibers surrounded by the nine fiber doublets and nine coarse outer fibers (Fig. 1; Table 4). The most severe abnormality noted in the axoneme of spermatozoa from smokers was the complete disappearance of one or more of the nine fiber doublets and one or more of the central fibers (Figs. 2 and 3; Table 4). Variations in these abnormalities were noticeable within the same spermatozoon or within the same sperm sample.

Axonemal deficiencies are often the cause of lowered motility, progressive motility, and fertility in spermatozoa with a high incidence of defects such as those observed in asthenozoospermic specimens (23–26). Abnormalities or reduction in the number of the axonemal fibers could have significant effects on the progressive motility of the spermatozoon.

The results of several studies suggest that cigarette smoking is associated with a higher incidence of gross sperm abnormalities and other spermatogenic deficiencies (5, 11–14, 16–18, 21, 22). Factors such as the number of cigarettes smoked per day, years of smoking (duration), and levels of nicotine by-products present in body fluids have correlated negatively with semen and sperm quantity and quality (22). Recently, we have shown that reconstitution of spermatozoa from smokers with seminal plasma from nonsmokers resulted in significant improvements in sperm quantity and viability. However, exposure of spermatozoa from the nonsmokers to seminal plasma from the smoker subjects yielded significant reductions in sperm viability.

This study shows for the first time that smoking clearly has a degenerative effect on spermatozoa. Most important, one of the direct effects of smoking that can be qualitatively measured is the production of abnormalities induced on the flagellar structure, which tend to bring about deficiencies in the motility characteristics of the sperm (4, 5, 11, 17). Lack of progressive motility (asthenozoospermia) has been associated with decreased pregnancy rates in humans as well as decreased levels of fertilization with the use of IVF procedures (6, 26, 28).

We believe that smoking presents a challenging dilemma for the physician and the smoking public. The aim of this study was to provide evidence that smoking adversely affects male fertility. The results showed that a high intensity of cigarette smoking, under the conditions of the current study, severely affected the ultrastructure of the flagellum and more specifically the axoneme of the human spermatozoon. We have also provided one mechanism of action that cigarette smoke and its components may have on sperm physiology and ultrastructure.

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