Infertility

Effect of Smoking on Sperm Vitality, DNA Integrity, Seminal Oxidative Stress, Zinc in Fertile Men

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OBJECTIVE
To assess the effect of smoking on sperm vitality, sperm DNA integrity, semen reactive oxygen species, and zinc levels in fertile men.

METHODS
One-hundred sixty men were investigated. They were divided into 2 equal groups: healthy fertile nonsmokers and healthy fertile smokers. They were subjected to history taking, clinical examination, and semen analysis. In their semen, sperm hypo-osmotic swelling test, sperm DNA fragmentation test, seminal reactive oxygen species, and zinc were assessed.

RESULTS
Compared with fertile nonsmokers, fertile smokers were significantly associated with lower hypo-osmotic swelling test and seminal zinc levels and significantly associated with higher sperm DNA fragmentation percent and seminal reactive oxygen species levels.

CONCLUSION
Smoking (cigarettes/day and duration) has detrimental effects on sperm motility, viability, DNA fragmentation, seminal zinc levels, and semen reactive oxygen species levels, even in fertile men, and it is directly correlated with cigarette quantity and smoking duration. UROLOGY 80: 822–825, 2012. © 2012 Elsevier Inc.

Cigarette smoking is among the most important modifiable risk factors for adverse health outcomes and a major cause of morbidity and mortality.1 Different groups reported the negative impact of smoking on sperm count, motility, and morphology, concomitant with increased seminal reactive oxygen species (ROS).2,3 However, the negative impact of smoking on fertile men is denied by some because many fertile men have children and also are normozoospermic.4 In many studies, semen samples that had been designated normozoospermic in conventional analysis were seen to be influenced by the exposed risk factors.5 Mostafa2 concluded that although some smokers may not have reduced fertility, and men with marginal semen quality may benefit from quitting smoking. Also, smokers should quit smoking for the sense of responsibility for their future generation because tobacco smoke contains numerous mutagenic substances.

Sperm DNA is recognized as an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters, especially with assisted reproductive techniques.6 As an important source of DNA toxins, smoking was associated with DNA strand break formation in spermatozoa.7 Meanwhile, hypo-osmotic swelling (HOS) test was introduced for investigating sperm membrane integrity, where its application was extended further to include sperm viability assessment given the argument that sperm with damaged tails can be considered as nonviable and those with intact tails as viable.8

In addition, seminal plasma zinc (Zn) is an important antioxidant and antibacterial agent that provides a protective role against heavy metal accumulation in semen.9 Semen parameters as sperm count, motility, viability, pH, and viscosity are affected by variations of seminal plasma zinc.10

This study aimed to evaluate the effect of smoking on sperm vitality, DNA fragmentation, semen ROS, and Zn levels in fertile men.

MATERIALS AND METHODS
One-hundred sixty men recruited from the Andrology Unit, University Hospital, were investigated after institutional review board approval and informed consent. They were divided into healthy fertile nonsmokers (n = 80) and fertile smokers (n = 80). Inclusion criteria were fertile men fathering a child within the prior 2 years with normozoospermic semen analysis. Non-smokers were all chosen as never having smoked before. Exclusion criteria were fertile men fathering a child within the prior 2 years with normozoospermic semen analysis. Non-smokers were all chosen as never having smoked before.
PBS at a concentration of 20
centrifugation at 300
g for 5 minutes and resuspended in the

CA). The pellet was washed twice in PBS (pH 7.4) by

condensation (DNA damage), yielding unstable chromatin,

population representing a sperm group altered in the nuclear

by a peak followed by a shoulder, which is the marginal

of 2 distinct populations. The main population is represented

modal nonartifactual DNA pattern confirming the existence

accuracy of the analysis was tested with standard reference

Two determinations were made for each sample in which the

ode lamp, lamp current (8 mA), and wavelength (213.9 nm).

spectrophotometer (Buck model 210 VGP; Buck Scien-

tube. Seminal Zn level was determined by flame atomic absorp-

night and thoroughly washed with deionized distilled water

pyridium iodide (PI) and excitation with a 488-nanometer (nm) argon laser. The

was incubated with phosphor-buffered saline (PBS) (pH 7.4) to 2

sodium citrate dihydrate and 1.351 g fructose in 100 mL dis-

One mL of freshly prepared hypo-osmotic medium (0.735 g

tests, testicular atrophy, chronic systemic diseases, autoimmune
disorders, occupational exposure to DNA toxins, and antioxi-
dant and/or vitamin intake. All subjects were subjected to

history taking, clinical examination, and semen analysis according
to World Health Organization guidelines. The following

were assessed in semen: sperm HOS test, sperm DNA fragmen-
tation percentage, and seminal Zn levels.

Sperm DNA fragmentation assessment was performed on
fresh semen using flowcytometry (DAKO-Cytomation, Glostrup, Denmark) supplied by Coulter (Beckman Coulter, Fullerton, CA) based on the fluorescence emission from individual sperm stained with propidium iodide (PI) and excitation with a 488-nanometer (nm) argon laser. The measurement is based on the ability of PI to bind histochemically to DNA under appropriate staining conditions. Semen samples were diluted with phosphate-buffered saline (PBS) (pH 7.4) to 2 × 10⁶ sperm/mL. Fifty μL were incubated with 100 μL lysing reagent for 15 seconds and then 2 mL of PI was added and mixed with tube. Immediately after staining, tube acquisition was done by flowcytometry, where the intensity of its emission corresponds to the DNA content. Flowcytometric analysis displays a constant and characteristic bi-modal nonartifactual DNA pattern confirming the existence of 2 distinct populations. The main population is represented by a peak followed by a shoulder, which is the marginal population representing a sperm group altered in the nuclear condensation (DNA damage), yielding unstable chromatin, which appears more stainable. The percentage of sperm cells with DNA damage was automatically calculated by the flowcytometer after acquisition of 5000 spermatozoa.

Seminal ROS levels were measured in fresh semen by detecting the chemiluminescence activity using luminol (5-amino-2,3 dihydro-1,4 pthalazidine reagent) (MP Biomedicals, Irvine, CA). The pellet was washed twice in PBS (pH 7.4) by centrifugation at 300g for 5 minutes and resuspended in the PBS at a concentration of 20 × 10⁶ sperm/mL. Ten mL luminol, used as a probe, was added to the aliquot. Seminal plasma ROS levels were assessed by measuring chemiluminescence activity by Autolamat luminometer (Berthold Technologies, Bad Wildbad, Germany) in the integrated mode for 15 minutes.

HOS Test
One mL of freshly prepared hypo-osmotic medium (0.735 g sodium citrate dihydrate and 1.351 g fructose in 100 mL distilled water) was mixed with 0.1 mL liquefied semen and then incubated at 37°C for 30 minutes (minutes). Spermatozoa were examined under phase-contrast microscope, where the swelling of sperm tails was identified and counted in duplicate in 100 spermatozoa.

**Table 1. Data of the investigated groups**

| Age (y) | 35.07 ± 5.13 (28-48) | 36.47 ± 6.06 (28-45) |
| Smoked cigarettes/day | 0 | 0 |
| Smoking duration (y) | 0 | 0 |
| Semen volume (mL) | 2.7 ± 0.77 (1.5-4.0) | 2.2 ± 0.46 (1.5-3.0) |
| Sperm count (10⁶/mL) | 76.7 ± 26.6 (50-140) | 72.0 ± 27.9 (45-150) |
| Sperm motility (%) | 57.7 ± 6.8 (50-70) | 54.0 ± 5.4 (50-65) |
| Sperm abnormal forms (%) | 10.1 ± 1.6 (7-13) | 11.3 ± 1.8 (8-14) |
| Sperm HOS test (%) | 82.93 ± 5.24 (75-90) | 70.27 ± 5.38 (65-85)* |
| Sperm DNA fragmentation (%) | 5.86 ± 1.38 (3.6-8.1) | 10.85 ± 2.37 (7-15)* |
| Seminal ROS (RLU) | 436.5 ± 270.7 (190-990) | 1180.7 ± 633.18 (405-2687)* |
| Seminal Zn (μg/mL) | 139.51 ± 7.78 (127.4-153.6) | 101.21 ± 11.03 (80.4-121.4)* |

* Significant difference compared with fertile nonsmokers (P < .05).

Data are presented as mean ± SD (range).

**Seminal Plasma Zn Analysis**
Liquefied semen specimens were centrifuged at 300g for 7 minutes and seminal plasma was removed and frozen at −20°C. All glassware and plastics were rinsed with 10% nitric acid overnight and thoroughly washed with deionized distilled water before use. One mL seminal plasma was digested overnight in 2 mL concentrated nitric acid and 2 mL perchloric acid in a glass tube. Seminal Zn level was determined by flame atomic absorption spectrophotometer (Buck model 210 VGP; Buck Scientific, Inc., Norwalk, CT) with air-acetylene flame, hollow cathode lamp, lamp current (8 mA), and wavelength (213.9 nm). Two determinations were made for each sample in which the accuracy of the analysis was tested with standard reference materials.

**Statistical Analysis**
The data were analyzed and expressed as mean values ± standard deviations using SPSS version 17 program (SPSS, Inc., IBM, Armonk, NY). Unpaired t-test was used in comparisons of numerical parametric data. Pearson correlation test was applied to analyze correlations between quantitative variables. Statistical significance was set at P < .05.

**RESULTS**
Fertile nonsmokers demonstrated significantly higher progressive sperm motility, HOS test percentage, and seminal Zn level and demonstrated significantly lower sperm DNA fragmentation percentage and seminal ROS levels compared with fertile smokers (Table 1). Number of cigarettes smoked per day as well as smoking duration demonstrated significant positive correlation with seminal ROS (r = .849, P = .001; r = .944, P = .001, respectively); sperm DNA fragmentation percentage (r = .801, P = .001; r = .880, P = .001); and significant negative correlation with sperm count (r = −.514, P = .004; r = −.548, P = .002); sperm motility (r = −.789; P = .001; r = .890, P = .001); sperm normal forms
percentage \((r = -0.457, P = 0.011; r = -0.569, P = 0.001)\); HOS test \((r = -0.789, P = 0.001; r = -0.890, P = 0.001)\); and seminal Zn \((r = -0.359, P = 0.001; r = -0.671, P = 0.001)\).

**COMMENT**

The present study demonstrates the direct detrimental impact of cigarettes smoked and smoking duration on different semen parameters, including impaired sperm motility, sperm vitality, sperm DNA integrity, seminal Zn level, and increased seminal ROS correlated with daily number of smoked cigarettes and smoking duration. Previously published literature also supported the negative impact of smoking on semen parameters, including sperm count and sperm motility as well as sperm normal forms even on the ultrastructural level.\(^{12,16-18}\) It appears that all affected parameters were connected to the increased seminal ROS correlated with smoking behavior.\(^{3,19}\) Nevertheless, the mechanism by which smoking can affect semen quality did not exclude direct involvement of toxic substances in cigarette smoke as nicotine, carbon monoxide, tar, etc., affecting male gametes.\(^{20}\)

Ramirez et al\(^{21}\) observed that sperm plasma membrane integrity is necessary for motility and fertilization because semen samples with abnormal motility exhibited a HOS test indicative of a defective plasma membrane. Belcheva et al\(^{22}\) demonstrated that although the sperm DNA integrity of healthy smokers remains in the normal range, a clear negative trend is observed, especially with respect to disturbance of plasma membrane phospholipid asymmetry. Horak et al\(^{23}\) showed that the levels of bulky DNA adducts was 1.2-fold higher in smokers than non-smokers, where a significant difference existed between current smokers and never-smokers (1.7-fold increase). Chohan and Badawy\(^{24}\) showed that the rate of sperm aerobic respiration is significantly lower in smokers. In addition, Linschooten et al\(^{25}\) indicated that spermatozoa of smokers encounter higher levels of oxidative stress. Niu et al\(^{26}\) showed that smoking >20 cigarettes per day or for more than 10 years has deleterious effects on sperm DNA integrity and nuclear maturation. Perrin et al\(^{27}\) demonstrated that tobacco consumption is associated with benzo[α]pyrene-diol-epoxide-DNA adducts in spermatozoa. However, Sergerie et al\(^{28}\) did not associate smoking with DNA fragmentation in the spermatozoa of healthy men.

Seminal Zn level was significantly decreased in fertile smokers compared with fertile nonsmokers being correlated with daily number of smoked cigarettes and smoking duration. Olderede et al\(^{29}\) showed that the total quantity of Zn in the ejaculate of smokers was significantly lower than nonsmokers, jeopardizing the content of chromatin zinc and the stability of the sperm chromatin contributing to reproductive failures. Zhang et al\(^{30}\) demonstrated a negative effect of smoking on seminal Zn levels, which was correlated with quantity and duration of cigarette smoking. Kumasani et al\(^{20}\) demonstrated that cigarette smoking affects both Ca\(^{2+}\)-ATPase activity and sperm motility attributed to reduced seminal Zn level. Liu et al\(^{31}\) correlated decreased seminal Zn in smokers and the extent of smoking, suggesting a part of associated increased seminal oxidative stress that is also responsible for the impaired sperm motility, vitality, and sperm DNA integrity. Dissanayake et al\(^{10}\) showed that deterioration of semen parameters, semen pH, and semen viscosity is correlated with decreased seminal Zn level.

**CONCLUSIONS**

Smoking has a negative effect, even in fertile men. The negative effects are directly correlated with sperm progressive motility, HOS, DNA fragmentation percentages, Zn level, and increased semen ROS related with daily number of cigarettes smoked as well as duration of smoking. Therefore, effective interventions targeted at helping patients quit smoking should be addressed for the benefit of general health and fertility potential.

**References**


