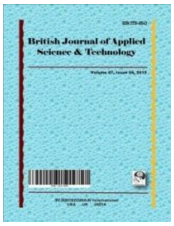

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Morphological Changes in the Testis of *Rattus norvegicus* Exposed to Cigarette Smoke

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JJLSS, KSA and JLOS designed the study, wrote and developed the protocol, wrote the first draft of the manuscript, performed the first part of the microscopic analysis and managed literature searches. Authors MBC, JDSP, WBLF and ALMMF developed the protocol. Author IMSPS performed part of the microscopic analysis. Author KSA performed the statistical analysis, and managed all group. All authors read and approved the final manuscript.

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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

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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 300 g, 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.

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 [1].

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. 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: [2] the interstitial compartment and the seminiferous tubules compartment. The interstitial compartment has slight variations from species to species components, but generally consists of a thin layer of loose connective tissue, predominantly Leydig cells.

The seminiferous tubules are most of the testicular parenchyma, and are made up of their own tunic, germinal epithelium and tubular lumen [3,4]. In them, there are the Sertoli cells and germ line cells, important for the production of sperm, the male reproductive cells. As it is being produced, through a process named spermatogenesis, cells move away from the germinal epithelium basal lamina, and approach the lumen of the seminiferous tubule, until they are thrown therein and then conducted, for straight and efferent ducts, the epididymis, where they will be stored [4].

Leydig cells located on the thin layer of loose connective tissue are responsible for the production of androgens. In addition, this layer, being abundantly vascularized, is critical for nutrition of the seminiferous tubules. There is, on this layer, blood and lymph vessels, nerves and connective tissue [4].

Also according to some authors [5], each seminiferous tubule is approximately 50 cm in length, ranging from 30 to 80 cm, which is important in spermatogenesis process time, and its overall yield. Some animals, for example, the Rodentia (rodents) order, which belongs Rattus norvegicus, have a higher productivity and shorter duration of spermatogenesis process, associated with a high percentage of seminiferous tubules, and a high number of Sertoli cells per gram of testicles, which makes the spermatogenic efficiency in these animals very high [6].

In rats, the seminiferous tubules diameter is uniform and around 250 micrometers, with an average length of 32 cm [7]. The size and thickness of the germinal epithelium, in the seminiferous tubules, thus has a strong

correlation with the reproductive function of animals.

Smoking is one of the problems still quite plaguing humanity. According some reports [8], smoking remains the leading cause of preventable deaths worldwide. More than 6 million deaths per year worldwide are due to the direct or indirect use of tobacco. Also according to the report, it is believed that smoking has caused more than 100 million deaths during the twentieth century.

1 billion and 200 million people are smokers, of which 200 million are women, and that 49% of the male population in the world is smoking. [8] In Brazil, a study conducted by Instituto Nacional do Câncer (INCA) indicates that 18.8% of the population smokes, with 22.7% of men and 16% of the country's female population [9]. Due to intense efforts, the prevalence of smoking in Brazil dropped from 35% to 18% in the period between 1989 and 2003, remaining slightly stable thereafter. [10]

Smoking is a framework that is unfavorable to longevity, and greatly speeds the aging process, a risk factor for numerous diseases in all age groups. Therefore, this habit has a devastating extent even greater than the effect on the duration of life: still affects the quality of life [11].

The main causes of death from smoking are cardiovascular diseases (1.69 million), chronic obstructive pulmonary disease (970,000) and lung cancer (850,000) Smoking is responsible for 90% of lung cancers, and it is an important risk factor for the emergence of malignant tumors in eight organs: mouth, larynx, pancreas, kidney, bladder, lung, cervix and esophagus, being the carcinogenic substances eliminated by cigarette smoke [12].

The cigarette is composed of several harmful to health, especially nicotine, carbon dioxide (CO2) and tar. [13] Various organs and systems suffer from the effects of nicotine, which, with CO2 acts on the cardiovascular system, raising the thromboxane levels and promoting increased platelet adhesiveness. Poisoning of the central nervous system by nicotine can trigger vomiting, tremor, seizures, respiratory depression and even death [14].

Studies have shown that tobacco burning generates more than five thousand toxic substances been identified. Varieties are described gas mixtures such as carbon monoxide, nitrogen oxides, nitrosamines, etc. Even solids such as nicotine, phenols, organic acids, aldehydes, ketones and aromatic polycyclic hydrocarbons can be obtained. And tar, one of the products most commonly found in cigarettes, has proven to have carcinogens such as arsenic, nickel, benzopyrene, cadmium and polonium [15-17].

Currently, there is a tendency of scientific papers to relate smoking to the testicular diseases and infertility. It is known that infertility may develop from a number of different causes, such as in a varicocele situation, infection, duct obstruction, cryptorchidism, or by hormonal, immunological or genetic causes. Many of them are related to testicular atrophy, since testicular parenchyma plays a fundamental role in spermatogenesis [18].

In another study [19], an association between exposure to tobacco and infertility was shown, with a higher degree of oligospermia/ azoospermia in subjects who underwent drug use. Regardless of the amount of tobacco consumed, there were changes in the composition of the seminal fluid of the subjects participants on the research. The result strongly suggests an important correlation between smoking and infertility.

Recent studies [20] have also shown that there is passage of various components through hematotesticular barrier, which consists of the junctional complex between the Sertoli cells, and, among other functions, protects the content of seminal fluid in the seminiferous tubules, preserving the foreign components present in the blood or lymph [5]. The passage of these cigarette components through the barrier affects the final content of semen and may cause degradation of seminal parameters and also according to these studies [20], cause changes in sperm nuclear, with possible impairment of fertility.

In mice, in vitro fertilization impairment was observed after exposure of the animals to tobacco smoke and high serum levels of nicotine [21]. Furthermore, there were adverse effects observed not only in spermatogenesis Rattus norvegicus, but also in fertilizing potential of sperm [22]. It is suggested that, a significant correlation between testicular diseases and tobacco consumption in rats.

In humans, similar studies were also conducted. There was an association between cigarette

smoking and decreased sperm density, mobility and increased anomalous forms [23]. Furthermore, in quantifying tobacco consumption, it is possible to reveal more deep changes in the sperm of heavy smokers [24].

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

The present study is justified, therefore, because of the growing importance of pathological conditions caused by tobacco, seeking to reach a greater number of research on the topic, and most health logistics action to avoid complications. Hence, the question of this study: "Does the cigarette smoke exposure promote morphological changes in the testicles of rats?" On the assumption that the effects of chronic exposure to cigarette smoke induces histopathological changes in the testicles.

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

2. MATERIALS AND METHODS

2.1 Ethical Procedures

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

2.2 Search Method

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

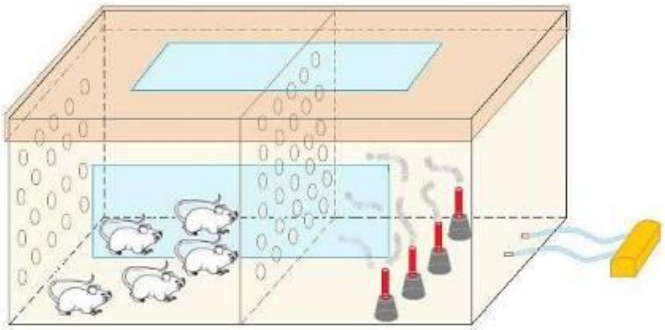
2.3 Selection and Handling of Animals

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

2.4 Experimental Procedure

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

To the animal's exposure to cigarette smoke, an inhalation chamber built of wood was used (100x44x44 cm) sealed with a glass cover, divided into two compartments by a metal screen, as shown in Scheme 1. One of the compartments was used for combustion four cigarettes which were placed in a plaster support; and the other, for the accommodation of animals that were within his cage. In the cigarette burning compartment, an air source was connected (air compressor with a flow of 10 L / min) allowing the combustion of the cigarette smoke and for driving the exposure chamber. This compartment on the other hand, contained small holes for the drainage of air, through which the exhaust was carried out.



Scheme 1. Graphic representation of the box used to expose animals to cigarette smoke

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

The cigarettes were purchased commercially used, each producing 10 mg of tar burning; 0.8 mg nicotine and 10 mg of carbon monoxide (according to the manufacturer). This product was chosen by the great support among smokers and have high levels of nicotine in their composition.

2.5 Euthanasia and Disposal of Animals

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

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

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

2.6 Data Collection

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

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

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

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

2.7 Statistical Analysis

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

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 Fig. 1. Seminiferous tubule of the animal consists of two structures [25]: 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.

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

Statistical analysis of the data showed that after the exposure period, the average cell area of the germinal epithelium of the seminiferous tubules of the control group animals was significantly higher ($p < 0.05$) compared to the average cell area of the germinal epithelium of seminiferous tubules of animals exposed to cigarette smoke. Thus, it can be seen that exposure of mice to cigarette smoke promoted reduction of the cell area of the germinal epithelium in the seminiferous tubules, with consequent reduction of the volume of the testicles of the animals. These findings are consistent with those found in another work [26], that observed histomorphological changes of the testicles, weight loss, oxidative stress and DNA damage, documented by the use of electrophoresis gel after exposure to cigarette smoke in newborn rats, in the first 70 days of life. Oxidative stress and damage to the genetic material are important factors in apoptosis induction and cell necrosis, which may cause reduction of germ cells of the seminiferous tubules.

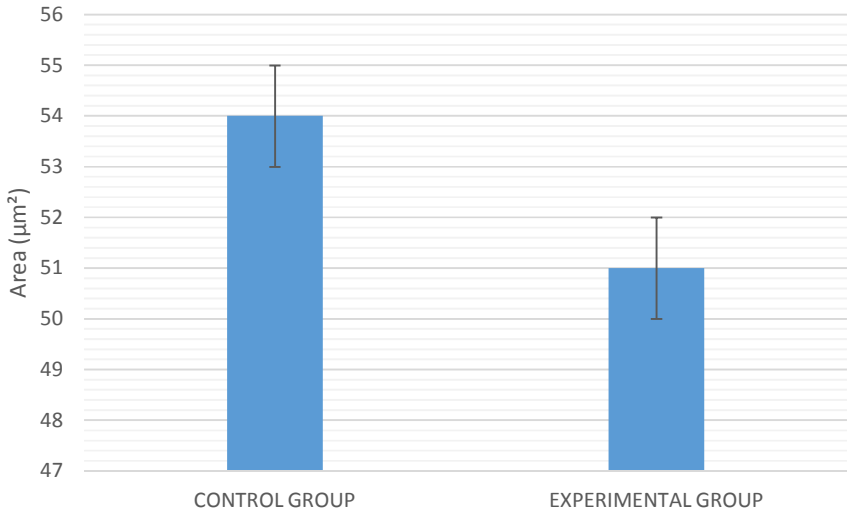


Fig. 1. Means and standard deviations of the areas (µm2) of the germinal epithelium of the seminiferous tubules of animals in the control and experimental group - Teresina, PI – 2014.
Source: Research data

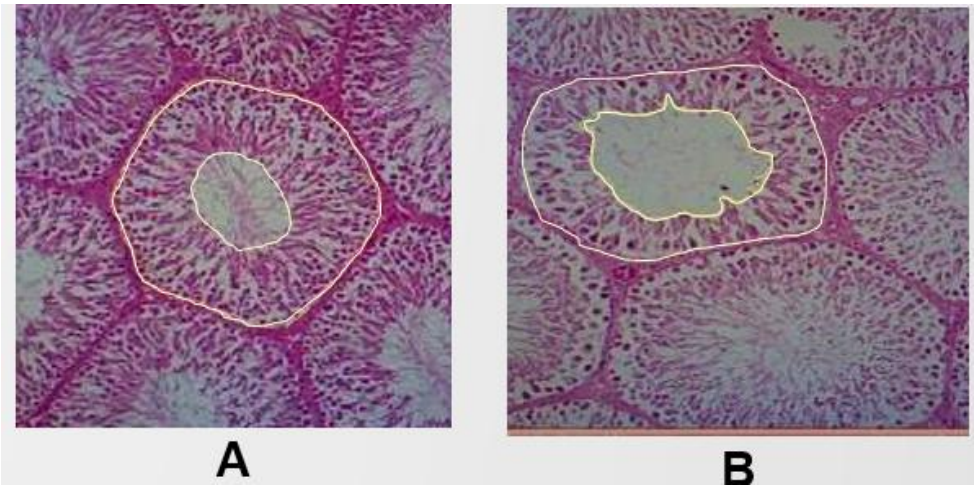


Fig. 2. Area of the germinative epithelium of the seminiferous tubules: control group (a) and experimental group (b). Software imagej® (h.e.; 100x)

Another study [27] observed the exposure of rats to cigarette smoke still in the intrauterine period. Pregnant rats were divided into two groups, with one of them, exposed to cigarette smoke and the other, control, exposed to the ambient air. The newborn rats, in turn, were divided into two groups: group 1 consists of those who were exposed to cigarette smoke in the intrauterine period (children of pregnant rats in group 1) and group 2, which does not had this exposure (children of pregnant rats in group 2). The study showed that animals exposed to cigarette smoke during the intrauterine period showed significant changes in the germinal epithelium. The histopathological analysis showed that the cells showed apoptotic morphology with pyknotic nuclei and condensed. Immunofluorescent techniques showed increased DNA damage these germinal epithelium cells of the seminiferous tubules in group 1. In addition, the researchers did a quantitative analysis of apoptosis (both Sertoli cells, the germ cells) that was different significantly greater ($p < 0.001$) in the group exposed to cigarette smoke when compared to the unexposed group. These data corroborate the findings of this study, since apoptosis and DNA damage are factors that can change the tropism of cells and tissues, which can be directly related to a decrease in cell area of the germinal epithelium of the seminiferous tubules of rats which was evidenced in this research.

Also in another publication [28], was observed the macro and microscopic appearance of testicles and epididymis after administration of nicotine, one of the main products of combustion of the cigarette. The authors applied nicotine diluted in saline subcutaneously in the abdominal region, and compared the appearance of the sexual organs to the group that received only saline solution application. In that work it was observed that there was no macroscopic changes of testicles and epididymis. However, histologically analysis of both organs, circulatory changes were found between animals such as congestion and hemorrhages in both parenchymal areas and in outlying areas. Circulatory disorders can promote alterations in the cellular area of the germinal epithelium, since adequate nutrition for these, blood vessels of the region are crucial.

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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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