The Effects of Passive Smoking on Laryngeal and Tracheal Mucosa in Male Wistar Rats During Growth: An Experimental Study

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Summary: Cigarettes contain toxic and carcinogenic substances. In this context, cigarette smoking, and similar activities, are associated with numerous pathologies, being considered a risk factor in up to 10% of the total number of deaths in adults. Recent evidence suggests that the exposure of children to smoking in the early days of their development causes many diseases. Using light microscopy, this study aims to analyze the possible histopathological effects of an experimental model of chronic inhalation of cigarette smoke (passive smoking) on the laryngeal and tracheal mucosa of young Wistar rats. A total of 24 young Wistar rats were studied for a period of 120 days. The animals were divided into two groups: passive smoking (n = 16) and control (n = 8). The level of exposure to cigarette smoke was evaluated from the urinary cotinine level. Although no cancerous lesions were identified, histopathological analysis in the laryngeal and tracheal mucosa of all the animals in the experimental group showed that the proportion of moderate and focal inflammation was higher in animals exposed to chronic inhalation of cigarette smoke ($P = 0.041$). Histopathologic analysis revealed moderate and focal inflammatory lesions in the region of the infraglottic mucosa in exposed animals, although without dysplastic or neoplastic lesions in the laryngeal and tracheal mucosa.

Key Words: Cigarette–Larynx–Trachea–Young rats–Histopathology.

INTRODUCTION

The ambient smoke (AS) released by burning tobacco is a mixture of smoke directly exhaled by smokers (primary stream smoke) and smoke released by the burning distal portion of the cigarette (secondary stream smoke). AS can cause harmful effects in people who inhale it. People exposed to AS are called passive smokers.[1–3]

Among the diseases most frequently induced by cigarette smoking in adults are oral, oropharyngeal, and esophageal cancer,[4] with squamous cell carcinoma being the most common histological form. Cigarette smoke is thought to induce laryngeal tumors in hamsters,[5] and at least one study has shown an increase of 1% to 9% in the incidence of adenomatous lung tumors.[6]

Our hypothesis is that during development, the mucosa in different glottal spaces and the tracheal mucosa show different responses to the aggression promoted by cigarette smoke. Therefore, this study aims to qualitatively analyze the possible histopathological effects on laryngeal and tracheal mucosa in young Wistar rats subjected to an experimental model of chronic cigarette smoke inhalation.

We divided the larynx into three internal spaces with different functions: the supraglottic, which separates the gastrointestinal tract from air; the glottal, with voice function; and the infraglottic, which connects to the trachea and is comparable to the adjacent tracheal lumen.[7]

METHODOLOGY

Induction of experimental model

This study was approved by the Ethics Committee on Animal Use (CEUA) of the Universidade Federal do Rio Grande do Sul under protocol n° 19.127 and the Ethics Committee on Animal Use (CEUA) of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Brazil, under protocol 084/12.

A total of 24 male Wistar rats (*Rattus norvegicus*) were obtained from the research in animals sector of the UFCSPA, RS, Brazil. The animals were randomly divided into two groups: Group F (passive smoking; n = 16) and group C (control; n = 8). The number of animals for this type of experiment has been statistically validated.[8] The induction of the animals to passive smokers was conducted in accordance with previous experimental protocols,[5,9–11] as follows.

Exposure to smoke

The animals in group F were placed in plastic boxes, measuring approximately $30 \times 40 \times 16$ cm^3 (four per box), with a wire mesh platform to access food and water. The boxes containing the animals were transferred to a laminar flow hood (CQ 800, Union equipment, laboratorial), measuring 60×80 cm, with a 20×20 cm pyramid-shaped exhaust pipe.

The concentration of Carbon Monoxide (CO) in the exhaust hood was adjusted according to previous protocols[9,11] (CO

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concentration between 100 and 300 ppm). The hood had a single overhead outlet, and the airflow was passive, with an average temperature of 22 °C and average humidity of 60%.

The concentration of particulate smoke was measured with a nephelometer (DustTrakII (Almont, Brazil), model 8532, serial number 8.53E+09), with a tube including a 2.4 μg/m^3/minute air filter. This device was used from 5 minutes after onset of exposure until cycle completion.

Over a period of 120 days, from the 12th day after birth, the animals were exposed daily to the smoke generated by burning 16 cigarettes per day (four cigarettes per session, with four sessions per day, at 8, 12, 16, and 20 hours). Each exposure session lasted for 20 minutes, whereas cigarette combustion lasted for 10 minutes on average. In the intervals between sessions, the animals that had not yet been weaned were placed together with their mothers.

The smoke was administered in the absence of the mother until the pups were weaned (at 21 days) to avoid contaminating the breast milk. During exposure to the smoke environment, the boxes containing group F were transferred to a laminar flow hood with four cigarettes in combustion. The AS (in the hood) dissipated passively through the extract vent to the exterior. We used commercial filter cigarettes with the following composition: 10 mg of tar, 0.8 mg of nicotine, and 10 mg of carbon monoxide.

The animals from group C were housed in two open boxes in the same room as the animals exposed to secondhand smoke, at a distance of 5 m from the hood. The animals from both groups had free access to water and feed.

After 120 days of exposure, following all the protocols required by Resolution n° 1000 of the Federal Council of Veterinary Medicine, all animals were sacrificed using the appropriate dose use of ketamine (75–90 mg/kg) and xylazine (10 mg/kg), after which the larynx and trachea were removed in block section.

Cotinine levels analysis

Exposure to cigarette smoke in animals was assessed by urinary concentrations of cotinine (a biomarker for passive tobacco consumption). Urine samples were collected at the end of the exposure time (120 days) *via* suprapubic puncture, while they were alive under anesthesia, immediately before the animals being sacrificed. The cotinine levels were quantified using high-performance liquid chromatography according to the protocol established by Ceppa et al.[12]

Histological processing

After sacrificing the animals, the organs were fixed in 4% paraformaldehyde (16 hours at 4 °C), dehydrated in ethanol, diaphanized in xylene, embedded in Paraplast (Sigma Chemical Company, St. Louis, MO). After which, 5 μm semi-serial sections were obtained using a microtome and plated onto polylysine (Sigma) (Sigma Chemical Company, St. Louis, MO) coverslips. The sagittal sections were then stained with hematoxylin and eosin (HE) for morphological analysis.

Pathological analysis

The histological slides were numbered and then analyzed by experienced pathologists who were blinded to the origin of the images. Any identified alterations were classified according to the severity observed (qualitative analysis). The presence or absence of inflammation in the mucosa, dysplasia, and carcinoma was assessed, as well as the degree of differentiation in such lesions.

TABLE 1.
Initial and Final Weight of the Control Group (C) and Passive Smoking Group (F)

	Initial Avarage (g)	Final Avarage (g)
C	20.1	363.2
F	21.9	376.4

Statistical analysis

The statistical analysis was performed with the aid of *GraphPad Prism 5.0* software (GraphPad Software, Inc., USA). A *t* test for independent samples was used to compare differences in the final weight of the animals and cotinine urine concentrations between the two groups ($P < 0.05$). In addition, because of the small sample, the chi-square test of independence with the calculated significance was used to detect differences in the proportion of light and focal inflammation between group F and group C.

RESULTS

At the end of the experiment, the analysis of animal weight showed no significant differences between group F (376.4 ± 52.8 g) and group C (363.2 ± 35.8 g) ($P = 0.532$) (Table 1). Moreover, the analysis of the urinary cotinine concentration in the rats from group F (4.72 ± 0.89 ng/ml) was significantly higher than the concentration in group C (0.65 ± 0.12 ng/ml) ($P = 0.0001$) (Figure 1).

The exposure to the particulate smoke, measured by the nephelometer, showed an average exposure of 98.4 μg/m^3 between the 6th and 10th minute of exposure, 86.6 μg/m^3, between the 11th and 15th minute of exposure, and an abrupt fall to 2.8 μg/m^3 in the last 5 minutes of exposure (Figure 2).

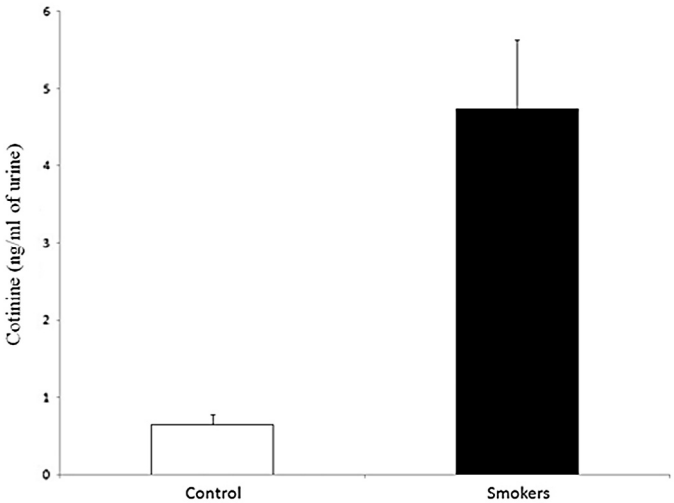

FIGURE 1. Cotinine levels (nanogram per milliliter of urine) in the control and passive smoking groups.