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Original Research Article

Morphological Changes in the Testis of *Rattus norvegicus* Exposed to Cigarette Smoke

ABSTRACT

Smoking has become a major cause of diseases worldwide. Cigarette smoking is associated with an increase in the development of a number of conditions which represent significant impact on public health. In this light the problem arises: Does exposure to cigarette smoke cause morphological changes in the testicles of rats? The present study is justified because of the growing importance of pathological conditions caused by exposure to substances in the cigarette and the scarcity of literature to correlate exposure to cigarette smoke to the emergence of testicular diseases that can promote infertility. The objective of this study was to analyze the morphology of the rat testicles exposed or not to cigarette smoke by checking the cell area of the germinal epithelium and comparison of histomorphometry of the seminiferous tubules. The study was approved by the "Comitê de Ética em Uso de Animais" (Ethics Committee on Animal Use) - CEUA / FACID under protocol 038/13. A total of 14 animals of the species *Rattus norvegicus* were selected, males, with 60 days of age and weighing 300g, divided into two groups (G1 and G2), with seven animals each group. The G1 animals were exposed to smoke produced by the combustion of four cigarettes for 30 minutes, twice a day, six days a week for 60 days. The G2 animals were not exposed. After 60 days, animals in both groups were euthanized and samples of the testicles were dissected and sent for routine processing for further microscopy light analysis. In each testicle sample was randomly calculated the cell area of the germinal epithelium of the seminiferous tubules, with the help of "ImageJ" software. Then the mean areas and standard deviations of each group of animals were calculated, for later histomorphometric comparison between groups. The results were statistically analyzed. Student T test was used, and the level of significance was 5% ($p < 0.05$). Statistical analysis of the data showed that after the exposure period, the average cell area of the seminiferous tubules of the unexposed group is significantly higher than the cell area of the seminiferous tubules of the above group ($p < 0.05$). It can be concluded that exposure of rats to cigarette smoke triggered histomorphometric changes in the testicles, by promoting a reduction of the cell area of the seminiferous tubules, indicating that smoking may result in atrophy of these structures.

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Keywords: Testicles. Seminiferous tubule. Cigarette smoke.

1. INTRODUCTION

The male reproductive system of mice is very similar to the human morphofunction way. Both have two testicles, genital ducts, accessory glands and penis, with similar functions in both species. The testicles are important for the production of testosterone and sperm, and because of this, are essential not only in the reproduction process, but also in sexual differentiation of these beings (Cormack, 2003).

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The mouse testicle is rounded to the cross section and oval to the cut in the side view. The upper limit of the length is 20 mm and diameter of 4 mm. The weight of the adult is 2.0 to 3.5 g (Caster 1956 cited Hebel; Stromberg 1976). Located in an external pouch (the scrotum) to the abdominal cavity, the testicles are organs with exocrine and endocrine functions, which can be divided into two compartments: (Russell et al, 1990) the interstitial compartment and the seminiferous tubules compartment . The interstitial compartment has slight variations

24 from species to species components, but generally consists of a thin layer of loose
25 connective tissue, predominantly Leydig cells.

26 The seminiferous tubules are most of the testicular parenchyma, and are made up of their
27 own tunic, germinal epithelium and tubular lumen (Hess, France, 2007 Junqueira, Carneiro,
28 2013). In them, there are the Sertoli cells and germ line cells, important for the production of
29 sperm, the male reproductive cells. According to Junqueira and Carneiro (2013), as it is
30 being produced through a process named spermatogenesis, cells move away from the
31 germinal epithelium basal lamina, and approach the lumen of the seminiferous tubule, until
32 they are thrown therein and then conducted, for straight and efferent ducts, the epididymis,
33 where they will be stored.

34 Leydig cells located on the thin layer of loose connective tissue are responsible for the
35 production of androgens. In addition, this layer, being abundantly vascularized, is critical for
36 nutrition of the seminiferous tubules. There is, on this layer, blood and lymph vessels, nerves
37 and connective tissue. (Junqueira, 2013)

38 Also according to Ross (2012), each seminiferous tubule is approximately 50 cm in length,
39 ranging from 30 to 80 cm, which is important in spermatogenesis process time, and its
40 overall yield. Some animals, for example, the Rodentia (rodents) order, which belongs
41 *Rattus norvegicus*, have a higher productivity and shorter duration of spermatogenesis
42 process, associated with a high percentage of seminiferous tubules, and a high number of
43 Sertoli cells per gram of testicles, which makes the spermatogenic efficiency in these
44 animals very high (Cordeiro Jr, 2009).

45 In rats, the seminiferous tubules diameter is uniform and around 250 micrometers, with an
46 average length of 32 cm (Muller, 1957 apud Stegani 2001). The size and thickness of the
47 germinal epithelium, in the seminiferous tubules, thus has a strong correlation with the
48 reproductive function of animals.

49 Smoking is one of the problems still quite plaguing humanity. According to the Global
50 Tobacco Epidemic (WHO, 2013) report, smoking remains the leading cause of preventable
51 deaths worldwide. More than 6 million deaths per year worldwide are due to the direct or
52 indirect use of tobacco. Also according to the report, it is believed that smoking has caused
53 more than 100 million deaths during the twentieth century.

54 WHO estimates further that 1 billion and 200 million people are smokers, of which 200
55 million are women, and that 49% of the male population in the world is smoking. In Brazil, a
56 study conducted by INCA indicates that 18.8% of the population smokes, with 22.7% of men
57 and 16% of the country's female population (INCA, 2013). Due to intense efforts, the
58 prevalence of smoking in Brazil dropped from 35% to 18% in the period between 1989 and
59 2003, remaining slightly stable thereafter (Vigitel, 2006).

60 Smoking is a framework that is unfavorable to longevity, and greatly speeds the aging
61 process, a risk factor for numerous diseases in all age groups. Therefore, this habit has a
62 devastating extent even greater than the effect on the duration of life: still affects the quality
63 of life (Bratzler, 2002; Goulart, 2014).

64 The main causes of death from smoking are cardiovascular diseases (1.69 million), chronic
65 obstructive pulmonary disease (970,000) and lung cancer (850,000) Smoking is responsible
66 for 90% of lung cancers, and it is an important risk factor for the emergence of malignant
67 tumors in eight organs: mouth, larynx, pancreas, kidney, bladder, lung, cervix and
68 esophagus, being the carcinogenic substances eliminated by cigarette smoke (Ezzati, 2003).

69 According to Cunha et. al. (2007), the cigarette is composed of several harmful to health,
70 especially nicotine, carbon dioxide (CO₂) and tar. Various organs and systems suffer from
71 the effects of nicotine, which, with CO₂ acts on the cardiovascular system, raising the
72 thromboxane levels and promoting increased platelet adhesiveness. Poisoning of the central
73 nervous system by nicotine can trigger vomiting, tremor, seizures, respiratory depression
74 and even death (Jorenby et al., 1999).

75 Studies have shown that tobacco burning generates more than five thousand toxic
76 substances been identified. Varieties are described gas mixtures such as carbon monoxide,
77 nitrogen oxides, nitrosamines, etc. Even solids such as nicotine, phenols, organic acids,
78 aldehydes, ketones and aromatic polycyclic hydrocarbons can be obtained. And tar, one of
79 the products most commonly found in cigarettes, has proven to have carcinogens such as
80 arsenic, nickel, benzopyrene, cadmium and polonium (Kodama et al., 1997; Pryor, 1997;
81 Park, 1998).

82 Currently, there is a tendency of scientific papers to relate smoking to the testicular diseases
83 and infertility. It is known that infertility may develop from a number of different causes, such
84 as in a varicocele situation, infection, duct obstruction, cryptorchidism, or by hormonal,
85 immunological or genetic causes. Many of them are related to testicular atrophy, since
86 testicular parenchyma plays a fundamental role in spermatogenesis (Netto Jr, 2007).

87 In the study by Coelho et al. (2009), an association between exposure to tobacco and
88 infertility was shown, with a higher degree of oligospermia / azoospermia in subjects who
89 underwent drug use. Regardless of the amount of tobacco consumed, there were changes in
90 the composition of the seminal fluid of the subjects participants on the research. The result
91 strongly suggests an important correlation between smoking and infertility.

92 Recent studies developed by Sepaniak et al. (2004) have also shown that there is passage
93 of various components through hematotesticular barrier, which consists of the junctional
94 complex between the Sertoli cells, and, among other functions, protects the content of
95 seminal fluid in the seminiferous tubules, preserving the foreign components present in the
96 blood or lymph (Ross, 2012). The passage of these cigarette components through the
97 barrier affects the final content of semen and may cause degradation of seminal parameters
98 and also according to Sepaniak (2004), cause changes in sperm nuclear, with possible
99 impairment of fertility.

100 In mice, in vitro fertilization impairment was observed after exposure of the animals to
101 tobacco smoke and high serum levels of nicotine (Reddy, 1995). Furthermore, there were
102 adverse effects observed not only in spermatogenesis *Rattus norvegicus*, but also in
103 fertilizing potential of sperm (Yamamoto, 1998). It is suggested that, a significant correlation
104 between testicular diseases and tobacco consumption in rats.

105 In humans, similar studies were also conducted. There was an association between
106 cigarette smoking and decreased sperm density, mobility and increased anomalous forms
107 (Wang Xing-Run, 2001). Furthermore, Rantala (1987) also noted that in quantifying tobacco
108 consumption, it is possible to reveal more deep changes in the sperm of heavy smokers.

109 Smoking has become a major cause of diseases in the world, disturbing factor in the health
110 strategy. Studies have shown the tobacco association with cardiovascular, pulmonary and
111 cancer diseases, and suggest testicular diseases that can promote infertility.

112 The present study is justified, therefore, because of the growing importance of pathological
113 conditions caused by tobacco, seeking to reach a greater number of research on the topic,

114 and most health logistics action to avoid complications. Hence, the question of this study:
115 "Does the cigarette smoke exposure promote morphological changes in the testicles of
116 rats?" On the assumption that the effects of chronic exposure to cigarette smoke induces
117 histopathological changes in the testicles.

118 The objective of this study was to determine the morphology of *Rattus norvegicus* testicles
119 after exposure to cigarette smoke, through the histomorphometric analysis of the cell area of
120 the seminiferous tubules.

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122 **2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY**

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124 **2.1 Ethical Procedures**

125 This work is in accordance with the Universal Declaration of Animal Rights and ethical
126 principles of animal experimentation issued by the "Conselho Nacional De Experimentação
127 Animal" (National Board of Animal Testing) (CONEA) as Federal Law No. 11.794, of October
128 08, 2008. The research protocol was forwarded to the Comissão de Ética em Uso de
129 Animais (Ethics Committee on Animal Use) - CEUA / FACID, and obtained a favorable
130 opinion, under protocol number 038/13.

131 **2.2 Search Method**

132 The research was experimental quantitative way, intended to analyze, highlight and confirm
133 the variables of interest in the study of animals studied.

134 **2.3 Selection and handling of animals**

135 The research site was 14 rodent species *Rattus norvegicus*, males, kept since birth in the
136 animal house of Faculdade Integral Diferencial - FACID / DEVRV, 60 days old and weighing
137 300g.

138 **2.4 Experimental Procedure**

139 The animals were randomly divided into two groups of seven animals, and the G1 group
140 exposed to cigarette smoke; and G2 used as control, not being exposed.

141 To the animal's exposure to cigarette smoke, an inhalation chamber built of wood was used
142 (100x44x44cm) sealed with a glass cover, divided into two compartments by a metal screen,
143 as shown in Scheme 1. One of the compartments was used for combustion four cigarettes
144 which were placed in a plaster support; and the other, for the accommodation of animals that
145 were within his cage. In the cigarette burning compartment, an air source was connected (air
146 compressor with a flow of 10 L / min) allowing the combustion of the cigarette smoke and for
147 driving the exposure chamber. This compartment on the other hand, contained small holes
148 for the drainage of air, through which the exhaust was carried out.

149 The animals in group G1 have been exposed to burning cigarettes 04 for about 30 minutes
150 twice a day, six days a week for a period of 60 consecutive days. Animals in the G2 group
151 were not exposed to cigarette smoke, and therefore considered the control group.

152 The cigarettes were purchased commercially used, each producing 10 mg of tar burning; 0.8
153 mg nicotine and 10 mg of carbon monoxide (according to the manufacturer). This product

154 was chosen by the great support among smokers and have high levels of nicotine in their
155 composition.

156 **2.5 Euthanasia and Disposal Of Animals**

157 After 60 days, the animals were sacrificed according to ethical principles suitable for the
158 dissection and the samples were analyzed.

159 The animals received the application of anesthetic thiopental Sodium, intraperitoneally at a
160 dose of 0.05 ml / 100g. After 5 minutes the application of potassium chloride 19.1% was
161 performed intraperitoneally in a single dose of 0.4 ml / 100g.

162 Then the animals were frozen in freezer to after be discarded by the technician responsible
163 for the animal facilities of the institution.

164 **2.6 Data Collection**

165 Soon after euthanasia, dissection and removal of the testicles was held. Each sample
166 received an ID, and then the specimens were submitted to fixing in formalin 10% for 48
167 hours and then forwarded to include procedures in histological paraffin. Cross-sections were
168 carried out on the blocks with a thickness of 04 micrometers on a rotary microtome, resulting
169 in semi-serial sections that were stained with hematoxylin-eosin (HE) for histomorphological
170 study by light microscopy. The slides were numbered according to the number and subset of
171 the animal to which it belonged.

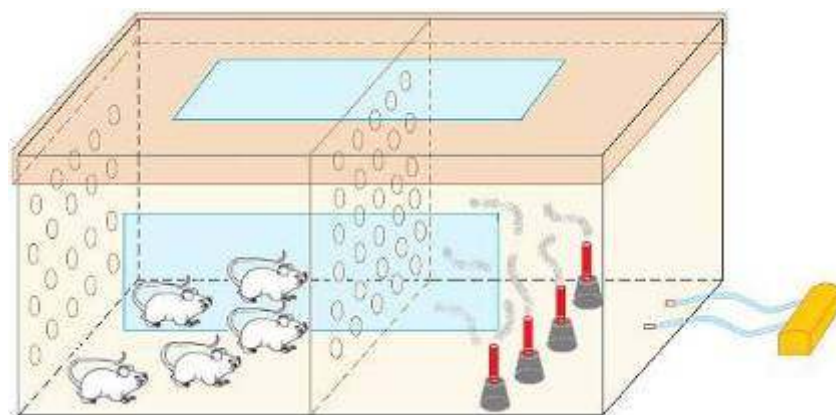
172 After the preparation and collection of histological slides, the analysis and the photographic
173 record of them in light microscope coupled to a computer was performed. After acquisition of
174 digital photomicrographs of the testicles, the images were imported into Image J software,
175 version 1:47 (Micronal).

176 In each testicular sample was calculated randomly cell area of the germinal epithelium of the
177 seminiferous tubules. Then the data were imported into Excel® software for Calculation of
178 the average cell area of the germinal epithelium of the seminiferous tubules of each group
179 (control and experimental), for comparison between groups.

180 The results relating to histomorphometric analysis of the seminiferous tubules were
181 expressed as mean $\pm$ standard deviation ($M \pm SD$). These results were presented in tables
182 and graphs and statistically analyzed.

183 **2.7 Statistical Analysis**

184 The variables obtained in the study were analyzed with the GraphPad InStat® program. The
185 **Student's t test** was carried out for data analysis. The significance level was 5% ($p < 0.05$).



Scheme 1

3. RESULTS AND DISCUSSION

The histomorphometric analysis of data from the cell area of the seminiferous tubules of the control group animals of the testicles and the group exposed to cigarette smoke are shown in Figure 1. According Yan et. al. (2008), seminiferous tubule of the animal consists of two structures: a germinal epithelium with germ cells arranged in concentric manner, and a lumen in the center, which generates the classification of these cells based on their location. The basal compartment of the cells are more immature and are more distant from the lumen, while the compartment adluminal cells are more mature and are closer to the lumen. The cell area, which was calculated in this work, it is therefore the total area of a seminiferous tubule subtracted area of the central lumen thereof.

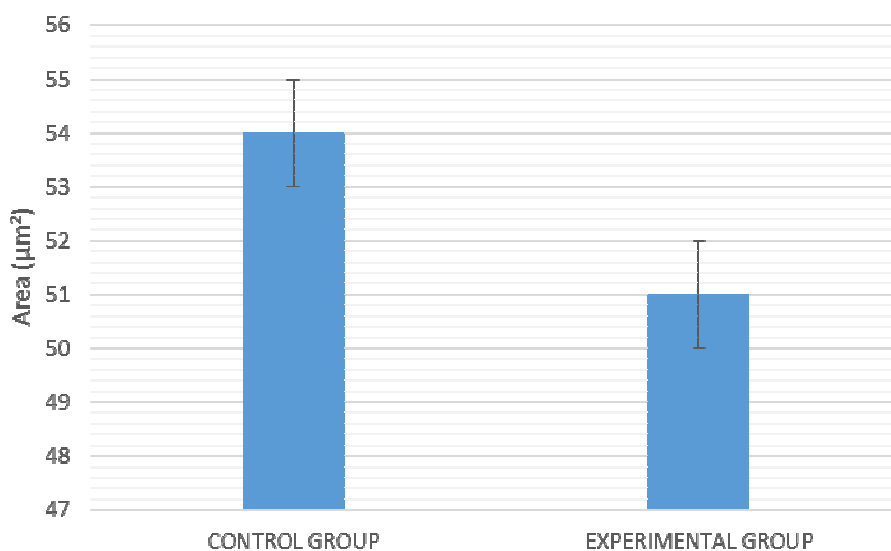


FIGURE 1 - Means and standard deviations of the areas (µm²) of the germinal epithelium of the seminiferous tubules of animals in the control and experimental group - Teresina, PI – 2014.
Source: Research Data

After analysis and comparison of data on the histomorphometry of the cell area of the germinal epithelium of the seminiferous tubules of the testicles, it can be observed that the

209 animals in the control and experimental group showed average cell area of the germinal
210 epithelium of the seminiferous tubules. It can be seen in the Figure 2, in which the first
211 picture (A) shows the cell area of germinal epithelium in the control group, while the other
212 picture (B), in which the epithelium is clearly smaller, shows the result in the experimental
213 group.

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215 Statistical analysis of the data showed that after the exposure period, the average cell area
216 of the germinal epithelium of the seminiferous tubules of the control group animals was
217 significantly higher ($p < 0.05$) compared to the average cell area of the germinal epithelium of
218 seminiferous tubules of animals exposed to cigarette smoke. Thus, it can be seen that
219 exposure of mice to cigarette smoke promoted reduction of the cell area of the germinal
220 epithelium in the seminiferous tubules, with consequent reduction of the volume of the
221 testicles of the animals. These findings are consistent with those found in the work of La
222 Maestra et al. (2015), who observed histomorphological changes of the testicles, weight
223 loss, oxidative stress and DNA damage, documented by the use of electrophoresis gel after
224 exposure to cigarette smoke in newborn rats, in the first 70 days of life. Oxidative stress and
225 damage to the genetic material are important factors in apoptosis induction and cell
226 necrosis, which may cause reduction of germ cells of the seminiferous tubules.

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228 Yüksel et. al. (2014) studied the exposure of rats to cigarette smoke still in the intrauterine
229 period. Pregnant rats were divided into two groups, with one of them, exposed to cigarette
230 smoke and the other, control, exposed to the ambient air. The newborn rats, in turn, were
231 divided into two groups: group 1 consists of those who were exposed to cigarette smoke in
232 the intrauterine period (children of pregnant rats in group 1) and group 2, which does not had
233 this exposure (children of pregnant rats in group 2). The study showed that animals exposed
234 to cigarette smoke during the intrauterine period showed significant changes in the germinal
235 epithelium. The histopathological analysis showed that the cells showed apoptotic
236 morphology with pyknotic nuclei and condensed. Immunofluorescent techniques showed
237 increased DNA damage these germinal epithelium cells of the seminiferous tubules in group
238 1. In addition, the researchers did a quantitative analysis of apoptosis (both Sertoli cells, the
239 germ cells) that was different significantly greater ($p < 0.001$) in the group exposed to
240 cigarette smoke when compared to the unexposed group. These data corroborate the
241 findings of this study, since apoptosis and DNA damage are factors that can change the
242 tropism of cells and tissues, which can be directly related to a decrease in cell area of the
243 germinal epithelium of the seminiferous tubules of rats which was evidenced in this research.

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245 Lima et. al. (2012) also observed the macro and microscopic appearance of testicles and
246 epididymis after administration of nicotine, one of the main products of combustion of the
247 cigarette. The authors applied nicotine diluted in saline subcutaneously in the abdominal
248 region, and compared the appearance of the sexual organs to the group that received only
249 saline solution application. In that work it was observed that there was no macroscopic
250 changes of testicles and epididymis. However, histologically analysis of both organs,
251 circulatory changes were found between animals such as congestion and hemorrhages in
252 both parenchymal areas and in outlying areas. Circulatory disorders can promote alterations
253 in the cellular area of the germinal epithelium, since adequate nutrition for these, blood
254 vessels of the region are crucial.

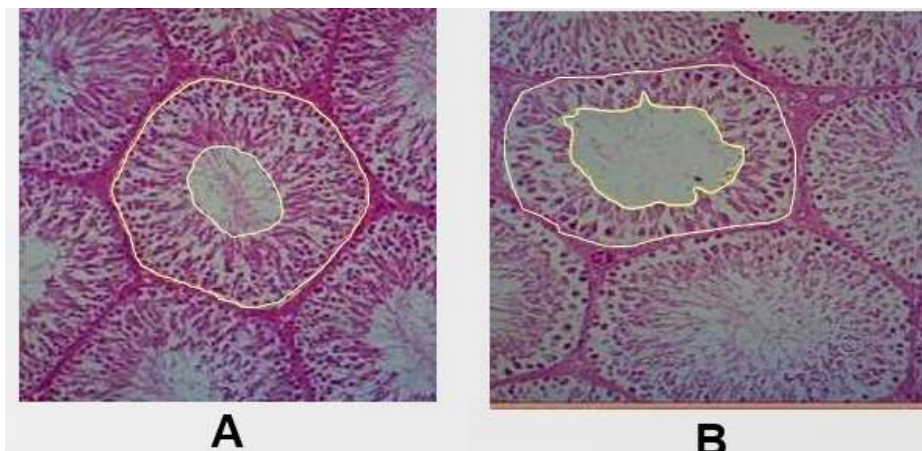


FIGURE 2: AREA OF THE GERMINATIVE EPITHELIUM OF THE SEMINIFEROUS TUBULES: CONTROL GROUP (A) AND EXPERIMENTAL GROUP (B). SOFTWARE IMAGEJ® (H.E.; 100X)

4. CONCLUSION

After application of the proposed methodology, and analysis and discussion of the results of this study, it can be concluded that exposure of rats to cigarette smoke was able to trigger histomorphometric changes in the cell area of the germinal epithelium of the seminiferous tubules, showing that smoking can trigger testicular atrophy.

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