



Possible mechanism by which zinc protects the testicular function of rats exposed to cigarette smoke

Michele K. Sankako¹, Patricia C. Garcia¹, Renata C. Piffer¹,
Bruna Dallaqua², Débora C. Damasceno², Oduvaldo C. M. Pereira¹

¹Department of Pharmacology, Institute of Biosciences, ²Department of Gynecology and Obstetrics, Botucatu Medical School, UNESP – Univ. Estadual Paulista, 18618-970 Botucatu, Sao Paulo, Brazil

Correspondence: Michele K. Sankako, e-mail: michelesankako@gmail.com

Abstract:

Background: The aim of this study was to evaluate the changes in testicular function of rats due to cigarette smoke exposure and the possible mechanism by which zinc protects against these alterations.

Methods: Male Wistar rats (60 days old) were randomly divided into 3 groups: control (G1, n = 10); exposed to cigarette smoke (G2, n = 10; 20 cigarettes/day/9 weeks) and exposed to cigarette smoke and supplemented with zinc (G3, n = 8; 20 cigarettes/day/9 weeks; 20 mg/kg zinc chloride daily for 9 weeks, by gavage). After the treatment period, the animals were euthanized, and materials were collected for analyses.

Results: G2 rats showed a reduction in body mass; impaired sperm concentration, motility, morphology and vitality; and increased malonaldehyde and thiol group levels and superoxide dismutase activity as compared to G1. Zinc prevented the reduction of sperm concentration and the excessive increase of lipid peroxidation and induced an increase in plasma testosterone levels, wet weight of testis and thiol group concentration.

Conclusions: Exposure to cigarette smoke led to harmful effects on testicular function at least partially due to the exacerbation of oxidative stress. Supplementary zinc had an important modulator/protector effect on certain parameters. The mechanism of zinc protection can be through an increase of SH concentration. Thus, zinc supplementation may be a promising addition to conventional treatments for male infertility related to smoking.

Key words:

oxidative stress, rats, semen, smoking, spermatozoa, testis, zinc

Abbreviations: AR – androgen receptor, CYC – cyclophilin, GSH-Px – glutathione peroxidase, Hb – hemoglobin, MDA – malonaldehyde, ROS – reactive oxygen species, SOD – superoxide dismutase, SH – thiol group

Introduction

Infertility is defined as the absence of conception after at least 12 months of unprotected intercourse in couples with a regular sex life [57]. In humans, infertility affects up to 15% of the sexually active population,

and 50% of these cases are due to a factor affecting the male [52]. It is important to consider that infertility can cause emotional disorders, including stress, low self-esteem and social isolation [11], which result from the inability of couples to generate offspring.

In recent decades, there has been a decline in the concentration and quality of human sperm in association with environmental and lifestyle factors [17, 45]. Of note, the available biological, experimental, and epidemiological data indicate that up to 13% of human infertility may be attributable to cigarette smoking [38]. Cigarette smoke contains a large number of

substances, including nicotine, carbon monoxide and other recognized carcinogens and mutagens [48]. Some of these substances and/or their metabolites can be detected in seminal plasma [21], indicating the passage of these substances through the blood-testis barrier. Therefore, cigarette smoking can damage sperm function and even the genetic material. Moreover, people exposed passively to cigarette smoke may suffer reproductive consequences similar to active smokers [38].

The exact molecular mechanisms by which cigarette smoke exposure damages reproductive parameters are not understood, but one possibility is *via* oxidative stress [36, 42] caused by the increased generation of free radicals as reactive oxygen species (ROS) [50]. Physiologically, there is a balance between ROS generation and antioxidant protection. The blood antioxidant system can be represented mainly by the antioxidant enzymes: superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Increased ROS generation will cause lipid peroxidation, and cytotoxic products like malonaldehyde (MDA) will be produced. As a consequence, ROS can trigger deleterious events that can affect sperm chromatin integrity [3] and cause a high frequency of DNA strand breaks [4].

Zinc is a structural and/or functional component of numerous metalloenzymes and metalloproteins [13]. It is involved in immune function [18], cell growth and proliferation [28], and DNA replication and transcription [53]. Moreover, zinc is important for reproduction due to its essential role in germ cell development [6]. Zinc also has antioxidant properties [56], for which three possible mechanisms have been described, including *via* synthesizing metallothionein, which inhibits the propagation reactions of free radicals [31]; acting as a structural and catalytic component of SOD, which reduces the toxicity of reactive oxygen species by catalyzing the dismutation of O_2^- into H_2O_2 and O_2 [15]; and preventing oxidation by binding to sulfhydryl (SH) groups in cell membrane proteins by occupying the binding sites of iron and copper, which are pro-oxidants [56].

A previous study from our laboratory revealed a correlation between smoking and possible male infertility and subfertility and that some of the smoking-induced changes in spermatozoa were prevented by zinc treatment [20]. Therefore, the present study aimed to evaluate changes in testicular function of rats exposed to cigarette smoke by hormonal, seminal, histopathological and molecular analyses, to in-

vestigate the possible mechanism for these alterations and the role of zinc in protecting against them.

Materials and Methods

Animals

Wistar male rats (*Rattus norvegicus albinus*), weighing approximately 250 g, were obtained from the Central Animal House of University Estadual Paulista (UNESP), Botucatu, Brazil, and maintained under standard conditions (temperature $25 \pm 1^\circ C$, humidity $55 \pm 5\%$ and lights on from 06:00 to 18:00 h) with free access to food and water in accordance with the Ethical Principles in Animals Research adopted by the Brazilian College of Animal Experimentation. All procedures were approved by the Institute of Biosciences/UNESP Committee for Animal Research (Protocol no. 114). For each set of experiments, one male sibling was chosen from each litter to decrease litter effects.

Drugs and chemicals

Acetic acid (Dinamica Quimica, Brazil); ethidium bromide (Amresco, USA); formaldehyde (Dinamica Quimica, Brazil); potassium chloride (Chemco, Brazil); sodium dodecyl sulfate (Sigma Chemical Co., USA); sodium pentobarbital (Hypnol 3%, Fontoveter, Brazil); thiobarbituric acid (Sigma Chemical Co., USA) and zinc chloride (Synth, Brazil) were used. The reagents used for oxidative stress status were acquired from Sigma Chemical Co. (USA).

Experimental groups

At 60 days of age, the animals were randomly divided into 3 groups:

- Group 1 (G1): non-smoking control group (n = 10);
- Group 2 (G2): smoking group (n = 10; 20 cigarettes per day for 9 weeks);
- Group 3 (G3): smoking/zinc group (n = 8; 20 cigarettes per day for 9 weeks plus supplementation with 20 mg/kg zinc chloride [20] daily for 9 weeks by gavage).

We did not include a zinc-only group because the aim of this study was to evaluate the role of zinc during exposure to cigarette smoke and not the effect of zinc in normal conditions.

Exposure to cigarette smoke

Cigarette smoke exposure was achieved by mainstream smoke generated by a mechanical smoking device and delivered into a chamber [14]. Smoke was released at a rate of 20 cigarettes/day divided into two repetitions of 10 cigarettes/30 min/day, at 10-min rest intervals, in the morning until the end of the experimental period. This procedure occurred every day for 63 consecutive days. This period included a complete spermatogenic cycle, which is approximately 52 days [19]. According to the manufacturer, each cigarette used in the experiment provided 10 mg tar, 0.7 mg nicotine, and 10 mg carbon monoxide.

Body weight and wet weights of testis, epididymis, seminal vesicle and prostate

After finishing the treatment, the rats were weighed and anesthetized with sodium pentobarbital (40 mg/kg, *ip*). The right testis, epididymis, seminal vesicle and prostate were identified, removed and weighed. The amount of seminal secretion was also determined.

Plasma testosterone concentration

Blood from the abdominal aorta was collected between 7:30 and 9:00 h. Samples were immediately placed in heparinized tubes, centrifuged at 2,500 rpm for 20 min at 2°C, and the plasma was stored at -20°C until assayed. Plasma testosterone level was determined by means of automatic equipment (VITROS ECi-Johnson and Johnson Ultra-Sensitive Chemiluminescence analysis; Johnson & Johnson Family of Companies, Langhorne, PA, USA) in a renowned laboratory for clinical analysis by using specific reagents (streptavidin, biotinylated antibody mouse anti-testosterone, horseradish peroxidase-labeled testosterone conjugate, and luminogenic substrate) supplied by Johnson and Johnson Orthoclinical. The sensitivity of this assay was 0.02 ng/dl. The intra- and inter-assay variations for the assay were 5.36% and 5.10%, respectively.

Histopathological testis evaluation

The right testes were fixed in Alfac fixing solution (80% ethanol, formaldehyde and glacial acetic acid, 8.5 : 1.0 : 0.5, v/v/v) for 24 h. The pieces were embedded in Paraplast Plus (Sigma Chemical Co., USA) and sectioned at 5 µm. The sections were stained with he-

matoxylin and eosin (HE). Analysis by light microscopy was performed following specific guidelines for toxicological studies [16]. The seminiferous tubules were classified as normal or abnormal (presence of acidophilic cells, multinucleated cells, spermatids retained in the epithelium, intraepithelial vacuolization, germinal lineage cells into the tubular lumen, degeneration or depletion of any cell of the tubules).

Sperm quality

All spermatozoa present in the vas deferens were collected and diluted in 1.0 ml culture medium (Ham Nutrient F-10 Modify; Cultilab, Brazil) for the evaluation of sperm concentration (luminal sperm content in the vas deferens), motility, vitality, and morphology. Sperm concentration and motility were analyzed in a Makler counting chamber (Sefi-Medical, Israel). Sperm motility was assessed by counting both motile and immotile spermatozoa in at least 10 separate randomly selected fields. At least 100 spermatozoa were evaluated per 10-µl drop in the five drops analyzed from each sample under a bright-field microscope (magnification 125×). Then, the proportion of spermatozoa that exhibited flagellar activity was recorded. Sperm vitality was analyzed after staining with eosin Y-nigrosin. One hundred spermatozoa were evaluated using phase contrast microscopy at 400× magnification and were classified as either dead (if they were orange-red in color, indicating that the stain had passed through the membrane) or alive (if they were not stained). The results were expressed as the percentage of live sperm. This staining technique makes it possible to differentiate spermatozoa that are immotile but alive from those that are dead. A reduction in the percentage of motile spermatozoa associated with a high percentage of viable spermatozoa may reflect structural or metabolic abnormalities that are derived from abnormalities in testicular function or antimotility factors in the seminal plasma [52]. To evaluate sperm morphology, smears were prepared on histological slides, stained with hematoxylin and Shorr stain and observed under a phase contrast microscope (magnification 1000×). Spermatozoa were classified as normal or abnormal [43, 52].

Assay for oxidative stress status

The tissue concentration of malonaldehyde was determined as an index of lipid peroxidation [58]. Testicu-

lar tissue samples were homogenized in a 1.15% KCl solution. The homogenates were centrifuged at 9,000 rpm for 10 min at 4°C. An aliquot (400 µl) of the supernatant was added to a reaction mixture containing 200 µl of 8.1% sodium dodecyl sulfate, 1,500 µl of 20% acetic acid (pH 3.5), 1,500 µl of 0.8% thiobarbituric acid, and 400 µl distilled water. Samples were then boiled for one hour at 95°C and centrifuged at 4,000 rpm for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm and compared with a standard curve obtained with different known concentrations of malonaldehyde. Data were expressed as nM/g tissue.

Blood samples were put into tubes containing anticoagulant. These tubes were centrifuged at 1,200 rpm for 10 min at 4°C to assay for oxidative stress status. The oxidative stress biomarkers estimated were SOD, SH groups, GSH-Px and MDA. All parameters were estimated in the washed erythrocytes according to those described by De Souza et al. [14].

Relative gene expression of androgen receptor

Tissue samples (prostate and testis) weighing 100 mg were homogenized in Trizol. Total RNA was extracted according to Trizol protocol. Androgen receptor (AR) expression was determined by semiquantitative RT-PCR, validated by choosing the number of PCR cycles and amount of RNA within the linear range of amplification curve. Total RNA (1 µg) was incubated with DNase (Invitrogen, USA) then reverse transcribed with SuperScript III (Invitrogen, USA) and oligo-d(T) primer (Invitrogen, USA). PCR was performed on 1 µl cDNA in a PCR mastermix containing 1.25 units Taq DNA polymerase (Invitrogen, USA), 0.4 µM primers (Invitrogen, USA), 0.2 mM dNTPs (Invitrogen, USA), and 1.5 mM MgCl₂ (Invitrogen, USA) in a total volume of 25 µl. Cyclophilin (CYC) was used as the internal control gene. For AR, the amplified fragment size was 227 bp, the sequence of forward primer was 5'-AAGCAGGGATGACTCTGGGA-3' and the sequence of the reverse primer was 5'-GGAATCAGGCTGGTTGTTGT-3'; for CYC, the amplified fragment size was 440 bp, the sequence of forward primer was 5'-ACGCCGCTGTCTCTTTTC-3' and the reverse primer was 5'-TGCCTTCTTTCACCTTGC-3'. Samples were denatured for 3 min at 94°C, followed by 26 (CYC) or 28 (AR) cycles of denaturing at 94°C for 45 s, annealing at 57°C (prostate) or 61°C (testis) for 45 s and extension at 72°C for 1 min. All PCR reactions were performed with positive (prostate

or testis) and negative (water replacing cDNA) controls. PCR products were separated on 2.0% agarose gels and stained with ethidium bromide, and specific bands were quantified by densitometry (Image Gauge, Fuji Photo Film Co., Japan).

Statistical analysis

The data were analyzed using GraphPad InStat version 3.0 for Windows (GraphPad software, USA).

For comparison of the results from the three experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the *a posteriori* Tukey-Kramer test or the non-parametric Kruskal-Wallis test, with the *a posteriori* Dunn test, according to the characteristics of each variable. Differences were considered significant at $p < 0.05$.

Results

Body weight; wet weights of testis, epididymis, seminal vesicle and prostate; and plasma testosterone concentration

Table 1 shows significant reductions in the body weight in rats in the cigarette smoke exposure group (G2) compared to the control group (G1). The wet weight of testis in the smoking/zinc group (G3) was increased compared to G2 (Tab. 1), and the testosterone plasma level was significantly increased in G3 compared to G2 (Tab. 2).

Tab. 1. Wet weight of testis, epididymis, ventral prostate, seminal vesicle and seminal secretion and body weight of the experimental groups

	Experimental groups		
	G1 (n = 10)	G2 (n = 10)	G3 (n = 8)
Testis (g)	1.74 ± 0.04	1.69 ± 0.04	1.92 ± 0.07 §
Epididymis (g)	0.65 ± 0.02	0.63 ± 0.03	0.65 ± 0.04
Ventral prostate (g)	0.47 ± 0.03	0.48 ± 0.04	0.43 ± 0.04
Seminal vesicle (g)	0.76 ± 0.04	0.88 ± 0.03	0.85 ± 0.05
Seminal secretion (g)	0.47 ± 0.03	0.47 ± 0.03	0.41 ± 0.04
Body weight (g)	474.69 ± 9.93	431.93 ± 11.38*	424.45 ± 9.22

G1 = control; G2 = smoking; G3 = smoking/zinc group. Values are expressed as the mean ± SEM; * $p < 0.05$ (G1 × G2); § $p < 0.05$ (G2 × G3) by ANOVA, followed by Tukey-Kramer test

Tab. 2. Sperm parameters (motility, vitality, morphology and concentration) and plasma testosterone concentration of the experimental groups

	Experimental groups		
	G1 (n = 10)	G2 (n = 10)	G3 (n = 8)
Progressive sperm (%)	25.50 (22.75 – 28.75)	18.50 [#] (15.00 – 23.25)	22.00 (18.00 – 23.00)
Alive sperm (%)	98.50 (98.00 – 99.00)	96.00 [#] (95.00 – 97.00)	97.00 (96.00 – 97.00)
Normal sperm morphology (%)	99.00 (98.00 – 99.00)	95.50 [#] (93.00 – 96.75)	97.00 (97.00 – 98.00)
Sperm concentration ($\times 10^6$ /ml)	76.60 \pm 2.22	65.90 \pm 2.94 [*]	77.88 \pm 2.26 [§]
Plasma testosterone concentration (ng/dl)	384.76 \pm 44.06	323.97 \pm 37.85	772.87 \pm 115.75 [§]

G1 = control; G2 = smoking; G3 = smoking/zinc group. Values are expressed as median (Q₁–Q₃) or as the mean \pm SEM; [#] p < 0.05 (G1 \times G2) by Kruskal-Wallis test, followed by Dunn test; ^{*} p < 0.05 (G1 \times G2); [§] p < 0.05 (G2 \times G3) by ANOVA, followed by Tukey-Kramer test

Tab. 3. Histopathological evaluation of testis of the experimental groups

	Experimental groups		
	G1 (n = 7)	G2 (n = 7)	G3 (n = 7)
Normal seminiferous tubule (%)	97.00 (94.00 – 97.00)	96.00 (92.50 – 96.00)	95.00 (93.50 – 97.00)

G1 = control; G2 = smoking; G3 = smoking/zinc group. Values are expressed as the median (Q₁–Q₃), p > 0.05 by Kruskal-Wallis test. There was no significant difference among the groups

Sperm quality, histopathological testis evaluation and androgen receptor relative gene expression

Table 2 shows reductions in the sperm concentration of G2 rats in comparison with G1 and G3. The percentages of progressive, alive and normal morphology sperm decreased in G2 compared to G1 (Tab. 2). No significant difference was observed among groups in the testis histopathology evaluation (Tab. 3). There was no statistically significant difference among groups in relation to the gene expression of androgen receptor (Tab. 4).

Oxidative stress status

Table 5 shows the oxidative stress status of the experimental groups. The concentration of malonaldehyde in testis was increased in G2 compared to G1 and G3. The MDA concentration in erythrocytes was increased in G2 in comparison with G1. The smoking group also showed an increase in SH group concentration compared to G1 and a reduction of this parameter in relation to G3. Superoxide dismutase activity was increased in G2 compared to G1.

Tab. 4. Relative gene expression of androgen receptor in testis and ventral prostate of the experimental groups

	Experimental groups		
	G1 (n = 5)	G2 (n = 5)	G3 (n = 5)
Testis	1.77 \pm 0.12	1.86 \pm 0.14	1.96 \pm 0.15
Ventral prostate	1.36 \pm 0.09	1.24 \pm 0.10	1.12 \pm 0.07

G1 = control; G2 = smoking; G3 = smoking/zinc group. Values are expressed as the mean \pm SEM, p > 0.05 by ANOVA. There was no significant difference among the groups

Discussion

In humans, it is well documented that cigarette smoking can decrease weight and that some people, mainly adolescents, smoke to try to lose weight [23]. In the present study using rats as animal model, a reduction in body weight was observed in the cigarette smoke-exposed group (G2) compared to the control group

Tab. 5. Concentration of Hb, MDA, SH, GSH-Px and SOD in erythrocytes and concentration of MDA in testes of the experimental groups

	Experimental groups		
	G1 (n = 7)	G2 (n = 7)	G3 (n = 7)
Hb (g/dl)	1.10 ± 0.04	1.10 ± 0.01	1.16 ± 0.02
MDA (nM/g Hb)	751.79 ± 54.26	1213.01 ± 181.11*	1060.25 ± 109.87
SH (µM/g Hb)	16.22 ± 0.78	21.29 ± 0.55*	25.73 ± 0.71 [§]
GSH-Px (U/mg Hb)	3.82 ± 0.34	3.44 ± 0.21	2.61 ± 0.33
SOD (U/mg Hb)	9.18 ± 0.14	10.16 ± 0.14*	9.78 ± 0.28
MDA in testis (nM/g tissue)	29.35 ± 4.54	57.80 ± 4.25*	41.00 ± 3.53 [§]

G1 = control; G2 = smoking; G3 = smoking/zinc group. Values are expressed as the mean ± SEM; * p < 0.05 (G1 × G2); [§] p < 0.05 (G2 × G3) by ANOVA, followed by Tukey-Kramer test

(G1). These alterations may be due to metabolic effects of nicotine [33]. Bellinger et al. [7] showed that administration of nicotine to rats induced a decrease in body weight that was not the result of an increased metabolic rate but by reduced food intake. The exact mechanism by which nicotine affects feeding behavior needs to be elucidated; however, there are some suggestions. Neuropeptides, like neuropeptide Y [9], and peptide hormones, like leptin [25], could be involved in the feeding-related actions of nicotine. Neuropeptide Y is a potent stimulator of feeding and could be decreased by nicotine. In contrast, leptin is a negative regulator of food intake hormone, that acts as a signaling molecule and communicates the level of body fat reserves to the hypothalamus, could be increased by nicotine. In addition, nicotine can also increase the release of neurotransmitters, including dopamine and serotonin, which inhibit food intake [55].

The plasma testosterone concentration was increased in G3 as compared to G2, indicating an effect of zinc to modulate this parameter. This result reinforces the finding of Garcia et al. [20], which showed an increase in the level of plasma testosterone in rats exposed to cigarette smoke and supplemented by zinc. Although we did not detect a significant difference between G1 and G2, Yamamoto et al. [54] and Kapawa et al. [24] observed lower levels of plasma testosterone in rats exposed to cigarette smoke. The short time of cigarette smoke exposure in our study was most likely not enough to cause this alteration.

An increase in the testicular wet weight of G3 rats as compared to G2 was observed, and this result is consistent with plasma testosterone level: testosterone is produced in testes, which require this hormone to

develop and perform their functions. Experimental studies and studies in human [30, 39] have demonstrated that a zinc-deficient diet leads to testosterone reduction, showing that there is a direct action of zinc on testicular steroidogenesis and corroborates the suggestion that hypogonadism associated with zinc deficiency is resulting from changes in testicular steroidogenesis or failure in Leydig cells. In addition, Mansour et al. [30] verified that zinc-deficient rats show a reduction of testosterone levels and reduced activity of 3 β-hydroxysteroid dehydrogenase, an important enzyme involved in the biosynthesis of testosterone, when compared to control and zinc-supplemented rats. Hesketh [22] observed that a zinc-deficient diet in boars caused ultrastructural changes in Leydig cells, indicating that zinc may be involved in the function of these testosterone-producing cells. Merrells et al. [32] reported that a zinc-deficient diet leads to a reduction in testis weight and an increase in morphologically abnormal sperm, suggesting that this association is preceded by the altered composition of fatty acids in the membranes of these cells.

Moreover, during the last several years, many papers have been presented that indicate an important role of zinc in the psychopathology and therapy of depression and anxiety. Zinc deprivation influences brain zinc homeostasis and lead to behavioral disturbances, such as anorexia, dysphoria, impaired learning and cognitive function. Clinical observations demonstrated a reduced serum zinc concentration in the depressed patients, which was normalized after successful antidepressant therapy [12, 37].

In this study, there were reductions in sperm concentration, percentages of progressive and alive sperm,

and sperm with normal morphology in G2 when compared to G1. Sperm parameters can provide valuable information regarding potential reproductive toxicity [43]. In human smokers, there are reports that cigarette smoking can reduce sperm concentration [27, 41], motility [26, 27] and morphology [27]. Cigarette smoking was also correlated with an abnormal retention of cytoplasmic residues in sperm that characterizes an abnormal morphology, thereby impairing their function [29]. In rats, Yamamoto et al. [54] and Kapawa et al. [24] observed that long-term exposure to cigarette smoke led to reductions in sperm concentration and/or motility. An impairment of seminal parameters by cigarette smoke was also observed in a previous study from our laboratory [20]. The potential correlation between semen parameters and an agent that alters male reproductive function is greater in humans than in animals. Therefore, is safe to assume that many perturbations of reproductive function that will not affect birth rate in a population of infrahuman mammals could cause reproductive failure in humans [5].

We also confirmed a positive effect of zinc on sperm concentration: the smoking/zinc G3 group had an increase in concentration compared to rats exposed to cigarette smoke (G2). Interestingly, the serum concentration of zinc in smokers appears to be reduced [51]. Furthermore, the zinc concentration in seminal plasma also appears to be reduced in smokers, and this is related to the damage found in semen parameters [27]. Abbasi et al. [1] verified that a restriction of zinc in the diet causes a reduction in sperm count in human, and this oligospermia is reversible with zinc supplementation.

In contrast, we did not detect significant differences in the histopathological evaluation of testis. However, there are reports that rats exposed to cigarette smoke had alterations in their testes [2, 40]. This contradictory result may be due to the difference between the protocols: the studies performed previously that exposed the animals to cigarette smoke at a higher period and rate than our study. The non-observation of such differences does not exclude cell dysfunctions in testis, as well as an important zinc role in these cells.

Androgens are critical steroid hormones that determine the male phenotype. Their actions are mediated by androgen receptors that, when linked to androgen, translocate to the nucleus and regulate the expression of responsive genes. The gene encoding AR is present on the X chromosome [10] and therefore exists in

both sexes. We analyzed the relative gene expression of AR by semiquantitative RT-PCR. We did not detect statistically significant differences in this evaluation. Although we found significant alterations in the testis, as discussed earlier, we have an indication that the protocol used does not generate changes at the level of gene expression.

In erythrocytes, we verified that the cigarette-smoke exposure group (G2) had a higher amount of MDA than control group (G1). In the testis, this same change was observed; moreover, a lower concentration of MDA in G3 in relation to G2 was detected. These results indicate that exposure to cigarette smoke leads to increased lipid peroxidation, and zinc supplementation can prevent this change in the testis. This increased lipid peroxidation had been reported in active and passive smokers of both sexes [35, 47] and in animals exposed to cigarette smoke [36]. In addition, some studies have drawn a correlation between lipid peroxidation and seminal parameters. Ben Abdallah et al. [8] observed a positive correlation between levels of MDA with acrosome abnormalities and the presence of residual cytoplasmic droplets in spermatozoa. Shamsi et al. [44] found a positive correlation between the level of MDA in blood and the percentages of dead sperm and abnormal sperm morphology and also detected a negative correlation between MDA in blood and in seminal plasma with the progressive sperm. According to these authors, the increase in lipid peroxidation can result in loss of integrity and fluidity of the membrane, increase in cell permeability, damage to the structure of DNA, inactivation of enzymes, reduction of sperm fertilizing potential and cell death.

The concentration of SH groups and SOD activity was higher in G2 rats than in G1. According to the results, we suggest that, due to exacerbated lipid peroxidation promoted by exposure to cigarette smoke observed by the increase in MDA, there was an activation of the antioxidant system, evidenced by increases in the concentration of SH groups and SOD activity in cigarette-smoke exposure group. However, this higher activation of the antioxidant system did not prevent exacerbated lipid peroxidation in G2. On the other hand, G3 rats had a high concentration of SH groups when compared to G2, demonstrating that zinc supplementation induced this increase and consequently reduced the testis oxidative stress. The mechanism whereby the cigarette smoke induces oxidative stress is not clear. Some studies suggest that nicotine and

cadmium could be involved [34, 49]. Smoking also causes the stimulation of inflammatory response [46], and it can increase Cu/Zn-SOD activity. According to Shamsi et al. [44], a positive correlation between sperm count and blood SOD can be established, which indicates that the SOD evaluation could be an indicator of sperm oxidant environment and could also be a biochemical parameter to quantify sperm concentration. Ozyurt et al. [36] reported increases in MDA concentration in testis and in SOD activity in rats exposed to cigarette smoke as compared to the control group, indicating that cigarette smoke causes exacerbated lipid peroxidation, which is in agreement with our results.

In conclusion, exposure to cigarette smoke can have harmful effects on testicular function. This occurs, at least partially, due to oxidative stress induced by cigarette smoke, increasing MDA concentration in blood and testicular tissue. Zinc supplementation can play an important modulator/protector role for certain parameters. The mechanism of zinc protection can be through an increase of SH concentration. Thus, zinc supplementation may be a promising addition to conventional treatments for male infertility related to smoking.

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