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Original Article

The Effect of Different Doses of Cigarette Smoke in a Mouse Lung Tumor Model

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Abstract: Few studies have used Balb/c mice as an animal model for lung carcinogenesis. In this study, we investigated the effect of different doses of cigarette smoking in the urethane-induced Balb/c mouse lung cancer model. After injection of 3mg/kg urethane intraperitoneally, the mice were then exposed to tobacco smoke once or twice a day, five times a week, in a closed chamber. The animals were randomly divided into four groups. The control group (G0) received urethane only. The experimental groups (G1, G2 and G3) received urethane and exposure to the smoke of 3 cigarettes for 10 minutes once a day, 3 cigarettes for 10 minutes twice a day, and 6 cigarettes for 10 minutes twice a day, respectively. The mice were sacrificed after 16 weeks of exposure, and the number of nodules and hyperplasia in the lungs was counted. The results showed no statistically significant difference in the mean number of nodules and hyperplasia among the different groups, suggesting that the Balb/c mice are not suitable to study the pathogenesis of tobacco smoking-induced tumor progression in the lungs.
Key Words: experimental carcinogenesis, lung cancer, Balb/c mice, tobacco smoking, urethane

Introduction

Lung cancer is the most common cause of cancer-related death in both men and women in the developed world [1]. In 2000, there were 10 million new cases of cancer in the world, and lung cancer was the most prevalent, corresponding to 1.2 million new cases per year. In Brazil, lung cancer accounted for 14,715 deaths in 2000 and it was the leading cause of cancer death. There were estimated 27,000 new cases of lung cancer for 2006 in Brazil [2].

Epidemiological studies have firmly established that smoking is the most important risk factor in the development of lung cancer, and smoking is related to 85-90% of lung cancer deaths [3-6]. Worldwide, cigarette consumption has resulted in a lung cancer epidemic with projections that tens of millions of cases will develop in the coming years [7]. It has also become obvious that exposure to environmental tobacco smoke (ETS), i.e. 'passive' or involuntary smoking, increases the risk of nonsmokers to develop lung cancer [8].

ETS is composed of exhaled mainstream smoke (MS) from the smoker, sidestream smoke (SS) emitted from the smoldering tobacco between puffs, contaminants emitted into the air during the puff, and contaminants that diffuse through the cigarette paper and mouth end between puffs [9, 10].

Animal modeling of lung cancer is imperative to evaluate chemopreventive agents before conducting human trials. Over the years, many attempts to reproduce lung cancer in experimental animals exposed to tobacco smoke have been made, most often with negative or only marginally positive results. Coggins [11-13] reviewed several chronic inhalation studies using rodents, dogs, and nonhuman primates, and concluded that no study has produced a statistically significant increase in lung tumors. Witschi *et al*

successfully developed a model of cigarette smoking lung carcinogenesis in A/J mice strain [8], but not in Swiss and Balb/c strains [14].

There are also other well-defined experimental lung carcinogenesis models, such as urethane-induced lung cancer [15, 16]. Our group has been studying the interaction between cigarette smoking, lung cancer and beta-carotene ingestion in Balb/c mice (data not published), and we did not find in the literature any successful experimental model of lung carcinogenesis in Balb/c mice or any information about how much different doses of cigarette smoking could interfere in this process. The aim of this study is to evaluate the effect of different doses of cigarette smoke in the urethane-induced lung cancer in Balb/c mice.

Material and Methods

Exposure Protocol

Seven to 13 weeks-old Balb/c male mice were employed. Lung tumors were induced with urethane, a genotoxic-mitogenic ethylic ester of carbamic acid previously used in other studies of chemical carcinogenesis in mice [17, 18]. In this experiment, the animals were treated with two intraperitoneal injections of urethane (1.5g/kg), with an interval of 48 hours.

Water and food were given *ad libitum* and no replacement of animals was done in case of death. Cigarette smoking experiment was performed in a closed chamber, once or twice a day, five days a week. The mice were randomly divided into four groups (G0, G1, G2 and G3) as follows: G0 (n=18) received only urethane; G1 (n=17) was exposed to the smoke of three cigarettes for 10 minutes once every day; G2 (n=28) was exposed to the smoke of three cigarettes for 10 minutes twice a day, leading to six cigarettes in 20 minutes; and the G3 group (n=18) was exposed to six

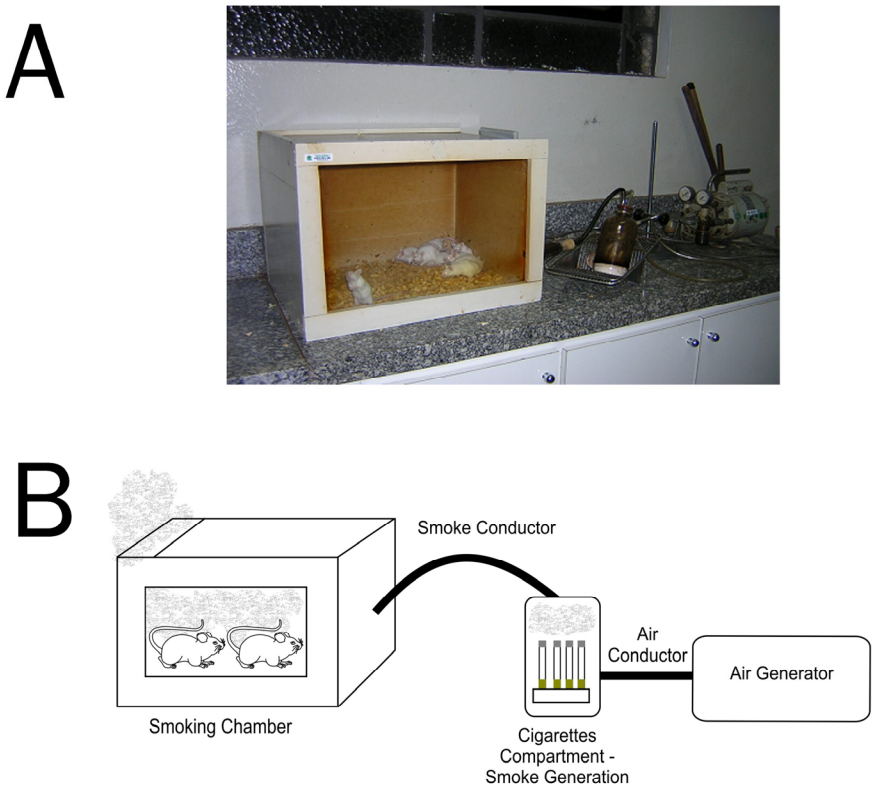

Figure 1 Photograph (**A**) and graphic description (**B**) of the experimental system. The cigarettes were lightened in a glass and left to glow in the compartment; the produced smoke was drawn into the chamber where the mice were placed only for the exposure.

cigarettes for 10 minutes twice a day, leading to 12 cigarettes in 20 minutes.

Exposure System

The sidestream smoke was generated by burning cigarettes Marlboro™ (Philip Morris) in a smoking chamber. The cigarettes were lightened in a glass and left to glow in the compartment and the produced smoke was drawn into the chamber where the mice were placed only for the exposure. The chamber atmosphere was monitored for carbon monoxide (CO). A graphic description and a picture of the experimental system are shown in **Figure 1**.

Mice in all three experimental groups were exposed whole body to the sidestream cigarette smoke once or twice a day, five days a week for 16 weeks. The animals were treated in accordance with the institutional and governmental guiding principles in the use of animals, and the study was approved by the Ethics in Animal Research Committee of the Institution.

Pathological Studies

After 16 weeks of the exposure protocol, the animals were anaesthetized with ether and sacrificed by sectioning the caudal aorta. The lungs were carefully dissected and fixed in 10% buffered formalin and embedded in paraffin. The abdominal organs were analyzed macroscopically, an in case of any alteration, they were also studied histologically. After routine processing, 5 µm thick sections were cut, each slide containing the whole cross-sectional area of the lungs and mediastinal lymph nodes of each mouse, were stained with hematoxilin-eosin. The entire lungs were available to histological analysis.

The lesions were submitted to histopathological analysis and classified as hyperplasia and nodules (**Figure 2**). We preferred to use this classification as in

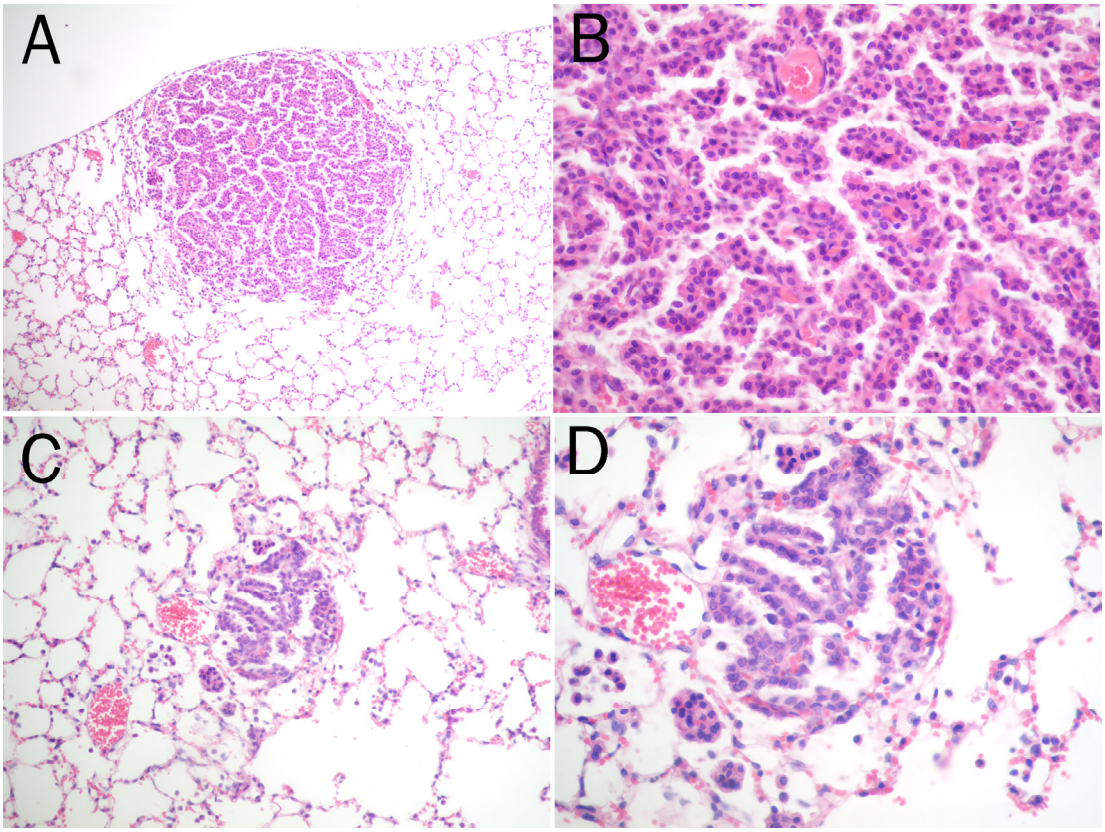

Figure 2 Photomicrograph of a representative lung nodule (**A** and **B**, H&E stain, 100x and 400x, respectively) and hyperplasia (**C** and **D**, H&E stain, 100x and 400x, respectively).

previous works [16, 18] rather than to classify in adenoma or cancer, as we did not see any metastases in this model to confirm the cancer diagnosis, although the histological characteristics strongly suggest that the lesions found are adenomas, as there was not much atypia in the nodules. The criterion for classification as hyperplasia was the presence of alveolar cell proliferation without obliteration of more than four intra-alveolar spaces; nodule was designed as proliferation with obliteration of more than four intra-alveolar spaces. All the lesions from both lungs were counted.

Statistical analysis was performed used the non-parametric method Mood Test. Statistical significance was tested and the minimum criterion was set at a value of p=0.05 for all comparisons.

Results

The mice tolerated the tobacco smoke exposure well, and no exposure-related deaths were observed. The body weight during the experiment was similar in all groups (data not shown). The mean [CO] was 540 ppm in the exposed groups, i.e., G1, G2 and G3, and was also similar in all those groups. The histological finding of pigmented macrophages proved the cigarette smoke inhalation.

The number of nodules and hyperplasia were counted in the histological sections. The statistical analysis showed no significant difference when comparing the median number of nodules and hyperplasia among the studied groups. There were no significant macro- or microscopic findings in other organs. **Figure 3** is a boxplot of nodules per groups.

Discussion

This study showed that different doses of tobacco exposures of Balb/c strain do not contribute to lung carcinogenesis. Our findings are similar to the few data using Balb/c mice reported in the literature [14], suggesting that this strain may be resistant to lung tumor progression induced by cigarette smoke. It is also interesting to notice the difference of response between Balb/c and A/J mouse strains.

Witschi *et al* examined the plausibility of rodent models for assessing tobacco

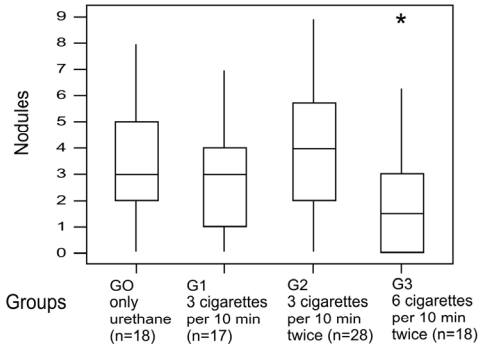

Figure 3 Boxplot presentation of lung nodules in each study group. The figure shows the most extreme values in the data set (maximum and minimum values), the lower and upper quartiles, the median and the outlier (*).

smoke-induced lung tumor formation and found that Balb/c mice exhibited only modest increases in tumor multiplicity and incidence in response to tobacco smoke while A/J mice demonstrated strong results [14]. On the contrary, in a previous study of our group using Balb/c strain, we observed an unexpected statistically significant reduction of the number of pulmonary nodules in mice exposed to the cigarette smoke (data not shown). One possible explanation for this finding is the dose response effect called hormesis [19], but the present study did not support this hypothesis. Witschi *et al* also found an increase in the lung tumor nodules in A/J strain, although not statistically significant [8]. In a later study of lung carcinogenesis in a A/J mouse model induced by ETS using food restriction regimens, Stinn *et al* found a reduction of the urethane-induced lung tumors accompanied by a threefold increase in blood corticosterone, suggesting a stress-induced inhibition of carcinogenesis [20].

A relevant methodological difference among the recent studies that would be considered a bias is that we have not submitted the animals to a recovery period after the exposure period, and the time of exposure. However, De Flora *et al* used the same protocol and failed to reproduce the effects. Another important fact is that our groups had smaller number of animals [21].

It is interesting to notice that in a previous work, Cury *et al* observed an effect of urban air pollution in the same urethane model, but in a

Swiss mice model [16]. The difference of susceptibility between mouse strains and human to tobacco smoke could be explained by molecular mechanisms or by response to stress [22].

Another important factor is the high level of [CO] observed in our study. Hypoxic effect could influence the tumor progression, but the results in the literature are controversial [23].

In conclusion, in mouse lung tumor models, Balb/c mouse appears to have no response to tobacco smoke toxicity, and may be resistant to tumor progression of the lung induced by cigarette smoke.

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