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RESEARCH ARTICLE

Low dose of fine particulate matter (PM2.5) can induce acute oxidative stress, inflammation and pulmonary impairment in healthy mice

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Abstract
Air pollution is associated with morbidity and mortality induced by respiratory diseases. However, the mechanisms therein involved are not yet fully clarified. Thus, we tested the hypothesis that a single acute exposure to low doses of fine particulate matter (PM2.5) may induce functional and histological lung changes and unchain inflammatory and oxidative stress processes. PM2.5 was collected from the urban area of São Paulo city during 24 h and underwent analysis for elements and polycyclic aromatic hydrocarbon contents. Forty-six male BALB/c mice received intranasal instillation of 30 µL of saline (CTRL) or PM2.5 at 5 or 15 µg in 30 µL of saline (P5 and P15, respectively). Twenty-four hours later, lung mechanics were determined. Lungs were then prepared for histological and biochemical analysis. P15 group showed significantly increased lung impedance and alveolar collapse, as well as lung tissue inflammation, oxidative stress and damage. P5 presented values between CTRL and P15: higher mechanical impedance and inflammation than CTRL, but lower inflammation and oxidative stress than P15. In conclusion, acute exposure to low doses of fine PM induced lung inflammation, oxidative stress and worsened lung impedance and histology in a dose-dependent pattern in mice.
Keywords: Particulate matter; PM2.5; lung injury; oxidative stress; respiratory mechanics

Introduction

Particulate matter (PM) is a heterogeneous mixture of gases, liquid and solid particles of different origins and sizes in suspension in the air, keeping close physical and chemical interaction. PM is classified as coarse (2.5–10 µm aerodynamic diameter, PM10), fine (0.1–2.5 µm aerodynamic diameter, PM2.5), and ultrafine (≤0.1 µm aerodynamic diameter) (Donaldson et al., 2001).

Fine particles remain suspended in the atmosphere for a long time (days and weeks), disperse uniformly, and deposit in the extrathoracic airways or, depending on air flow and diffusion, penetrate deeper into the smaller airways and alveoli, where they can remain for weeks or months. Particles can be eliminated by the mucociliary system in the conductive airways and/or undergo phagocytosis by macrophages, when deposited in the alveolar regions (Donaldson & Stone, 2003). Moreover, components leached from PM2.5 can enter the circulation and modify circulatory homeostasis, thus increasing the risk factors for adverse cardiovascular events, such as arrhythmias, sudden death, increased plasma viscosity, change in blood parameters such as fibrinogen levels or red blood cells counts (Seaton et al., 1999; Stone & Godleski, 1999; Gardner et al., 2000; Schwartz, 2001; Kodavanti et al., 2002).

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Saldiva et al. (1992a) observed that chronic exposure to the air of São Paulo significantly compromises the properties of the mucociliary defense and predisposes experimental animals to respiratory infection. Acutely, increases in PM2.5 concentration are associated with exacerbation of asthmatic bouts and respiratory symptoms, as well as with higher hospital admissions by respiratory causes, such as rhinitis, sinusitis, bronchitis, asthma and pneumonia.

The mechanisms involved in the relationship between PM and adverse health effects are not well understood. Nevertheless, it is believed that an association between inflammatory process and oxidative stress exists (Donaldson & Tran, 2002). PM inhalation or instillation promotes inflammatory responses in animals and humans (Clarke et al., 1999; Ghio & Devlin, 2001) characterized by cytokine release, increased oxidative stress and vascular permeability with concomitant neutrophil recruitment (Li et al., 1996), as well as increased expression of genes related to NF-κB activation, including TNF-α, TGF-β and IL-6 (Shukla et al., 2000).

Considering that exposure to high or even daily allowed doses of PM2.5 can induce pulmonary dysfunction as well as systemic oxidative damage, we tested the hypothesis that acute instillation of low doses of PM2.5 may trigger pulmonary alterations in mice, mainly at the lung periphery. To that end, central airways and lung periphery mechanics and histology as well as inflammatory and biochemical markers were analyzed after exposure to PM2.5.

Materials and methods

PM sampling

PM samples were collected from downtown São Paulo, Brazil, by an impactor sampler located at the intersection of two very busy avenues between August 2005 and April 2006. In this place, 89% of fine particles are generated by traffic (CETESB, 2006). PM was collected during 24 h on polycarbonate filters (cat. no. ATTP03700, Millipore Corporate, Billerica, MA) using a Harvard impactor (Air Diagnostics, Harrison, ME) with a flow of 10 $L \cdot min^{-1}$. The filters were dried (24 h at 50°C) and weighed, before and after PM collection. The weight difference corresponded to the total collected mass. Particles were extracted in distilled water by ultrasonic sonication during 8 h, to a final particle:volume ratio of 1:2 (1 μg:2 μL), based on the total collected mass of PM2.5.

Animals and experimental groups

BALB/c mice were provided by the animal facilities of the Carlos Chagas Filho Institute of Biophysics, Federal University of Rio de Janeiro. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "*Guide for the Care and Use of Laboratory Animals*" prepared by the National Academy of Sciences, USA. The work was carried out in accordance with EC Directive 86/609/EEC. The experiments were approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol IBCCF 046).

Forty-six male BALB/c mice (25–30 g BW) were randomly assigned to three main groups. Control group (CTRL $n = 9$) received an intranasal (i.n.) instillation of 30 μL (3 × 10 μL) of saline (0.9% NaCl). Groups P5 and P15 ($n = 16$ each) received PM as extracted by sonication. The solution concentration was 1 μg:2 μL. Thus, P5 group was instilled with 10 μL of the extract plus two 10-μL doses of saline solution. P15 received three 10-μL doses of the extract. Five animals (FILTER group) were instilled (i.n.) with 30 μL of a solution obtained after the extraction procedure from naive polycarbonate filters, as described earlier. For i.n. instillation, mice were anesthetized with sevoflurane, and saline or suspended particles were gently instilled into their snouts with the aid of a precision pipette. The animals recovered rapidly after instillation.

Particle composition

Elements content (Na, Al, Si, P, S, K, Ca, Ti, V, Fe, Ni, Cu, Zn and Pb) of PM2.5 was analyzed by spectrometry (EDX 700HS, Shimadzu Co, Kyoto, Japan) (Mauad et al., 2008). Polycyclic aromatic hydrocarbons (PAHs) were determined by liquid chromatography (LC-10AS, Shimadzu Co., Kyoto, Japan) with a fluorescence detector (RF-10 AxL, Shimadzu Co., Kyoto, Japan), as previously described (Mazzoli-Rocha et al., 2008). Briefly, PAHs were extracted consecutively with an acetone/n-hexane mixture in an ultrasonic bath (90°C for 20 min). Isooctane (1 mL) was added prior to each PAH extraction step. Then, extracts were filtered, combined, and concentrated (1 mL) by vacuum rotatory evaporation. Reagents were supplied by Tedia Co. (Fairfield, OH).

The combined extracts were passed through a chromatography column filled with 7 g of Al2O3/Na2SO3 deactivated with 11% of water, and n-hexane (20 μL) was used to elute PAHs. Cleaned extracts were concentrated and eluted with 35 mL of n-hexane/ethyl ether (3:1, v/v). The eluents were concentrated and then diluted with 0.5 mL of acetonitrile.

A 20-μL aliquot of the acetonitrile extract was analyzed by liquid chromatography (LC-10AS, Shimadzu Co., Kyoto, Japan) with a fluorescence detector (RF-10 AxL, Shimadzu Co., Kyoto, Japan). The separation was performed on a Shimadzu CLC-ODS II column (180.0 × 4.1 mm i.d.), isocratic run was completed with a mobile phase composed of water:acetonitrile mixture (20:80, v/v), and detection was determined at 255/325, 253/350, 333/390, 287/462, 280/430, 294/404, 300/500, and 300/421 Ex/Em wavelength steps. The following PAHs were analyzed: naphthalene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene (B[a]P), and benzo[ghi]perylene. The software Borwin 1.2 (Jmbs Developments, Le Fontanil, France) was used for the quantification.

Pulmonary mechanics

Twenty-four hours after instillation the animals were anesthetized (diazepam 1 mg and pentobarbital sodium 20 mg·kg·BW^{-1} i.p.), paralyzed (pancuronium bromide 0.1 mg·kg^{-1} i.p.), and ventilated (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) with constant tidal volume (0.2 mL) and flow (1 mL·s^{-1}). A positive end-expiratory pressure (2 cmH_2O) was applied to the expiratory limb of the ventilator, and the anterior chest wall was surgically removed. A pneumotachograph was connected to the tracheal cannula for airflow measurements. Lung volume was determined by digital integration of the flow signal. The pressure gradient across the pneumotachograph was determined by a differential pressure transducer (Validyne MP45-2, Engineering Corp., Northridge, CA). Transpulmonary pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp. Northridge, CA). Signals were amplified (Beckman type R Dynograph, Schiller Park, IL), low-pass filtered (8-pole Bessel filters, 902LPF, Frequency Devices, Haverhill, MA) at 100 Hz, sampled at 200 Hz and analog-to-digital converted (DT2801A, Data Translation, Marlboro, MA). All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung mechanics static elastance (E_{st}), and elastic component of viscoelasticity (ΔE), as well as resistive (ΔP_1), viscoelastic (ΔP_2), and total (ΔP_{tot}) pressures was determined by the end-inflation occlusion method (Bates et al., 1985a, 1988).

Pulmonary histology and immunohistochemistry

Heparin (1000 IU) was intravenously injected immediately after the determination of pulmonary mechanics. The trachea was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. Thereafter, the lungs were removed *en bloc*.

The left lung was fixed by immersion in modified Millonig's buffered formaldehyde solution for 24 h and routinely processed for histological analyses. Four-μm-thick lung slices were stained with hematoxylin and eosin. The analyses were blindly done by two independent researchers. Morphometric analysis was performed with an integrating eyepiece with a coherent system made of a 100-point and 50-line (known length) grid coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The fractional areas of collapsed and normal alveoli were determined by the point-counting technique at a magnification of ×400 across 10 random non-coincident microscopic fields per animal. Points falling on normal or collapsed alveoli were expressed as percentage of the total number of points in the grid (Weibel, 1990).

For the determination of macrophages, neutrophils, IL-6 and TNF-α, the lung sections were deparaffinized and hydrated. After blocking the endogenous peroxidase, antigen retrieval was performed with high temperature citrate buffer (pH = 6.0). The following primary antibodies were used in the study: rat monoclonal antibody anti-mouse F4/80 and anti-mouse neutrophil (1:100, AbD Serotec, Raleigh, NC); goat polyclonal anti-mouse TNF-α and anti-mouse IL-6 (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA). ABC staining system (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as secondary antibody; 3′3 diaminobenzidine (DAB, Sigma, St. Louis, MO) was used as chromogen. The sections were counterstained with hematoxylin. For negative controls, the first antibody was omitted from the procedure and BSA was used instead. Analysis was performed at ×400 magnification on an Olympus microscope (model BH2, Tokyo, Japan). Fifty to sixty fields of 26,000 μm^2 (10 fields/animal in 5–6 animals from each group) were counted by two investigators at different occasions. The investigators counted non-identified sections. Total number of macrophages and neutrophils in air spaces were expressed as total cells per μm^2. The intensity of the parenchymal staining for TNF-α and IL-6 was semi-quantitatively analyzed by means of a visual analog scale (0 = absent; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong) (Lopes et al., 2009).

For the determination of 8-isoprostane, 4-μm-thick lung slices, mounted on silanized slides, were deparaffinized and hydrated. After blocking the endogenous peroxidase, antigen retrieval was performed with trypsin. Polyclonal goat anti-8-epi-PGF2α (1:1200, Oxford Biomedical Research, Oxford, England) was used as primary antibody. The Vectastin ABCKit (Vector Laboratories, Burlingame, CA) was used as secondary antibody; DAB (Sigma, St. Louis, MO) was used as chromogen. The sections were counterstained with hematoxylin. For negative controls, the first antibody was omitted from the procedure and BSA was used instead. The expression of 8-isoprostane was assessed at ×400 magnification. Two different observers performed independent measurements. The intensity of the parenchymal staining for 8-isoprostane was semi-quantitatively analyzed by means of a visual analog scale (0 = absent; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong) (Lopes et al., 2009).

Lung biochemical analysis

The right lung was removed, homogenized (model NT 136, Novatécnica, São Paulo, Brazil) in a buffer solution (potassium phosphate 100 mM + EDTA 5 mM, final solution volume: 1 mL), centrifuged at 7000*g* (model 243M, FANEM, São Paulo, Brazil) for 10 min, and the supernatants were collected for biochemical analysis. Protein concentration was estimated by the Bradford's method (Bradford, 1976).

Myeloperoxidase

Inflammatory changes were examined by myeloperoxidase (MPO) activity in the supernatant of lung homogenates, by using hydrogen peroxide (H_2O_2), hexadecyltrimethylammonium bromide, and 3,3′,5,5′-

tetramethylbenzidine. Absorbances were determined at 655 nm using a plate reader (Model 550, Bio-Rad, Hercules, CA) (Suzuki et al., 1983). MPO activity was expressed in mU/mg protein.

Malondialdehyde assay

As an index of lipid peroxidation, we used the thiobarbituric acid reactive substances (TBARS) method for analyzing malondialdehyde products during an acid-heating reaction (Draper & Hadley, 1990). Briefly, samples from lung homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid. The samples were then heated in a boiling water bath for 30 min. TBARS were determined by absorbance at 532 nm and expressed as malondialdehyde equivalents (nm/mg protein).

Catalase

Catalase (CAT) activity was measured by the rate of decrease in hydrogen peroxide content at 240 nm (Aebi, 1984). Final activity values were corrected by the protein content in each sample and expressed as U/mg protein.

Reduced glutathione/oxidized glutathione

The assay was based on the reaction of glutathione (GSH) or oxidized glutathione (GSSG) with 5,5-dithiobis-(2-nitrobenzoic acid) that produces the 2-nitro-5-thiobenzoate (TNB) chromophore (Rahman et al., 2006). To determine GSSG, samples of lung homogenate were treated with 2-vinylpyridine, which covalently reacts with GSH (but not GSSG). The excess 2-vinylpyridine was neutralized with triethanolamine. The rate of formation of TNB, measured at 412 nm, is proportional to the concentration of GSH or GSSG in the sample. The concentration of an unknown sample was calculated from the linear equation or the regression curve generated from several standards of GSH or GSSG. The final result was presented as GSH/GSSG ratio.

Statistical analysis

SigmaStat 9.0 statistical software package (SYSTAT, Point Richmond, CA) was used. The normal distribution of the data (Kolmogorov–Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were tested. In all instances, both conditions were satisfied, and one-way analysis of variance was used followed by Tukey test for multiple comparisons when needed. The morphometric parameters underwent an arcsine transformation so that a normal distribution resulted. The significance level was always set at 5% ($p < 0.05$).

Results

Analysis of the PM

Table 1 shows element and PAH composition of PM2.5. It can be seen that iron and zinc were the prevalent metals, whereas phenanthrene represents the most abundant PAH.

Pulmonary mechanics

Figure 1 depicts ΔP_1, ΔP_2, ΔP_{tot}, E_{st} and ΔE measured in all groups. ΔP_2, ΔP_{tot}, E_{st} and ΔE were significantly higher in P15 than in CTRL and FILTER. P5 showed significantly larger ΔP_2, ΔP_{tot} and ΔE than CTRL and FILTER. No significant difference was detected between P5 and P15 in all instances, although P15 always tended to show higher values than P5. No functional difference was observed between FILTER and CTRL groups, supporting the absence of any bias due to the extraction process. Therefore, the other parameters were determined only in the three main groups, i.e. CTRL, P5, and P15.

Histopathological analysis

Figure 2 depicts photomicrographs of pulmonary parenchyma in CTRL, P5 and P15 mice. Normal areas in CTRL, P5 and P15 amounted to 91.0 ± 2.7%, 90.4 ± 2.7%, and 86.8 ± 1.8% (SEM) of tissue area, respectively, whereas alveolar collapse was detected in 9.0 ± 2.7%, 9.4 ± 2.5%, and 13.1 ± 1.8% (SEM) of tissue area, respectively. P15 displayed a significantly higher amount of collapsed areas than P5 and CTRL. No difference was observed between CTRL and P5.

Inflammatory signaling

P15 presented significantly higher macrophage and neutrophil content in lung parenchyma than CTRL. Neutrophil influx was higher in P15 than in P5, whereas no difference was observed between CTRL and P5 for both cell types (Figure 3A). The activity of MPO, a marker of neutrophil activation, was higher in P5 and P15 than in CTRL (Figure 3B). P15 also showed significantly

Table 1. Polycyclic aromatic hydrocarbons and elements in PM2.5.

PAH	ng/g	Element	PPM
Naphthalene	798.0	Na	3615 ± 3802
Acenaphthylene	483.9	Al	157 ± 789
Fluorene	233.6	Si	1300 ± 2697
Phenanthrene	3309.9	P	0.23 ± 0.22
Fluoranthene	1157.4	K	1884 ± 4313
Pyrene	127.9	Ca	861 ± 1894
B[a]anthracene	5.0	Ti	242 ± 359
B[b]fluoranthene	9.4	V	99 ± 65
B[k]fluoranthene	1.4	Fe	3595 ± 4342
B[a]pyrene	5.8	Ni	67 ± 69
B[ghi]perylene	0.4	Cu	322 ± 253
		Zn	1620 ± 2164
		Pb	172 ± 225

Concentrations of polycyclic aromatic hydrocarbons (PAHs) quantified using the software Borwin 1.2 by integration of the chromatograms and calculation the concentration of composites. For the identification of composites, the time of retention of each one was compared with the chromatogram of a standard solution. 102 samples were used in the calculation of the concentration of elements (mean ± SD).

higher staining scores for IL-6 and TNF-α in lung tissue than CTRL and P5, whereas no difference was observed between the latter groups (Figure 3C and 3D).

Oxidative damage and stress

P15 showed significantly higher staining scores for 8-isoprostane and TBARS expression than CTRL (Figure 4A and 4B). Both parameters were slightly increased in P5 group, but without significant differences in relation to CTRL or P15. CAT activity increased progressively after PM2.5 exposure, being significantly higher in P15 than in CTRL and P5. GSH/GSSG decreased only in P15 group compared with CTRL and P5 (Figure 4C and 4D).

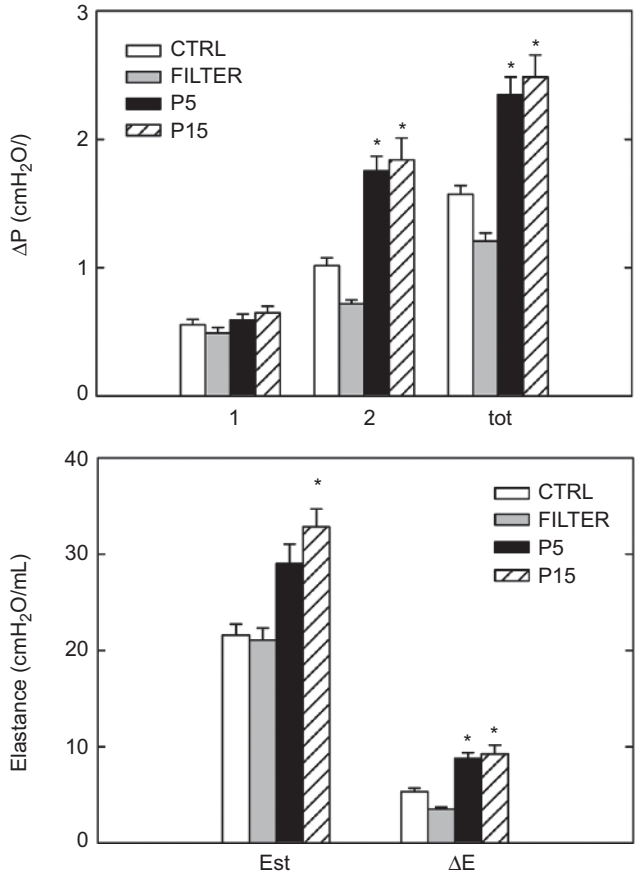

Figure 1. Pressures dissipated to overcome pulmonary viscous (ΔP_1), viscoelastic/inhomogeneous (ΔP_2), the sum of both pressures (ΔP_{tot}), static elastance (Est), and the elastic component of viscoelasticity (ΔE) in groups that did not receive PM2.5 (CTRL, $n=9$, and FILTER, $n=5$) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively, $n=16$ in each). Values are mean + SEM. *Significantly different from CTRL and FILTER groups ($p<0.05$).

Discussion

Our results showed that acute exposure to low doses of urban PM2.5 (5 and 15 μg of PM2.5) impaired lung function, characterized by increased elastic and viscoelastic components of lung mechanics. Low dose exposure to PM2.5 also induced lung inflammation and oxidative damage. Lung inflammation was evidenced by increased MPO activity and neutrophil influx into lung parenchyma, as well as increased expression of proinflammatory cytokines such as TNF-α and IL-6. Oxidative damage was expressed by increased reactive substances to thiobarbituric acid and 8-isoprostane, whereas oxidative stress was characterized by increased expression of CAT and reduction in GSH/GSSG.

The used doses were based on the mean concentration of PM2.5 in São Paulo (23 μg/m³), and considering that on a peak pollution day, this amount increases considerably reaching a four-fold value (CETESB, 2008). Furthermore, it should be noted that only a fraction of the nasally instilled PM2.5 reaches the alveolar region (Sabaitis et al., 1999). Indeed, our doses were lower than those usually found in experimental models of air pollution (Gavett et al., 2003; Rivero et al., 2005; Kooter et al., 2006; Mantecca et al., 2010), hence allowing the observation of pulmonary effects induced by ambientally relevant levels of PM2.5. It is noteworthy that even a single low dose exposure to PM2.5 yielded both functional and histological/biochemical pulmonary changes (Figures 1–4).

We focused our measurements on the responses present at 24 h after exposure, because epidemiological studies indicate a 24-h lag between exposure and respiratory symptoms (Braga et al., 2002). Indeed, previous experimental studies from our group using total suspended particles (Mazzoli-Rocha et al., 2008) and diesel PM (Laks

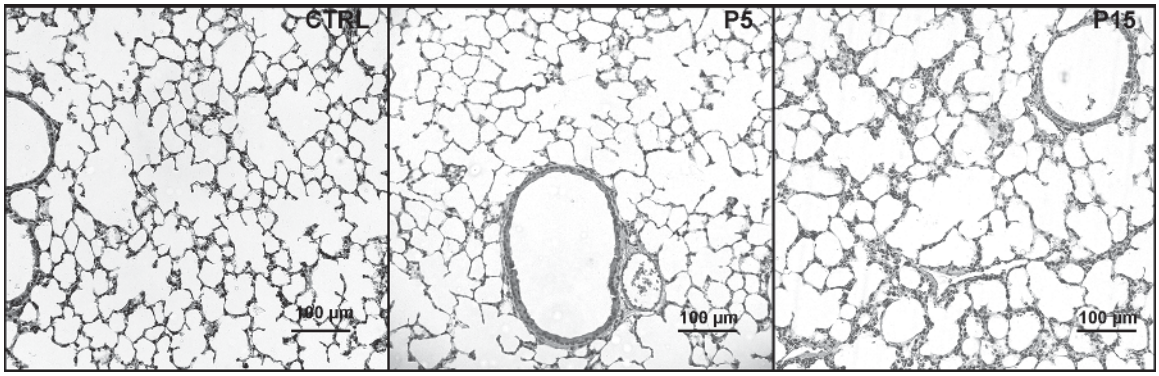

Figure 2. Photomicrographs of pulmonary parenchyma. Representative images from a mouse that did not receive PM2.5 (CTRL) and from those that were instilled with 5 or 15 μg of PM2.5 (P5 and P15, respectively). Original magnification: ×200.

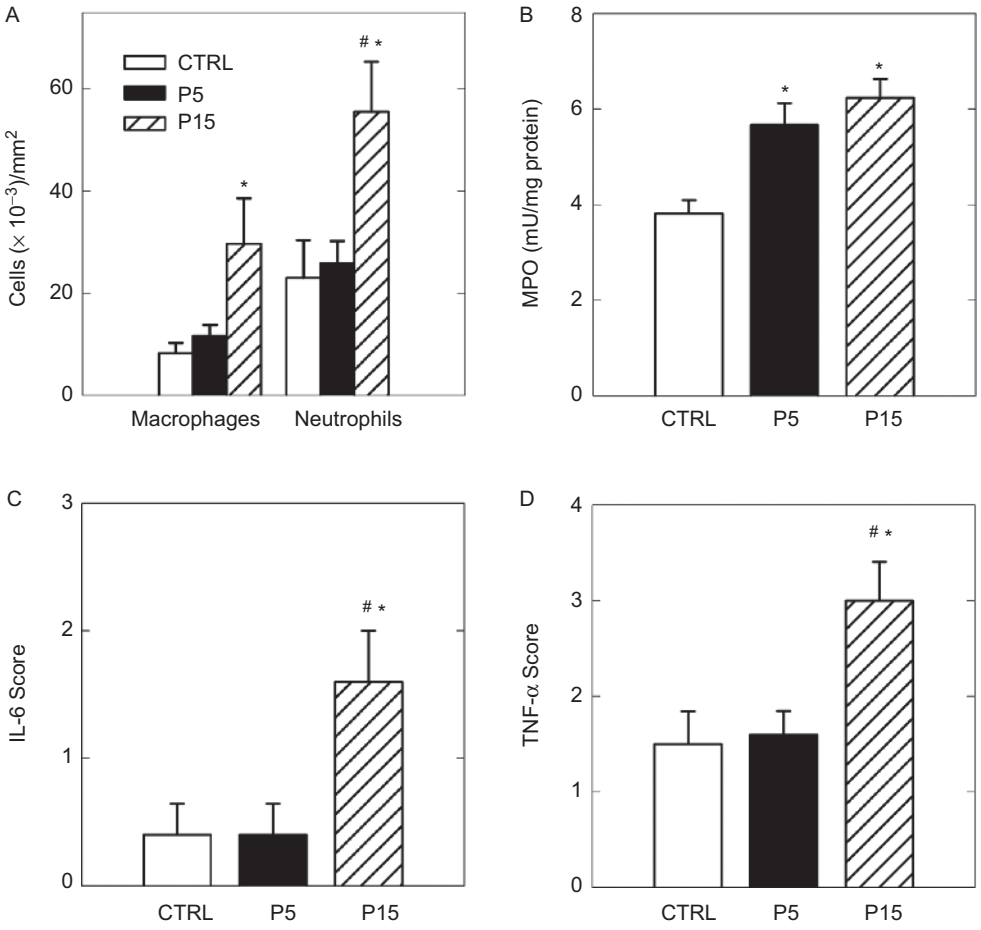

Figure 3. Inflammatory signaling in mice that did not receive PM2.5 (CTRL) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively). In panel A, the amounts of macrophages (mononuclear cells) and neutrophils (polymorphonuclear cells) per area of lung parenchyma in CTRL ($n=6$), P5 ($n=5$) and P15 ($n=5$) groups are shown. Values are mean ± SEM of 5–10 fields per slide. Panel B depicts the activity of myeloperoxidase (MPO) in the samples from lung homogenates of CTRL ($n=5$), P5 ($n=5$) and P15 ($n=5$) groups. Panels 2C and 2D show that tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were higher in the lung tissue of P15 mice ($n=5$), than in P5 ($n=5$) and CTRL ($n=6$) that did differ between them. Values are mean ± SEM of 5–10 fields per slide. *, # indicate values significantly different from CTRL and P5, respectively ($p<0.05$).

et al., 2008) have already demonstrated adverse respiratory effects 24 h after exposure to PM, thus supporting the epidemiological findings.

The suspended PM (extract of sonicated PM2.5) was administered to mice by i.n. instillation. Nasal breathing is characterized by the retention of large particles in the extrathoracic regions of the respiratory system. This model of administration affects the distribution of particles according to their aerodynamic size and, thus, our results may not necessarily reflect the interactions of particles with the respiratory system as if they were inhaled by aerosol. Nevertheless, it is very useful for comparative studies of particle toxicity (Terashima et al., 1997; Laks et al., 2008; Mazzoli-Rocha et al., 2008). It should be stressed that we used PM2.5 suspended in distilled water and, thus, the sonication and dilution processes may have somewhat modified the particle characteristics.

Previous studies disclosed that PMs from different sources are able to induce inflammatory processes triggered by their components (Sørensen et al., 2003; Park et al., 2006). It is also well determined that PM composition varies according to different factors, such as source of air pollution, humidity, and temperature. PM2.5 from the urban area of São Paulo city is mainly produced by traffic and showed high concentration of elements and PAHs with inflammatory characteristics (Table 1). Our results support the concept that ambient particles induce acute respiratory inflammation and oxidative damage (Figures 3 and 4).

The variety of pollutants found in ambient air and their capacity to interact synergistically render difficult the establishment of a cause-effect relationship between a specific compound and the observed toxicity of a given air sample. Previous studies show that the metal composition of the PM can generate pulmonary injury (Prahalad et al., 2001). The most commonly found elements in airborne PM are Cr, Co, Ni, Mn, Zn, Cu, and, mainly, Fe (Billet et al., 2007). The latter appears in higher concentrations in particles generated by fossil fuel burning than in PMs from other origins (Park et al., 2006). In this context, sodium and iron were the most abundant elements present in the PM2.5 used in this study (Table

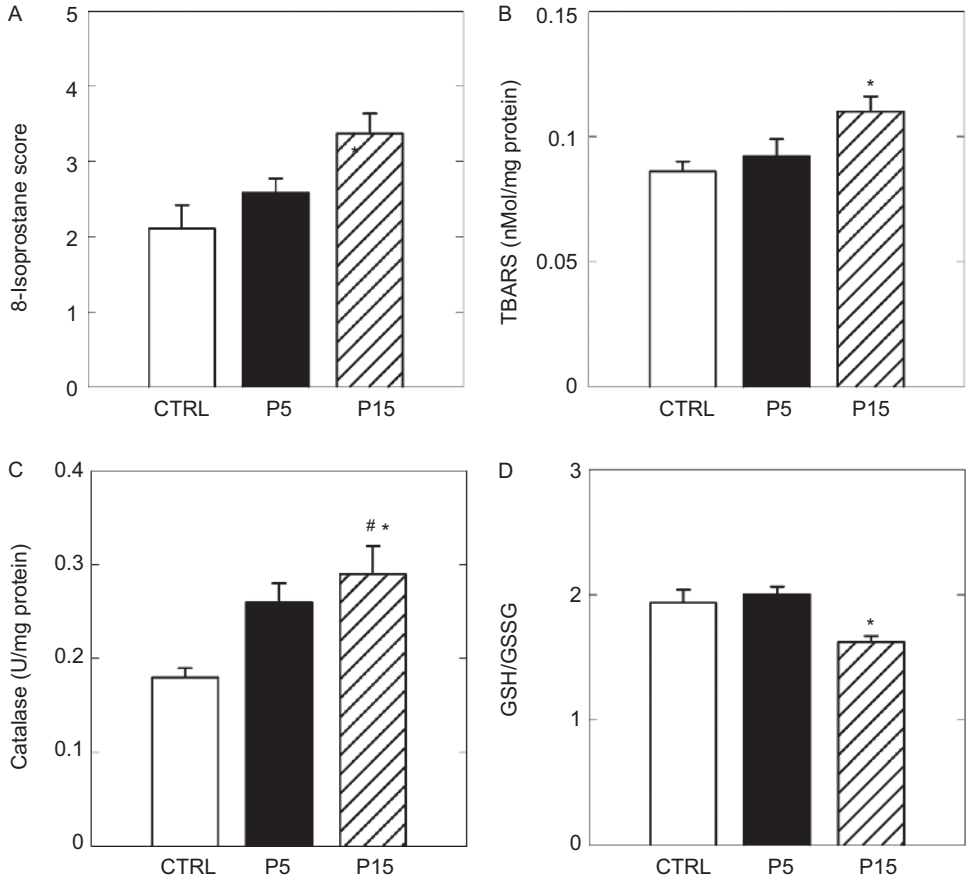

Figure 4. Markers of oxidative damage and stress in mice that did not receive PM2.5 (CTRL) and in those that received 5 or 15 μg of PM2.5 (P5 and P15, respectively). Panel A: semi-quantitative score of staining intensity for 8-isoprostane in CTRL ($n=6$), P5 ($n=5$) and P15 ($n=5$). Values are mean + SEM of 5–10 fields per slide. Panel B shows the values of thiobarbituric acid reactive substances (TBARS) in CTRL, P5, and P15 animals ($n=7$, 10, and 11, respectively). Panel C shows catalase activity in the lungs of CTRL ($n=7$), P5 ($n=10$), and P15 ($n=10$) groups. Panel D shows the relationship between the activities of reduced (GSH) and oxidized (GSSG) glutathione in the lungs of mice in CTRL ($n=8$), P5 ($n=13$) and P15 ($n=11$) groups. In panels B–D values are mean + SEM of samples from lung homogenates. *, # indicate values significantly different from CTRL and P5, respectively ($p<0.05$).

1). Fe is described as deeply linked to the production of oxidative stress (Park et al., 2006) by generating reactive oxygen species (ROS) and facilitating superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) conversion to hydroxyl ions (OH^-) (Stohs & Bagchi, 1995; Park et al., 2006). The resultant pulmonary effects would include surfactant dysfunction (Chauhan & Misra, 1991), epithelial damage, increased vascular permeability and inflammatory response, followed by impaired pulmonary function (Soukup et al., 2000; Dye et al., 2001). Other elements, such as V, Mn, Zn, Cu, Ni, Co, and Cr, also present in our sample, are known to generate ROS (to a lesser extent than Fe) that lead to inflammation and oxidative stress (Stohs & Bagchi, 1995; Sørensen et al., 2005). The initial phase of the pulmonary response to PM exposure seems to be influenced by single elements, while a persistent response would reflect complex interactions among different components present in a composite (Dreher et al., 1997; Antonini et al., 2004).

In the present study, PM concentrations of PAH (Table 1), particularly benzo[a]anthracene, benzo[b] fluoranthene, benzo[k]fluoranthene, and benzo[a] pyrene, are considered of potential carcinogenic risk, in accordance with IARC (Castaño-Vinyals et al., 2004). Mazzoli-Rocha et al. (2008) detected benzo[a]pyrene and benzo[a]anthracene in samples of total suspended PM from São Paulo, confirming the high levels of PAH in this city. The presence of PAH in PM has been associated with the triggering of inflammation, generation of ROS, and lipid peroxidation (Sørensen et al., 2003), especially in alveolar macrophages and epithelial cells (Li et al., 2002). In this line, our study detected markers of inflammation (Figures 2 and 3) and production of ROS (Figure 4) in P15 mice. According to Caricchia et al. (1999), the amount of PAH can decrease in a sample of PM when the analysis takes place 24 h after collection. This was exactly the case in the present study, thus implying that the ambient levels of PAH (and their effects) may have been underestimated.

Although the simple chemical analysis of particles, as in the present work, does not warrant the establishment of a direct correlation between composition and effect, the presence of metals and PAHs in our PM2.5 could have induced a pulmonary impairment similar to that reported in a study with diesel particles (Laks et al., 2008) and also in the face of total suspended particles and

biomass burning-derived particles (Mazzoli-Rocha et al., 2008). Studies on pulmonary retention of PAH in solution, after intratracheal instillation in rats, indicate that PAHs are quickly absorbed by the respiratory tract (Wolff et al., 1989). Noteworthy, inhaled PAH is predominantly adsorbed to ultrafine particles, penetrating in the cells of the bronchial epithelium, where it is metabolized, activated and biochanged (Sørensen et al., 2003; Castaño-Vinyals et al., 2004). Finally, it is not possible to exclude the contribution of other unmeasured compounds to our results.

To discard any eventual bias in our data secondary to the extraction process itself, which could potentially remove and suspend undesired filter components, an extra control group was analyzed (FILTER group). In this group, animals were instilled with a solution obtained from naïve filters submitted to the same extraction protocol used for PM filters. FILTER and CTRL groups presented similar mechanical results, thus excluding the possibility of a biased conclusion due to the extraction process. It should be stressed that ΔP_2, ΔP_{tot} and ΔE pertaining to FILTER mice seem smaller than those in CTRL group, although no statistically significant difference was disclosed. The smaller number of mice in FILTER group could have induced the apparent differences.

To our knowledge, there are few studies describing the independent contributions of central and peripheral airways/tissue to pulmonary resistance, as previously characterized (Bates et al., 1988; Saldiva et al., 1992b), after exposure to pollutants. Our results, as well as those obtained from total suspended particles (Mazzoli-Rocha et al., 2008), revealed that central airway resistance was not modified after particle exposure, as indicated by ΔP_1. In contrast, ΔP_1 was increased after diesel exposure in mice (Laks et al., 2008). TSP samples used by Mazzoli-Rocha et al. (2008) were represented mainly by coarse particles, but originated from the same place as our PM2.5 samples. On the other hand, diesel particles, usually characterized mainly by fine particles, came from a different source showing different metal and PAH contents. E_{st} and ΔE increased in a dose-dependent manner (Figure 1). Taken together, these findings may suggest that the apparent discrepancies among ΔP_1 behavior could result from particle composition rather than particle size. The resistive component of tissue viscoelasticity (ΔP_2) was increased in both PM5 and PM15 groups (Figure 1), in accordance with previous reports after TSP and diesel exposure (Mazzoli-Rocha et al., 2008; Laks et al., 2008). These mechanical alterations could be attributed to the PMs capacity to penetrate to the alveolar region, triggering an inflammatory reaction (Figure 3), and generating oxidative stress and damage in lung tissue (Figure 4) (see below). These functional changes induced by i.n. instillation of low doses of PM2.5 are herein reported for the first time in the literature.

The aforementioned functional findings were accompanied by morphologic alterations in pulmonary parenchyma (Figure 2). The alveolar collapse and septal distortion observed in P15 (Figure 2) may have caused mechanical parenchyma heterogeneity, potentially contributing to the increase in ΔP_2, E_{st} and ΔE (Bates et al., 1985a, 1985b). Additionally, inflammation and eventual surfactant dysfunction may have contributed to morphological and, thus, functional changes (Liu et al., 1996). In this line, airborne metal pollutants (as Fe, Cd, Ni and Co) interact with proteins and phospholipids of the surfactant, modifying its composition and physicochemical properties, besides jeopardizing its secretion by type II pneumocytes. As a result, alveolar surface tension may increase yielding alveolar collapse (Srivastava & Misra, 1986; Chauhan & Misra, 1991), as also present in our study.

P15 group presented a significant inflammatory response, as indicated by MPO activity (Figure 3B), influx of defense cells, as well as IL-6 and TNF-α expression (Figure 3A). Although inflammation could be observed in P5 group too, a milder response was found: only MPO was higher in P5 than in CTRL. It should be noted that even though the amount of neutrophils in the lung did not differ between CTRL and P5 groups (Figure 3A), these cells were probably activated in the latter mice, since MPO was expressed by P5 animals but not expressed by CTRL (Figure 3B). Other experimental studies report recruitment of neutrophils and alveolar macrophages 24 h after intratracheal instillation of 500 μg (Oberdörster et al., 1992) or 380 μg/m^3 (André et al., 2006) of ultrafine particles, as well as i.n. instillation of 100 and 500 μg of PM2.5 from São Paulo in healthy rats (Rivero et al., 2005). In this context, the exposure to PM increases the expression of genes related to NF-κB activation and TNF-α, TGF-β and IL-6 (Shukla et al., 2000; Ding et al., 2010), as depicted in Figure 3C and 3D. Finally, inflammation per se not only modifies the structure of the alveolar septa, but also jeopardizes surfactant action, thus yielding an increased percentage of collapsed alveoli, as aforementioned.

Free radicals and oxidative stress are extensively implicated in the inflammatory response associated with exposure to PM (Donaldson & Stone, 2003; Pereira et al., 2007). Oxidative stress mediated by PM can originate from different sources, involving: (i) direct generation of ROS in the presence of free and oxidant radicals on the particle surface, (II) soluble fractions as organic components and transition metals, (III) alteration in mitochondrial function or NADPH-oxidase, and (IV) activation of inflammatory cells able to generate ROS and reactive nitrogen species (Risom et al, 2005). Many inflammatory mediators, induced by PM exposure, are regulated by transcription factors susceptible to oxi-reduction, as NF-κB, AP-1 and C/EBP, suggesting an increased production of ROS after PM exposure (Li et al., 2002; González-Flecha, 2004; Ohyama et al., 2007). The capacity to generate ROS would be related with particle size and surface area, independently of its composition (Brown et al., 2000). However, evidence suggests that metals and PAH can act synergistically, producing

ROS (Park et al., 2006; Ohyama et al., 2007). To assess the possible involvement of PM2.5 in the genesis of oxidative stress and damage to the pulmonary tissue, we measured some compounds involved in its initial (CAT and GSH/GSSG ratio) and late stages (TBARs and 8-isoprostane). In all instances, P15 presented higher values than CTRL and P5 displayed a nonsignificant, not so intense response (Figure 4). Supporting our findings that P15 mice presented pulmonary oxidative damage (Figure 4A and 4B), it has been shown that soluble and insoluble substances present in PM are able to increase TBARS (Ghio et al., 1996; Bae et al., 2010). Moreover, recent studies (Ding et al., 2010; Martin et al., 2010) showed that low doses of PM were able to increase oxidative stress and induce an inflammatory process in the lung.

Oxidative stress was addressed by the determination of reduced and oxidized glutathione (GSH and GSSG, respectively), since GSH formation falls while GSSG production rises, leading to a reduction in intracellular GSH/GSSG ratio in that condition (Rahman et al., 1999). We found a reduced GSH/GSSG in P15 group (Figure 4C) in accordance with Li et al. (2003), who studied pulmonary alveolar macrophages and bronchial epithelial cells in culture and found similar results, and Ding et al. (2010), who reported a diminished GSH/GSSG in the face of intratracheally instilled low doses of PM2.5 in neonates rats. Additionally, Li et al. (2000) showed that PAH present in diesel particles is related to the reduction in GSH/GSSG. Interestingly, Powell et al. (1994) described reduced GSH levels in adults and asthmatic children, stressing its relation with the inflammatory process. Finally, there may be three possible mechanisms accounting for the decline in GSH in response to PM exposure: (i) via GSH peroxidase (GPx) reaction, (ii) via the GSH transferase reaction, and (iii) via GSH efflux (Deneke & Fanburg, 1989).

The present work disclosed an increased CAT activity in P15 group (Figure 4D). In this context, many authors report consumption of antioxidant enzymes and increased lipid peroxidation in the liver and kidney in diverse species (Weng et al., 2007) in the face of oxidative stress (Sicinska et al., 2006), supporting our results. Under physiological conditions, GSH and CAT play a key antioxidant role, which protects against oxidative damage especially mediated by free radicals and lipid peroxidation. Their decrease induced by fine PM may reduce the protection against oxidative stress (Ding et al., 2010).

In conclusion, this study evaluated for the first time early alterations in the elastic and viscoelastic pulmonary mechanical components of mice exposed to low doses of PM2.5 gathered in a densely populated urban center. These mechanical modifications could probably be explained by histological, inflammatory, and oxidative derangements induced by these fine particles. Our results provide experimental support to epidemiological findings of an association between respiratory impairment and acute exposure to PM2.5.

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Declaration of interest

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