

BASIC RESEARCH

Urban, traffic- related particles and lung tumors in urethane treated mice

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OBJECTIVE: The present study was designed to evaluate the effects of urban, traffic-related, fine particulate matter (PM_{2.5}) on mice lung tumorigenesis under controlled exposure conditions.

METHODS: Four groups of female Swiss mice were treated with intraperitoneal injections of urethane and saline solution. Urethane was used to start the carcinogenesis process. The animals were housed in two chambers receiving filtered and polluted air. In the polluted air chamber, pollutant levels were low. After two months of exposure, the animals were euthanized and lung tumoral nodules were counted.

RESULTS: Saline-treated animals showed no nodules. Urethane-treated animals showed 2.0+2.0 and 4.0+3.0 nodules respectively, in the filtered and non-filtered chambers ($p=0.02$), thus showing experimental evidence of increased carcinogenic-induced lung cancer with increasing PM_{2.5} exposure.

CONCLUSION: Our data support the concept that low levels of PM_{2.5} may increase the risk of developing lung tumors.

KEYWORDS: Air Pollutants; Particulate Matter; Lung Cancer; Lung Nodules; Urethane.

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INTRODUCTION

Chronic diseases of cardiovascular and respiratory systems and cancer are the major causes of death in São Paulo. A study conducted in Brazilian Cities to evaluate cancer mortality rates showed that in São Paulo City deaths due to cancer increased from 35 to 70 per 100,000 inhabitants between 1980 and 2000, and might become the major cause of death in next years.¹

Pollution is implicated in lung cancer development by the presence of mutagens and carcinogens in ambient air, such as mineral fibers and polycyclic aromatic hydrocarbons.² The mechanisms by which air pollution might increase the risk of cancer are related to formation of DNA adducts and oxidative damage.³

The major source of air pollution in São Paulo is vehicular.⁴ Previous studies of our group found difference in cancer rates among districts of São Paulo, showing higher incidence of lung and/or larynx cancer in more polluted districts.⁵ In addition, experimental studies showed that São Paulo "real-world"

urban air pollution has promoter effect on cancer development, and can modify the progression of lung tumors.^{6,7}

Urban particulate matter (PM) was associated with lung cancer mortality rates⁸ and prolonged exposure to fine particulate air pollution was associated with significant increase in lung cancer mortality even after controlling for cigarette smoking, diet, occupational exposure, other individual risk factors, and regional and spatial differences.⁹ Carcinogenic effects of PM can be explained by the mutagenic polycyclic aromatic hydrocarbons (PAHs) adsorbed to its surface, the oxidative damage of the DNA caused by metals and/or benzene, or inflammation induced by particulate matter.^{9,10}

Urethane is a chemical carcinogen used in several studies to induce mice tumors similar to human lung adenocarcinomas.¹¹ Since we have previously showed that chronic exposure to São Paulo air pollution promotes a higher number of lung tumor nodules in urethane-treated mice, here the goal was to evaluate urban, traffic-related, particulate matter ≤ 2.5 micron in aerodynamic diameter (PM_{2.5}) on mice lung tumorigenesis under controlled exposure conditions.

METHODS

The study was conducted in accordance with National and Institutional guidelines for animal welfare. All animals used in the research were treated humanely, with due

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consideration to the alleviation of distress and discomfort. The study was approved by the institutional ethics committee of the São Paulo University Medical School, Brazil.

Local and environmental conditions

Exposures were performed using two Open-top Chambers installed at the campus of the Medical School.

The exposure site was located 20 meters away from the roadside, 160 meters from a monitoring station of the State of São Paulo Sanitation Agency (CETESB), and 150 meters from a busy traffic intersection in downtown São Paulo. At this intersection, it is estimated that approximately 83,941 cars, 9,936 diesel vehicles, and 6,321 motorcycles circulate daily on the main street, and 25,590 cars, 5,299 diesel vehicles and 808 motorcycles circulate on the lateral street of the crossing. There are no industries or significant biomass burning sources on the surroundings.^{12,13}

Previous characterization of PM_{2.5} mass collected at the monitoring station and from the roof of the Medical School has shown that approximately 67% of the PM_{2.5} mass is derived from vehicular sources, with a black carbon/organic carbon ratio ranging between 40-70% throughout the day.¹²⁻¹⁴

Exposure Chambers

The exposure system was described in detail elsewhere.¹² Briefly, the side-by-side chambers are cylindrical aluminum frames measuring 1.5 meters of diameter and 2.5 meters of height, and covered with a plastic UV film. Airflow is forced at 50 L/min from the base of the cylinder, is uniformly distributed throughout the chamber, and exits at the top through a wide opening. This is a normobaric system where the pressure inside the chambers does not exceed the atmospheric pressure by more than 3 cm H₂O. In one of these chambers, here named as filtered chamber, a three-stage filtering system was designed to trap large particles (stage 1, plain and bag filters) and fine particles (stage 2: model JFL-90), and stage 3: (High Efficiency Particulate Air). The other chamber, here named as non-filtered chamber, was equipped only with the stage 1.¹²⁻¹⁴ Filters were purchased from Purafil (São Paulo, SP, Brazil).

Exposure assessment

The 24-hour concentration of PM_{2.5} was gravimetrically determined using Harvard impactors (Air Diagnostics, Harrison, ME) at a flow rate of 10 L/min. Impactors were equipped with polycarbonate filters and results were expressed in µg/m³.

Hourly concentrations of CO (Gas Filter Correlation, 48 series, Thermo Scientific, Waltham, Massachusetts), PM₁₀ (PM ≤10 µm) (FH62 I-n Beta Attenuation Monitor, Graseby Andersen, Smyrna, GA), NO₂ (Chemiluminescence, 42 Series, Thermo Scientific, Waltham, Massachusetts) and SO₂ (Pulsed Fluorescence, 43 Series, Thermo Scientific, Waltham, Massachusetts) were obtained from the monitoring station of CETESB located nearby, as described above. Temperature and relative humidity (inside chambers and outdoor) were daily recorded.

Experimental Animals

One hundred six-week-old female Swiss mice were randomly housed in the chambers for 60 days. Animals

received the same balanced food (Nuvital-Nutrients Ltda, Colombo, Brazil), water *ad libitum*, and were kept at the same ambient conditions of temperature, humidity and noise. Half of the animals (urethane group) received the carcinogen divided in two doses of 1 g/Kg i.p. with 48-hour interval, being the second dose immediately before the housing in the exposure chambers. The other half received saline solution i.p. with the same time interval.

Thus, four groups of 25 animals were formed: urethane and saline-treated mice were housed in the non-filtered chamber (UNF and SNF, respectively), and urethane and saline-treated mice were housed in the filtered chamber (UF and SF, respectively). At the end of the exposure, the animals were deeply anesthetized with pentobarbital 25mg/Kg and euthanized by exsanguination of the abdominal aorta. Lungs were fixed by intratracheal instillation of buffered formalin solution at constant pressure of 20 cm of H₂O for 24 hours.

Lung nodules present at the pleural surface were counted by 3 independent observers who were unaware of the animal groups. A tissue section was done in the fixed lungs of urethane-treated animals and stained with Hematoxylin and Eosin.

Statistical analysis

Statistical analysis was performed using SPSS version 13. Data are presented as means and standard deviations (SD) for each group. To compare number of nodules between groups we applied Student's t-test for Independent samples. One-way ANOVA followed by the Bonferroni post-hoc test were applied for comparison of lung weights. The level of significance was set at 5%.

RESULTS

During the course of this study, both chambers were subjected to equal conditions of temperature and relative humidity. The mean outdoor concentrations of pollutants recorded by CETESB station was of 24.50 µg/m³ for PM₁₀ (24-hour mean), 1.93 ppm of CO (8-hour mean), 116.72 µg/m³ of NO₂ (1-hour mean), and 14.47 µg/m³ of SO₂ (24-hour mean). Gaseous pollutants could not be retained by the filtering systems, thus the concentrations of CO, NO₂, and SO₂ were assumed to be similar in both chambers. The World Health Organization (WHO) recommends that the annual average ambient PM_{2.5} levels be <10 µg/m³, NO₂:<200 µg/m³ 1-hour mean and SO₂ <20 µg/m³ 24-hour.¹⁵ The CETESB recommends that the levels of CO should not exceed 9 ppm by 8-hour mean (C ONAMA).¹⁶

PM_{2.5} mean values were 4.54 µg/m³ and 17.66 µg/m³, respectively in the filtered and non-filtered chambers (p<0.001). Therefore, the 2-month levels obtained in this experiment in the non-filtered chamber are above the recommended by the WHO. As expected, levels in the filtered chamber were far below this number.

Saline-treated animals did not present lung nodules, irrespective of which chamber the animals were hosted in. In urethane treated animals, nodules were randomly located in the lungs, having a white color. Histological samples revealed that the nodules could be classified as adenomas (Figure 1). The number of nodules counted in urethane-treated animals was significantly larger in the non-filtered chamber (UNF 4.0 +/- 3.0 s) in comparison with the filtered chamber (UF 2.0 +/- 2.0) p=0.02 (Figure 2).

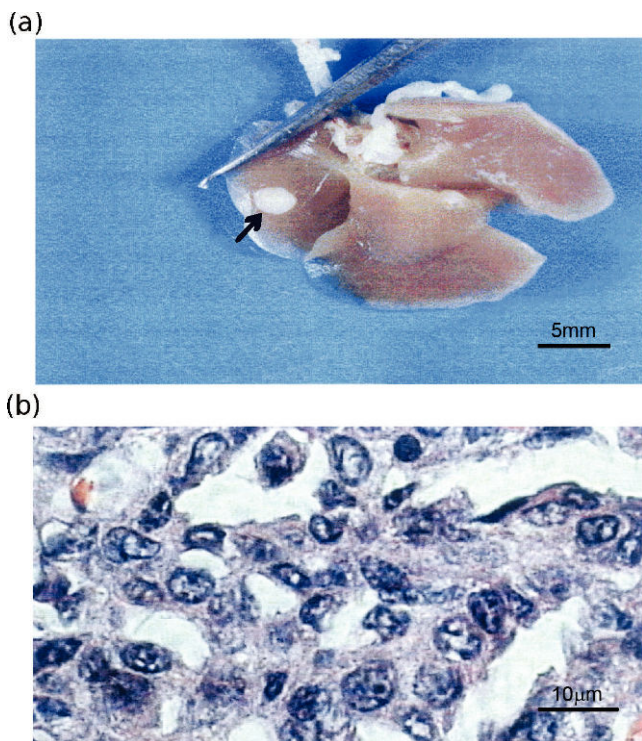


Figure 1 - (a) Photograph of lung obtained from urethane-treated animals showing nodule formation; (b) Photomicrograph of tissue section obtained from fixed lungs of urethane-treated animals and stained with Hematoxylin and Eosin showing atypical adenoma. x 1000.

DISCUSSION

The results suggest that increased exposure to PM2.5 may increase carcinogenic-induced lung cancer. As such, mice treated with urethane and exposed to a higher concentration of urban, traffic- related PM2.5 during a 2-month period developed more lung nodules than those exposed to lower

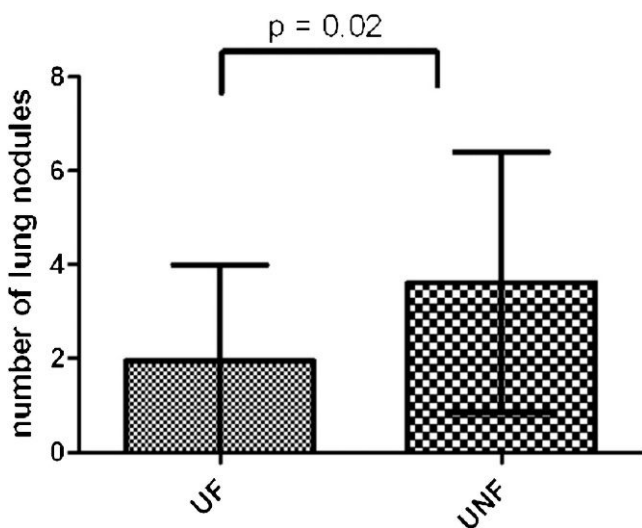


Figure 2 - Graph showing number of nodules counted in urethane-treated animals which was significantly larger in the non-filtered chamber (UNF) in comparison with the filtered chamber (UF) (p=0.02).

concentration. Although there is epidemiological evidence of an association between lung cancer incidence and air pollution in São Paulo,⁵ there are few experimental studies exploring the association of urban air pollution and lung tumorigenesis. It was previously shown that chronic exposure to ambient air pollution enhanced the formation of adenomas in urethane treated mice, with changes in phenotype towards malignancy,^{6,7} while the present study shows association between ambient levels of urban, traffic-related PM2.5 and lung nodules development.

Researchers found that PM greatly inhibits nucleotide excision repair for ultraviolet (UV) light and benzo(a)pyrene diol epoxide-induced DNA damage in human lung cells. They further demonstrated that PM exposure could significantly increase both spontaneous and UV-induced mutagenesis. These results together suggest that the carcinogenicity of PM may act through its combined effect on suppression of DNA repair and enhancement of DNA replication errors.¹⁷ In addition, extractable organic matter induced cell morphological transformation and played a role in tumor-initiating carcinogenesis.¹⁸

Urethane is a rapid model of lung carcinogenesis, since the formation of nodules occurs within two months.⁵⁻⁷ Several lines of evidence indicated that ethyl carbamate (urethane) induction of lung tumor formation involves activation of NF-κB, a major effector of inflammatory responses.¹⁹

It is possible that the mechanism by which urban, traffic-related PM promoting lung tumorigenesis in this study is also dependent on the activation of inflammatory pathways. Diesel exhaust exposure has been shown to induce a pronounced inflammatory response in the airways, together with an enhanced epithelial expression of cytokines such as interleukin (IL)-8, Gro-α, IL-13 and activation of redox sensitive transcription factors (NF-κB, AP-1), and MAP kinases idem (p38, JNK)²⁰. Biopsies from 15 healthy subjects exposed to diesel exhaust showed enhanced epidermal growth factor receptor (EGFR) expression and phosphorylation of the tyrosine residue (Tyr 1173) which is in accordance with the previously demonstrated activation of the JNK, AP-1, p38 MAPK and NF-κB pathways and associated downstream signaling and cytokine production. These findings suggest that exposure to diesel exhaust can activate *in vivo* redox-sensitive transcription factors, which is consistent with oxidative stress triggering the increased synthesis of pro-inflammatory cytokines.²⁰

It is important to note that this study reports tumorigenic effects of ambient concentrations of air pollution, in levels that are similar to those observed in several cities across the globe. Indeed, the study of Pope⁹ disclosed an increased risk of lung cancer due to long term exposure to PM2.5 at concentrations of 10 µg/m³ and beyond, without any evidence of a safety threshold. Our data support the concept that low levels of PM2.5 may increase the risk of developing lung tumors. Although we have not studied the specific components of the PM involved in tumorigenesis or the mechanisms related to this process in urethane-induced tumors, it is the first time that the specific influence of PM2.5 obtained from São Paulo’s air pollution was related to tumor promotion using a very simple experimental model assessing the relationship between lung cancer and air pollution.

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