ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

# Late reproductive analysis in rat male offspring exposed to nicotine during pregnancy and lactation

M. Miranda-Spooner, C. C. Paccola, F. M. O. Neves, S. U. de Oliva and S. M. Miraglia

Laboratory of Developmental Biology, Department of Morphology and Genetics, Federal University of Sao Paulo – UNIFESP, Sao Paulo, Brazil

**Correspondence:**
Sandra M. Miraglia, Laboratório de Biologia do Desenvolvimento, Departamento de Morfologia e Genética, Universidade Federal de São Paulo-Escola Paulista de Medicina/UNIFESP-EPM; Rua Botucatu, 740, Edificio Leitão da Cunha, 2º andar, CEP 04023-900, São Paulo/SP, Brasil.
E-mail: miraglia.sm@gmail.com

**Keywords:**
epididymis, nicotine, pregnancy, rats, spermatozoa, testis

Received: 26-Dec-2014
Revised: 20-Jul-2015
Accepted: 31-Jul-2015

doi: 10.1111/andr.12100

## SUMMARY

We previously observed that nicotine, administered to rats (Wistar) during pregnancy and lactation periods, provokes, in the progeny, late morphofunctional alterations in Leydig cell, body weight increase in adulthood (90 days post partum, dpp) as well as seminiferous epithelium injury. Aiming to investigate whether the spermatogenic damage previously observed in adult progenies from pregnant and lactating nicotine-exposed rat dams are maintained or whether it is worsened in older rats, we analyzed the morphological testicular alterations after up to two complete periods of spermatogenesis (53 days each), spermatic parameters, and sperm DNA fragmentation. Pregnant and lactating rats were nicotine-exposed (2 mg/kg/day) through an osmotic minipump implanted on the first day of pregnancy and replaced after birth. Absolute Control (no minipump) and Sham Control (minipump without nicotine) groups were established. The offspring were killed at 90, 143, and 196 dpp. Significant alterations in morphometric and stereological testicular parameters, such as concentration of sperm number, daily sperm production, and plasma and intratesticular levels of cholesterol and testosterone were not observed in nicotine-exposed rats. Testicular histopathological analysis showed small intraepithelial vacuolization and an accentuated germ cell desquamation in exposed rats. However, the offspring from nicotine-exposed dams exhibited higher frequency of morphologically abnormal spermatozoa and lower sperm motility in comparison with control groups. In addition, nicotine-exposed groups showed a significant reduction in sperm mitochondrial activity and an increased sperm DNA fragmentation (Comet assay). These results indicate a late reproductive damage in the male progeny caused by maternal nicotine exposure, related to the decrease in sperm quality.

## INTRODUCTION

The human fertility potential has been depreciated because of exposure to pollutants, changes in lifestyle, and xenobiotics (Arabi, 2004). In this context, male infertility accounts for 40% of infertility problems (Agarwal *et al.*, 2008). Because spermatogenesis involves several steps such as mitosis, meiosis, differentiation, and maturation, the male germinal lineage cells are highly susceptible to endogenous and exogenous agents (Zenzes, 2000). In addition, the complex regulation of spermatogenesis by endocrine and testicular paracrine/autocrine factors (Huleihel & Lunenfeld, 2004) makes the reproduction vulnerable to disruption.

Around one-third of the world's population smokes regularly (World Health Organization, 2010). Moreover, clinical studies have revealed that 10.4% of women report smoking during pregnancy in United States (Martin *et al.*, 2010). In the United States, the prevalence of smoking 3 months before pregnancy has not changed since 2000 (23%). However, a statistically significant decrease in the prevalence of smoking during the last 3 months of pregnancy (from 13.3 to 12.3%) and during the 4 months after delivery (from 18.6 to 17.2%) was noticed. In addition, other countries have reported similar declining rates of smoking during pregnancy over the past 20 years. On the other hand, certain populations still have very high prenatal and postnatal smoking rates (reviewed by Meernik & Goldstein, 2015).

Cigarette tobacco has more than 4000 chemicals and around 60 carcinogens, according to the International Agency for Research on Cancer – IARC, 2004). Nicotine, a very toxic alkaloid,

is one of the major hazardous components of tobacco and is responsible for most of the deleterious effects caused by cigarettes. This is an important subject to be considered because nicotine can adversely cause deleterious effects on the organism, affecting health in several ways including alterations in the fertility of both men and women, in gestation, and in embryo development. Indeed, nicotine is responsible for many harmful effects of cigarette smoking on reproductive health (Zenzes, 2000).

The toxicity caused by nicotine on the spermatozoa arises not only from testicular damage but also from injury on the epididymis, which could reduce the number of mature spermatozoa and increase the percentage of immature forms. It is also well known that nicotine harmfully affects sperm quality parameters in humans and rats exposed to this xenobiotic during adulthood (Sofikitis *et al.*, 1995; Arabi, 2004; Ravnborg *et al.*, 2011).

Several studies have emphasized the relationship between nicotine and male reproductive damage in adulthood (Sofikitis *et al.*, 1995). Conversely, experimental studies focusing on the impact of nicotine exposure during pregnancy and lactation on the late reproductive function and fertility of the male progenies are scarce (Lagunov *et al.*, 2011; Sobinoff *et al.*, 2014). Because of its high lipid solubility, nicotine is absorbed by the pregnant mother, as reported in human and animal studies, and readily crosses the placental tissue into the bloodstream of the fetus (Shea & Steiner, 2008). Recent studies showed that maternal nicotine exposure during pregnancy and lactation can induce structural changes in either the testis or epididymis of the male offspring (Lagunov *et al.*, 2011), as well as changes in somatic and germ cell epigenome (Leslie, 2013). In a previous study performed by our group, we observed that nicotine, when administered in rats during whole pregnancy and lactation periods, provokes morphofunctional alterations in Leydig cells and body weight increase in adulthood (90 days post partum, dpp) as well as an increase in cholesterol and testosterone plasma levels (Paccola *et al.*, 2014). High levels of intratesticular testosterone and an accentuated sloughing of germ cells into the lumen were observed by Narayana *et al.* (2005) in rats after treatment with methyl parathion. Thus, we decided to investigate whether high plasmatic testosterone levels are still maintained in older offspring from nicotine-exposed rat dams as well as whether the intratesticular fluid (ITF) testosterone levels are changed, and accompanied by relevant alterations in spermatogenesis.

We proposed to investigate whether the alterations in testosterone levels would occur for longer periods in the nicotine-exposed offspring and whether a relevant spermatogenetic damage would also take place in these older rats. In addition, we investigated the impact of this possible damage on the production and quality of spermatozoa. Thus, to better scrutinize this reproductive damage, we evaluated sperm quantitative and qualitative parameters as well as intratesticular and plasmatic cholesterol levels.

To simulate the usual exposure of pregnant and lactating smoking women, nicotine was chronically administered during pregnancy and breastfeeding using suitable methodology.

## MATERIAL AND METHODS

The experimental protocol followed the ethical principles adopted by the Brazilian College of Animal Experimentation and it was approved by the Institutional Ethics Committee (protocol number 077/2012).

### Animals

Female ($n$ = 36) and male ($n$ = 18) Wistar rats were housed in polypropylene cages under controlled conditions: hygiene, photoperiod (12 h light/dark cycle), humidity (50%–60%), and temperature (22–23 °C). They had free access to tap water and commercial laboratory chow (Nuvilab CR1, Nuvital Nutrientes, Brazil). Female rats in estrous and pro-estrous were mated overnight with males (two females per male); in the following morning, vaginal smears were examined for the presence of spermatozoa and when spermatozoa were present, this day was defined as the first day of pregnancy. The pregnant rats were housed individually and observed daily for delivery. From the progenies obtained, six newborn rats (preferentially males) were kept with their dams throughout the breastfeeding period to obtain better and equal feeding for all litters. After weaning (21 days), the male offspring were maintained in the cages (four per cage) at the same controlled conditions.

### Experimental protocol

The rat dams were randomly distributed into three groups ($n$ = 12): Absolute Control (AC), no minipump implanted; Sham Control (SC), minipump implanted only with bacteriostatic water (Abbott Laboratories, Illinois, USA) and Nicotine-exposure group (Ni), in which a minipump filled with a dose of nicotine (Sigma–Aldrich Co, St. Louis, MO, USA) sufficient to release 2 mg/kg/day for 28 days was fixed; nicotine was dissolved in bacteriostatic water (Abbott Laboratories). This dosage corresponds to the daily human consumption of one packet (20 cigarettes) per day and is equivalent to the human moderate daily consumption (Roy *et al.*, 2002). To this end, on the first day of pregnancy, Ni and SC group animals were anesthetized with ketamine–xylazine (100 mg/kg of body weight) before the subcutaneous implantation of the osmotic minipump (2ML4, Alzet, Cupertino, CA, USA) in the middle of the backside of the body of each rat. It was replaced with a new nicotine-filled osmotic minipump (Ni group) just after birth and removed at the weaning day (21st dpp) (Paccola *et al.*, 2014). Thus, the progenies of each group were redistributed into subgroups according to the following euthanasia ages: 90 dpp (young adult rats), 143 dpp and 196 dpp (adult rats), that is, after, respectively, one and two complete periods of spermatogenesis in Wistar rats (around 53 days – one period).

### Blood collection and histological procedures

At the ages previously specified, the rats were weighed and submitted to euthanasia through $CO_2$ inhalation. Heparin (130 UI/kg, Clexane, Sanofi Winthrop Industrie, Maisons-Alfort, France) was administered 10 min before the euthanasia. Blood of the rats was collected from the inferior vena cava and plasma separated and stored at −20 °C for further hormonal analyses. The testes and epidydimides were removed and weighed. The volume of testes (Vt) was determined according to the Scherle's method (Scherle, 1970). In sequence, the left testis was adequately fixed by immersion in Bouin's liquid, according to the procedures of Russell *et al.* (1990), for 48 h and processed for histopathological analysis. To this end, specific fragments of the left testis were paraffin-embedded (P-3683, Sigma Chemical Co.,

St. Louis, MO, USA). Two non-consecutive isotropic 4-μm-thick testicular sections (Mandarim-de-Lacerda, 2003) per rat (10 cross sections of same thickness aside from each other) were submitted to the periodic acid-Schiff histochemical method with Harris's hematoxylin counterstaining (PAS + H).

#### Testicular morphometric and stereological analysis

The diameter of androgen-dependent tubules (stages VII and VIII) and the seminiferous epithelium height were determined by direct measurement utilizing the Leica QWin V3 (Leica Cambrige, UK) image analysis system coupled to an Olympus BX-50 light microscope (Tokyo, Japan). Fifty seminiferous tubule cross sections per testis (4μm-thick) were measured at 20× magnification. When the sections were lightly oblique, only the minor axis was considered to measure the tubular diameter (Miraglia & Hayashi, 1993). The stereological testicular analyses consisted in determining the volume density (Vv) and volume (V) of the seminiferous epithelium (lumen excluded), interstitial tissue, and lymphatic space. To calculate the volume densities, 30 fields per testis were systematically randomly sampled using an integrating ocular lens with 25 points connected to a light microscope (Weibel, 1963; Miraglia & Hayashi, 1993), at 100× magnification. For this parameter, two non-consecutive cross sections (10 cross sections of the same thickness aside from each other) per animal were analyzed (30 fields per animal); the total number of points counted for each animal was 750. As volume density is expressed in percentage, the volume of the main testicular components could be obtained using these stereological data because we also had the total volume of the testes (see section: Blood collection and histological procedures). Thus, the volumes of the seminiferous epithelium, interstitial tissue, and lymphatic space were estimated by multiplying the respective volume density (in percentage) by the testis total volume and dividing each result by 100.

#### Histopathological analysis

Histopathological analysis of the testicular sections (4μm-thick) from the left testes of the subgroups at 143 and 196 dpp was performed under a light microscope. Two non-consecutive testicular sections (Mandarim-de-Lacerda, 2003) were analyzed per animal and scrutinized using a binocular light microscope (Bx51; Olympus) under 40× and 100× magnifications. Thus, the histopathological alterations in the seminiferous epithelium were categorized and the images captured using an image analysis system (Leica Qwin-V3, Leica®; Cambridge, UK) coupled to the light microscope (Bx 51; Olympus), at 10× and 40× magnifications. Analysis and calculation of the frequency of seminiferous tubule sections with sloughed epithelial cells (germ and Sertoli cells) into the lumen were performed on the testicular sections (4μm-thick) stained by the PAS + H histochemical method. All sections of seminiferous tubules containing cells inside the lumen (which were not spermatozoa) were computed. We only included cross sections whose tubular lumen was well defined.

#### Enumeration of sperm number and daily sperm production

Homogenization-resistant testicular spermatids (step 19) and spermatozoa were, respectively, collected from right testes and caput/corpus and cauda of the right epididymides for counting, as previously described (Robb *et al.*, 1978). The testis and epididymal sperm number were expressed by organ and gram of organ. Daily sperm production (DSP) was obtained by dividing the total number of homogenization-resistant spermatids per testis by 6.1 days, as this is the length of time for which spermatids are kept in the seminiferous epithelium during its cycle (Robb *et al.*, 1978). The sperm transit time through the portions of the epididymis was determined by dividing the number of spermatozoa in each portion by the DSP (Robb *et al.*, 1978).

#### Sperm morphology

Sperm samples were obtained from the cauda of the left epididymis of all subgroups. Samples of 2 μL of the epididymal fluid were homogenized in 2 mL of bidistilled water. One drop of the solution was smeared onto a glass slide and air-dried. The smears were stained by the Shorr/hematoxylin method. For morphological evaluation, 200 spermatozoa were randomly analyzed and the percentage of abnormal spermatozoa was obtained. The classification of morphological abnormalities consisting of the head and tail of the spermatozoa was performed according to the modified descriptions, based on Filler (1993) and on the World Health Organization (WHO, 2010), adapted for the experimental model used. The abnormal characteristics considered were as follows: (i) shape of spermatozoa head; (ii) defects in tails; (iii) multiple abnormalities.

#### Sperm motility

Sperm motility parameters of the right epididymis were analyzed using the Computer-Assisted Sperm Analysis (CASA) system, according to the modified methodology previously described (Klinefelter *et al.*, 1991). Quickly, the cauda of the epididymis collected and punctured was introduced into 2 mL of Hank's balanced salt solution (HBSS) supplemented with 2.0 g/L bovine serum albumin, pH 7.3, maintained at 37 °C. The sperm suspension media were maintained at 37 °C for 30 min to homogeneously distribute spermatozoa in the media; an aliquot of the suspension was drawn by capillary action into a prewarmed chamber slide with a depth of 100 μm (LEJA Standart Count, Nieuw-Vennep, the Netherlands). The slides containing the spermatozoa were placed into a Hamilton Thorne Integrated Visual Optical System (HTM-IVOS, Hamilton-Thron Research, Inc., Beverly, MA, USA) with a software, which is specific for rat sperm analyses (Toxicology System, version 12, USA). Motility parameters observed were as follows: percentage of motile spermatozoa, curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), and straightness (STR).

#### Sperm mitochondrial activity

Sperm mitochondrial activity was analyzed using the method proposed by Hrudka (1987), based on the oxidation of 3,3-diaminobenzidine (DAB) by cytochrome *c*-oxidase, through a chain reaction in which the reagent is polymerized and deposited in the midpiece of the spermatozoa. Initially, 50 mL of the sample collected from the left epididymis was added to 100 mL of a solution containing 1 mg/mL of DAB diluted in phosphate-buffered saline and incubated for 1 h at 37 °C. After the incubation period, smears were then prepared on microscope slides and air-dried. The slides were fixed in 10% formaldehyde for 5 min, washed, and air-dried again. A total of 200 spermatozoa

were counted at 100× magnification, using a differential interference contrast Olympus BX-51TF microscope; male gametes were classified as class I (100% of the midpiece was stained), class II (more than 50% of the midpiece was stained), class III (less than 50% of the midpiece was stained), or class IV (absence of staining in the midpiece).

### Sperm DNA fragmentation

Sperm nuclear DNA fragmentation was evaluated by Comet assay as previously described (Codrington *et al.*, 2004) with some minor changes. To assess single and double-stranded DNA breaks, sperm samples were thawed at 37 °C for 2 min in a water bath and diluted in pre-warmed (37 °C) 0.5% low melting point (LMP) agarose (LGC Laboratories, Sao Paulo, Brazil) at a concentration of $4 \times 10^6$ cells/mL. The solution (125 μL) was placed on slides pre-coated with 1.5% normal melting point agarose (LGC Laboratories); cover slips were used to flatten the LMP agarose containing the spermatozoa and the slides were stored at 4 °C for 15 min. To avoid further damage to the sperm DNA, the following steps were carried out in the dark. First, each slide was covered with 1.5 mL of pre-chilled lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris–HCl; final pH 10) containing 10% dimethylsulfoxide, 1% Triton X-100 and 40 mM dithiothreitol under 4 °C for 1 h. After washing the slides with distilled water ($dH_2O$), a second lysis buffer, pre-warmed (37 °C) and containing proteinase K (0.1 mg/mL), was used to cover the slides for 2 h and 30 min in a 37 °C incubator. The slides containing the spermatozoa were then gently washed in chilled $dH_2O$ and covered with freshly prepared alkaline solution (1 mM EDTA and 0.05 M NaOH, pH 12.1) for 45 min. In sequence, the slides were washed twice with Tris/borate/EDTA (TBE) buffer (0.89 M Tris, 0.89 M boric acid and 0.5 M EDTA, pH 8) and then placed inside a horizontal electrophoresis box filled with TBE buffer, where they were submitted to electrophoresis at 1.4 V/cm for 25 min. Finally, the slides were fixed in pre-chilled 70% alcohol, air-dried, and stored in the dark until analysis. On the day of analysis, the slides were stained with ethidium bromide (20 mg/mL in $dH_2O$; 1.5 mL/slide) under an epifluorescence microscope (Nikon Eclipse CI; Nikon, Tokyo, Japan); 50 cells were analyzed per slide (two slides/animal) using the specific image analysis system for Comet assay (LUCIA *Comet Assay*, v.7.02.00, Praha, Czech Republic). The following parameters were analyzed: tail DNA (%), tail length (μm), tail moment (tail length × tail DNA% /100) and Olive Tail moment (Olive *et al.*, 1991; Vendramini *et al.*, 2014).

### Plasmatic and intratesticular testosterone and cholesterol levels

The plasma and ITF testosterone levels were obtained from rats at 143 and 196 dpp. Once the plasma testosterone level at 90 dpp was previously obtained by our group (Paccola *et al.*, 2014). So, at the time of euthanasia, the male offspring were anesthetized with sodium thiopental (89 mg/kg; Thiopentax, Cristália Produtos Químicos e Farmacêuticos Ltda., Itapira, SP, Brazil). Heparin (100 IU/kg of body weight, Clexane, Sanofi Winthrop Industrie) was administered 10 min before the euthanasia, for obtaining adequate volumes of blood and ITF. The left testis of these animals ($n$ = 14) was quickly removed and prepared for extraction of ITF, which was collected according to the method described by Rehnberg (1993). The fluid obtained was centrifuged at 4 °C (model 5430R; Eppendorf, Hamburg, Germany) and stored at −20 °C for subsequent dosages of testosterone and cholesterol. Blood was collected from the inferior vena cava and the plasma was separated and also stored at −20 °C for further hormonal analyses. Thereafter, the animals were killed by rupture of this vein. Plasmatic and ITF testosterone levels were determined by the radioimmunoassay method using the Coat-A-/ucodep>Count Total Testosterone kit (Siemens, Los Angeles, CA, USA). In sequence, the values were obtained using a radiation accountant (Cobra II, auto-gamma; Packard Instrument Company, Downers Grove, IL, USA). The sensitivity of the testosterone kit was 4 ng/dL. The plasmatic and intratesticular cholesterol levels were obtained by enzymatic methodology using a Cholesterol Liquiform Kit (Labtest, Lagoa Santa, MG, Brazil). The values were read at 402 nm utilizing APOLO-LB-912 (Berthold Technologies, Bad Wildbad, Germany) coupled to a Mikro Win 2000 System. Analytical sensitivity of the cholesterol kit was 1.04 mg/dL.

### Statistical analysis

The data were submitted to statistical tests using *SigmaPlot* software 12.0 (Systat Software Inc., San Jose, CA, USA). Data obtained from the treated and control groups were compared using one-way ANOVA parametric test followed by the post hoc Student–Newman–Keuls multiple comparison test, if necessary, or the Kruskal–Wallis non-parametric test followed by the post hoc Dunn's test. Differences were considered statistically significant when $p \leq 0.05$.

## RESULTS

### Biometric analysis: body weight, absolute and relative weights of the testis and epididymis and testicular volume

At three different ages studied, significant alterations in the body weight, testicular volume as well as of testis and epididymis absolute and relative weights did not occur in rats of the nicotine-exposed subgroups (Ni90, Ni143, Ni196) in comparison with the Absolute Control (AC90, AC143, AC196) and Sham Control (SC90, SC143, SC196) subgroups (Table 1).

### Histopathological analysis

The AC and SC groups showed normal histological characteristics of the seminiferous epithelium at 143 and 196 dpp. On the other hand, rats of the Ni143 and Ni196 subgroups showed intense desquamation of the seminiferous epithelium resulting in cellular debris and large quantity of germinal lineage cells, mainly primary spematocytes and round spermatids detached into the tubular lumen (Figs 1 and 2). The quantification of tubular sections with epithelial cell desquamation (germ cells and Sertoli cell nuclei) into the lumen of the rats at 143 and 196 dpp was assessed in all groups. Regarding this issue, there was an evident increase in the frequency of cellular desquamation in the N143 and N196 subgroups in comparison with those observed in control subgroups at the corresponding ages (Table 2).

### Morphometric analysis: seminiferous epithelium height and androgen-dependent tubular diameter

Seminiferous epithelium height and tubular diameter of androgen-dependent tubule sections (stages VII and VIII) did not show significant statistical differences (Table S1) in

**Table 1** Body weight and male reproductive organ weights of rats pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at 90, 143, and 196 dpp

| Parameters | Subgroups ($n$ = 14) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| Body weight (g) | 370.4 ± 43.3 | 379.3 ± 30.9 | 371.3 ± 22.9 | 468.8 ± 41.9 | 469.8 ± 49.5 | 460.2 ± 54.0 | 482.2 ± 42.3 | 490.1 ± 44.6 | 490.6 ± 50.1 |
| Absolute testis weight (g) | 1.70 ± 0.12 | 1.66 ± 0.05 | 1.65 ± 0.06 | 1.81 ± 0.10 | 1.84 ± 0.06 | 1.78 ± 0.10 | 1.84 ± 0.08 | 1.76 ± 0.09 | 1.79 ± 0.06 |
| Relative testis weight (g/100 g) | 0.450 ± 0.03 | 0.465 ± 0.05 | 0.444 ± 0.03 | 0.399 ± 0.04 | 0.396 ± 0.04 | 0.390 ± 0.04 | 0.376 ± 0.02 | 0.352 ± 0.03 | 0.362 ± 0.03 |
| Absolute epididymis weight (mg) | 648.3 ± 56.0 | 611.6 ± 31.2 | 641.4 ± 36.7 | 704.0 ± 38.0 | 697.0 ± 53.6 | 689.2 ± 3.8 | 716.0 ± 56.9 | 680.0 ± 38.9 | 715.0 ± 29.5 |
| Relative epididymis weight (mg/100 g) | 170.6 ± 10.2 | 167.1 ± 20.5 | 169.3 ± 13.3 | 151.2 ± 15.8 | 152.2 ± 14.3 | 151.7 ± 20.2 | 146.3 ± 9.5 | 142.7 ± 10.0 | 145.4 ± 14.8 |

Values are expressed in mean ± standard deviation (SD). ANOVA test: no significant differences were observed.

comparison with the respective Absolute and Sham Control subgroups, at the three ages studied.

### Stereological testicular analysis

The volume density (Vv) and the volume (V) of testicular components (seminiferous epithelium, interstitial tissue, lymphatic space) did not show significant alterations in rats of the nicotine-exposed subgroups at 90, 143, and 196 dpp compared with the control subgroups at the corresponding ages (Table S2).

### Sperm quantitative analysis

The quantitative sperm parameters did not change significantly among the groups at all ages studied. The count of mature spermatids (step 19), the DSP per testis as well the sperm number and transit time through the epididymal caput/corpus and cauda did not vary significantly when nicotine-exposed subgroups (Ni90, Ni143, Ni196) were compared with the Absolute Control and Sham Control subgroups at the three respective ages (Tables 3 and 4).

### Sperm qualitative analysis

Nicotine exposure during pregnancy and lactation provoked a significant ($p \leq 0.05$) decrease in sperm quality parameters at 90, 143, and 196 dpp compared with the AC and SC Control groups at the respective ages.

#### *Sperm morphology*

The abnormalities of the sperm head and tail (Table 5; Fig. 3A–P) were significantly ($p \leq 0.05$) more frequent in the nicotine-exposed rats and this phenomenon was observed in the offspring at 90, 143, and 196 dpp. However, backward bent sperm head (Table 5; Fig. 3A) and detached sperm head (Table 5; Fig. 3B) were observed ($p \leq 0.05$) only in the Ni90 subgroup. Spermatozoa with multiple abnormalities were also more numerous in the nicotine subgroups (90, 143, and 196 dpp) when compared with the AC and SC groups of the same ages (Table 5; Fig. 3J–P). Thus, a slower significant ($p \leq 0.05$) frequency of morphologically normal spermatozoa was noted in nicotine-exposed rats at all ages studied compared with the respective control rats (Fig. 3Q).

#### *Sperm motility (CASA)*

The results of the analysis of the sperm motility parameters are displayed in Table 6. The Ni90, Ni143, and Ni196 subgroups showed a significant ($p \leq 0.05$) decrease in the percentage of motile spermatozoa in comparison with the control subgroups (from AC and SC groups) at the corresponding ages. In addition, Ni90 rats displayed a significant ($p \leq 0.05$) decrease in three sperm vigor parameters (VCL, ALH, and BCF) and two sperm progression parameters (VAP and VSL). In addition, Ni143 and Ni196 rats also demonstrated a significant ($p \leq 0.05$) decline in two vigor parameters (ALH and BCF) (Table 6).

#### *Sperm mitochondrial activity (DAB)*

The DAB reaction for mitochondrial activity showed statistically significant ($p \leq 0.05$) alterations among AC, SC, and Ni groups at 90, 143, and 196 dpp. These changes were observed at all levels of classification evaluated: classes I, II, III, and IV (Table 7; Fig. S1A).

#### *Sperm DNA fragmentation (Comet assay)*

The nicotine-exposed group also exhibited a higher significantly ($p \leq 0.05$) percentage of sperm nuclear DNA fragmentation (Table 8, Fig. S1B) as regards the percentage of tail DNA and the tail length of the Comet and consequently, an increase in the tail extent moment at 90, 143, and 196 dpp.

#### *Plasma and intratesticular levels of cholesterol and testosterone*

The plasma and intratesticular levels of cholesterol (Fig. 4A–B) and testosterone (Fig. 4C–D) did not vary significantly in the nicotine subgroups (Ni143 and Ni196) in comparison with their respective control subgroups.

## DISCUSSION

Placenta provides a gateway to oxygen and nutrients from mother to fetus, produces hormones to support the pregnancy and serves as an excretion pathway for various metabolites; it

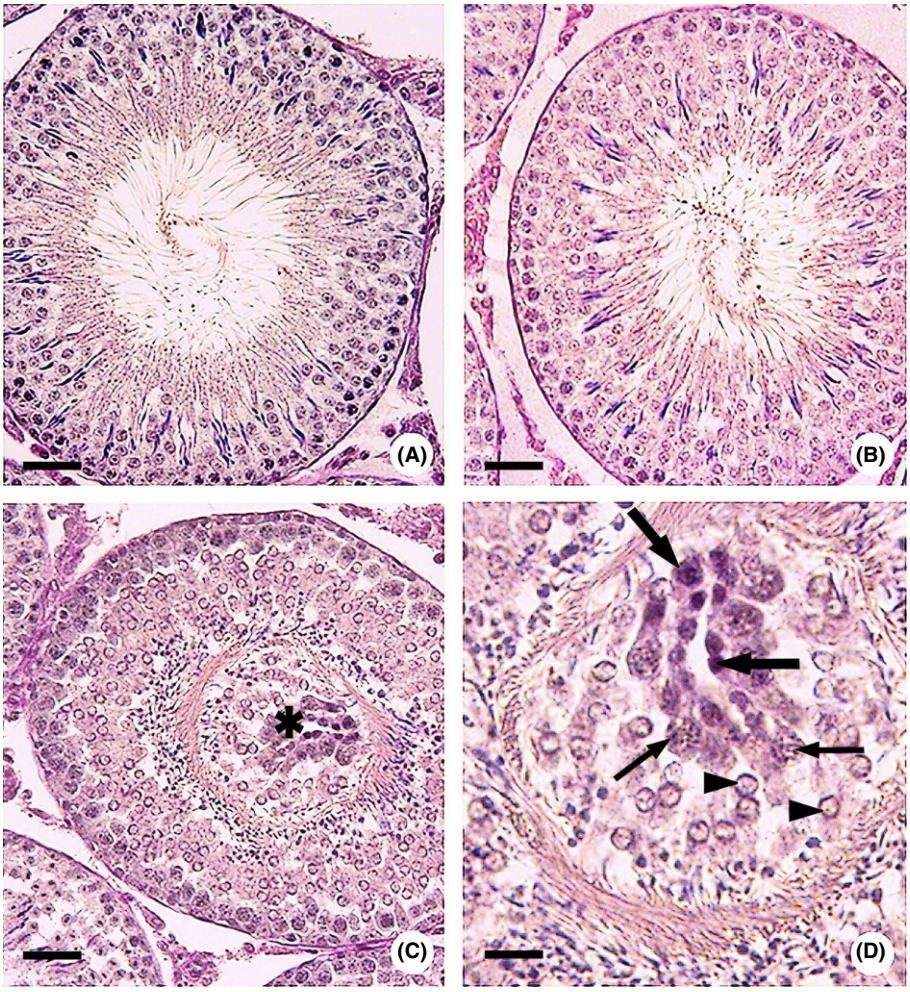



**Figure 1** Photomicrographs of testicular sections of 143 dpp rats submitted to the periodic acid-Schiff histochemical method and counterstained with Harris's hematoxylin (PAS + H). Observe the normal morphology of the seminiferous epithelium of rats from AC143 (A) and SC143 (B) subgroups, respectively. Bar: 32 μm. In the testicular section of the Ni143 rat, note the large quantity of germinal lineage cells in the tubular lumen (asterisk – C), including round spermatids (arrowheads – D), primary spermatocytes (thin arrows – D), and cells in degeneration (thick arrows – D). Bar: 37 μm and 16 μm, respectively.

plays an important role in the exposure of the fetus to potential toxicants via maternal circulation (Myllynen & Vähäkangas, 2013). Nicotine rapidly crosses the placental membrane into the fetal bloodstream (Lips *et al.*, 2005), the blood–testis barrier (Zenzes, 2000), and is present in breast milk of nicotine-exposed rats (Paccola *et al.*, 2014). The decrease in blood flow and the increase in vascular resistance caused by nicotine deprive the fetus of nutrients and oxygen, creating a state of hypoxia and malnutrition, thereby affecting fetal development (Shea & Steiner, 2008). There are overwhelming lines of evidence indicating that nicotine has an unfavorable impact on fertility but the mechanisms involved in spermatogenesis alterations are complex. Reduction in the Sertoli-cell-specific desert hedgehog gene expression may be part of a mechanism linking maternal gestational smoking with an increased incidence of impaired reproductive development in male offspring (Fowler *et al.*, 2008). In addition, a Sertoli cell dysfunction and a compromised spermatogonial stem cell population have been observed in adult offspring from smoking-exposed mouse dams (Sobinoff *et al.*, 2014).

As previously commented, the current experimental model attempts to simulate the nicotine exposure experienced by pregnant and lactating smoking women. However, the rat dams were only exposed to Ni from the first day of pregnancy; thus, the present research does not mimic the human situation because mothers rarely start smoking during pregnancy. Considering all these background data, we studied the adverse effects on male reproduction of offspring from nicotine-exposed rat dams throughout pregnancy and lactation. We observed that nicotine exposure had a negative late impact on sperm qualitative parameters of the offspring. It is known that measurements of nicotine and cotinine in amniotic fluid and fetal plasma have indicated that the fetus is actually exposed to higher nicotine concentrations than the smoking mother (Luck *et al.*, 1985).

Because in this study only rat dams (generation 0) were exposed to nicotine during pregnancy and breastfeeding and their progeny (F1 generation) had the quality of spermatozoa significantly altered in both early and late adulthood, we should reflect about the possible epigenetic mechanisms involved, although we have not studied them. There is strong evidence that miRNAs play an important role in male germ cell development, regulating mitosis, meiosis, spermiogenesis, and modulating the self-renewal and differentiation of spermatogonial stem cells in rodents (reviewed by Yao *et al.*, 2015). Concerning this, Rehan *et al.* (2012) commented that the germ line epigenetic marks imposed by nicotine exposure during pregnancy can become permanently programmed and transferred through the cell lineage to the subsequent generations. This is an interesting matter that deserves further investigation considering the imminent impact on the general and reproductive health of the progeny (first generation) of smoker couples. On the other hand, the toxic effect of nicotine in vitro on spermatozoa of normal nonsmoker donors was also observed. The most common alteration

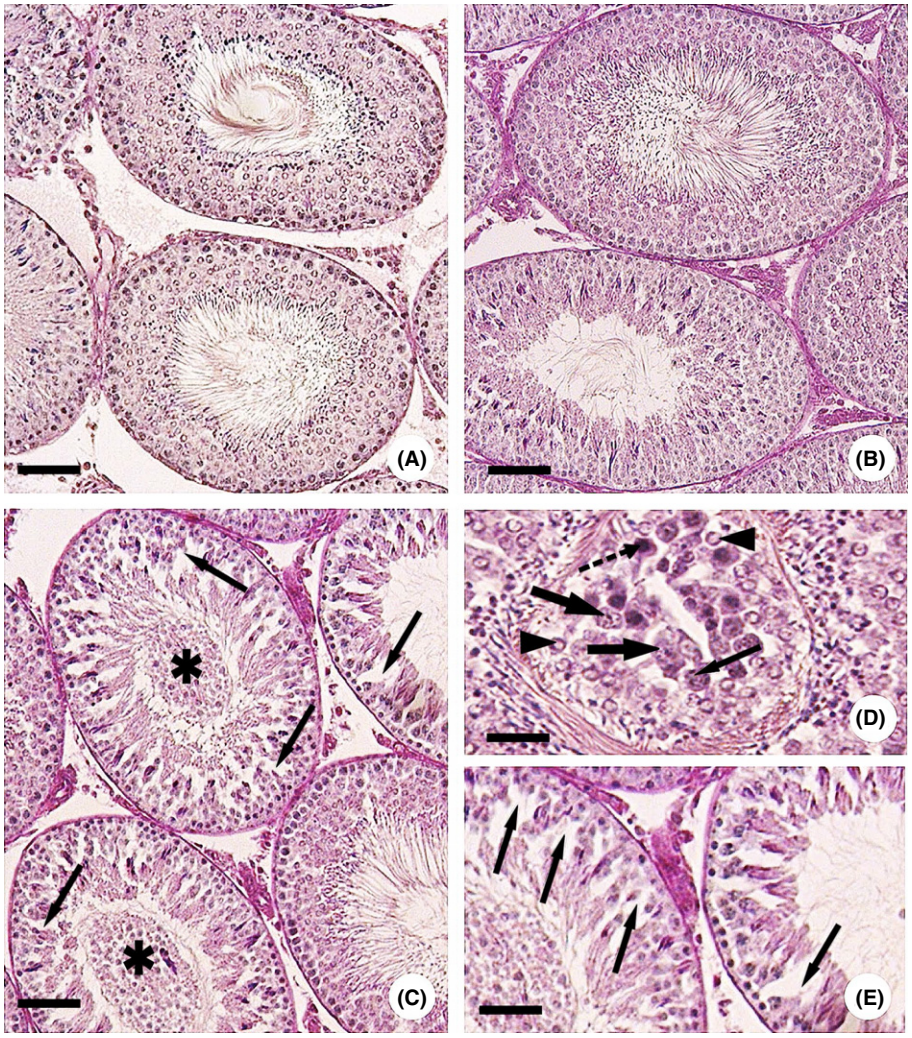



**Figure 2** Photomicrographs of tubular sections of 196 dpp rats submitted to the periodic acid-Schiff histochemical method and counterstained with Harris's hematoxylin (PAS + H). Seminiferous tubule sections of AC196 (A) and SC196 (B) rats, respectively, showing organized seminiferous epithelium containing various cell types until round and elongated spermatids. Bar: 70 μm in both. Note the large quantity of cellular debris and germinal lineage cells in the tubular lumen (asterisks) and the absence of germ cells resulting in partial depletion of the seminiferous epithelium (arrows) of a Ni196 rat (C). Bar: 70 μm. Observe, in detail (D), the large quantity of germinal lineage cells in the tubular lumen, including degenerating cells (dotted arrow), round spermatids (arrowheads), primary spermatocyte (thin arrow), and Sertoli cell nuclei (thick arrows). Bar: 11 μm. Note the absence of germinal lineage cells resulting in partial depletion of the seminiferous epithelium (arrows – E). Bar: 23 μm.

was the decrease in motility parameters (Reddy *et al.*, 1995; Condorelli *et al.*, 2013; Oyeyipo *et al.*, 2014). However, increase in the occurrence of late apoptotic spermatozoa with altered chromatin compactness or DNA fragmentation (Condorelli *et al.*, 2013), reduced sperm viability (Condorelli *et al.*, 2013; Oyeyipo *et al.*, 2014), and premature induction of the acrosome reaction (Oyeyipo *et al.*, 2014) have also been described in this type of experiment. Toxicants (such as nicotine) may directly or indirectly target the DNA through oxidative stress (OS) and DNA strand breaks, which are observed in mature spermatozoa; this might injure mitotic spermatogonia or meiotic germ cells, as well as post-meiotic spermatids undergoing spermiogenesis (reviewed by Delbès *et al.*, 2010). In addition, protamine deficiency, increased reactive oxygen species (ROS) production and abortive apoptosis are known to cause sperm DNA damage (Zini *et al.*, 2014). Overall, DNA damage in spermatozoa can occur during either the production or the transport of sperm cells (Sakkas & Alvarez, 2010).

Studies in vitro have focused on the toxic effects of nicotine. In this study, albeit we must also consider this aspect, the sperm quality alteration observed at adulthood, in different ages (F1 generation was not directly exposed to Ni), also suggests a genotoxic effect as previously commented. There is a positive association between heavy paternal smoking and generation of

**Table 2** Frequency (%) of seminiferous tubule sections with sloughing of epithelial cells, observed in adults rats pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at 90, 143, and 196 dpp

| Subgroups | Frequency of tubular sections with epithelial cell sloughing (%) | | |
|---|---|---|---|
| ($n$ = 6) | 90 dpp | 143 dpp | 196 dpp |
| AC | – | 3.82 (2.79–4.31) | 2.3 (1.79–2.84) |
| SC | – | 5.07 (2.83–5.26) | 2.93 (2.64–3.93) |
| Ni | – | 13.27 (10.47–16.50)[a] | 8.62 (7.23–17.24)[b] |

Values are expressed in median and interquartile ranges (Q1–Q3); Kruskal–Wallis test and Dunn's test: [a]Ni143 > AC143 and SC143; [b]Ni196 > AC196 and SC196 ($p \leq 0.05$).

oxidative DNA damage in the germ line, with suppression of antioxidant mechanisms in seminal plasma (reviewed by Aitken & Roman, 2008). The data from the current research reinforce that nicotine seems to be toxic and genotoxic and suggest that it probably induces ROS production, as observed by Al-Malki & Moselhy (2013). Although substantial evidence indicates that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities, the excess of ROS causes DNA damage in the form of modification of all bases, production of base free sites, deletions, DNA cross-link, and chromosomal

**Table 3** Spermatic quantitative parameters in rats at 90, 143, and 196 dpp, pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups

| Parameters | Subgroups ($n = 6$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| Step 19 spermatid count in the testis ($\times 10^6$/organ) | 62.8 (54.1–82.3) | 60.8 (46.6–83.6) | 59.6 (50.9–70.0) | 95.3 (84.6–116.7) | 100.5 (71.6–120.4) | 91.9 (73.4–126.0) | 99.0 (88.8–124.7) | 99.1 (82.5–130.1) | 83.1 (66.3–106.6) |
| Relative step19 spermatid count in the testis ($\times 10^6$/g of organ) | 71.4 (41.4–80.7) | 52.7 (39.1–71.8) | 57.5 (45.7–76.9) | 66.1 (51.5–75.9) | 70.0 (50.8–75.7) | 62.9 (51.1–75.1) | 65.6 (53.5–83.6) | 66.9 (50.0–83.2) | 53.2 (41.7–72.1) |
| Daily sperm production ($\times 10^6$/testis/day) | 10.3 (8.9–13.5) | 10.0 (7.6–13.7) | 9.75 (8.3–11.5) | 15.6 (13.8–19.1) | 16.5 (11.7–19.7) | 15.0 (12.0–20.6) | 16.2 (14.6–20.4) | 16.2 (13.5–21.3) | 13.6 (10.8–17.5) |

Values are expressed in median and interquartile ranges (Q1–Q3). Kruskal–Wallis test. No significant differences were observed.

**Table 4** Spermatic quantitative parameters in rats at 90, 143, and 196 dpp, pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups

| Parameters | Subgroups ($n = 6$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| Sperm count in the epididymal caput/corpus ($\times 10^6$/organ) | 61.2 (53.8–72.0) | 60.1 (52.2–72.3) | 60.8 (53.3–67.7) | 98.8 (87.7–106.1) | 77.9 (73.2–87.0) | 82.4 (50.7–99.0) | 102.2 (94.4–115.5) | 103.3 (89.9–109.2) | 89.0 (82.8–99.1) |
| Relative sperm count in the epididymal caput/corpus ($\times 10^6$/g of organ) | 240.5 (208.5–325.5) | 291.3 (234.1–312.8) | 240.0 (231.6–275.0) | 277.5 (246.9–295.0) | 242.5 (217.5–257.5) | 226.3 (215.0–243.8) | 272.5 (260.5–277.5) | 284.5 (248.8–298.4) | 240.6 (226.7–264.6) |
| Sperm count in the epididymal cauda ($\times 10^6$/organ) | 97.9 (63.7–124.0) | 98.5 (79.2–112.3) | 74.4 (52.4–92.3) | 150.2 (138.8–157.3) | 129.2 (120.7–162.5) | 118.1 (98.0–139.5) | 141.0 (131.3–158.8) | 165.0 (148.2–168.0) | 151.5 (134.8–152.4) |
| Relative sperm count in the epididymal cauda ($\times 10^6$/g of organ) | 532.5 (468.7–562.5) | 622.5 (506.2–670.0) | 520.0 (500.0–553.7) | 525.0 (508.7–590.0) | 472.5 (440.6–540.6) | 478.7 (416.2–519.3) | 517.5 (442.5–600.0) | 550.0 (543.7–576.2) | 482.5 (442.5–511.2) |
| Sperm transit time in the epididymal caput/corpus (days) | 10.6 (6.0–19.6) | 11.0 (7.3–18.2) | 10.0 (8.3–12.0) | 8.1 (5.2–10.3) | 8.6 (5.9–11.0) | 7.5 (6.5–9.6) | 9.1 (7.5–11.2) | 6.0 (5.6–9.8) | 10.0 (7.3–13.9) |
| Sperm transit time in the epididymal cauda (days) | 5.4 (4.3–9.3) | 7.4 (4.5–11.6) | 6.9 (5.5–9.7) | 6.5 (4.3–7.0) | 5.2 (3.4–6.3) | 5.8 (5.3–6.2) | 5.8 (4.5–6.8) | 5.4 (3.9–7.1) | 7.0 (5.0–8.8) |

Values are expressed in median and interquartile ranges (Q1–Q3). Kruskal–Wallis test. No significant differences were observed.

rearrangements; OS is also associated with high frequencies of single and double-stranded DNA breaks and gene mutations. In addition, the overproduction of ROS initiates many oxidative reactions that lead to OS resulting in damage of not only the basic constitutive molecules (cellular proteins and lipids) but also mitochondrial and nuclear DNA, ultimately facilitating an altered sperm function (Simmons *et al.*, 2005; Colley *et al.*, 2013). In the present work, increased sperm nuclear DNA fragmentation was observed in nicotine-exposed offspring at different ages; so, we suppose that this occurrence should be associated with changes in the early stages of germinal lineage; however, we did not evaluate the level of ROS, neither in the blood nor in the testis. Some studies have shown that nicotine may have a direct cytotoxic effect on spermatozoa through DNA damage (Jana *et al.*, 2010). Conversely, the germ cells were exposed to nicotine during intrauterine life and lactation and there was no direct exposure of the offspring to nicotine. Sobinoff *et al.* (2014) described that male offspring of mice show gonocyte apoptosis, reduced spermatogonial stem cells, meiotic germ cell apoptosis, abnormal Sertoli cell formation, impaired seminiferous tubule organization and germ cell development after maternal smoke exposure during pregnancy and lactation. The outcome was compromised adult sperm production/function, decreased male fertility, and a suggestive high DNA damage. The extension of DNA fragmentation might also be an indicative assessment in unexplained infertility (Sepaniak *et al.*, 2006). As commented by Sobinoff *et al.*, (2012), the persistence of increased DNA damage in adult offspring, as observed in this study, suggests continued vulnerability of germinal cells during spermatogenesis. Indeed, low sperm quality observed after exposure to nicotine has not ceased for a long time. Significant re-arrangements of the testicular mitochondrial genome could lead to significant alterations in mitochondrial function/energy production and OS, resulting in continued DNA damage long after maternal exposure to nicotine (Sobinoff *et al.*, 2014). Besides, further causes of the enhanced DNA damage may involve other components of cigarette smoke that can become highly reactive originating compounds and molecular adducts with DNA (Sobinoff *et al.*, 2012).

In the current research, there was a reduction in motility and mitochondrial activity and an increased frequency of spermatozoa with tail abnormalities in all nicotine-exposed rat offspring,

**Table 5** Frequency of sperm morphological abnormalities (%) in rats at 90, 143, and 196 dpp, pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups

| Subgroups ($n$ = 7) | Morphological abnormalities of spermatozoa (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Head | | | Tail | | | | Multiple |
| | Banana-shaped | Detached | Backward bent | Coiled | Bent | Broken | Detached | |
| AC90 | 1.0 (0.0–2.0) | 1.0 (0.0–2.0) | 3.0 (0.2–3.0) | 2.0 (1.0–3.0) | 0.0 (0.0–3.2) | 3.0 (1.2–3.0) | 2.0 (1.2–3.7) | 0.0 (0.0–0.0) |
| SC90 | 1.0 (0.0–2.0) | 0.0 (0.0–1.0) | 2.0 (2.0–3.0) | 2.0 (1.0–3.0) | 0.0 (0.0–1.0) | 3.0 (2.0–4.0) | 1.0 (0.0–1.0) | 0.0 (0.0–1.0) |
| Ni90 | 5.0 (2.0–5.0)[a] | 4.0 (3.0–4.0)[a] | 6.0 (3.0–7.0)[a] | 9.0 (6.0–9.0)[a] | 1.0 (0.0–5.0) | 6.0 (5.0–6.0)[a] | 4.0 (2.0–8.0)[a] | 1.0 (0.0–3.0)[a] |
| AC143 | 1.0 (1.0–3.5) | 1.0 (0.0–2.0) | 2.0 (0.0–3.0) | 2.0 (1.0–6.0) | 0.0 (0.0–0.2) | 5.0 (2.0–6.0) | 1.0 (0.0–2.0) | 0.0 (0.0–1.0) |
| SC143 | 4.0 (2.0–4.0) | 1.0 (0.7–2.5) | 2.5 (1.0–4.0) | 3.0 (1.7–5.7) | 0.0 (0.0–0.5) | 1.5 (1.0–5.2) | 1.0 (0.0–2.0) | 0.0 (0.0–0.0) |
| Ni143 | 7.0 (4.0–8.0)[b] | 2.0 (1.0–5.2) | 3.0 (2.0–4.0) | 5.5 (4.0–9.0) | 0.0 (0.0–0.7) | 4.0 (3.0–7.7) | 0.5 (0.0–5.0) | 1.5 (0.5–3.0)[b] |
| AC196 | 5.0 (3.5–5.5) | 0.0 (0.0–2.0) | 4.0 (3.0–5.5) | 4.0 (2.5–7.0) | 0.0 (0.0–0.0) | 3.0 (1.2–3.7) | 0.0 (0.0–0.5) | 0.0 (0.0–1.0) |
| SC196 | 4.0 (3.0–7.7) | 1.0 (0.0–2.0) | 2.5 (1.0–5.0) | 3.5 (1.75–7.5) | 0.0 (0.0–0.2) | 3.0 (2.0–4.0) | 0.5 (0.0–1.2) | 0.0 (0.0–0.2) |
| Ni196 | 9.0 (6.0–11.0)[c] | 1.0 (0.0–2.5) | 3.0 (3.0–6.0) | 5.0 (4.0–8.0) | 0.0 (0.0–0.5) | 7.0 (4.7–8.2)[c] | 1.0 (0.0–2.5)[c] | 2.0 (1.0–4.0)[c] |

Values are expressed in median and interquartile ranges (Q1–Q3). Kruskal–Wallis one-way analysis of variance test and Dunn's test: [a]Ni90 > AC90 and SC90; [b]Ni143 > AC143 and SC143; [c]Ni196 > AC196 and SC196 ($p \leq 0.05$).

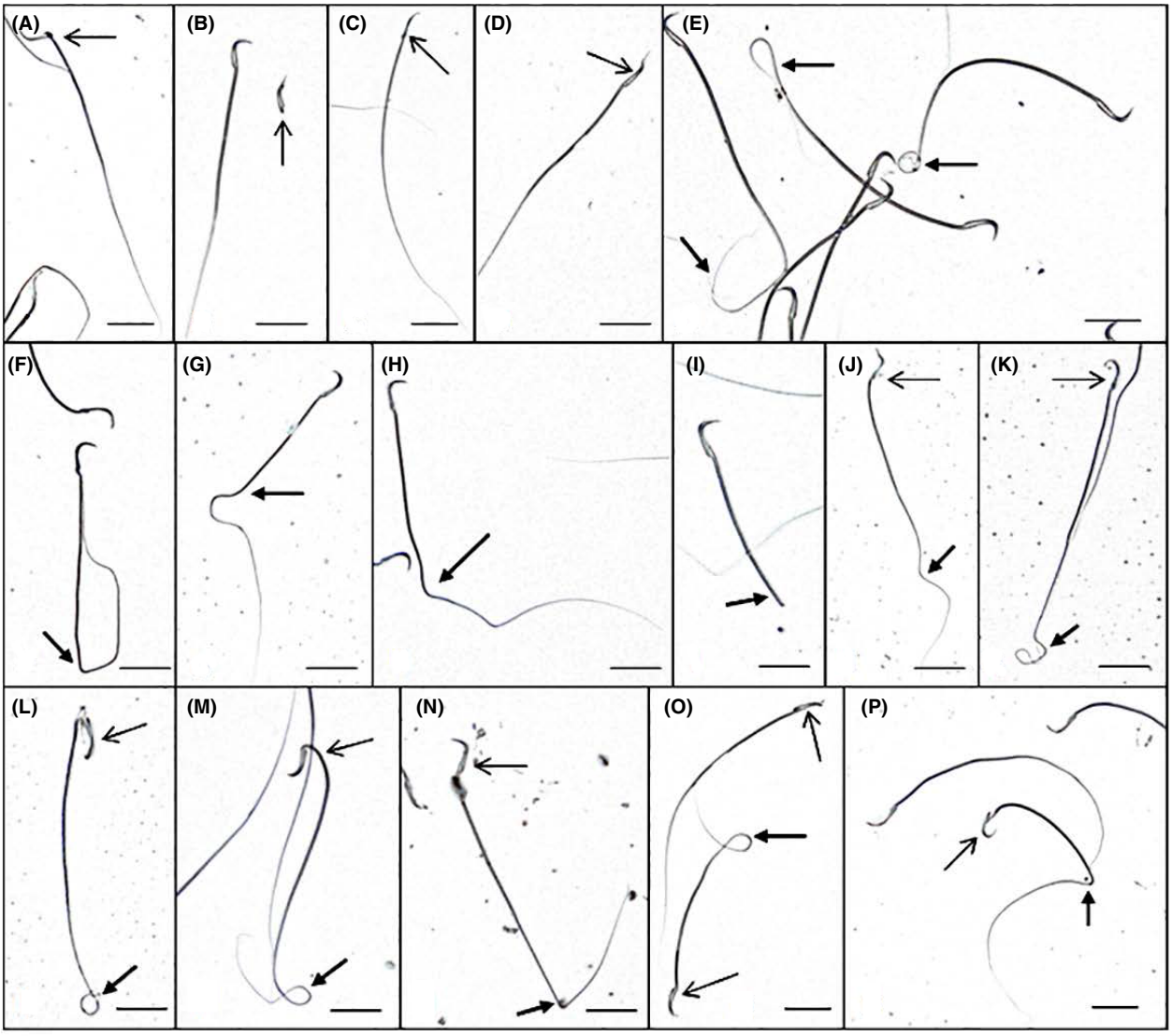
at 90, 143, and 196 dpp. All the alterations related to sperm motility observed here may be involved with a reduction in the expression of genes that participate in flagellum development and/or the other features linked to motility effectiveness (Miki *et al.*, 2002; Sobinoff *et al.*, 2014). According to Ballinger *et al.* (1996), optimal mitochondrial function is essential for fertility as spermatozoa require energy for surviving and for their motility; besides, even throughout germ cell maturation, the harmful action of nicotine on mitochondrial DNA may also be responsible for the abnormal functioning of sperm mitochondrial structures. Conversely, studies have found that mitochondria-derived ATP is not crucial for sperm motility, as glycolysis is the main mechanism utilized for acquiring energy and motility. Thus, the role of mitochondria in sperm function might be predominantly related to other physiological aspects (Amaral *et al.*, 2013). However, it seems that there is no final conclusion about the energy source of sperm motility, that is, oxidative phosphorylation or glycolysis. In addition, as previously commented, it has been suggested that small quantities of mitochondrial ROS are important for spermatozoa to acquire fertilizing competence (Piomboni *et al.*, 2012). The appropriate mitochondrial functionality has been associated with the sustenance of motility, extended to the hyperactivation as well as to the strictly associated phenomenon of sperm capacitation (Amaral *et al.*, 2013). Besides, mitochondria are the main source of ROS produced by spermatozoa, notably via the formation of superoxide in the electron transfer chain, although NADPH oxidase may also be an additional source. Considering that damage produced by ROS can occur during germ cell development and even though mitochondria are compartmentalized organelles, an increased ROS production has been associated with sperm mitochondrial membrane permeabilization, which is accompanied by mitochondrial membrane potential dissipation, uncoupling of oxidative phosphorylation, failure to synthesize ATP and OS, culminating in oxidative DNA adduct formation, DNA strand breakage, and cell death (Oyeyipo *et al.*, 2011; Aitken *et al.*, 2012; Galluzzi *et al.*, 2012; Marchetti *et al.*, 2012; Abdul-Ghani *et al.*, 2014; La Maestra *et al.*, 2015; Yousefniapasha *et al.*, 2015). Treulen *et al.* (2015) proposed that the mitochondrial permeability transition, because of pores opening in sperm inner mitochondrial membrane, is an important mechanism of ROS overproduction and DNA fragmentation.

Different from the qualitative parameters, our results showed that maternal nicotine exposure did not cause significant quantitative alterations in spermatozoa from the different epididymal segments. On the contrary, Sobinoff *et al.* (2014) observed a reduction in sperm counts in adult offspring of smoke-exposed mouse; however, they carried out the experiment using maternal smoking starting 6 weeks before pregnancy. Lagunov *et al.* (2011) administered nicotine to rat dams daily, for 2 weeks prior to mating until weaning by the subcutaneous route; in sequence, they studied sperm quantitative parameters in the offspring but they did not evaluate qualitative parameters. As previously commented, in the current experiment we established an experimental model to simulate the situation of a moderate habitual smoker (one pack of cigarettes per day) (Roy *et al.*, 2002; Paccola *et al.*, 2014). However, although we have used a different methodology from Lagunov and collaborators relating to nicotine administration, we did not find results different from their results concerning some sperm quantitative parameters of the progenies in adulthood.

In the present research, nicotine-exposed rats during the intrauterine and lactation phases showed some disturbances in spermatogenesis at the 143 and 196 dpp. In an ongoing study, our group observed in 90 dpp rats, which were nicotine-exposed to conditions similar in this study, a very high frequency of seminiferous tubule sections containing a large quantity of desquamated cells that almost filled their lumen. Whether this damage was transitory or not will be the subject of future research. Alterations in spermatogenesis after nicotine exposure were also observed by Lagunov *et al.* (2011). In this study, the negative impact of nicotine on the integrity of the seminiferous epithelium in offspring probably involved a causal relationship between nicotine exposure of mothers and a significant reproductive damage, even after one or up to two completed periods of spermatogenesis. Surprisingly, the histological damage previously observed at 90 days of age was still maintained in late adulthood (196 dpp). These detrimental effects are in accordance with the sperm qualitative parameters that were impaired in the nicotine-exposed group in comparison with the control groups. It is possible that a morphofunctional alteration in Sertoli cells has occurred that could be the main cause of the accentuated sloughing observed by us (Sobinoff *et al.*, 2014; Paccola & Miraglia, 2016).

**Figure 3** Representative photomicrographs of sperm smear collected from epididymal cauda in nicotine-exposed rats, showing head abnormalities (thin arrows) and tails abnormalities (thick arrows). Note a backward bent sperm head (A), sperm head detachment from its tail (B), banana-shaped head spermatozoa (C and D); and several changes in sperm tails as coiled tail (E), bent (E–H) and broken (I). Observe also the multiple alterations in sperm head and tails (J–P). Bar = 20 μm. (Q) Frequency of spermatozoa with normal morphology (in percentage) of rats pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at 90, 143, and 196 dpp. Values expressed in mean ± SD; ($n$ = 7). ANOVA test and post hoc Student–Newman–Keuls test: *Ni90 < AC90 and SC90; Ni143 < AC143 and SC143; Ni196 < AC196 and SC196 ($p < 0.001$).



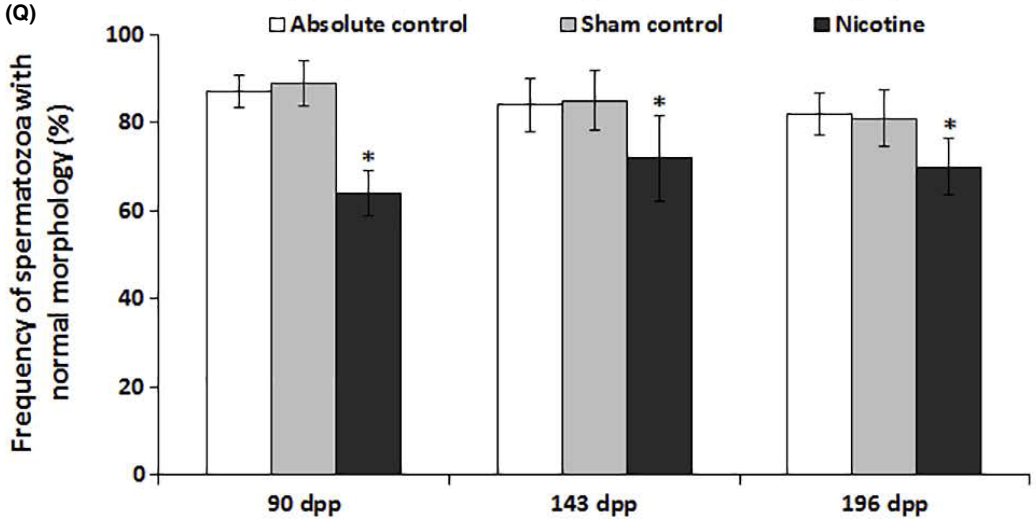



**Table 6** Sperm motion parameters (CASA HTM-IVOS) of rats pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups at different ages

| Parameters | Subgroups ($n$ = 6) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| Motility | 98 (96.7–99.0) | 97 (94.5–99.0) | 77 (68.7–88.5)[a] | 98.0 (97.5–99.0) | 98.0 (97.5–99.2) | 84.5 (79.0–90.0)[b] | 81.5 (76.2–89.2) | 81.5 (76.0–89.2) | 59.0 (52.0–64.5)[c] |
| VAP (μm/s) | 275.4 (252.8–284.0) | 264.2 (244.4–284.0) | 182.1 (161.5–206.8)[a] | 224.0 (195.0–249.0) | 220.0 (192.0–247.1) | 213.8 (199.5–223.7) | 269.4 (265.9–276.5) | 268.0 (265.0–275.5) | 255.0 (230.5–270.0) |
| VSL (μm/s) | 207.5 (197.8–228.4) | 207.4 (197.0–228.4) | 159.4 (131.2–182.0)[a] | 155.8 (143.2–188.9) | 155.0 (143.0–186.0) | 169.9 (165.8–183.3) | 204.2 (199.2–207.3) | 203.0 (198.0–206.1) | 197.4 (175.1–211.9) |
| VCL (μm/s) | 412.8 (371.9–422.0) | 390.6 (329.4–420.0) | 276.5 (237.9–295.0)[a] | 330.9 (292.2–374.9) | 333.8 (292.1–370.0) | 300.1 (286.4–329.4) | 435.1 (425.0–445.8) | 435.3 (425.5–446.8) | 430.3 (381.2–448.8) |
| ALH (μm) | 14.2 (12.8–14.4) | 14.2 (12.8–14.5) | 10.8 (10.4–12.8)[a] | 15.0 (13.7–16.1) | 14.8 (13.7–15.3) | 13.2 (9.8–14.0)[b] | 15.0 (14.0–15.3) | 14.8 (14.0–15.5) | 12.4 (12.2–13.4)[c] |
| BCF (Hz) | 11.9 (10.5–13.5) | 12.0 (10.5–13.5) | 9.2 (7.7–10.5)[a] | 15.5 (13.6–17.2) | 14.6 (12.9–17.2) | 12.1 (10.5–13.2)[b] | 10.7 (9.8–11.7) | 10.7 (9.1–11.7) | 8.5 (5.4–9.7)[c] |
| STR (%) | 76.0 (72.7–76.5) | 76.5 (74.2–76.5) | 73.0 (68.0–79.0) | 73.0 (72.7–74.5) | 73.0 (72.5–74.5) | 73.0 (71.5–74.7) | 73.5 (72.7–74.7) | 73.0 (72.0–74.5) | 73.0 (72.7–75.5) |
| LIN (%) | 54.0 (50.0–55.2) | 54.0 (49.7–55.2) | 55.5 (50.2–63.0) | 51.0 (51.0–52.0) | 51.5 (50.7–52.2) | 52.5 (51.7–53.5) | 49.0 (47.7–50.7) | 49.0 (47.7–51.0) | 48.0 (46.7–51.7) |

Values are expressed in median and interquartile ranges (Q1–Q3); Kruskal–Wallis test and Dunn's test: [a]Ni90 < AC90 and SC90; [b]Ni143 < AC143 and SC143; [c]Ni196 < AC196 and SC196 ($p \leq 0.05$).

**Table 7** Sperm mitochondrial activity in rats of the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at different ages

| Classes | Subgroups ($n$ = 7) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| Class I | 79 (77–81) | 79 (78–81) | 40 (35–42)[a] | 80 (80–81) | 78 (75–84) | 32 (32–34)[b] | 82 (79–84) | 80 (76–84) | 30 (24–38)[c] |
| Class II | 18 (17–21) | 18 (13–19) | 41 (38–44)[d] | 18 (17–20) | 19 (14–22) | 46 (45–49)[e] | 15 (14–19) | 18 (15–21) | 48 (35–48)[f] |
| Class III | 2 (2–3) | 2 (1–4) | 17 (11–20)[d] | 2 (1–2) | 2 (1–5) | 16 (12–20)[e] | 2 (2–3) | 2 (1–3) | 20 (16–25)[f] |
| Class IV | 0 (0–0) | 0 (0–1) | 4 (2–5)[d] | 0 (0–0) | 0 (0–1) | 5 (4–5)[e] | 0 (0–1) | 0 (0–1) | 5 (2–6)[f] |

Values are expressed in median and interquartile ranges (Q1–Q3); Kruskal–Wallis test and Dunn's test: [a]Ni90 < AC90 and SC90; [b]Ni143 < AC143 and SC143; [c]Ni196 < AC196 and SC196 ($p \leq 0.05$). [d]Ni90 > AC90 and SC90; [e]Ni143 > AC143 and SC143; [f]Ni196 > AC196 and SC196 ($p \leq 0.05$).

**Table 8** Sperm DNA fragmentation (Comet assay) in rats of the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at different ages

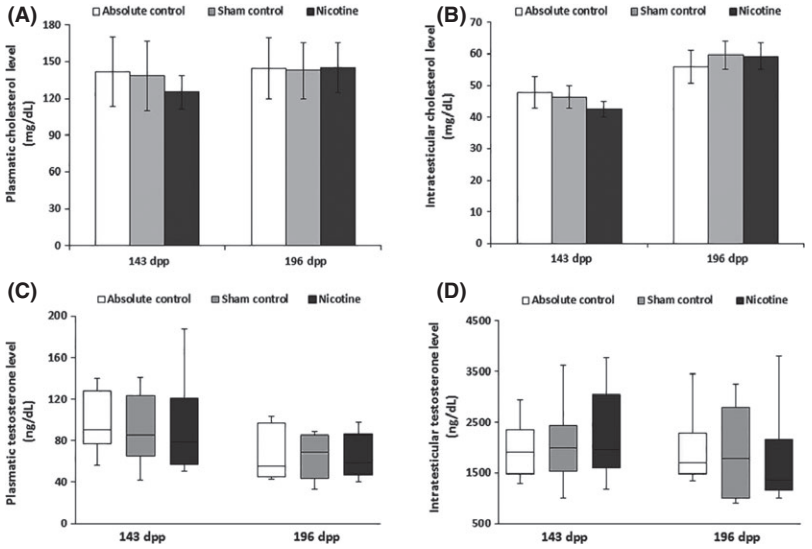
| Parameters | Subgroups ($n$ = 6) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | AC90 | SC90 | Ni90 | AC143 | SC143 | Ni143 | AC196 | SC196 | Ni196 |
| % Tail DNA | 0.7 (0.3–0.9) | 0.7 (0.3–1.0) | 2.3 (1.6–2.8)[a] | 0.6 (0.5–0.8) | 0.7 (0.5–0.9) | 3.2 (2.3–4.5)[b] | 0.7 (0.4–1.2) | 0.7 (0.4–1.1) | 2.6 (2.2–3.9)[c] |
| Tail length (μm) | 7.6 (4.6–8.3) | 7.5 (4.5–8.2) | 21.8 (15.9–29.9)[a] | 7.4 (5.6–9.6) | 7.3 (5.6–9.1) | 26.8 (15.3–54.9)[b] | 6.5 (5.6–9.1) | 6.4 (5.5–9.2) | 28.3 (17.7–36.8)[c] |
| Tail extent moment | 0.06 (0.03–0.09) | 0.06 (0.02–0.08) | 0.67 (0.52–1.19)[a] | 0.07 (0.05–0.12) | 0.07 (0.04–0.13) | 1.21 (0.55–3.45)[b] | 0.07 (0.04–0.13) | 0.07 (0.03–0.14) | 0.98 (0.63–1.95)[c] |
| Olive Tail moment | 0.12 (0.05—0.21) | 0.11 (0.04–0.19) | 0.29 (0.14–0.62) | 0.11 (0.07–0.27) | 0.12 (0.08–0.29) | 0.58 (0.07–1.06) | 0.10 (0.02–0.17) | 0.11 (0.03–0.18) | 0.32 (0.13–0.61) |

Values are expressed in median and interquartile ranges (Q1–Q3). Kruskal–Wallis test and Dunn's test: [a]Ni90 > AC90 and SC90; [b]Ni143 > AC143 and SC143; [c]Ni196 > AC196 and SC196 ($p \leq 0.05$).

In a previous study undertaken by us, an increase in the plasma cholesterol and testosterone levels was observed in offspring (at 90 days of age) from rat dams exposed to nicotine during pregnancy and breastfeeding (Paccola *et al.*, 2014). It is important to remind that Leydig cell steroidogenesis like spermatogenesis is also vulnerable to OS (Aitken & Roman, 2008). Interestingly, the drop in cholesterol and testosterone levels was not maintained throughout the ages, after, respectively, one and two complete periods of spermatogenesis (143 and 196 dpp), as shown in this study. Yamamoto *et al.* (1998) found no significant alteration in the serum levels of testosterone in smoking-exposed adult rats compared with control groups. Nevertheless, a large number of studies seem to confirm the association between smoking and disturbance of male reproductive hormone levels (Kapoor & Jones, 2005), although showing conflicting results. The variations among the reports may be because of the different protocols used including the different exposure phases of reproductive development. However, it is well known that nicotine alters the endocrine system causing endocrine diseases. Significant higher plasmatic levels of total and free testosterone and of LH have been noticed in male smokers. In addition, testosterone, estradiol, and SHBG levels were higher in current smoking post-menopausal women than in non-smokers; after those women stopped smoking for a year, the levels of

**Figure 4** Plasmatic (A) and intratesticular (B) cholesterol levels (mg/dL) of rats pertaining to the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups, at 90, 143, and 196 dpp. Values expressed in mean ± SD ($n$ = 14). ANOVA test. Plasmatic (C) and intratesticular (D) testosterone levels (ng/dL) of rats pertaining to the AC, SC, and Nicotine (Ni) groups, at 90, 143, and 196 dpp. Values are expressed in median and interquartile ranges (Q1–Q3); ($n$ = 14). Kruskal–Wallis test: No significant differences were observed.




estradiol and of total and free testosterone returned to those of non-smokers (reviewed by Tweed *et al.*, 2012). Nicotine also inhibits pulsatile LH secretion in males, and the tolerance developed by nicotine exposure also disappears within a week of quitting (Funabashi *et al.*, 2005). Chronic exposure of female offspring to the low dose of nicotine during lactation tended to increase plasma LH levels at 20 and 40 days (Meyer & Carr, 1987). Considering these available data and taking into account the increased testosterone plasma level occurred in male offspring at 90 dpp from nicotine-exposed rat dams (Paccola *et al.*, 2014), we suppose that nicotine has caused a transitory deregulation of the hypothalamic–pituitary–thyroid axis (HPT) because, in this study, the older adult rats did not show significant changes in hormone level after maternal nicotine exposure; besides, it is likely that this alteration occurred during lactation. Indeed, relevant changes in the plasmatic testosterone level were not observed in nicotine-exposed neonate and late pubertal rats but it did in young adult rats (Paccola *et al.*, 2014). Regarding the possible HPT transitory deregulation, an exception must be considered because we studied the effect of nicotine in rats and other available data are referring to the use of cigarette smoke in humans. Alterations of the hormonal profile of gonadotropins in offspring from nicotine-exposed dams were previously observed by our group (Paccola & Miraglia, 2016).

In summary, our results indicate the occurrence of late reproductive damage evidenced by a decrease in the sperm quality in male progeny whose rat dams were exposed to nicotine during pregnancy and breastfeeding. The complex mechanism by which nicotine damages the male gonad puts nicotine as the foremost reproductive risk factor. The reproductive damage presented here can be considered as another significant health risk to the offspring from expectant smoking mothers. The ad libitum use of nicotine replacement therapies may account for one-third to two-thirds the concentration of Ni that is reached by cigarette smoking, depending on the type of therapy. For instance, when 4 mg-nicotine gum is used, the nicotine level can exceed that absorbed by smoking (reviewed by Hukkanen *et al.*, 2005). Moreover, nicotine is one of the major hazardous components of tobacco (Zenzes, 2000). It crosses the placenta barrier and can result in fetal concentrations that are 15% higher than maternal concentrations (Lambers & Clark, 1996). Despite the well-documented benefits of smoking cessation, the efficacy and safety of nicotine replacement therapy use for smoking cessation in pregnant women has not been clearly demonstrated (reviewed by De Long *et al.*, 2014). Similar to the findings of Leslie (2013), our findings are also concerned about the safety of using nicotine replacement therapy during pregnancy. The current results point to the harmful effect of nicotine on not only the health and now, on the quality of spermatozoa, but also on the general reproductive health of the offspring of smoking mothers.

## ACKNOWLEDGMENTS

The authors thank Dr. Deborah Spaine from the Division of Urology, Human Reproduction Section – UNIFESP, for supporting the motility analysis; Professor Catarina Segretti Porto from the Institute of Pharmacology (INFAR), Laboratory of Experimental Endocrinology – UNIFESP for the supervision and assistance in the hormone dosage; Dr. Eliane Beraldi Ribeiro and Valter Tadeu Boldarine, from the Laboratory of Nutrition Physology – UNIFESP, for helping in the cholesterol dosage; and Dr. Vanessa Vendramini Vilela for the English review.

## FUNDING

This work was financially supported by Coordination for the Improvement of Higher Level Personnel (CAPES/Brazil).

## REFERENCES

Abdul-Ghani R, Qazzaz M, Dabdoub N, Muhammad R & Abdul-Ghani AS. (2014) Studies on cigarette smoke induced oxidative DNA damage and reduced spermatogenesis in rats. *J Environ Biol* 35, 943–947.

Agarwal A, Makker K & Sharma R. (2008) Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 59, 2–11.

Aitken RJ & Roman SD. (2008) Antioxidant systems and oxidative stress in the testes. *Adv Exp Med Biol* 636, 154–171.

Aitken RJ, Jones KT & Robertson SA. (2012) Reactive oxygen species and sperm function–in sickness and in health. *J Androl* 33, 1096–1106.

Al-Malki AL & Moselhy SS. (2013) Protective effect of vitamin E and epicatechin against nicotine-induced oxidative stress in rats. *Toxicol Ind Health* 29, 202–208.

Amaral A, Lourenço B, Marques M & Ramalho-Santos J. (2013) Mitochondria functionality and sperm quality. *Reproduction* 146, R163–R174.

Arabi M. (2004) Nicotinic infertility: assessing DNA and plasma membrane integrity of human spermatozoa. *Andrologia* 36, 305–310.

Ballinger SW, Bounder TG, Davis GS, Judice SA, Nickals JA & Albertini RJ. (1996) Mitochondrial genome damage associated with cigarette smoking. *Cancer Res* 56, 5692–5697.

Codrington AM, Hales BF & Rabire B. (2004) Spermiogenic germ cell phase-specific DNA damage following cyclophosphamide exposure. *J Androl* 25, 354–362.

Colley SM, Wintle L, Searles R, Russell V, Firman R, Smith S, Deboer K, Merriner DJ, Genevieve B, Bentel JM, *et al.* (2013) Loss of the nuclear receptor corepressor SLIRP compromises male fertility. *PLoS ONE* 8, e70700.

Condorelli RA, La Vignera S, Giacone F, Iacoviello L, Vicari E, Mongioi' L & Calogero AE. (2013) In vitro effects of nicotine on sperm motility and bio-functional flow cytometry sperm parameters. *Int J Immunopathol Pharmacol* 26, 739–746.

De Long NE, Barra NG, Hardy DB & Holloway AC. (2014) Is it safe to use smoking cessation therapeutics during pregnancy? *Expert Opin Drug Saf* 13, 1721–1731.

Delbès G, Hales BF & Robaire B. (2010) Toxicants and human sperm chromatin integrity. *Mol Hum Reprod* 16, 14–22.

Filler R. (1993) Methods for evaluation of rat epididymal sperm morphology. In: *Methods in Toxicology - Part A: Male Reproductive Toxicology* (eds. RE Chapin & JJ Heindel), pp. 335–343. Academic Press, California.

Fowler PA, Cassie S, Rhind SM, Brewer MJ, Collinson JM, Lea RG, Baker PJ, Bhattacharya S & O'Shaughnessy PJ. (2008) Maternal smoking during pregnancy specifically reduces human fetal desert hedgehog gene expression during testis development. *J Clin Endocrinol Metab* 93, 619–626.

Funabashi T, Sano A, Mitsushima D & Kimura F. (2005) Nicotine inhibits pulsatile luteinizing hormone secretion in human males but not in human females, and tolerance to this nicotine effect is lost within one week of quitting smoking. *J Clin Endocrinol Metab* 90, 3908–3913.

Galluzzi L, Kepp O, Trojel-Hansen C & Kroemer G. (2012) Mitochondrial control of cellular life, stress, and death. *Circ Res* 111, 1198–1207.

Hrudka F. (1987) Cytochemical and ultracytochemical demonstration of cytochrome c oxidase in spermatozoa and dynamics of its changes accompanying ageing or induced by stress. *Int J Androl* 10, 809–828.

Hukkanen J, Jacob P & Benowitz NL. (2005) Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* 57, 79–115.

Huleihel M & Lunenfeld E. (2004) Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian J Androl* 6, 259–268.

IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. (2004) Tobacco smoke and involuntary smoking. *Monogr Eval Carcinog Risks Hum* 83, 1–1438.

Jana K, Samanta PK & De DK. (2010) Nicotine diminishes testicular gametogenesis, steroidogenesis, and steroidogenic acute regulatory protein expression in adult albino rats: possible influence on pituitary gonadotropins and alterations testicular antioxidant status. *Toxicol Sci* 116, 647–659.

Kapoor D & Jones TH. (2005) Smoking and hormones in health and endocrine disorders. *Eur J Endocrinol* 152, 491–499.

Klinefelter GR, Gray LE Jr & Suarez JD. (1991) The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. *Reprod Toxicol* 5, 39–44.

La Maestra S, De Flora S & Micale RT. (2015) Effect of cigarette smoke on DNA damage, oxidative stress, and morphological alterations in mouse testis and spermatozoa. *Int J Hyg Environ Health* 218, 117–122.

Lagunov A, Anzar M, Sadeu JC, Khan MIR, Bruin JE, Woynillowicz AK, Buhr M, Holloway ALC & Foster WG. (2011) Effect of in utero and lactational nicotine exposure on the male reproductive tract in peripubertal and adult rats. *Reprod Toxicol* 31, 418–423.

Lambers DS & Clark KE. (1996) The maternal and fetal physiologic effects of nicotine. *Semin Perinatol* 20, 115–126.

Leslie FM. (2013) Multigenerational epigenetic effects of nicotine on lung function. *BMC Med* 11, 27–30.

Lips KS, Bruggmann D, Pfeil U, Vollerthun R, Grando SA & Kummer W. (2005) Nicotinic acetylcholine receptors in rat and human placenta. *Placenta* 26, 735–746.

Luck W, Nau H, Hansen R & Steldinger R. (1985) Extent of nicotine and cotinine transfer to the human fetus, placenta and amniotic fluid of smoking mothers. *Dev Pharmacol Ther* 8, 384–395.

Mandarim-de-Lacerda CA. (2003) Stereological tools in biomedical research. *An Acad Bras Cienc* 75, 469–486.

Marchetti P, Ballot C, Jouy N, Thomas P & Marchetti C. (2012) Influence of mitochondrial membrane potential of spermatozoa on in vitro fertilisation outcome. *Andrologia* 44, 136–141.

Martin JA, Osterman MJ & Sutton PD. (2010) Are preterm births on the decline in the United States? Recent data from the National Vital Statistics System. *NCHS Data Brief* 39, 1–8.

Meernik C & Goldstein AO. (2015) A critical review of smoking, cessation, relapse and emerging research in pregnancy and post-partum. *Br Med Bull* 114, 135–146.

Meyer DC & Carr LA. (1987) The effects of perinatal exposure to nicotine on plasma LH levels in prepubertal rats. *Neurotoxicol Teratol* 9, 95–98.

Miki K, Willis WD, Brown PR, Goulding EH, Fulcher KD & Eddy EM. (2002) Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. *Dev Biol* 248, 331–342.

Miraglia SM & Hayashi H. (1993) Histomorphometry of immature rats testis after eating. *J Morphol* 217, 65–74.

Myllynen P & Vähäkangas K. (2013) Placental transfer and metabolism: an overview of the experimental models utilizing human placental tissue. *Toxicol In Vitro* 27, 507–512.

Narayana K, Prashanthi N, Nayanatara A, Kumar HH, Abhilash K & Bairy KL. (2005) Effects of methyl parathion (o, o-dimethyl o-4-nitrophenyl phosphorothioate) on rat sperm morphology and sperm count, but not fertility, are associated with decreased ascorbic acid level in the testis. *Mutat Res* 588, 28–34.

Olive PL, Wlodek D, Durand RE & Banáth JP. (1991) DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res* 51, 4671–4676.

Oyeyipo IP, Raji Y, Emikpe BO & Bolarinwa AF. (2011) Effects of nicotine on sperm characteristics and fertility profile in adult male rats: a possible role of cessation. *J Reprod Infertil* 12, 201–207.

Oyeyipo IP, Maartens PJ & du Plessis SS. (2014) In vitro effects of nicotine on human spermatozoa. *Andrologia* 46, 887–892.

Paccola CC, Neves FM, Cipriano I, Stumpp T & Miraglia SM. (2014) Effects of prenatal and lactation nicotine exposure on rat testicular interstitial tissue. *Andrology* 2, 175–185.

Paccola CC & Miraglia SM. (2016) Prenatal and lactation nicotine exposure affects Sertoli cell and gonadotropin levels in rats. *Reproduction* 151, 117–133.

Piomboni P, Focarelli R, Stendardi A, Ferramosca A & Zara V. (2012) The role of mitochondria in energy production for human sperm motility. *Int J Androl* 35, 109–124.

Ravnborg TL, Jensen TK, Andersson A, Toppari J, Skakkebæk NE & Jørgensen N. (2011) Prenatal and adult exposures to smoking are associated with adverse effects on reproductive hormones, semen quality, final height and body mass index. *Hum Reprod* 26, 1000–1011.

Reddy A, Sood A, Rust PF, Busby JE, Varn E, Mathur RS & Mathur S. (1995) The effect of nicotine on in vitro sperm motion characteristics. *J Assist Reprod Genet* 12, 217–223.

Rehan VK, Liu J, Naeem E, Tian J, Sakurai R, Kwong K, Akbari O & Torday JS. (2012) Perinatal nicotine exposure induces asthma in second generation offspring. *BMC Med* 10, 129–142.

Rehnberg GL. (1993) Collection of interstitial fluid and seminiferous tubule fluid from the rat testis. In: *Methods and Toxicology - Part A: Male Reproductive Toxicology* (eds RE Chapin & JJ Heindel), pp. 265–273. Academic Press, California.

Robb GW, Amann RP & Killian GJ. (1978) Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *J Reprod Fertil* 54, 103–107.

Roy TS, Seidler FJ & Slotkin TA. (2002) Prenatal nicotine exposure evokes alterations of cell structure in hippocampus and somatosensory cortex. *J Pharmacol Exp Ther* 300, 124–133.

Russell LD, Ettlin RA, Sinhahikim AP & Clegg ED. (1990) *Histological and Histopathological Evaluation of the Testis*, 1st edn. Cache River Press, Florida.

Sakkas D & Alvarez JG. (2010) Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 93, 1027–1036.

Scherle W. (1970) A simple method for volumetry of organs. *Mikroskopie* 26, 57–60.

Sepaniak S, Forges T & Monnier-Barbarino P. (2006) Cigarette smoking and fertility in women and men. *Gynecol Obstet Fertil* 34, 945–949.

Shea A & Steiner M. (2008) Cigarette smoking during pregnancy. *Nicotine Tob Res* 10, 267–278.

Simmons RA, Suponitsky-Kroyter I & Selak MA. (2005) Progressive accumulation of mitochondrial DNA mutations and decline in mitochondrial function lead to beta-cell failure. *J Biol Chem* 28, 28785–28791.

Sobinoff AP, Bernstein IR & McLaughlin EA. (2012) All your eggs in one basket: mechanisms of xenobiotic induced female reproductive senescence¦ NOVA. *The University of Newcastle's Digital Repository*, 559–584.

Sobinoff AP, Sutherland JM, Beckett EL, Stanger SJ, Johnson R, Jarnicki AG, McCluskey A, St John JC, Hansbro PM & McLaughlin EA. (2014) Damaging legacy: maternal cigarette smoking has long-term consequences for male offspring fertility. *Hum Reprod* 29, 2719–2735.

Sofikitis N, Miyagawa I, Dimitriadis D, Zavos P, Sikka S & Hellstrom W. (1995) Effects of smoking on testicular function, semen quality and sperm fertilizing capacity. *J Urol* 154, 1030–1034.

Treulen F, Uribe P, Boguen R & Villegas JV. (2015) Mitochondrial permeability transition increases reactive oxygen species production and induces DNA fragmentation in human spermatozoa. *Hum Reprod* 30, 767–776.

Tweed JO, Hsia SH, Lutfy K & Friedman TC. (2012) The endocrine effects of nicotine and cigarette smoke. *Trends Endocrinol Metab* 23, 334–342.

Vendramini V, Cedenho AP, Miraglia SM & Spaine DM. (2014) Reproductive function of the male obese Zucker rats: alteration in sperm production and sperm DNA damage. *Reprod Sci* 21, 221–229.

Weibel ER. (1963) Principles and methods for the morphometric study of the lung and other organs. *Lab Invest* 12, 131–155.

World Health Organization. (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th edn. World Health Organization, Geneva.

Yamamoto Y, Isoyama E, Sofikitis N & Miyagawa I. (1998) Effects of smoking on testicular function and fertilizing potential in rats. *Urol Res* 26, 45–48.

Yao C, Liu Y, Sun M, Niu M, Yuan Q, Hai Y, Guo Y, Chen Z, Hou J, Liu Y & He Z. (2015) MicroRNAs and DNA methylation as epigenetic regulators of mitosis, meiosis and spermiogenesis. *Reproduction* 150, R25–R34.

Yousefniapasha Y, Jorsaraei G, Gholinezhadchari M, Mahjoub S, Hajiahmadi M & Farsi M. (2015) Nitric oxide levels and total antioxidant capacity in the seminal plasma of infertile smoking men. *Cell J* 17, 129–136.

Zenzes MT. (2000) Smoking and reproduction: gene damage to human gametes and embryos. *Hum Reprod Update* 6, 122–131.

Zini A, Albert O & Robaire B. (2014) Assessing sperm chromatin and DNA damage: clinical importance and development of standards. *Andrology* 2, 322–325.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Photomicrographs of rat spermatozoa submitted to DAB method.

**Figure S2.** Photomicrographs of rat spermatozoa submitted to the Comet assay.

**Table S1.** Diameter of seminiferous tubule sections in androgen-dependent stages and height of the seminiferous epithelium in rats of the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups.

**Table S2.** Volume density (Vv) and volume (V) of the seminiferous epithelium, testicular interstitial tissue, and lymphatic space in rats of the Absolute Control (AC), Sham Control (SC), and Nicotine (Ni) groups.