Prenatal and lactation nicotine exposure affects Sertoli cell and gonadotropin levels in rats

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Abstract

Nicotine is largely consumed in the world as a component of cigarettes. It can cross the placenta and reach the milk of smoking mothers. This drug induces apoptosis, affects sex hormone secretion, and leads to male infertility. To investigate the exposure to nicotine during the whole intrauterine and lactation phases in Sertoli cells, pregnant rats received nicotine (2 mg/kg per day) through osmotic minipumps. Male offsprings (30, 60, and 90 days old) had blood collected for hormonal analysis (FSH and LH) and their testes submitted for histophatological study, analysis of the frequency of the stages of seminiferous epithelium cycle, immunolabeling of apoptotic epithelial cells (TUNEL and Fas/FasL), analysis of the function and structure of Sertoli cells (respectively using transferrin and vimentin immunolabeling), and analysis of Sertoli-germ cell junctional molecule (β-catenin immunolabeling). The exposure to nicotine increased the FSH and LH plasmatic levels in adult rats. Although nicotine had not changed the number of apoptotic cells, neither in Fas nor FasL expression, it provoked an intense sloughing of epithelial cells and also altered the frequency of some stages of the seminiferous epithelium cycle. Transferrin and β-catenin expressions were not changed, but vimentin was significantly reduced in the early stages of the seminiferous cycle of the nicotine-exposed adult rats. Thus, we concluded that nicotine exposure during all gestational and lactation periods affects the structure of Sertoli cells by events causing intense germ cell sloughing observed in the tubular lumen and can compromise the fertility of the offspring.

Introduction

Nicotine, extracted from Nicotiana tabacum plant (‘tobacco’), is one of the most widely consumed legal drugs in the world through cigarettes. This drug causes changes in the synthesis of sexual hormones (Dempsey & Benowitz 2001) and reduces fertility rate in smokers, either men or women (Shiverick & Salafia 1999, Mello et al. 2001). It also provokes, in adult rats, reduction of gonadotrophic hormones- follicle-stimulating hormone (FSH) and luteinizing hormone (LH), disorders on the spermatogenesis, as well as numerical and morphological abnormalities in sperm (Jana et al. 2010).

In the first decade of the 21st century alone, ~8% of mothers kept smoking during pregnancy (WHO, 2011) and the number increased during breastfeeding because many mothers returned to smoking after the delivery (Meernik & Goldstein 2015); this is of great concern since nicotine is a highly soluble drug (Shea & Steiner 2008) that is able to cross the placental membrane and also reach the breast milk (Lambers & Clark 1996). Nicotine can also be considered an endocrine disruptor (Kanungo et al. 2012); so we have to consider that endocrine disruptors acting in the fetal stage can cause reproductive damage, which will be observed in adulthood due to injury to not only Leydig cells but also to Sertoli cells (Richburg & Dwyer 2010). Our group has found that the exposure to nicotine early in life causes morphological changes of the Leydig cells in the adult offspring (Paccola et al. 2014).

Studies have shown that secretory disorders in Leydig or Sertoli cells can be the cause of disorders in spermatogenesis, changes in sperm morphology, and sperm motility which can happen during maturation in the epididymis of smoker patients (aging from 21 to 38 years) who smoked over 20 cigarettes daily for more than three years (Sofikitis et al. 1995). Indeed, Sertoli cells play a key role in the control of spermatogenesis and in germ cell development (Fritz 1973, Carreau et al. 1994) and any alteration in these cells can cause severe damage to spermatogenesis (Hess et al. 1991, Brilhante et al. 2012). However, there are very few studies about the effects of nicotine on late spermatogenesis (Lagunov et al. 2011) in animals exposed during whole intrauterine (21–22 days in rats) period and lactation (from the birth to weaning).

As Sertoli cells are the first cells of embryonic male gonads to differentiate, they play a key role in testis development and any event that affects the Sertoli cells during the fetal and neonatal periods can lead to...
functional changes that will compromise the spermatogenesis in adulthood (Sharpe et al. 2003). Also, cigarette smoking by women during pregnancy may affect the future fertility of the son (Sharpe 2010). So, with the intent to mimic as closely as possible what happens with women who smoke during pregnancy and breastfeeding, the experiments were performed during the whole intrauterine and lactation phases.

In rats, the Sertoli cell proliferation takes place during the fetal period (Wang et al. 1989, Sharpe et al. 2003) and the blood-testicular-barrier (BTB) is morphologically structured from 16 to 19 days post-partum (dpp) (Bergmann & Dierichs 1983). However, a functional BTB is not fully established until 25 dpp. This phenomenon is closely associated with the onset of meiosis I and II (Mok et al. 2011). At around 60 dpp, rats are in late puberty (Stumpp et al. 2006) when spermatozoa begin to be found in the epididymis (Robb et al. 1978). At 90 dpp, rats are considered to be fertile young adults. Without the physical and metabolic support provided by Sertoli cells, germ cell differentiation, meiosis, and transformation into spermatozoa and spermatogenesis can be compromised (Russell et al. 1990, Sharpe et al. 2003).

Therefore, considering that any exposure to toxicants during the fetal period can lead to reproductive impairment that may only be diagnosed later in life (Richburg & Dwyer 2010), rats aged 30, 60, and 90 dpp were chosen to evaluate the effect of nicotine in different stages of the sexual maturation focusing on the morphofunctional alterations caused in the Sertoli cells and its impact on the spermatogenesis.

The synchronized movement of germ cells throughout the seminiferous epithelium up to the spermatogenesis involves the disruption and reorganization of Sertoli-Sertoli and Sertoli-germ cells junctions (Cheng & Mruk 2002, Xia et al. 2005, Kopera et al. 2010). Among the several molecules that compose the adherens junctions in the seminiferous epithelium, there is the catenin family (α and β). β-catenin participates in the Sertoli-germ cell anchoring junction in the apical ectoplasmic specialization and also in the restructuring of the BTB at the basal ectoplasmic specialization between Sertoli cells (Kolas et al. 2011). In this dynamic process, the cytoskeleton of Sertoli cells also undergoes structural changes (Upadhya et al. 2011).

As an important component of the cytoskeleton, the intermediate filaments (type III) of vimentin assist in anchoring the germ cells into ‘crypts’ in the Sertoli cell apical cytoplasm (Franke et al. 1979, Vogl et al. 2008). Not surprisingly, the loss of these filaments caused by toxic agents can result in rupture of the junctions and sloughing of immature germ cells into the tubular lumen (Richburg & Boekelheide 1996, Kopec et al. 2005), thus disrupting the spermatogenesis.

Considering that the vimentin intermediate filaments of muscle fibers are vulnerable to nicotine (Cucina et al. 2000, Calore et al. 2003), we decided to investigate the possible structural changes produced in the Sertoli cells regarding the vimentin distribution and β-catenin availability in the seminiferous epithelium of rats that were exposed to nicotine during intrauterine and lactation periods. Different stages of the seminiferous epithelium cycle were investigated, since they are controlled and regulated by Sertoli cells (Hess & Renato de Franca 2008). The hormonal levels are also investigated since Sluka et al. (2006) showed that FSH regulates the formation of junctions in ectoplasmic specializations (with the participation of vimentin) and also stimulates the organization of adherens junctions in Sertoli cells (which involves β-catenin).

In order to better understand the effects of nicotine on Sertoli cell function, we proposed to analyze the expression of the protein transferrin in the seminiferous epithelium, which is considered a good marker of the Sertoli cell function (Skinner & Griswold 1980, Holmes et al. 1983). Besides, the ‘Fas/Fasl’ system was also included in our study, as it is a useful tool for investigating the physiology of Sertoli cells, since it participates in the recognition/elimination of damaged germ cells and in the control of the germ cell population size (Lee et al. 1997). The apoptosis occurrence in the seminiferous epithelium was also analyzed by the TUNEL method.

Thus, we proposed the first experimental study focusing on the direct damage on Sertoli cells caused by nicotine exposure and its importance to the spermatogenic process. The relationship between the morphological and functional damage in the Sertoli cells is discussed.

Materials and methods

This study was approved by the Animal Research Ethics Committee of the Federal University of São Paulo, Brazil (CEP 0136/11).

Animals

Male offsprings (n = 90) were harvested from 45 female rats (Rattus norvegicus albinus) obtained from the Center for the Development of Experimental Models for Medicine and Biology (CEDEME/UNIFESP, Sao Paulo, Brazil). Female rats in estrous and proestrous were mated with males overnight (two females per male). The pregnancy test was performed the following morning. When the test was positive, it was considered day 1 of gestation. The rat dams were distributed into three groups according to the treatment applied: nicotine-treated (N), absolute control (C), and sham control (S). During the experiments, one rat dam plus six pups were housed per cage (40×30×15 cm). Rats were maintained under the conditions of 12 h light:12 h darkness cycles, at 21–23 °C room temperature; standardized lab chow (Nuvilab CR1, Nuvital, Curitiba, PR, Brazil) and water were provided from which they were allowed to feed ad libitum.
**Experimental protocol**

At the first day of pregnancy, rat dams of the N and the S groups were anesthetized with ketamine-xilazine (100 mg/kg of body weight). Then, they had an osmotic minipump carefully and subcutaneously implanted in the middle back of their body (2ML4 – Alzet, Durect Corporation, Cupertino, CA, USA) following the standard surgical procedures. In addition, the incision was sealed with cyanoacrylate tissue adhesive. Minipumps were filled with 2 mg/kg per day of nicotine (Nicotine hydrogen tartrate salt, Sigma–Aldrich Co, St Luis/MI) dissolved in bacteriostatic water (Abbott). Doses of 1.5–2.0 mg/kg per day of nicotine are similar to those contained in 20 cigarettes (one packet) and equivalent to the human moderate daily consumption (Levin et al. 1996, Slotkin 1998, Roy et al. 2002, Matta et al. 2007).

As the minipump was programmed to release nicotine during 28 consecutive days and the rat pregnancy lasts around 21–22 days, the minipump was replaced within the first 24 h after birth to assure nicotine presence in the milk throughout the lactation period (22 days). The rat dams of the S group received the same treatment except that the minipump was filled only with bacteriostatic water. The rat dams of the C group had no osmotic minipump implanted.

Male offspring were distributed into the subgroups according to each treatment applied and the age at killing. The male offspring from 15 nicotine-treated rat dams (N group) were divided according to the age of killing scheduled and named as follows: N30 (prepubertal rats killed at 30 dpp), N60 (pubertal rats killed at 60 dpp), and N90 (young adult rats killed at 90 dpp). In the same manner, the male offspring from 15 rat dams from the S group and from the 15 rat dams of the C group were distributed into the following subgroups: S30, S60, and S90; C30, C60, and C90.

**Blood and testis sampling**

The male rats from all groups (30, 60, and 90 dpp) had their plasma collected and submitted to hormonal dosages. The animals were intraperitoneally injected with heparin (Liquemin, Roche; 130 UI/kg b.w) and anesthetized with thiopental (Thiopentax, Cristália Produtos Químicos Farmaceúticos Ltda, Sao Paulo, Brazil; 89 mg/kg b.w); then they had their blood collected from the inferior vena cava. After that, the animals were killed by the rupture of this vein and their testes were collected. The blood was then centrifuged and the plasma obtained was used for the hormonal analyses. The testes were weighed and immersion fixed in Bouin’s solution for 24 h obtained was used for the hormonal analyses. The testes were collected. The blood was then centrifuged and the plasma collected from the inferior vena cava. After that, the animals were killed by the rupture of this vein and their testes were collected. The blood was then centrifuged and the plasma obtained was used for the hormonal analyses. The testes were weighed and immersion fixed in Bouin’s solution for 24 h.

**Hormonal dosages**

Plasma concentrations of LH and FSH were performed by the company Genese Products Diagnostics Ltd, using the Multiplex Map Rat Pituitary Magnetic Bead Panel test. The analysis was performed by xPonent Software/Analyst, version 4.2. The specific assay for rats has 3.28 pg/ml sensitivity for LH and 7.62 pg/ml for FSH. For estradiol analysis, we used the ELISA kit for estradiol (CEA461Ge code, Cloud Clone Corp., Houston, Texas, USA). The microplates reading were held in 450 nm Expert Plus reader (Biochrom Co., Cambridge, UK) and the analysis of the results was performed using the Galapagos Software version 10.0.

These analyses were performed in uniplicate, i.e. a single sample per animal. Although, it is recommended to analyze plasma hormones in duplicate, we have adopted the uniplicate analysis because the samples of control rats analyzed in duplicate had a very low coefficient of variation (CV). From an essay of ten duplicated samples on the same plate (a total of 20 assays), an intra-assay CV was calculated as s.d./mean expressed in percentage (CV = s.d./Mean×100). The intra-assay precision (%CV) was 3.3 for LH and 2.8 for FSH. A CV of 10% or less is considered satisfactory (Murray et al. 1993). The intra-assay precision (%CV) was 12.8 for LH and 12.3 for FSH.

**Histopathological analysis**

Two testicular cross-sections from the paraffin-embedded specimens (4 μm; with an interval of ten sections between them), were obtained per rat; they were submitted to the Periodic Acid Schiff (PAS) histochemical method and counterstained with Hematoxylin (H). The analysis of the tubular sections was performed systematically to identify the stage of the seminiferous epithelium cycle (Hess et al. 1990). In addition, the histopathological alterations of the seminiferous epithelium were categorized and the images were captured using an image analysis system (Leica Qwin-V3, Leica, Cambridge, UK) coupled to a light microscope.

**Calculation of the frequency of seminiferous tubules with sloughed cells**

The analysis and the calculation of the frequency of seminiferous tubule sections with sloughed epithelial cells (germ and Sertoli cells) into the lumen were performed on testicular sections (4 μm) stained by the PAS-H method. Two testicular sections (at an interval of ten sections between them), from each animal were fully scrutinized using the previously cited image analysis system coupled to a light microscope at ×10 magnification objective lens. All seminiferous tubule sections containing cells inside the lumen (which were not filled only with bacteriostatic water) were stained with Hematoxylin (H) and periodic acid Schiff (PAS) histochemical methods and counterstained with Hematoxylin (H). The analysis of the tubular sections was performed systematically to identify the stage of the seminiferous epithelium cycle (Hess et al. 1990). In addition, the histopathological alterations of the seminiferous epithelium were categorized and the images were captured using an image analysis system (Leica Qwin-V3, Leica, Cambridge, UK) coupled to a light microscope.

**Table 1 FSH and LH plasma levels in prepubertal, pubertal, and adult rats pertaining to absolute control (C30, C60, C90), sham control (S30, S60, S90) and nicotine-exposed (N30, N60, N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).**

<table>
<thead>
<tr>
<th>Subgroups (n)</th>
<th>FSH (pg/ml)</th>
<th>LH (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C30 (9)</td>
<td>11 362.18 (7569.44–22 580.85)</td>
<td>228.93 (160.44–525.12)</td>
</tr>
<tr>
<td>S30 (9)</td>
<td>17 146.82 (11 837.11–18 930.43)</td>
<td>590.37 (370.41–920.89)</td>
</tr>
<tr>
<td>N30 (10)</td>
<td>16 833.85 (11 837.11–18 930.43)</td>
<td>472.07 (301.79–1126.91)</td>
</tr>
<tr>
<td>C60 (9)</td>
<td>5897.44 (4085.57–6665.37)</td>
<td>697.68 (423.77–1441.99)</td>
</tr>
<tr>
<td>S60 (9)</td>
<td>14 312.36 (2765.62–7960.34)</td>
<td>356.57 (204.70–764.27)</td>
</tr>
<tr>
<td>N60 (10)</td>
<td>7023.00 (5740.99–8577.98)</td>
<td>524.45 (385.30–602.85)</td>
</tr>
<tr>
<td>C90 (9)</td>
<td>31 500.47 (2596.56–5602.93)</td>
<td>568.83 (323.71–648.41)</td>
</tr>
<tr>
<td>S90 (9)</td>
<td>41 166.50 (3258.24–5874.34)</td>
<td>418.54 (290.25–854.14)</td>
</tr>
<tr>
<td>N90 (10)</td>
<td>6967.62 (5645.68–9010.68)</td>
<td>4110.35 (748.24–1744.49)</td>
</tr>
</tbody>
</table>

Statistical analyses: Kruskal–Wallis’s one-way ANOVA and Dunn’s test. aN90 > C90. bN90 > C90 and S90 (P < 0.05).
were deparaffinised, hydrated, and submitted to the inactivation of ten sections per animal from the paraffin-embedded testes. Intraepithelial vacuoles (D and E – thin arrows), detachment of a strip of cells from the basal tubular region (F – arrow), and degenerating binucleated (arrow). In D, E, F and G: note the tubular sections from N30 rats showing sloughing of degenerating germ cells into the lumen (D – thick arrow), many showing normal and organized epithelium in accordance with the expected ones for each age. In A: note the small and rare intraepithelial vacuole region of the seminiferous tubule section (stage XI of cycle) (L – thick arrow); epithelial disorganization and presence of germ cell types from different stages, i.e., uncommon cellular associations (M – thick arrows); cells displaying pyknotic nucleus either displaying an intense eosinophilic cytoplasm (J – arrows), or a clear halo around of the nucleus suggesting cellular death (K – arrows). In H, I, J and K: observe tubular sections from N60 rats with large amount of sloughed germ cells (H – thick arrow) together with some Sertoli cell nuclei (H – thin arrow and inset) into the lumen, large amount of intraepithelial vacuoles (L – arrow), germ cells presenting pyknotic nucleus either displaying an intense eosinophilic cytoplasm (J – arrows), or a clear halo around of the nucleus suggesting cellular death (K – arrows). In L, M and O: note tubular sections from N90 rats showing retention of step 19 elongated spermatids, which are usually not seen in that position (L and M – thin arrows and inset); peculiarly elongated Sertoli cell nuclei in its major axis uncommonly placed perpendicular to the basal region of the seminiferous tubule section (stage XI of cycle) (L – thick arrow); epithelial disorganization and presence of germ cell types from different stages, i.e., uncommon cellular associations (M – thick arrows); cells displaying pyknotic nuclei (N – thin arrow); cellular depletion and intraepithelial vacuoles (N and O – thick arrows); and finally, binucleated formation of round spermatids (O – thin arrow).

**Calculation of the frequency of the stages of the seminiferous epithelium cycle**

The analysis of the frequencies of the 14 stages of the rat seminiferous epithelium cycle was carried out using the adapted methodologies described by Hess et al. (1990) and Nakai et al. (2004). The frequencies of the sections at the specific stages were obtained in pubertal and adult rats (50 and 90 dpp). Two PAS + H-treated testicular sections (4 μm), with an interval of ten sections between them, were analyzed per animal under a light microscope at ×50 magnification objective lens. Two hundred cross-sections per animal were evaluated, as that is the minimum number of sections that must be evaluated when the number of animals per group is equal to 10 (Hess et al. 1990). Lightly oblique sections were only computed if they showed well-defined characteristics of a particular stage. The identification of the 14 stages of the seminiferous epithelium cycle (I–XIV) followed the classification established by Leblond & Clermont (1952). The determination of stages from I to IV depended on the clear vision of the region corresponding to the ‘Golgi apparatus’ in the round spermatids. Considering the difficulty in distinguishing the stages from II to III, they were grouped in a single stage (II–III), as indicated by Russell et al. (1990). The same occurred with the stages XII and XIII, which could also be grouped (Hoyt et al. 1995).

The frequency of stages was calculated as follows (Nakai et al. 2004): (frequency of the stage (%) = number of tubular sections in a determined stage ×100/total number of tubular sections analyzed).

**Immunolabeling of the proteins Fas, FasL, Transferrin, Vimentin and β-catenin**

Two non-consecutive testicular sections (7 μm) with an interval of ten sections per animal from the paraffin-embedded testes were deparaffinised, hydrated, and submitted to the inactivation of the endogenous peroxidase (3% H2O2). After that, the sections were submitted to antigen retrieval when necessary, depending on the primary antibody. For inhibiting background, the sections were treated with BSA and incubated with the primary antibody. For inhibiting background, the sections were treated with BSA and incubated with streptavidin-peroxidase (kit LSAB-K0690, DAKO (Carpinteria, CA, USA), 30 min each one) and treated with 3,3’-diaminobenzidine tetrachloride (DAB-K3468, DAKO). The incubations were performed at room temperature, and between them the slides were rinsed with phosphate buffer (0.05 M, pH 7.4). Harris’ Hematoxylin was used for counterstaining. Only cells showing intense dark brown staining were considered positive. Negative controls were carried out for each immunohistochemical reaction by omitting each respective primary antibody.

All slides were treated identically, with the same antibody concentration and development time applied to all samples for each antibody used. For the Fas protein labeling, the sections were submitted to proteinase K (20 μg/ml) for 12 min to antigen retrieval and, after that, treated with 10% BSA for 10 min and incubated with anti-Fas primary antibody (rabbit anti-rat, X-20: sc-1024, Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:500) for 1 h. For the FasL protein labeling, the sections were immersed in sodium citrate buffer (10 mM, pH 6.0) in microwave (15 min, 600 w); then they were treated with 10% BSA for 10 min and incubated with anti-FasL primary antibody (rabbit anti-rat, N-20: sc-820, Santa Cruz Biotechnology, 1:300) for 1 h.

In the transferrin labeling on Sertoli cells, the sections were treated with 15% BSA for 20 min and incubated with anti-transferrin primary antibody (rabbit anti-rat, A110-124, Bethyl Laboratories, Inc., Montgomery, TX, USA, 1:7500) for 1 h. In the vimentin labeling, the sections were immersed in citrate buffer in microwave (15 min), treated with 10% BSA for 20 min and incubated with anti-vimentin primary antibody (mouse anti-rat, sc-6260, Santa Cruz Biotechnology, 1:150) for 2 h. In the β-catenin labeling, the sections were also immersed in citrate buffer in microwave for 15 min and treated with 7% BSA for 10 min and incubated with anti-β-catenin (rabbit anti-rat, sc-7199, Santa Cruz Biotechnology, 1:100) for 1 h.

**Labeling of apoptotic cells (TUNEL)**

Two testicular sections (5 μm) from paraffin-embedded specimens, per animal and with an interval of ten sections between them, were deparaffinised, hydrated, and submitted to the Apoptag Plus *in situ* apoptosis Fluorescein Detection Kit (S7111, Chemicon, Millipore Temecula, CA, USA), according to the datasheet procedure. Then, the sections were washed and following a specification described. After this, all sections were incubated with the biotin secondary antibody (anti-rabbit and anti-mouse) and with streptavidin-peroxidase (kit LSAB-K0690, DAKO (Carpinteria, CA, USA), 30 min each one) and treated with 3,3’-diaminobenzidine tetrachloride (DAB-K3468, DAKO). The incubations were performed at room temperature, and between them the slides were rinsed with phosphate buffer (0.05 M, pH 7.4). Harris’ Hematoxylin was used for counterstaining. Only cells showing intense dark brown staining were considered positive. Negative controls were carried out for each immunohistochemical reaction by omitting each respective primary antibody.

All slides were treated identically, with the same antibody concentration and development time applied to all samples for each antibody used. For the Fas protein labeling, the sections were submitted to proteinase K (20 μg/ml) for 12 min to antigen retrieval and, after that, treated with 10% BSA for 10 min and incubated with anti-Fas primary antibody (rabbit anti-rat, X-20: sc-1024, Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:500) for 1 h. For the FasL protein labeling, the sections were immersed in sodium citrate buffer (10 mM, pH 6.0) in microwave (15 min, 600 w); then they were treated with 10% BSA for 10 min and incubated with anti-FasL primary antibody (rabbit anti-rat, N-20: sc-820, Santa Cruz Biotechnology, 1:300) for 1 h.

In the transferrin labeling on Sertoli cells, the sections were treated with 15% BSA for 20 min and incubated with anti-transferrin primary antibody (rabbit anti-rat, A110-124, Bethyl Laboratories, Inc., Montgomery, TX, USA, 1:7500) for 1 h. In the vimentin labeling, the sections were immersed in citrate buffer in microwave (15 min), treated with 10% BSA for 20 min and incubated with anti-vimentin primary antibody (mouse anti-rat, sc-6260, Santa Cruz Biotechnology, 1:150) for 2 h. In the β-catenin labeling, the sections were also immersed in citrate buffer in microwave for 15 min and treated with 7% BSA for 10 min and incubated with anti-β-catenin (rabbit anti-rat, sc-7199, Santa Cruz Biotechnology, 1:100) for 1 h.
Table 2 Frequency (%) of seminiferous tubule sections with sloughing of epithelial cells observed in prepubertal (30 dpp), pubertal (60 dpp), and adult (90 dpp) rats pertaining to absolute control (C30, C60, C90), sham control (S30, S60, S90), and nicotine-exposed (N30, N60, N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>(n = 10)</th>
<th>30 dpp</th>
<th>60 dpp</th>
<th>90 dpp</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.0 (1.5–22.0)</td>
<td>3.0 (1.25–4.75)</td>
<td>0.0 (0.0–3.0)</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>5.0 (3.0–18.5)</td>
<td>19.0 (0.0–24.0)</td>
<td>4.0 (2.0–10.0)</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>21.0 (12.5–45.0)</td>
<td>38.0 (24.0–41.0)</td>
<td>6.0 (4.0–13.0)</td>
<td></td>
</tr>
</tbody>
</table>

Statistical analysis: Kruskal–Wallis’s one-way ANOVA and Dunn’s test. N60 > C60. N90 > C90 (P < 0.05).

and mounted using mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). Negative control was performed by omitting the incubation with TdT, which was replaced by PBS. Mammary gland section slides, provided by the Apoptag Kit, were used as positive control.

**Stereological analysis**

**Volume density of the immunolabeling in seminiferous epithelium**

The stereological analysis of Fas, FasL, transferrin, vimentin, and β-catenin labeling in the seminiferous epithelium was performed separately for each protein studied, using the computerized image analysis system (Leica Qwin-V3, Leica) coupled to a light microscope. The volume density (Vv) in the seminiferous epithelium was calculated as the ratio between immune-positive epithelial area (Ae +) and total area of tubules analyzed (Att), i.e. (Ae +/Att) (Stumpp et al. 2006); according to the principle of stereology, the area measures can be taken to infer the data on the volume of the analyzed tissue (Gundersen et al. 1988). Two testicular sections were analyzed per animal, computing 50 fields per section. For the analysis of the Fas and FasL positive tissue was performed under a light microscope at ×40 magnification objective lens; the transferrin, vimentin, and β-catenin labeling was performed using a ×20 objective lens; the expression of these proteins was also observed in the testicular interstitium, but we considered only the epithelial area for the stereological analysis.

As the distribution pattern of vimentin in Sertoli cells (Amlani & Vogl 1988) and the β-catenin expression in Sertoli-germ cell junctions (Lee et al. 2003) changes according to the phases of the cycle of the seminiferous epithelium, the volume density of each immunolabeling for both pubertal and adult rats (60 and 90 dpp) were performed taking into account each stage of the cycle, whenever possible. To obtain these stereological data at different stages of the cycle of the seminiferous epithelium we also used the referred image analysis system coupled with a light microscope using a ×20 objective lens; when more details were needed to confirm a specific stage, higher magnifications were applied to complement the analysis. In addition, while stages VI–XI and XIV were individually analyzed stages I–V and XII–XIII were grouped, since the expression of vimentin is similar among these stages (Zhu et al. 1997, Upadhyay et al. 2011).

**Numerical densities of apoptotic cells in seminiferous epithelium**

The numerical density (Nv) of TUNEL-positive cells was obtained using a computerized analysis system (Leica Qwin-V3, Leica) coupled to a light microscope. Two non-consecutive testicular sections per animal were analyzed under ×40 objective lens. Fifty fields were randomly analyzed in each testicular section, totaling a 100 fields per rat (Stumpp et al. 2004).

**Statistical analysis**

The data were submitted to either parametric or non-parametric tests using SigmaPlot software 11.2. Data that passed the normality test were submitted to one-way ANOVA to compare data among the groups followed by Student–Newman–Keuls multiple comparison test, while there were significant differences. The non-parametric Kruskal–Wallis test

Table 3 Frequencies (%) of the stages of the seminiferous epithelium cycle in pubertal and adult animals pertaining to absolute control (C60 and C90), sham control (S60 and S90), and nicotine-exposed (N60 and N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

<table>
<thead>
<tr>
<th>Subgroups</th>
<th>(n = 10)</th>
<th>I (%)</th>
<th>II–III (%)</th>
<th>IV (%)</th>
<th>V (%)</th>
<th>VI (%)</th>
<th>VII (%)</th>
<th>VIII (%)</th>
<th>IX (%)</th>
<th>X (%)</th>
<th>XI (%)</th>
<th>XII–XIII (%)</th>
<th>XIV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C60</td>
<td>4.5</td>
<td>15.9</td>
<td>6.3</td>
<td>8.4</td>
<td>7.3</td>
<td>17.5</td>
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Statistical analysis: Kruskal–Wallis’s one-way ANOVA and Dunn’s test. *N60 < C60 and S60. *N60 > S60. *N90 > S90 (P < 0.05).
was used to compare data that failed the normality test followed by the Dunn's test when it was significant. Differences were considered significant when \( P \leq 0.05 \).

**Results**

Pregnant rat dams and their offspring were monitored daily throughout the experiments. There were no cases of stillbirth, birth defects, or other signs of toxicity that could be attributed to nicotine. Behavioral changes in the rat dams that could be attributed to the stress caused by the osmotic minipump implant were also not evidenced. Body weights were obtained at every chosen age of killing and no differences were found when nicotine and control groups were compared, at any age-equivalent subgroup. The absolute and relative testicular weights (testicular weight/100 g body weight) obtained immediately before the killing showed no significant differences when nicotine-exposed rats were compared to the control rats at the same age (data not shown).

**Plasma levels of FSH and LH**

At 30 dpp and 60 dpp, the FSH and LH plasma levels did not vary significantly when subgroups were compared; however, it seems that there was a subtle tendency of rising FSH level in N60 rats in comparison to those from the control subgroups. In contrast, a significant increase \((P\leq 0.05)\) of FSH and LH plasma levels was observed at 90 dpp in nicotine-exposed rats (N90 subgroup) in comparison with the control subgroups (Table 1).

**Testicular histopathology**

The cross sections of the seminiferous tubules of prepubertal, pubertal, and adult rats of control groups (C and S) displayed seminiferous epithelium with dynamic spermatogenesis, since they were organized in concentric layers containing Sertoli cells in close contact with differentiating germ cells, which were distributed in accordance with the normal aspects described for each age. Intraepithelial vacuoles or sloughing of epithelial cells into the lumen were rarely observed in the control animals (Fig. 1A, B and C). On the contrary, in N30, N60, and N90 rats, an intense sloughing of epithelial cells was observed. At other times, in some animals of N30 and N60 subgroups, large portions of seminiferous epithelium were disconnected and detached from the basal lamina, constituting true epithelial folds toward the tubular lumen. Sertoli cell nuclei were also commonly identified in such epithelial folds. In addition, intraepithelial vacuoles were noted very often in nicotine-exposed rats, which were at all ages analyzed. They were generally larger than some rare vacuoles observed in control rats and located in regions commonly occupied by spermatocytes and round spermatids (Fig. 1).

**Figure 2** Photomicrographs of portions of rat testicular sections submitted to immunolabeling procedure, with omission of primary antibody (negative control). Note the absence of labeling in 30 dpp (A), 60 dpp (B), and 90 dpp (C) rats.
Table 4 Total volume densities (Vv) of Fas and FasL immunolabeling in the seminiferous epithelium of prepubertal, pubescent, and adult rats pertaining to absolute control (C30, C60, C90), sham control (S30, S60, S90) and nicotine-exposed (N30, N60, N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

<table>
<thead>
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<th>Subgroups (n = 5)</th>
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<th>Vv – FasL protein</th>
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<td>N30</td>
<td>7.14 (3.63–20.07)</td>
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<td>C60</td>
<td>56.02 (16.97–72.26)</td>
<td>0.81 (0.32–3.90)</td>
</tr>
<tr>
<td>S60</td>
<td>7.42 (6.00–13.82)</td>
<td>1.27 (0.27–3.01)</td>
</tr>
<tr>
<td>N60</td>
<td>4.06 (1.91–31.10)</td>
<td>1.30 (0.75–6.28)</td>
</tr>
<tr>
<td>C90</td>
<td>26.96 (16.08–81.88)</td>
<td>0.86 (0.20–2.55)</td>
</tr>
<tr>
<td>S90</td>
<td>32.95 (16.39–165.13)</td>
<td>0.66 (0.28–0.98)</td>
</tr>
<tr>
<td>N90</td>
<td>20.99 (7.50–30.20)</td>
<td>0.49 (0.20–0.76)</td>
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</tbody>
</table>

Statistical analysis: Kruskal–Wallis’s one-way ANOVA. No significant differences.

Germ cells showing abnormally condensed and pyknotic nuclei and usually strong eosinophilic cytoplasm (Fig. 1J, K and N) were noticed in N60 and N90 rats in the region generally occupied by spermatocytes. Step 19 spermatids (elongated spermatids) could be observed in tubular sections in stages IX–X of epithelium seminiferous cycle in these rats. Binucleated formation and oversized cytoplasmic area were also detected in round spermatids of N90 rats (Fig. 1O).

Generally, the profiles of Sertoli cell nuclei are usually ovoid and their major axis is parallel in relation to the basal region of the tubules. However, in N90 rats, the Sertoli cell nucleus profiles showing peculiarly elongated shapes were frequently observed (as shown in the stage XI, in the Fig. 1L) and their major axis was uncommonly and perpendicularly oriented in relation to the basal region of the tubular section.

Frequency of seminiferous tubule sections containing sloughing of epithelial cells

The histopathological analysis also included the quantification of tubular sections with epithelial cell desquamation (germ cells and Sertoli cell nuclei) into the lumen of prepubertal, pubertal, and adult rats (30, 60, and 90 dpp).

Regarding this issue, there was an evident increase in the frequency of cellular desquamation in the subgroups N60 and N90 in comparison with those observed in control subgroups at corresponding ages (Table 2).

Frequency of stages of the seminiferous epithelium cycle in pubertal and adult animals

The evaluation of the frequencies of the 14 stages of the seminiferous epithelium cycle was carried out in pubertal (60 dpp) and adult (90 dpp) rats. At these ages, the animals already present all cellular associations of the seminiferous epithelium cycle (Table 3). Nicotine-exposed animals showed some changes in the frequency of specific stages, when compared to the control subgroups at the corresponding ages. N60 rats had a significant reduction in the frequency of tubular sections in the androgen-dependent stage VIII, and in those after the spermiation (stage IX). They also showed an evident increase in the frequency of tubular sections in stage XIV, when division of spermatocytes is happening. On the other hand, a significant increase in frequency of the stage IX was noticed in N90 rats. A suggestive increase in the frequency of stages X and XI also seems to have occurred at this same age, but it was not statistically significant.

Immunolabeling and stereological analysis

No labeling was observed on the negative control slides, in which the primary antibody was omitted. Images of the negative control were provided (Fig. 2).

Total Vv of Fas and FasL immunolabelings in the seminiferous epithelium

In this type of analysis, staging the seminiferous epithelium cycle was not considered and the tubules were indistinctly evaluated. The Fas and FasL proteins were detected in the seminiferous epithelium of prepubertal, pubertal and adult rats, as well in the interstitial tissue of these animals. The analyses of the total volume densities of the labeling, for each protein

Table 5 Total volume density (Vv) of transferrin, vimentin, and β-catenin immunolabeling in the seminiferous epithelium of prepubertal, pubertal, and adult rats pertaining to absolute control (C30, C60, C90), sham control (S30, S60, S90) and nicotine-exposed (N30, N60, N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

<table>
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<tr>
<th>Subgroups (n = 5)</th>
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<th>Vv – vimentin</th>
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<td>S60</td>
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<td>15.65 (12.05–31.77)</td>
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<tr>
<td>C90</td>
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<td>47.64 (36.98–58.13)</td>
<td>57.33 (55.51–68.28)</td>
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Statistical analysis: Kruskal–Wallis’s one-way ANOVA. No significant differences.
(Fas and FasL) in the seminiferous epithelium in nicotine-exposed rats showed no statistical differences, when compared to control animals at the same age (Table 4).

**Total Vv of transferrin, vimentin and β-catenin immunolabeling in the seminiferous epithelium**

The transferrin was detected in the seminiferous epithelium of prepubertal, pubertal and adult animals, as well as in the testicular interstitial tissue. There were no significant differences in the volume densities of transferrin labeling in the seminiferous epithelium at 30, 60, and 90 dpp when nicotine-exposed animals were compared to control rats of the same age (Table 5).

The seminiferous epithelium immunolabeling for the protein vimentin was detected in all animals studied. It was evident in the cytoplasm of the Sertoli cells of rats in all the groups, mainly in the perinuclear cytoplasmic region; the apical region of these cells also showed labeling although with less intensity. The analysis of volume densities showed that the expression of vimentin had a trend of reduction in N60 and N90 rats when compared to control animals at the same age (Table 5). In addition, an interesting differential aspect observed in nicotine-exposed adult animals (N90) was the more conspicuous distribution of vimentin labeling in Sertoli cells, since it was much more concentrated in the perinuclear region and even rarer in the adluminal region when compared to the ones observed in control animals at the same age (Fig. 3).

The β-catenin immunolabeling in the seminiferous epithelium was also detected in all animals studied. In general, this labeling was weaker in the cytoplasm of Sertoli cells than in spermatogonia and spermatocytes (Fig. 4). In the nicotine-exposed rats the labeling also occurred in cell layers misplaced towards the lumen and in areas where no germ cells were found (Fig. 4). Nevertheless the volume densities of β-catenin in the seminiferous epithelium of 30, 60, and 90 dpp rats showed no significant differences when compared to the data from the respective control subgroups (Table 5).

**Vv of vimentina immunolabeling in the different stages of the seminiferous epithelium cycle**

When the analysis of the vimentin labeling were carried out in relation to the stages of the seminiferous epithelium cycle, we observed, in the N90 rats, a reduced vimentin expression during early stages of the cycle, which was statistically significant ($P \leq 0.05$) in stages I–V, VI and VIII, compared to the C90 subgroup (Table 6).

**Vv of β-catenin immunolabeling at the different stages of the seminiferous epithelium cycle**

When the evaluation of the β-catenin immunolabeling was performed considering the stages of the seminiferous epithelium cycle, no significant differences were observed in nicotine-exposed rats in comparison to the corresponding control rats, either in puberty or in adulthood (Table 7).

**Nv of apoptotic epithelial cells (TUNEL-positive cells)**

TUNEL-positive cells were detected in the seminiferous epithelium of prepubertal, pubertal, and adult rats. However, significant differences were not observed when data from nicotine-exposed animals were compared to those of the control groups, at the corresponding ages studied (Table 8).

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**Figure 3** Photomicrographs of portions of rat testicular sections submitted to vimentin immunolabeling. In A, B and C: note in the tubular sections from C30 (A), S30 (B), and N30 (C) rats the Sertoli cell profiles showing brown golden labeling in the cytoplasm. Such pattern of labeling was seen in all the ages studied. In the tubular sections from C60 (D) and S60 (E) rats, note that both the perinuclear and the apical regions of the Sertoli cells are labeled, while in N60 rat (F) the labeling is concentrated in the perinuclear region (thin arrow). Also observe in N60 the labeled cells in the tubular lumen (thick arrow). In the C90 (G) and S90 (H) rats, labeling is also noticed in both perinuclear and the apical regions of the Sertoli cells, while in N90 (I) it was more concentrated in the perinuclear region (arrows). Bar = 10 μm.
Figure 4 Photomicrographs of portions of rat testicular sections submitted to β-catenin immunolabeling. Note, in the tubular sections from C30 (A), S30 (B), N30 (C), C60 (D), S60 (E), N60 (F), C90 (G), S90 (H), and N90 (I) the labeling in germ cells cytoplasm (thin arrows) and between elongated spermatids (thick arrow – G). In the nicotine-exposed rats, the labeling can also be observed in cell layers misplaced towards the lumen (thick arrows – C and F) and in areas where germ cells were depleted (thick arrow – I). Bar = 20 μm.
Table 6

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Statistical analysis: Kruskal–Wallis’s one-way ANOVA and Dunn’s test.

Discussion

Prenatal exposure to smoking can reduce the number of both germ and somatic cells in human embryos (Mamsen et al. 2010), as well as of Sertoli cells in adult rats (Ahmadnia et al. 2007). Although certain toxic agents disrupt the Sertoli cell function and reduce its ability to support spermatogenesis, increasing the elimination of germ cells via apoptosis (Richburg 2000), we show here that nicotine does not cause apoptosis of germ cells and/or somatic testicular cells. In this regard, nicotine was shown to inhibit apoptosis in several cell types (Wright et al. 1993).

Instead, in nicotine-exposed 60 and 90 dpp rats we observed cells with nuclear pyknosis, irregular chromatin condensation and an intense cytoplasmic eosinophilia, which are features suggestive of cell death by necrosis (Cotran & Robbins 1996). Besides apoptosis and necrosis, several types of cellular deaths have been described, such as necroptosis. In contrast to necrosis, which has been described to be a consequence of extreme physicochemical stress that leads to an accidental and uncontrolled phenomenon (Vanden Berghe et al. 2013), necroptosis is a programmed necrosis that promotes inflammation through leakage of cellular content from damaged plasma membranes (Chan et al. 2015). Interestingly, the exposure of pregnant rats to nicotine causes a dose dependent increase in the rate of inflammatory serum markers in the progeny (Mohsenzadeh et al. 2014). Thus, it is possible that some cells observed in the current study, which presented suggestive morphological characteristics of death, could be cells in necroptosis. This deserves further detailed investigation.

Sobinoff et al. (2014) recently described an increase in TUNEL but not in caspase labeling in testis of 21-day-old mice after maternal smoke exposure. They observed some Sertoli cell damage in the juvenile offspring, with persistent testicular pathology in the adult, which was associated with germ cell DNA damage and to a significant change in total testicular gene expression, including genes involved in the Sertoli function (tight junction signaling). In spite of the authors having used cigarette exposure, we believe that the main harmful factor was caused by nicotine, or even worse by the other components of the cigarette. Indeed, according to Zenzes (2000) nicotine is one of the major hazardous components of tobacco and is responsible for most of the deleterious effects caused by smoking cigarettes.

We must also consider that the germ cell loss might have occurred secondarily, due to the rupture of Sertoli–germ cell junctions, as Sertoli cells provide the required microenvironment for the development of the germ cells. Reinforcing this aspect, Aydos et al. (2001) observed ultrastructural changes in Sertoli cells of adult rats after nicotine injection as a response to the degeneration of Sertoli–Sertoli cell junctions. Güven

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Table 7 Volume density (Vv) of -catenin labeling according to the stage of the seminiferous epithelium cycle in pubertal and adult rats pertaining to absolute control (C60 and C90), sham control (S60 and S90) and nicotine-exposed (N60 and N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

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<th>VIII</th>
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<th>XI</th>
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</table>

Statistical analysis: Kruskal–Wallis’s one-way ANOVA. No significant differences.

There are numerous studies showing that toxic agents may inhibit the release of spermatids from the Sertoli cell cytoplasm (Richburg & Dwyer 2010). The 2,5-hexanedione, for e.g. induces deformation of epithelial vacuoles (related to the dilatation of endoplasm reticulum of Sertoli cells) and the retention of 19 step spermatids in stages IX and X. It is also reported to change the duration of the cycle of seminiferous epithelium in rats (Rosiepen et al. 1995) by inhibiting some Sertoli cell specific enzymes (as β-glucuronidase and γ-glutamyl transpeptidase), which is followed by an increase in FSH and LH levels (Chapin et al. 1982).

Any enzymatic modifications taking place before the morphological changes are noticed; therefore, further morphological changes can also result from the toxic

et al. (1999) exposed adult rats to inhalation of cigarette smoke, causing the disruption of tight junctional complexes between the Sertoli and germ cells, sloughing of spermatids into the lumen, disorganization in seminiferous tubules and the morphological alteration of germ cells.

Alterations in -catenin expression have been referred to as a cause of desquamation of immature germ cells in the seminiferous epithelium (Kolasa et al. 2011). However, in the current study, nicotine seems to have no specific action on the expression of -catenin. In the same way, Hutamekalin et al. (2008) demonstrated that nicotine, along with other hydrocarbons contained in the cigarette, affects junctional complex components in the brain and endothelial cells via oxidative stress, without affecting the expression of -catenin.

On the other hand, Wong et al. (2008) mentioned that the loss of round spermatids but not of elongating and elongated-spermatids, with no increase in germ cell apoptosis, suggests a disruption in desmosome-like junction. In the present research we noticed not only the loss of cells from the peripheral lines but also the detachment of some portions of seminiferous epithelium from the basal lamina, which fold into the tubular lumen, suggesting that the damage affected the distribution of vimentin, which is present in both desmosome and hemidesmosome-like junctions. Indeed, toxic agents disrupt vimentin filaments causing immature germ cell sloughing (Richburg & Boekelheide 1996, Kopecky et al. 2005). Thus, the increase in epithelial cell desquamation and the intraepithelial vacuoles observed in nicotine-exposed rats can be related to the reduced vimentin expression noticed in adult rats.

As the Sertoli cells are also responsible for the synchronization of the cycle of the seminiferous epithelium, alterations in their cytoskeleton may also be related to the interruption in the pace and/or in the progression of the cycle. Evidences of such effect are the disorganization of the cell associations within the seminiferous epithelium, retention of step 19 spermatids in the IX and X stages and alterations in the frequencies of seminiferous epithelium stages in N60 and N90 rats.

There are numerous studies showing that toxic agents may inhibit the release of spermatids from the Sertoli cell cytoplasm (Richburg & Dwyer 2010). The 2,5-hexanedione, for e.g. induces deformation of epithelial vacuoles (related to the dilatation of endoplasm reticulum of Sertoli cells) and the retention of 19 step spermatids in stages IX and X. It is also reported to change the duration of the cycle of seminiferous epithelium in rats (Rosiepen et al. 1995) by inhibiting some Sertoli cell specific enzymes (as β-glucuronidase and γ-glutamyl transpeptidase), which is followed by an increase in FSH and LH levels (Chapin et al. 1982).
effects caused in the distribution of actin and assembly of microtubules and vimentin in the Sertoli cells (Rosiepen et al. 1995). Thus, the changes in the cytoskeleton of these cells could be the cause of the histopathological alterations observed in our analysis; however, the secretory function of Sertoli cells seemed to be preserved, since transferrin expression did not change.

Structures formed by Sertoli cells, such as the tubulobulbar complex, work along with vimentin filaments in the cytoplasmic reduction of spermatids in steps 18 and 19 (Upadhay et al. 2011). So, the abundance of cytoplasmic area noticed in round spermatids of N90 rats might be a consequence of the nicotine effect on Sertoli cytoskeleton. Aydos et al. (2001) reported anomalies in spermatids under ultrastructural analysis in testis of rats exposed to nicotine in adulthood. Mak et al. (2000) also mentioned that the consumption of tobacco (cigarette) may be associated with the failure to remove residual cytoplasm in human sperm, which can lead to defects in the sperm function.

The reorganization/collapse of vimentin has also been correlated with loss of microtubules (Upadhay et al. 2011). Microtubules control the dynamics of cellular division (cytokinesis) and multinucleated formation is frequently observed in studies with toxic agents (Freitas et al. 2002). Here, we also found binucleated formation in round spermatids of N90 rats, which is probably a harmful effect of nicotine on the spermatids cytoskeleton and cytokinesis. In fact, nicotine has been showing effects on the cellular division in oocytes of cows by altering the microtubules (Liu et al. 2007).

The evident difference in the distribution of vimentin filaments throughout the cycle of seminiferous epithelium was observed in nicotine-exposed rats, especially such cells in the apical region, where the staining was weaker than in the perinuclear region. As previously described by Kopecky et al. (2005) in rats treated with busulfan, the perinuclear condensation and the disorganization of the filaments in the basal region can be a result of a breakdown of these filaments around the nuclei of Sertoli cells. So, it is likely that nicotine altered the intracellular organization of vimentin, which depends on the action of kinase and protein phosphatase, regulator enzymes of the reorganization of the filaments through phosphorylation (Upadhay et al. 2011).

The raise in FSH, modulated by thyroid hormones, induces vimentin phosphorylation and contributes to the change in cell shape (Spruill et al. 1983). Indeed, nicotine exposure during lactation causes changes in the hypothalamic–pituitary–thyroid axis, leading to hypothyroidism in adult rats (de Oliveira et al. 2011); also, the decrease in T3 levels is related to hyperphosphorylation of vimentin and concentration of this protein around the Sertoli cell nuclei (Zamoner et al. 2007). So, in the present research, the nicotine effects on vimentin expression possibly occurred due to a primary effect on the hypothalamic–pituitary axis. Thus, unbalanced levels of steroid hormones and FSH may have interfered in vimentin phosphorylation in the testes, that could have caused the collapse of vimentin network in the perinuclear region of Sertoli cells.

The decrease in T3 and T4 levels caused by nicotine (de Oliveira et al. 2011) can lead to exacerbated proliferation of Sertoli cells, but in this case, the cells display impaired function (Hess et al. 1993). So, nicotine might even have increased the number of Sertoli cells during the proliferative phase, contributing to a raise in the germ cell number. Sertoli cells could also fail to provide structural support to the germ cells, leading to desquamation. In this way, the balance between proliferation and increased cell loss causes no changes in testicular weight and volume. This could be the reason why even with the intense sloughing of germ cells, nicotine did not affect the testicular weight and volume (Paccola et al. 2014), as well as the sperm number, but compromised its quality (data not shown).

In relation to the secretory function of the Sertoli cell, although transferrin represents a good tool for indicating the functional state of Sertoli cells (Skinner & Griswold 1980), we must consider in future studies the immunoexpression of other important proteins, such as inhibin. The gonadal-derived inhibin A and B are essential factors in mammalian reproduction, negatively regulating pituitary production of FSH (Walton et al. 2015). Changes exhibited by chronic inhibin insufficiency on Sertoli and Leydig cells reflect impairment of: i) functionality of the testicular somatic environment; ii) their compromised capacity to support complete, ongoing spermatogenesis; and iii) daily sperm production and can cause testicular abnormalities, without affecting hormone concentrations (Itman et al. 2015). It is possible that a similar situation occurred in the present research, through the nicotine exposure. In addition, Storgaard et al. (2003) showed that pregnant humans who smoke more than ten cigarettes per day, have sons who present a 30–48% reduction in sperm counts and

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**Table 8** Numerical densities (Nv) of TUNEL-positive cells in the seminiferous tubules of prepubertal, pubertal, and adult rats pertaining to absolute control (C30, C60, C90), sham control (S30, S60, S90), and nicotine-exposed (N30, N60, N90) subgroups. Values are expressed as medians and interquartile ranges (Q1–Q3).

<table>
<thead>
<tr>
<th>Subgroups (n=5)</th>
<th>Nv – TUNEL-positive epithelial cells (cell number/mm³ × 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C30</td>
<td>18.22 (12.86–22.50)</td>
</tr>
<tr>
<td>S30</td>
<td>28.27 (17.53–29.75)</td>
</tr>
<tr>
<td>N30</td>
<td>20.56 (11.93–35.34)</td>
</tr>
<tr>
<td>C60</td>
<td>7.15 (4.62–7.60)</td>
</tr>
<tr>
<td>S60</td>
<td>10.09 (2.88–12.30)</td>
</tr>
<tr>
<td>N60</td>
<td>5.93 (2.53–12.53)</td>
</tr>
<tr>
<td>C90</td>
<td>3.15 (1.70–11.82)</td>
</tr>
<tr>
<td>S90</td>
<td>4.85 (4.24–12.55)</td>
</tr>
<tr>
<td>N90</td>
<td>5.25 (1.37–11.56)</td>
</tr>
</tbody>
</table>

Statistical analysis: Kruskal–Wallis’s one-way ANOVA. No significant differences.
testis size in adulthood, besides a 24% decrease of the inhibin B concentration in blood. However, we cannot forget that Stoggard et al. analyzed human smokers and cigarettes contain many other harmful substances besides nicotine. Thus, the inhibin expression could be affected not only by nicotine.

Still regarding the role of hormones, the damaged seminiferous tubules may produce metabolites which are capable of blocking the biosynthesis of testosterone by the Leydig cells, leading to high levels of LH and causing an increase in Leydig cell trophic stimulation (Rich et al. 1979). This could explain the increase in LH plasma levels noted in this research, associated with the tubular damage. Increased LH plasma levels after exposure to cigarette smoke were already reported (Mendelson et al. 2003, Svartberg & Jorde 2007). According to Reddy et al. (1998) and Tweed et al. (2012), this phenomenon could be the result of the nicotine stimulation on the hypothalamic–pituitary axis. Considering the increase in plasma testosterone in adult rats due to maternal exposure to nicotine (Paccola et al. 2014), a decrease in intratesticular testosterone should also be expected (McLachlan et al. 1994, Xia et al. 2005). The alterations in the frequencies of the tubules in androgen-dependent stages, observed in N60 rats and reflected in N90 rats may reinforce this hypothesis.

In adult animals, the intratubularic testosterone levels are usually 50–100-fold higher than that of the serum levels. On the other hand, intratubularic testosterone levels of 5–20% below normal levels still guarantees spermatogenesis; in some species, levels can be even lower (2% of normal adult levels) and still support a degree of spermatogenesis (McLachlan & O’Donnell 2012).

Conversely, androgens are involved in the development and maintenance of spermatogenesis, so a possible relationship between the effect of nicotine on Sertoli cell androgen receptors and the seminiferous epithelium damage cannot be excluded. Denolet et al. (2006) described how genes encoding proteases and proteases inhibitors, cell adhesion molecules, components of extracellular matrix, and cytoskeleton molecules of Sertoli cells can be controlled, in part, by androgens. A selective ablation of androgen receptors in Sertoli cells does not completely prevent the formation of a functional and anatomical barrier in the seminiferous epithelium; instead, it delays the barrier construction and causes defects in many tubules. The development of a defective barrier is accompanied by disturbances in the nuclear maturation and polarization in Sertoli cells, resulting in aberrant positioning of cytoskeleton elements, such as vimentin (Willems et al. 2010).

Corroborating these findings, Nakai et al. (1995) reported that the toxic agent carbendazim alters the shape of nuclei by causing modifications in the Sertoli cytoskeleton components, which is also related to sloughing of epithelial cells. In fact, we found Sertoli cells with unusual elongated nuclei, which were perpendicularly oriented in relation to the basal region of the seminiferous tubule in post-spermiation stages in the N90 rats. As previously reported by Leblond & Clermont (1952) and Griswold (2014), in stages IX–XIV of the seminiferous cycle, the Sertoli cells show nuclei usually oriented parallel to the tubular base and flattened along the basement membrane. Therefore, the difference in nuclear shape reinforces the alteration of intermediary filaments noticed in the Sertoli cells of adult rats, since this element of the cytoskeleton contributes to determining the nuclear shape and cytoarchitecture of these cells (Vogl et al. 2008).

In conclusion, nicotine exposure during intrauterine life and lactation caused the change in the pattern of vimentin expression, altering Sertoli-germ cell junctional complexes; this event may have greatly contributed to the seminiferous epithelium disorganization and to the intense sloughing of germ cell into the lumen, hence compromising the spermatogenesis in puberty and adulthood. Structural alterations and functional impairment of other cytoskeleton proteins under the action of nicotine, during phases of proliferation and differentiation of Sertoli cells, must be investigated in order to better understand the damage caused by nicotine on spermatogenesis and its consequences to the fertility potential of the offspring.

Compared to rats, sperm production in humans is low, magnifying the changes to fertility caused by toxicants that affect spermatogenesis (Working 1988). For this reason, we must take into account that maternal exposure to nicotine could be even worse in man. We strongly recommend that women avoid smoking cigarettes throughout pregnancy and lactation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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