Original Article

Lipid Peroxidation and Nitric Oxide Levels in Male Smokers' Spermatozoa and their Relation with Sperm Motility

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Abstract

Background: Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes known as nitric oxide synthases. Low concentrations of NO is essential in biology and physiology of spermatozoa, but high amounts of NO is toxic and has negative effects on sperm functions. Moreover, sperm membrane contains high concentrations of polyunsaturated fatty acids that are highly susceptible to oxidative damage that interferes with fertilization ability. Therefore, we investigated the correlation between levels of sperm malondialdehyde (MDA) and NO with sperm motility in male smokers.

Methods: Semen samples were collected from normozoospermic smoker (n=64) and nonsmoker (n=83) men. The content of sperm lipid peroxidation was determined by measuring malondialdehyde (MDA). The sperm NO were also measured using Griess reagent. Data was analyzed by SPSS, (version 15.0), using independent t-test and Pearson analysis.

Results: The mean MDA and NO concentrations in the sperm of normozoospermic male smokers were significantly higher than the control group or normozoospermic nonsmokers, ($p < 0.001$). A significant negative relationship was noted between sperm motility and sperm MDA levels ($r=-0.32$, $p=0.01$); and sperm motility and sperm NO concentration (for nitrite, $r=-0.34$, $p=0.006$ and for nitrate, $r=-0.38$, $p=0.002$).

Conclusion: It was concluded that the increase in MDA and NO production in sperm can influence sperm motility in normozoospermic smokers. Therefore, it seems that cigarette smoking may affect the fertility of male smokers via increasing the amount of sperm MDA/lipid peroxidation and NO concentrations.

Keywords: Cigarette smoking, Human sperm, Lipid peroxidation, Nitric oxide, Smoker men.
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Introduction

In mammalian germ cells, reactive oxygen species (ROS) production, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and/or hydroxyl radical ($^{\cdot}OH$), has been shown to be a physiological event required for maturation, capacitation, acrosomal reaction of spermatozoa, binding with the zona pellucida, and oocyte fusion (1). However, excessive generation of ROS damages the structural and functional parameters of sperm (2). Antioxidants, including superoxide dismutase, glutathione peroxidase, and catalase play an important defensive role in neutralizing ROS (3). The balance between ROS generation and antioxidants should be finely tuned in order to protect sperm (3). All cellular components, including polyunsaturated fatty acids, proteins, and nucleic acids, are potential targets of ROS. However, sperm's membrane polyunsaturated fatty

acids have a critical role in sperm function because lipid peroxidation leads to a loss of integrity in the sperm plasma membrane (4).

A lot of studies support the role of nitric oxide as a messenger in a wide array of biological processes such as neurotransmission (5), regulation of vascular wall tone (6), and immune system activity as an effector molecule with bactericidal and antiviral properties (7). NO also modulates sexual and reproductive functions in mammalian species (8). NO is a free radical which is generated from the oxidation of L-arginin to L-citrulline by nitric oxide synthase (NOS) isozymes (9). NOS activity has been detected in human and rat testis, epididymis, prostate, and seminal vescicles (10). Several studies have shown that this enzyme is associated with acrosome and tails of mouse and human sperm and it appears to be involved in sperm motility and acrosomal reaction that are the main factors in fertilization process (11–13).

Several studies have demonstrated that certain chemicals present in cigarette smoke can adversely affect male fertility (14, 15). Some of these compounds, especially nicotine and its metabolite cotenine, and cadmium have been found in seminal plasma at a concentration proportional to those in serum; therefore, these substances seem to be able to pass from blood-testis barrier (16). Consequently, seminal plasma of male smokers can be considered as a toxic environment for sperm. A relationship has also been found between cigarette smoking and alteration of sperm quality (14, 17, 18). Indeed, cigarette smoking is linked to increased levels of ROS in seminal plasma (15).

Although cigarette smoking is a hazardous habit but more people seem to consume cigarettes on a regular basis. The highest prevalence of smoking is observed in young males, aged 20–39 years, during their reproductive age (19).

Although a lot of studies have been done on the effects of smoking on male reproductive function (14, 15, 19, 21), but the role of cigarette smoking on nitric oxide and lipid peroxidation levels in sperm seem to be limited in the literature. Therefore, the objective of the present study was to evaluate the effects of cigarette smoking on the amount of sperm nitric oxide and sperm oxidation by measuring the levels of nitrite (NO_2^-), nitrate (NO_3^-), and malondialdehyde (MDA) in the sperm of normozoospermic men with the habit.

Methods

The study was approved by the institutional review board of the Institute Research Committee of Ahwaz Jundishapour University of Medical Sciences.

Semen samples were obtained from male partner of couples attending Razi Laboratory in Ahwaz, Iran for a routine semen analysis. Subsequently, a questionnaire was distributed to obtain demographic data, smoking habits, alcohol use, and use of other substances and drugs. Individuals who had consumed alcohol and/or narcotic drugs in the past three months were not eligible for the study. Other exclusion criteria included recent fever or exposure to gonadotoxins (*e.g.* chemotherapy or radiotherapy), to pesticides or heavy metals (professionally). None of the participants were receiving or had received any kind of vitamin treatment in the past three months.

A total of 147 men with a mean age of 30 years (ranging from 17–41 years) provided 147 semen sample for analysis. The 147 men categorized according to the number of cigarettes they usually smoked per day over three months before providing the sample, were divided into two groups of nonsmokers (n=83) and smokers (n=64).

Semen samples were collected by masturbation into a sterile container after 2–3 days of sexual abstinence. Following a ten-minute liquefaction period of semen at 37 ^oC and 5% CO_2, samples were examined for volume, sperm motility and count according to World Health Organization guidelines (22). All the collected samples had a volume of ≥3.0 *ml* and sperm concentration/ *mL* of $\geq 20\times10^6$. Sperm were separated from seminal plasma by centrifugation at 1000 *g* for 10 minutes at room temperature. The sperm were washed twice by adding phosphate buffer (20 *mM*, pH= 7.4) and centrifuged at 2500 *g* for 5 minutes. Finally, sperm samples were resolved in phosphate buffer and were aliquoted as a homogenous mixture with 1×10^6 sperm. The aliquots were stored at -80 ^oC for nitrite, nitrate, and malondialdehyde assays.

Sperm lipid peroxidation determination: Lipid peroxides, derived from polyunsaturated fatty acids, are unstable molecules which decompose to form a complex series of compounds, most abundantly malondialdehyde (MDA) (23). Therefore, content of sperm lipid peroxidation was determined by measuring MDA as described by Rao et al. (23). Sperm samples were lysed using rapid freeze and

thaw method, respectively at -80 ^oC and 35 ^oC, for least three times. The samples were centrifuged at 4000 *g* for 10 minutes, and the supernatant was used for the assays. The MDA level of each sample was measured by its color reaction with thiobarbituric acid reagent at 100 ^oC for one hour, which its maximum absorbance is at 534 *nm* (23). The concentration of MDA was calculated using the extinction coefficient of 1.56×10^5 $mol.l^{-1}.cm^{-1}$ and expressed as nmol of MDA equivalents per 10^6 sperm.

Sperm NO analysis: NO assay is difficult because it decomposes rapidly into nitrite (NO_2^-) and nitrate (NO_3^-) in biological solutions. Therefore, NO_2^- and NO_3^- assay is often used as a measure of NO radical production. Sperm specimens lysed through rapid freeze-thawing at -80 ^oC and 35 ^oC, for three times respectively. The sperm NO_2^- levels were measured using the Griess reagent, as previously described (24, 25). The Griess reagent consists of sulfanilamide (58 *mM* in 3 *M* HCl) and N-1-naphtylethylenediamine (722 μM). It is necessary to use protein-free samples for NO_2^- and NO_3^- assays; therefore, Somogyi method is used to eliminate protein interference (26). Briefly, 8 *ml* distilled water, 0.5 *ml* zinc sulphate (10%), and 0.5 *ml* NaOH (0.5 N), were added to 1 *ml* of semen sample. The mixture was later centrifuged for 10 minutes at 4000 *g*, and finally the supernatant was collected. NO_2^- concentration of sperm samples was determined by spectrophotometry at 540 *nm* with the addition of Griess reagent to deproteinized samples converting NO_2^- into a deep purple azo compound. In this study, 0.78–50 μM concentration of sodium nitrite was used for plotting the standard curve, and the results were reported as $nmol/10^6$ sperm.

NO_3^- measurement is based on a two-step procedure. The first step is the reduction of NO_3^- to NO_2^- using copper coated cadmium granules, as previously reported (25). The second step is the addition of Griess reagent, as described in above.

Statistical analysis: All assays were performed in triplicate and the mean±SD was used for the calculation. T-test was employed for comparisons between sperm nitrite, nitrate, and malondialdehyde levels in smoker and nonsmoker men. The coefficients of correlation were calculated analyzed by linear (Pearson) analysis. According to "one sample kolmogorov-smirnov test" data distribution are normal. Significance was defined as $p \leq 0.05$.

Results

The series studied included a total of 147 normozoospermic men who were, divided into two groups of smokers (n=64) and nonsmokers (n=83). Smokers had consumed 7 to 40 cigarettes per day (16±7.5 cigarettes/day) for a duration of 1 to 20 years (6±4 years). Comparing sperm motility, lipid peroxidation levels (using MDA assay), and NO levels (in forms of nitrite and nitrate) between the two groups showed a significant decrease in motility (p=0.004) and a significant increase in concentrations of MDA, nitrite, and nitrate (p <0.001) in sperm of smokers (Table 1). Correlations between sperm motility and levels of MDA, nitrite, and nitrate in sperm of smokers have been shown in Figure 1. This figure, shows a significantly negative correlation between sperm motility and concentrations of MDA (r=–0.32; p=0.01), nitrite (r=–0.34; p=0.006), and nitrate (r=–0.38; p=0.002) in sperm of male smoker. A significant positive relation was found between MDA levels in sperm of the smoker group with the number of cigarettes smoked per day (r=0.48; p <0.001) and/or duration of cigarette smoking per year (r=0.66; p <0.001) (Figure 2). However, the relationship was not statistically significant between nitrite and nitrate content of sperm cells with the number of consumed cigarettes per day (r=0.07; p=0.5 and r=–0.02; p=0.8, respectively) and/or cigarette smoking duration of per year (r=–0.04; p=0.7 and r=–0.01; p=0.9, respectively) (Data not shown). Finally, a positive but not significant relation was observed between concentration of MDA in sperm with both nitrite (r=0.20; p=0.1) and nitrate levels (r=0.19; p=0.1) in sperm of smoker men (Figure 3).

Table 1. Semen analysis and comparison of motility and concentration of MDA, nitrite, and nitrate in the sperm of normozoospermic smokers and non-smokers

Characteristics	Non-smoker (n=83)	Smoker (n=64)
Age (*year*)	29.27±4.96	29.70±4.45 [a]
Volume (*ml*)	4.10±1.40	3.20±1.06 [b]
pH	8.01±0.22	8.03±0.32 [c]
Sperm concentration (10^6/*ml*)	65.50±22.90	52.80±20.70 [b]
Sperm morphology (%)	47.50±18.90	37.00±21.20 [b]
Sperm motility (%)	46.50±13.80	42.50±20.20 [b]
Sperm MDA (*nmol*/10^6 *sperm*)	0.14±0.05	0.27±0.10 [b]
Sperm NO_2^- (*nmol*/10^6 *sperm*)	0.41±0.12	0.77±0.25 [b]
Sperm NO_3^- (*nmol*/10^6 *sperm*)	0.78±0.22	1.36±0.46 [b]

MDA: malondialdehyde concentration; NO_2^-: nitrite concentration; No_3^-: nitrate concentration. a: p=0.5, b: p ≤0.01, c: p=0.66

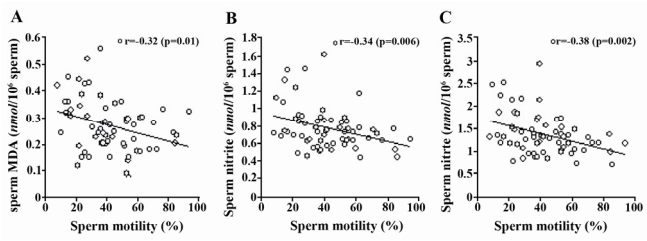

Figure 1. The relationship between sperm motility and concentration of sperm MDA, A) nitrite, B) and nitrate (C) in male smokers

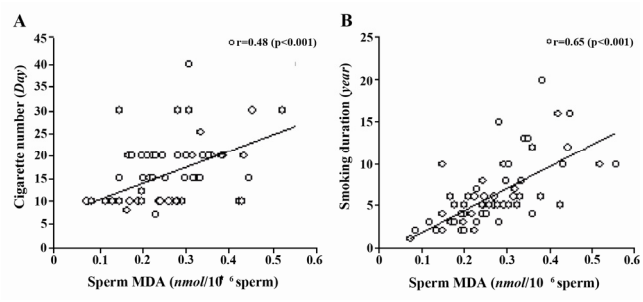

Figure 2. The relationship between sperm MDA concentration and the number of cigarette, A) and duration of smoking, B) in male smokers

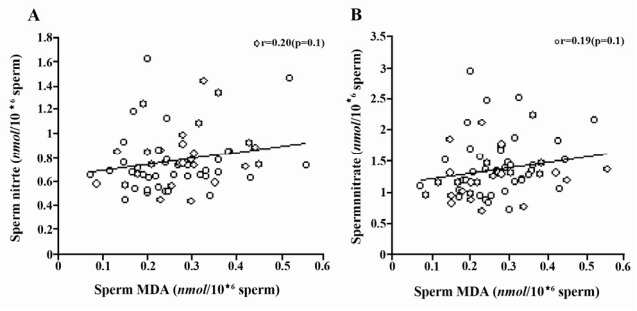

Figure 3. The relationship between sperm MDA and sperm nitrite, A) and nitrate concentrations, B) in male smokers

Discussion

Cigarette smoke contains several oxidant chemicals (15, 44), which could have an important role in lipid peroxidation processes within sperm.

In the present study, we observed a significantly decreased sperm motility (by 9%) and a significantly increased MDA, nitrite, and nitrate concentrations (by 48%, 47%, and 43%, respectively), in the sperm of normozoospermic smokers in comparison with non-smoker men. Several reports support the negative effect of cigarette smoke on sperm motility; for instance, Calogero et al. (27) demonstrated cigarette smoke extract suppressing sperm motility in a concentration- and time- dependent manner. Similarly, Hung et al. (28, 29) also reported that in vitro and/or in vivo tobacco smoke treatment decrease the percentage of motile sperm and motility parameters in adult rhesus monkeys. However, the literature concerning the effects of cigarette smoking on sperm MDA, nitrite, and nitrate concentration in fertile men on smoking is limited. Non the less, There were some studies on levels of these substances in semen and/or other parts of the reproductive system of smoker and/or non-smoker infertile men (30–33).

Numerous studies have shown oxidative stress to play a key role in the pathophysiology of sperm in human (4, 34, 35). Hsieh et al. (36) obtained a negative correlation between MDA concentration and motility of sperm in oligoasthenospermic men. They suggested that increased MDA levels could inhibit sperm motility by pathologically affecting sperm membrane. In contrast, some other researchers have reported that MDA levels in the seminal plasma were not correlated with motility or concentration of sperm (37, 38). Similarly, Aleksandra et al. (39) did not obtain any correlation between MDA concentration and sperm parameters.

Several reports have indicated that these deleterious reactive compounds are usually suppressed by the coordinated functioning of seminal enzymatic and nonenzymatic antioxidants. For instance, Geva et al. (3) demonstrated that use of antioxidants led to a reduction in MDA, which was correlated with improvement in the prevalence of fertilization. Other reports indicate that enzymatic antioxidants, such as glutathione peroxidase (40), and SOD (41), as well as nonenzymatic antioxidants, such as vitamin E (42), and vitamin C (43), could increase sperm motility by reducing MDA activity. In the present study, evaluation of the MDA content of sperm had a significant (p=0.01) negative correlation with sperm motility in normozoospermic smokers. In addition, we observed a positive and significant relationship between both the number of cigarette smoked per day ($p < 0.001$) and duration of cigarette smoking per year ($p < 0.001$) with concentration of MDA in sperm. These results suggest that cigarette smoke could increase sperm MDA levels in a concentration- and time-dependent manner.

Nitric oxide (NO) is a large molecule which plays an important role in sperm physiology (45). Lewis et al. (46) showed that sperm were sources of NO and constitutive nitric oxide synthase (NOS) which is present in two isoforms similar to those present in both endothelial (ecNOS) and brain (bNOS) cells. NO itself is a highly reactive,

short-lived, and lipophilic molecule with a half-life of just a few seconds which makes it difficult to measure (47). Therefore, its metabolites, nitrite and nitrate, were studied in the present study. Our study indicated a significant negative correlation between sperm motility and levels of both nitrite (p=0.006) and nitrate (p=0.002) in sperm of normozoospermic smokers. However, no significant correlation was found between sperm nitrite and/or nitrate concentration with the number and/or duration of cigarette smoking. Similarly, except one report (48) that demonstrated sperm motility could not be affected by NO levels, other investigations have shown decreased sperm motility in the presence of high concentrations of NO-releasing compounds, such as sodium nitroprussie (49–51). Thus, result of this study being in agreement with other reports, we suggest that cigarette smoking can increase NO concentration in sperm in a concentration- and time- independent manner.

Weinberg et al. (51) demonstrated that NO could reduce ATP levels in cells via a decrease in ATP content and/or production as approximately 90% of the energy in sperm is produced as ATP (51); therefore, this mechanism may probably be responsible for a significant negative correlation observed between sperm nitrite and nitrate levels with sperm motility, in this study.

Finally, this investigation indicated a positive but insignificant correlation between MDA content of sperm with nitrite and nitrate levels in male smokers. This suggests that the elevated lipid peroxidation in male smokers' sperm has not solely occured by NO production. Therefore, cigarette smoking probably could increase MAD/lipid peroxidation in sperm via production of NO and other oxidative agents. In the present study, we excluded samples presenting leukocytospermia, thereby, allowing us to demonstrate these molecular alterations had been caused by tobacco and not mediated by leucocytes. In support of our hypothesis, there are a lot of reports that demonstrate production of different oxidative agents, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), proxyl radical (ROO^-) and hydroxyl radical (OH^-) in the male reproductive system (52–54).

Conclusion

In conclusion, the present study indicate an association between cigarette smoking and an increase in MDA/lipid peroxidation levels duo to an increase in the concentration of NO and probably other oxidative factors in the sperm of normozoospermic cigarette smokers. In addition, it was also demonstrated that high levels of MDA and NO have a significant negative correlation with sperm motility. Therefore, we suggest cigarette components may potentially affect motility of sperm via increase in MDA/lipid peroxidation, and NO concentrations in sperm. Thus, high levels of sperm MDA and NO may be one of the possible factors of fertility in male smokers. However, further investigations are needed to elucidate the exact effect of cigarette smoking on sperm activity in male smokers.

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