

# Acute Effects of Inhalable Particles on the Frog Palate Mucociliary Epithelium

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This work was designed to evaluate the toxicity of inhalable particles  $\leq 10 \mu\text{m}$  in aerodynamic diameter ( $\text{PM}_{10}$ ) collected from the urban air in São Paulo, Brazil, to the mucociliary apparatus using the frog palate preparation. Seven groups of frog palates were immersed in different concentrations of  $\text{PM}_{10}$  diluted in Ringer's solution during 120 min: 0 (control,  $n = 31$ ); 50 ( $n = 10$ ); 100 ( $n = 9$ ); 500 ( $n = 28$ ); 1,000 ( $n = 10$ ); 5,000 ( $n = 11$ ); and 10,000  $\mu\text{g}/\text{m}^3$  ( $n = 10$ ). Mucociliary transport and transepithelial potential difference were determined at 0, 30, 60, and 120 min exposure. Additional groups (control and 500  $\mu\text{g}/\text{m}^3$ ) were studied by means of morphometric analyses (quantification of the amount of intraepithelial and surface mucins), measurement of cilia beat frequency, and quantification of total glutathione. Mucociliary transport and transepithelial potential difference were significantly decreased at higher concentrations of  $\text{PM}_{10}$  ( $p = 0.03$  and  $p = 0.02$ , respectively). Exposure to  $\text{PM}_{10}$  also elicited a significant decrease of total glutathione ( $p = 0.003$ ) and depletion of neutral intraepithelial mucins ( $p = 0.0461$ ). These results show that  $\text{PM}_{10}$  can promote significant alterations in ciliated epithelium *in vitro*. **Key words:** air pollution, cilia beat frequency, glutathione, mucociliary transport, morphometric analyses. *Environ Health Perspect* 107:829–833 (1999). [Online 9 September 1999] <http://ehpnet1.niehs.nih.gov/docs/1999/107p829-833macchione/abstract.html>

Acute nonepisodic variations of air pollution have been consistently associated with adverse health effects both in terms of mortality (1–4) and morbidity (5–8). The epidemiologic association between air pollution and health has been mostly described in terms of respiratory diseases. The air pollution–health relationship presents only a short time lag and does not seem to exhibit evidence of a safe threshold. In other words, adverse health effects are observed at concentrations below the air quality standards and are detectable a few days after the pollution increases (1). The pollutant most robustly associated with health impairment is the inhalable fraction of particles  $\leq 10 \mu\text{m}$  in aerodynamic diameter ( $\text{PM}_{10}$ ) that are generated by many processes, including industrial emissions (9,10), the transportation sector, soil dust, and organic emissions from cars and buses (3,4).

Mucociliary clearance is the main pulmonary defense mechanism against noxious inhaled agents (11–13). This system is based on the continuous transport of the airway mucus to the oropharynx by the coordinated beating of the ciliated cells. Considering the high degree of interaction between particles deposited on the airway epithelium and mucociliary clearance, it is logical to propose that abnormalities of mucociliary transport may play a role in the pathogenesis of  $\text{PM}_{10}$ -dependent pulmonary injury. In this context, studies focusing on the acute effects of low

levels of  $\text{PM}_{10}$  on mucociliary transport are necessary to provide biologic plausibility to the epidemiologic work relating air pollution to respiratory diseases.

In this study we performed a series of experiments and constructed dose–response curves to verify the effects of the soluble fraction of urban  $\text{PM}_{10}$  on different properties of the mucociliary system. We used the isolated frog palate preparation that presents a surface similar to human respiratory epithelium. The frog palate system allows the direct observation of mucociliary transport (MT), cilia beat frequency (CBF), and measurements of transepithelial potential difference (PD) across the epithelium. We also investigated the oxidative properties of urban  $\text{PM}_{10}$  on the mucociliary epithelium by measuring the concentrations of antioxidant total glutathione (GSH) and antioxidant response to  $\text{PM}_{10}$ . Last, we monitored the effects of  $\text{PM}_{10}$  on epithelial mucus extrusion by morphometric analysis.

## Materials and Methods

**Sampling campaign.** Inhalable particles ( $\text{PM}_{10}$ ) were collected at a downtown site in the urban area of São Paulo, Brazil, at the School of Medicine, University of São Paulo. The inhalable particle sampler was approximately 20 m from the main roads to minimize the contribution of resuspended soil dust from nearby streets. Sampling was performed during a 2-year period (October

1995–October 1997). The sampler was a Gent stacked filter unit (Nuclepore, Pleasanton, CA) (14,15) fitted with a specially designed inlet that provided a 50% cutoff diameter of 10  $\mu\text{m}$ . Aerosol particles were sampled with Teflon filter (Millipore, Bedford, MA) (14,15) fitted with a specially designed filters. The filters were weighed before and after collection and immediately used; they were agitated for 30 min in Ringer's solution. The difference in weight and the volume of air was used to estimate the quantity of  $\text{PM}_{10}$  in the sampled air accumulated in the filter.

**Analytical methodology.** The elemental concentration was measured with particle-induced x-ray emission (PIXE) (16,17). The elemental analysis was carried out on samples collected during winter 1994 as a preliminary approach to determine the toxic profile of the particle mixture we would use in the toxicologic experiment. A dedicated nuclear accelerator facility at the University of São Paulo, the LAMFI, was used for the PIXE analysis. A proton beam with energy of 2.4 MeV and a beam current of 30–50 nA was used; irradiation times were 600 sec. Detection limits are typically  $< 0.5 \text{ ng}/\text{m}^3$  for approximately 22 elements measured.

**Exposure protocol.** For the studies of MT rate, seven groups of frog palates were exposed and followed for 120 min after immersion in  $\text{PM}_{10}$  concentrations as follows: Ringer's solution containing 0 (control,  $n = 31$ ); 50 ( $n = 10$ ); 100 ( $n = 9$ ); 500 ( $n = 28$ ); 1,000 ( $n = 10$ ); 5,000 ( $n = 11$ ); and 10,000  $\mu\text{g}/\text{m}^3$  ( $n = 10$ ). The previously mentioned concentrations refer to micrograms of particles diluted in cubic meters of

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saline. Although it is not possible to extrapolate any given concentration made in a liquid system to air, we aimed to work with low concentrations of PM<sub>10</sub> on the same order of magnitude to that observed in the urban environment. Additional studies were performed in palates exposed either to 0 ( $n = 13$ ) or to 500  $\mu\text{g}/\text{m}^3$  ( $n = 16$ ) for measuring CBF. Morphometric analyses were carried out for control ( $n = 31$ ) and palates exposed to 500  $\mu\text{g}/\text{m}^3$  ( $n = 28$ ). In addition, GSH levels were determined in another group of frog palates exposed either to 0 ( $n = 6$ ) or to 500  $\mu\text{g}/\text{m}^3$  ( $n = 6$ ) of PM<sub>10</sub>. The equivalent particulate concentration could have a standard deviation (SD) of up to 20%.

**Frog palate preparation.** The frog palate is a simple and efficient preparation to study mucociliary function. The palate is lined with a pseudostratified epithelium composed of both ciliated and mucus-secreting cells, which is similar to the epithelium human conductive airways (12). Mature frogs (*Rana catesbiana*) weighing approximately 100 g were obtained from Ibiúna city vivarium. In our laboratory the animals received a balanced diet and water *ad libitum*, according to routine veterinary procedures, until their sacrifice.

The frogs were pitched, and their spinal cord was sectioned by bending the head forward and inserting a needle into the brain and then down to the spinal cord. The jaw was disarticulated and the upper portion of the head was removed by cutting it with scissors from the junction of the posterior pharynx and esophagus out to the skin of the back. The frog palate was kept in a refrigerator at 4°C for 2 days. It was covered with plastic wrap in a humidified chamber to allow for natural depletion of the palate mucus. On day 3 mucus samples were collected from the posterior edge of the palate with a needle. The samples were immediately immersed in mineral oil to prevent dehydration. All of the experiments were performed on day 3 after frog palate removal. Under these experimental conditions the mucus layer is depleted but the ciliary activity is maintained (18).

**Mucociliary transportability.** Mucus transportability by ciliary beating was evaluated using the *in vitro* frog palate preparation. The MT of autologous mucus samples placed on a mucus-depleted frog palate was determined with the aid of a stereoscopic microscope equipped with a reticulated eyepiece. MT was calculated by dividing the distance traveled (6 mm) by elapsed time (in minutes). At least five measurements were made for each dose and time of the study. The mucus samples were rinsed with petroleum ether to remove the oil prior to their placement on the surface of the palate. The experiments were carried out at room temperature (20°C).

During the measurements the frog palate was maintained inside an acrylic chamber, with a microenvironment of 100% humidity provided by ultrasonic nebulization of standard Ringer's solution mixed with distilled water (1:1) (12,19).

**Transepithelial electric potential difference.** The technique previously described by our laboratory (20) was used as follows. The PD was measured using two flexible agar bridges saturated with potassium chloride (21) and connected to calomel half-cells, one for reference and other for test, both connected to the high input of a grounded electrometer. Because of the saturated agar bridges, a correction for diffusion potential was not necessary when measuring at different ion concentrations (22). The reference electrode was placed in the submucosa of the frog palate and the test electrode on the epithelial surface at the intersection of the cephalad region. Thus, PD measurements were made well away from the cut edge of the palate to minimize possible artifacts due to electrical leaks.

**Cilia beat frequency.** CBF was measured by a modification of the videoscopic technique described by Braga (23) by focusing on a group of cilia through an optical microscope (10 × objective, 10 × ocular) connected to a video camera. The incident light illuminating the ciliated epithelium is reflected from the cilia packed together and from the thin layer of mucus covering the cilia. This reflection is cyclic; its direction changes according to movements of the underlying cilia. At a certain angle during their stroke, the cilia reflect the incident light into the microscope, and the area of that group of cilia appears as a light spot. A fraction of a second later, the cilia change their angle, the light falls outside the frontal lens of the objective, and this area appears dark. These rhythmic changes of flaring and fading intensity are assumed to represent ciliary activity, and each cycle of light fluctuation corresponds to a beat cycle. The observations using reflected light lead to the measurement of ciliary wave frequency, which is the same as CBF, because it was observed at any fixed point on the surface; the surface rises and falls at the frequency of the ciliary beat. The other parameter associated with the ciliary wave is the ciliary wavelength, which is a function of the degree of metachronism between neighboring cilia. The activity of the cilia was recorded on a videotape recorder capable of slow-motion recording and playback. The CBF was recorded at three locations per test area, then counted directly from the screen at slow motion, from which the real frequency is calculated (24).

**Morphometric analyses.** After frog palates were exposed to either 0 or approximately 500  $\mu\text{g}/\text{m}^3$  PM<sub>10</sub> in Ringer's solution for 120

min, the palates were fixed in buffered 4% formalin solution and processed according to routine histologic procedures for paraffin embedding and cutting. Transversally oriented 5- $\mu\text{m}$  thick slides were taken and stained with the combination of Schiff's periodic acid and Alcian Blue at a pH of 2.5. With this technique, neutral and acidic glycoproteins are stained in red and blue, respectively (25). Quantitative morphologic evaluation of frog palate epithelium was done by means of a point-counting technique (26). In the present investigation, a graticule of 100 points attached to the eyepiece of an optical microscope was used, and 400 × magnification was selected. For each slide, 4,000 points were counted in 10 randomly selected microscopic fields. This number was large enough to keep the coefficient of error (SE/mean) under 10%. The parameters evaluated were acid mucus (AM) intraepithelial volumetric fraction ( $F_v$ ), neutral mucus (NM) intraepithelial  $F_v$ , and the amount of mucus deposited on the mucociliary epithelium surface (MDS). Results were expressed as square micrometers per micrometer.

**GSH assay.** After exposure to PM<sub>10</sub> or Ringer's solution, the mucociliary epithelium was removed from the frog palate, cut into small pieces, and added to 2 M perchloric acid with 4 mM EDTA (1:4 weight/vol). This mixture was homogenized and centrifuged at 3,000 ×  $g$  for 5 min to remove protein (27). An aliquot of the deproteinized, acid-soluble extract was neutralized with a solution of 2 M KOH, 0.3 M MOPS, centrifuged, and the supernatant was assayed for total GSH (GSH + oxidized glutathione) using the technique of Tietze (28). A linear increase in absorbance at 412 nm was followed in a Beckman DU7500 spectrophotometer (Beckman Instruments, Fullerton, CA) after the addition of a 0.1-ml sample to the following reaction media: 0.02 ml 5,5'-dithiobis 2-nitrobenzoic acid (1.5 mg/ml in 0.5% NaHCO<sub>3</sub>), 0.05 ml reduced nicotinamide adenine dinucleotide phosphate (NADPH; 4 mg/ml in 0.5% NaHCO<sub>3</sub>), and 0.12 U glutathione reductase and 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA in a final volume of 1 mL. Data were expressed in mole of GSH equivalents per milligram of tissue.

**Statistical analyses.** The values of the relative change of MT (MT at 30, 60, and 120 min divided by the MT at 0 min, i.e., before the immersion) and absolute change of PD (PD at 30, 60, and 120 min subtracted by the PD at 0) were compared among the experimental groups by means of two-way analysis of variance (ANOVA) for repeated measures, with two factors: time of exposure and concentration of PM<sub>10</sub>. For MT, logarithm transformations were necessary to make

variances homogeneous. After this procedure, Dunnett's method was used to characterize this difference among the different concentrations of PM<sub>10</sub> versus control. The values of the relative change in CBF in respect to baseline were compared between 500 µg/m<sup>3</sup> and Ringer's solution by means of Kruskal-Wallis one-way ANOVA on ranks. The values of the AM, NM, MDS, and total GSH were compared among 500 µg/m<sup>3</sup> and Ringer's solution by means of the *t*-test. The level of significance was set at 5%.

## Results

PM<sub>10</sub> values for the urban area of São Paulo are high. Table 1 shows the average aerosol elemental concentrations for the fine mode particles (particle diameter < 2 µm). Black carbon has a high average concentration value of 7.97 µg/m<sup>3</sup>, showing the relatively high impact of diesel emissions in the São Paulo atmosphere.

Tables 2 and 3 show the mean (± SD) values for MT and PD, respectively, determined for the different concentrations of PM<sub>10</sub>. ANOVA treatment of data showed that MT was significantly decreased in the presence of PM<sub>10</sub> (*p* = 0.03) relative to control after exposure to concentrations of 1,000 µg/m<sup>3</sup> and higher. PD also presented significant changes (*p* = 0.02) at a concentration of 10,000 µg/m<sup>3</sup>. Table 4 shows the mean (± SD) values for the morphometric parameters, AM, NM, and M, and total

**Table 1.** São Paulo aerosol characterization study, 1994: fine mode (particle diameter < 2 µm) elemental concentrations in nanograms per cubic meter.<sup>a</sup>

Variable	Mean	SD	Minimum	Maximum	<i>n</i>
Al	115	69.5	4.48	253	67
Si	175	50.1	104	236	16
S	1,527	1,039	94.9	5,294	82
Cl	35.9	27.0	3.26	132	74
K	530	268	36.3	1,357	82
Ca	91.5	39.4	18.2	217	82
Ti	15.1	8.34	0.93	36.8	82
V	7.25	4.21	0.73	19.2	82
Cr	5.43	3.91	0.25	18.1	68
Mn	21.9	14.7	0.34	68.8	82
Fe	346	159	46.9	887	82
Ni	6.28	4.04	0.33	16.7	82
Cu	15.3	9.96	2.12	52.6	82
Zn	127	104	5.24	530	82
Br	7.77	4.24	1.25	17.4	82
Rb	2.34	1.09	0.76	5.33	34
Sr	1.43	0.47	1.06	2.12	6
Zr	4.84	2.86	1.98	9.37	8
Pb	44.4	35.6	3.16	178	82
FPM	30.9	14.9	3.64	79.8	82
BC	7.97	3.49	1.62	19.3	82

Abbreviations: BC, black carbon concentration (in µg/m<sup>3</sup>); FPM, aerosol fine mode mass concentration (in µg/m<sup>3</sup>); *n*, number of samples in which the variable appears above the analytical detection limit; SD, population standard deviation.

<sup>a</sup>Only values above detection limit were used in calculating average and standard deviation.

GSH determined after frog palate treatment with Ringer's solution and 500 µg/m<sup>3</sup> PM<sub>10</sub> for 120 min. A significant decrease in the content of NM (*p* = 0.0461) and in total GSH (*p* = 0.003) was observed in the frog palates after exposure to PM<sub>10</sub>. Values for CBF determined after exposure to Ringer's solution and 500 µg/m<sup>3</sup> of PM<sub>10</sub> were not different at 0 (6.51 ± 0.90 × 6.69 ± 0.94, Ringer's solution and PM<sub>10</sub>, respectively); 30 (6.11 ± 1.15 × 6.02 ± 0.47); 60 (5.98 ± 1.00 × 5.81 ± 0.54); and 120 min (5.74 ± 0.85 × 5.31 ± 0.84) (*p* = 0.144). When variations of MT and PD are analyzed in relation to both concentration of PM<sub>10</sub> and time of exposure (Figures 1 and 2), a significant correlation was observed for both (MT: *r* = 0.209, *p* = 0.0013; PD: *r* = 0.328, *p* < 0.0001).

## Discussion

Outdoor air pollution has been implicated as a significant cause of both morbidity and mortality in São Paulo (3,4) and in other urban areas (9,10). The adverse effects of air pollution on human health are mostly reflected in respiratory and cardiovascular diseases. Effects can be detected within a short time lag and do not exhibit evidence of a safe threshold. The harmful air pollution levels, especially those of inhalable particles, that have been reported by epidemiologic studies are considerably lower than those considered by the conventional toxicologic approaches (6). Thus, there is a gap of knowledge between epidemiologists and toxicologists. This clearly indicates the necessity of experiments that explore the short-term effects of PM<sub>10</sub> on biologic systems.

We used the frog palate assay as a model to detect functional and structural injuries to the ciliated epithelium because of the similarities between the epithelium of the frog palate surface with the epithelium that lines the respiratory tract of mammals. In addition, the frog palate model is sensitive to injury and allows the determination of different parameters of mucociliary transport (29). Frog palate preparation is an efficient experimental tool to assess the deleterious effects of oxidants on the ciliated epithelium (29).

The results obtained in the present investigation indicate that functional abnormalities of the mucociliary epithelium occur after short-term exposure to urban PM<sub>10</sub>. For some of the measured parameters, the effects were significant for concentrations as low as 500 µg/m<sup>3</sup>. However, it is difficult to extrapolate our exposure protocol to pollutant concentrations measured in urban air. The dosimetry of inhaled particles after dilution in the microenvironment surrounding the respiratory epithelium *in vivo* does not correspond to the concentration obtained by diluting particles in Ringer's solution. In addition, the diluting protocol used in this investigation shows that we are dealing primarily with the soluble fraction of PM<sub>10</sub>, which was, in the present study, 60% of the total mass diluted. Thus, our results should be interpreted with caution because we cannot directly extrapolate the concentrations used in our study to those measured in the aerial environment. Most of the studies focusing on *in vitro* toxicity of urban particles have difficulties in simulating the proper environmental conditions faced by the airway

**Table 2.** MT after exposure to different concentrations of PM<sub>10</sub> for different periods.

Concentration	MT (mm/sec) <sup>a</sup>			
	0 min	30 min	60 min	120 min
Ringer's solution	0.30 ± 0.10	0.42 ± 0.11	0.40 ± 0.10	0.38 ± 0.11
50 µg/m <sup>3</sup>	0.27 ± 0.10	0.32 ± 0.07	0.29 ± 0.05	0.33 ± 0.06
100 µg/m <sup>3</sup>	0.26 ± 0.10	0.32 ± 0.06	0.36 ± 0.08	0.37 ± 0.06
500 µg/m <sup>3</sup>	0.39 ± 0.14	0.45 ± 0.09	0.46 ± 0.13	0.42 ± 0.10
1,000 µg/m <sup>3</sup>	0.40 ± 0.09	0.42 ± 0.08	0.42 ± 0.06	0.36 ± 0.06
5,000 µg/m <sup>3</sup>	0.44 ± 0.14	0.44 ± 0.11	0.43 ± 0.10	0.38 ± 0.10
10,000 µg/m <sup>3</sup>	0.34 ± 0.08	0.37 ± 0.10	0.34 ± 0.07	0.31 ± 0.09

Abbreviations: MT, mucociliary transport; PM<sub>10</sub>, particulate matter ≤ 10 µm in aerodynamic diameter.

<sup>a</sup>Values are shown as mean ± standard deviation.

**Table 3.** PD after exposure to different concentrations of PM<sub>10</sub> for different periods.

Concentration	PD (mV) <sup>a</sup>			
	0 min	30 min	60 min	120 min
Ringer's solution	-3.13 ± 4.62	-4.68 ± 3.30	-5.00 ± 3.11	-4.23 ± 4.00
50 µg/m <sup>3</sup>	-4.40 ± 2.37	-6.10 ± 3.54	-3.40 ± 3.57	-3.60 ± 3.37
100 µg/m <sup>3</sup>	-0.70 ± 6.64	-2.60 ± 3.97	-1.60 ± 5.0	-2.80 ± 4.27
500 µg/m <sup>3</sup>	-4.50 ± 3.63	-5.21 ± 3.73	-5.46 ± 2.08	-4.75 ± 2.5
1,000 µg/m <sup>3</sup>	-5.10 ± 1.52	-5.60 ± 1.78	-5.00 ± 0.94	-3.40 ± 2.37
5,000 µg/m <sup>3</sup>	-6.63 ± 2.62	-6.27 ± 2.37	-5.09 ± 1.97	-4.36 ± 2.50
10,000 µg/m <sup>3</sup>	-4.50 ± 1.65	-3.50 ± 1.78	-2.80 ± 2.49	-1.40 ± 2.17

Abbreviations: PD, transepithelial potential difference; PM<sub>10</sub>, particulate matter ≤ 10 µm in aerodynamic diameter.

<sup>a</sup>Values are shown as mean ± standard deviation.



epithelium: the complex extraction and concentration procedures generally used to extract the organic fraction adsorbed to the particle surface are not present in living organisms. In the airway milieu, inhaled particles are either dissolved or absorbed into a watery environment. Findings of an effect on ciliated epithelium using a simple dilution procedure and within a short time lag such as in our study seem to be of toxicologic relevance. However, it is difficult to extrapolate our results to the real conditions of air pollution. Previous studies that used rats as biologic indicators demonstrated that animals exposed chronically to São Paulo atmosphere developed secretory cell hyperplasia in the airways and ultrastructural ciliary alterations. Also, the quantitative analysis of morphologic and histologic parameters of the nasal septum showed secretory hypertrophy, combined with a shift toward acidic mucus secretion and ciliary damage (30,31). More recently Souza et al. (32) reported similar findings regarding inflammatory changes in the airways of humans living for at least 5 years in areas with approximately  $90 \mu\text{g}/\text{m}^3$   $\text{PM}_{10}$ . The aforementioned data support the concept that low levels of air pollution impair mucociliary function.

The mechanisms involved in the mucociliary impairment determined by  $\text{PM}_{10}$  are not completely understood. Considering our protocol of extracting material from the filters, we are dealing mostly with the soluble fraction contained in  $\text{PM}_{10}$ , although we cannot rule out the possibility that some insoluble particles are present in our testing solutions. Air pollution particles have been associated with oxidant generation (33). In our study, the decrease in GSH supports the concept that the mechanism involved in the mucociliary impairment is at least in part mediated by oxidative stress. Among all of the elements present in  $\text{PM}_{10}$  (Table 1), the metals are the most likely involved in the generation of oxidative stress. Emission source particles of both natural

and anthropogenic origin can include soluble metal salts and insoluble components that may have the capacity to complex metals at the surface. Metals that exist in more than one stable valence state can participate in electron transfer reaction and subsequently possess a potential to generate oxidants (34,35). Among metals that can assume two stable valence states, the first-row transition metals titanium, vanadium, chromium, manganese, iron, cobalt, nickel, and copper are found in greatest concentrations in both the crust and atmosphere of the earth. Similarly, these metals can be found in significant concentrations in air pollution particles. Kadiiska and co-workers (33) found that the metal compounds responsible for free radical formation associated with exposure to the oil fly ash were contained in the water-soluble fraction of this emission source of air pollution particles.

The black carbon concentration in  $\text{PM}_{10}$  collected in São Paulo represents  $26.1 \pm 9.7\%$  of the fine mode aerosol mass (Table 1), suggesting that the transportation sector gives an important contribution to fine mode aerosol concentration, as previously reported by the Environmental Agency of São Paulo (36). This finding is particularly important when considering the increase in the numbers of cars and trucks in São Paulo in recent decades; this highlights the necessity for the establishment of public policies to control traffic and, therefore, particulate emissions.

Organic compounds may contribute to the burden of metals present in air pollution particulates through complexation because fuel combustion products are among the

organic components in air pollution particles (Table 1). Clearly, the present results encourage the use of our model to verify the contribution of soluble metal components for the pathogenesis of  $\text{PM}_{10}$ -induced mucociliary dysfunction.

In this work, we studied the short-term effects (up to 120-min exposure) of a  $\text{PM}_{10}$  solution on different parameters related to important mechanisms of mucociliary epithelium: ciliary transport, epithelial permeability, mucus discharge, and antioxidant defenses. For some of these studies, the effects of  $\text{PM}_{10}$  were explored across a wide range of concentrations. The basic idea was to investigate the coherence provided by different estimators of toxicity and, thus, to verify whether the adverse effects of  $\text{PM}_{10}$  were consistent enough to be seen using different methodologic approaches.

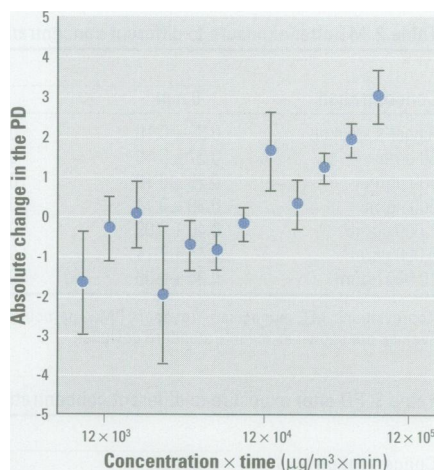
In this work we showed that both MT (Table 2) and PD (Table 3) were impaired by the exposure to high concentrations of  $\text{PM}_{10}$ . Similar effects have been recently observed in the same model exposed to  $\text{H}_2\text{O}_2$  (29). Another way of exploring the results obtained in our study is to plot the changes of MT or PD as a function of the product dose  $\times$  time. The results of this procedure are presented in Figures 1 and 2, and clearly suggest that the effects of  $\text{PM}_{10}$  on ciliary apparatus are dependent not only on concentration but on time as well. MT exhibited a biphasic response for lower doses and time of exposure, increasing in the early phase and decreasing for higher dose  $\times$  time products (Figure 1). This behavior represents the classical response of a ciliated epithelium

**Table 4.** Morphometric parameters and total glutathione of frog palate epithelium after Ringer's solution or approximately  $500 \mu\text{g}/\text{m}^3$   $\text{PM}_{10}$  exposure.

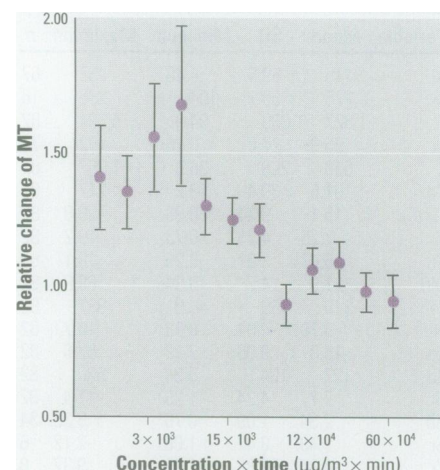
Parameters/ glutathione	Ringer's	$500 \mu\text{g}/\text{m}^3$ $\text{PM}_{10}$
AM ( $\mu\text{m}^2/\mu\text{m}$ )	$4.33 \pm 3.43$	$3.42 \pm 1.84$
NM ( $\mu\text{m}^2/\mu\text{m}$ )	$5.74 \pm 2.08$	$4.57 \pm 1.79^*$
MDS ( $\mu\text{m}^2/\mu\text{m}$ )	$2.13 \pm 2.85$	$1.50 \pm 0.71$
Total glutathione (nmol/g of tissue)	$263.80 \pm 99.94$	$94.97 \pm 30.43^*$

Abbreviations: AM, acid mucus intraepithelial volumetric fraction; MDS, deposited mucus on the mucociliary epithelium surface volumetric fraction; NM, neutral mucus intraepithelial volumetric fraction;  $\text{PM}_{10}$ , particulate matter  $\leq 10 \mu\text{m}$  in aerodynamic diameter.

\* $p \leq 0.05$ .



**Figure 1.** Absolute changes of PD as a function of the product of concentration of  $\text{PM}_{10}$  (in micrograms per cubic meter)  $\times$  time of exposure (in minutes). Abbreviations: PD, transepithelial potential difference;  $\text{PM}_{10}$ , particulate matter  $\leq 10 \mu\text{m}$  in aerodynamic diameter. Each point represents the average of all measurements for each time  $\times$  dose product and bars represent the standard error ( $r = 0.328$ ,  $p < 0.0001$ ).



**Figure 2.** Relative changes of MT as a function of the product of concentration of  $\text{PM}_{10}$  (in micrograms per cubic meter)  $\times$  time of exposure (in minutes). Abbreviations: MT, mucociliary transport;  $\text{PM}_{10}$ , particulate matter  $\leq 10 \mu\text{m}$  in aerodynamic diameter. Each point represents the average of all measurements for each time  $\times$  dose product and bars represent the standard error ( $r = 0.209$ ,  $p = 0.0013$ ).

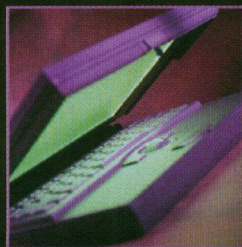
to injury (13,29), characterized by an increase, i.e., an adaptation to injury followed by a significant impairment when aggression becomes more prolonged or severe. Figure 2 shows that there is a progressive shift toward a more positive PD, suggesting that the epithelium becomes more leaky, i.e., that PM<sub>10</sub> may affect the integrity of epithelial tight junctional complexes (29).

Incubation of frog palate with PM<sub>10</sub> elicited a depletion of the GSH levels in mucociliary epithelium (Table 4). These data are indicative of an oxidant capacity of PM<sub>10</sub> particles, suggesting that oxidative mechanisms could be responsible for the observed adverse effects. In our study a depletion of neutral mucins was also observed, which again points to the fact that PM<sub>10</sub> possesses an irritative capacity that elicits mechanisms of epithelial protection. As previously reported (37–39), the epithelium extrudes mucus in response to injury in an attempt to eliminate the aggressor agent. Mucus also has antioxidant capabilities because the sugar moieties in mucus may act similarly to mannitol and glucose in scavenging toxic oxygen metabolites effectively (40–43). Thus, the simultaneous findings of GSH depletion and mucus discharge are compatible with oxidative injury to the ciliated epithelium promoted by PM<sub>10</sub>. In fact, urban PM<sub>10</sub> can elicit free radical activity through hydroxyl radicals generated by iron-dependent mechanisms (44).

In conclusion, our results indicate that in a short period of time, urban aerosol particles are able to induce alterations of functional, biochemical, and morphologic parameters of the ciliated frog palate. It is possible that these results may help to interpret the epidemiologic data relating changes of PM<sub>10</sub> levels to short-term health effects.

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