

# Anti-inflammatory Effects of Aerobic Exercise in Mice Exposed to Air Pollution

RODOLFO DE PAULA VIEIRA<sup>1,2</sup>, ALESSANDRA CHOQUETA TOLEDO<sup>3</sup>, LUCAS BOGAZ SILVA<sup>1</sup>, FRANCINE MARIA ALMEIDA<sup>3</sup>, NILSA REGINA DAMACENO-RODRIGUES<sup>4</sup>, ELIA GARCIA CALDINI<sup>4</sup>, ANGELA BATISTA GOMES SANTOS<sup>1</sup>, DOLORES HELENA RIVERO<sup>1</sup>, DEBORAH CAMARGO HIZUME<sup>3</sup>, FERNANDA DEGOBBI TENÓRIO QUIRINO SANTOS LOPES<sup>3</sup>, CLARICE ROSA OLIVO<sup>3</sup>, HUGO CAIRE CASTRO-FARIA-NETO<sup>5</sup>, MILTON ARRUDA MARTINS<sup>3</sup>, PAULO HILÁRIO NASCIMENTO SALDIVA<sup>1</sup>, and MARISA DOLHNIKOFF<sup>1</sup>

<sup>1</sup>Laboratory of Experimental Air Pollution (LIM 05), Department of Pathology, University of São Paulo, School of Medicine, São Paulo, BRAZIL; <sup>2</sup>Post Graduate Program in Biophotonics Applied to Health Sciences, Nove de Julho University, São Paulo, BRAZIL; <sup>3</sup>Laboratory of Experimental Therapeutics (LIM 20), Department of Clinical Medicine, School of Medicine, University of São Paulo, São Paulo, BRAZIL; <sup>4</sup>Laboratory of Cellular Biology (LIM 59), Department of Pathology, School of Medicine, University of São Paulo, São Paulo, BRAZIL; and <sup>5</sup>Laboratory of Immunopharmacology, Oswaldo Cruz Foundation, Institute Oswaldo Cruz, Rio de Janeiro, BRAZIL

## ABSTRACT

VIEIRA, R. D. P., A. C. TOLEDO, L. B. SILVA, F. M. ALMEIDA, N. R. DAMACENO-RODRIGUES, E. G. CALDINI, A. B. G. SANTOS, D. H. RIVERO, D. C. HIZUME, F. D. T. Q. S. LOPES, C. R. OLIVO, H. C. CASTRO-FARIA-NETO, M. A. MARTINS, P. H. N. SALDIVA, and M. DOLHNIKOFF. Anti-inflammatory Effects of Aerobic Exercise in Mice Exposed to Air Pollution. *Med. Sci. Sports Exerc.*, Vol. 44, No. 7, pp. 1227–1234, 2012. **Purpose:** Exposure to diesel exhaust particles (DEP) results in lung inflammation. Regular aerobic exercise improves the inflammatory status in different pulmonary diseases. However, the effects of long-term aerobic exercise on the pulmonary response to DEP have not been investigated. The present study evaluated the effect of aerobic conditioning on the pulmonary inflammatory and oxidative responses of mice exposed to DEP. **Methods:** BALB/c mice were subjected to aerobic exercise five times per week for 5 wk, concomitantly with exposure to DEP (3 mg·mL<sup>-1</sup>; 10 µL per mouse). The levels of exhaled nitric oxide, reactive oxygen species, cellularity, interleukin 6 (IL-6), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were analyzed in bronchoalveolar lavage fluid, and the density of neutrophils and the volume proportion of collagen fibers were measured in the lung parenchyma. The cellular density of leukocytes expressing IL-1 $\beta$ , keratinocyte chemoattractant (KC), and TNF- $\alpha$  in lung parenchyma was evaluated with immunohistochemistry. The levels of IL-1 $\beta$ , KC, and TNF- $\alpha$  were also evaluated in the serum. **Results:** Aerobic exercise inhibited the DEP-induced increase in the levels of reactive oxygen species ( $P < 0.05$ ); exhaled nitric oxide ( $P < 0.01$ ); total ( $P < 0.01$ ) and differential cells ( $P < 0.01$ ); IL-6 and TNF- $\alpha$  levels in bronchoalveolar lavage fluid ( $P < 0.05$ ); the level of neutrophils ( $P < 0.001$ ); collagen density in the lung parenchyma ( $P < 0.05$ ); the levels of IL-6, KC, and TNF- $\alpha$  in plasma ( $P < 0.05$ ); and the expression of IL-1 $\beta$ , KC, and TNF- $\alpha$  by leukocytes in the lung parenchyma ( $P < 0.01$ ). **Conclusions:** We conclude that long-term aerobic exercise presents protective effects in a mouse model of DEP-induced lung inflammation. Our results indicate a need for human studies that evaluate the pulmonary responses to aerobic exercise chronically performed in polluted areas. **Key Words:** AEROBIC TRAINING, IMMUNOLOGY, LUNG INFLAMMATION, DIESEL EXHAUST PARTICLES, NITROSATIVE STRESS, OXIDATIVE STRESS

Address for correspondence: Rodolfo de Paula Vieira, M.Sc., Ph.D., University of Sao Paulo, Avenida Doutor Arnaldo 455, 01246-903, São Paulo, Brazil; E-mail: rodrelena@yahoo.com.br.

Submitted for publication July 2011.

Accepted for publication December 2011.

0195-9131/12/4407-1227/0

MEDICINE & SCIENCE IN SPORTS & EXERCISE®

Copyright © 2012 by the American College of Sports Medicine

DOI: 10.1249/MSS.0b013e31824b2877

**D**iesel exhaust particles (DEP) emitted during the combustion of diesel fuel represent a major contributor to the airborne particulate matter mass in urban areas (8). DEP consists of metals, organic species, and polycyclic aromatic hydrocarbons and affects different aspects of health and disease (16,18). DEP and its compounds induce many proinflammatory effects in different organs (4,18), especially the respiratory tract. The inhalation and deposition of DEP result in the release of different proinflammatory molecules, contributing to the development and exacerbation

of pulmonary diseases, such as asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome (10,27,35).

Short-term and long-term administrations of DEP in rodents are related to the increased release of proinflammatory cytokines into the lungs, notably interleukin  $6\beta$  (IL- $6\beta$ ), IL-6, KC, and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) (4,11,31). The release of these cytokines in exposed animals is associated with the recruitment and activation of different types of leukocytes, which contribute to the development of pulmonary inflammatory diseases (28,41). A possible mechanism underlying DEP-induced cytokine release is the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that activate transcription factors, such as nuclear factor  $\kappa$ B (4,42,43).

Regular aerobic exercise results in multiple health benefits, including improvement of cardiorespiratory fitness and quality of life, reduction of obesity and blood pressure, and increased longevity (15,24). When performed chronically on a regular basis, aerobic exercise also reduces oxidative stress systemically (13) in different diseases, such as heart diseases, type 2 diabetes, rheumatic arthritis, and Alzheimer and Parkinson diseases (29), as well as in the airway epithelial cells of animals with long-term allergic lung inflammation (40). Chronic practice of regular exercise exerts a marked anti-inflammatory effect in different models of pulmonary diseases, such as in asthma models (25,37–40), acute respiratory distress syndrome (22,30), and chronic obstructive pulmonary disease (20,36).

Studies that have investigated the effects of exposure to air pollutants during exercise have suggested that people exercising in polluted environments are at increased risk of respiratory and cardiovascular morbidity related to air pollution owing to an exercise-induced amplification in respiratory uptake, lung deposition, and toxicity of inhaled pollutants (3,12,26,32–34). Exercise may increase the likelihood of an adverse effect by increasing the dose of pollutants delivered to target sites in the lungs as ventilation increases to meet metabolic demands (5). However, these studies do not take into account the potential anti-inflammatory effects of aerobic conditioning, which could inhibit the proinflammatory events induced by air pollution. Therefore, the aim of the present study was to investigate the effects of 5 wk of aerobic exercise performed in association with DEP exposure on pulmonary and systemic inflammation, oxidative, and nitrosative stress and remodeling.

## METHODS

In all experiments, the American College of Sports Medicine guidelines for animal care was followed. This study was approved by the review board for human and animal studies of the School of Medicine of the University of São Paulo (protocol 1225/09).

## DEP Collection, Analysis, and Suspension

A particle trap device was adapted to the exhaust pipe of a bus from the public transportation fleet of São Paulo. The bus was equipped with a Mercedes Benz MB1620, 210-hp engine, without electronic fuel injection control, which ran on diesel containing 500 ppm of sulfur. This particular type of bus was chosen because it is the most frequently operating bus in São Paulo, based on information provided by the municipality. Briefly, a stainless steel mesh was inserted into the exhaust pipe of the bus. Diesel particles were collected after 1 d of routine bus operation and stored for toxicological studies. Twenty-four hours after collection, particle composition analysis was performed following previously published methods (16). Following the analysis, DEP was suspended in 0.9% NaCl ( $3 \text{ mg}\cdot\text{mL}^{-1}$ ) for 1 h to obtain a homogeneous solution (16).

## Experimental Protocol

**Animals and experimental groups.** Thirty-two BALB/c male mice were obtained from the Animal Facility of the School of Medicine of the University of São Paulo and maintained under controlled conditions of temperature, humidity, and a dark–light cycle. The animals were divided into four groups ( $n = 8$  each group) as follows: control (non-exercised and non-DEP-instilled), exercise (Exe; exercised and non-DEP-instilled), DEP (nonexercised and DEP-instilled), and DEP + Exe (DEP-instilled + exercise).

**DEP instillation.** Aiming to mimic exercise performed in air-polluted areas, DEP ( $3 \text{ mg}\cdot\text{mL}^{-1}$ ) was intranasally instilled ( $10 \mu\text{L}$  per mouse) with a  $100\text{-}\mu\text{L}$  micropipette 10 min after the beginning of exercise. DEP instillation was performed five times per week, during 5 wk. The intranasal instillation induced a reflex of apnea followed by a deep inspiration. The noninstilled groups were administered vehicle (0.9% NaCl).

**Exercise treadmill test and training.** Animals were adapted to the treadmill for rodents (Super ATL; Imbramed, RS, Brazil) training for 3 d (15 min, 25% inclination,  $0.2 \text{ km}\cdot\text{h}^{-1}$ ). On the fourth day, the individual maximal exercise capacity test was performed with a 5-min warm-up (25% inclination,  $0.2 \text{ km}\cdot\text{h}^{-1}$ ) and followed by an increase in treadmill speed ( $0.1 \text{ km}\cdot\text{h}^{-1}$  every 2.5 min) until animal exhaustion (i.e., when they were not able to run voluntarily after 10 mechanical stimuli) (37–40). The test was repeated after 5 wk of training. The maximal exercise capacity (100%) was defined as the maximum speed reached by each animal. The physical test was performed for each mouse individually. The speed average of each group was calculated, and then the mice were submitted to treadmill training as a mean speed of the group workload. Mice were trained at low intensity, corresponding to 50% of the initial maximal speed obtained in the exercise test, for  $60 \text{ min}\cdot\text{d}^{-1}$ ,  $5 \text{ d}\cdot\text{wk}^{-1}$  for 5 wk, as previously described (37–40).

**Determination of exhaled nitric oxide levels.** Twenty-four hours after the last DEP instillation and exercise session,

mice were anesthetized with 50 mg·kg<sup>-1</sup> intraperitoneally of thiopental sodium, tracheostomized, and connected to a rodent ventilator (FlexiVent; Scireq, Montreal, Quebec, Canada) with a tidal volume of 10 mL·kg<sup>-1</sup> at a frequency of 2 Hz. The levels of exhaled nitric oxide (eNO) were measured after a 5-min collection at the expiratory port of the ventilator using a mylar bag. The concentrations of eNO were measured by chemiluminescence using a nitric oxide analyzer (NOA 280; Sievers Instruments, Boulder, CO) (30).

**ROS in bronchoalveolar lavage fluid.** Immediately after the collection of bronchoalveolar lavage fluid (BALF, 3 × 500 μL 0.9% sterile saline solution lung lavage), 400 μL was used for evaluation of the levels of ROS by chemiluminescence with luminol as previously described (1). The results were expressed as photons per minute per cell × 10<sup>5</sup>.

**Total and differential cells in BALF.** After BALF collection, the samples were centrifuged at 800 rpm, at 4°C for 10 min. The supernatant was stored at -70°C for cytokine analysis, the cellular pellet was resuspended in 1 mL of 0.9% sterile saline, and the total cell count was performed using a Neubauer chamber (Carl Roth GmbH, Karlsruhe, Germany) (30). For the differential cell count, cytopsin slides were prepared and stained with Diff Quick (Medion Diagnosis, Düringen, Switzerland), and 300 cells were counted in a blinded fashion (30).

**Cytokine analysis.** The levels of IL-1β, IL-6, KC, and TNF-α in BALF and in serum were measured using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

**Lung histology and immunohistochemistry.** All animals from each group were used for histological analysis. Four-micrometer-thick slides were stained with hematoxylin and eosin for the evaluation of the number of polymorphonuclear (PMN) cells in the lung parenchyma and with picrosirius for the evaluation of the collagen content in the lung parenchyma (30,38). The density of PMN cells in the lung parenchyma was assessed by conventional morphometric techniques (30,38). The content of collagen in the lung parenchyma was evaluated with image analysis, as previously described (30). Briefly, 20 aleatory fields of lung parenchyma at 400× magnification in each lung were photographed and analyzed using the software (Image Pro Plus 4.5; MediaCybernetics, Silver Spring, MD) (30). A color threshold (corresponding to positive area for collagen fibers) was established to be sure that all images were analyzed under the same color standard. The parenchymal tissue area was determined by subtracting the area of parenchymal airspaces from the total area in each field (30). The volume proportion of collagen fibers were determined as the relation between area of collagen fibers in total tissue area and were expressed as percentage (%) (30).

In addition, the number of cells positive for IL-1β, KC, and TNF-α in the lung parenchyma was evaluated with immunohistochemistry and conventional morphometric techniques (38). Briefly, lung sections were deparaffinized, and

a 0.5% peroxidase methanol solution was applied for 10 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed with a citrate solution for 30 min. The sections were incubated overnight at 4°C with anti-IL-1β (1:300), anti-KC (1:500), and anti-TNF-α (1:400) (Santa Cruz Biotechnology, Santa Cruz, CA). An ABC VECTASTAIN Kit (Vector Elite PK-6105; Vector Laboratories, Burlingame, CA) was used as a secondary antibody, and 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as a chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany).

## Statistical Analysis

The statistical analysis was performed using the software SPSS 13.0 (SPSS, Inc., Chicago, IL). Parametric and nonparametric data are expressed as the means ± SD or the median + interquartile range, respectively. Comparisons between the two outcomes (exercise and DEP) were performed by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test (parametric data) or by Dunn *post hoc* test (nonparametric data). Differences were considered statistically significant at  $P < 0.05$ . In addition, a multivariate ANOVA was performed to resolve the main effects of exercise and DEP for all outcomes evaluated. This test also produces an interaction term that identifies whether or not the effects of one factor (exercise) are influenced by the effects of the other (DEP exposure).

## RESULTS

**Effects of aerobic training and DEP on exercise capacity.** As demonstrated in Table 1, the exercised mice not subjected to DEP instillation (Exe group) significantly improved their physical capacity compared with all other groups ( $P < 0.01$ ). The multivariate ANOVA revealed that both exercise ( $F = 29.518$ ,  $P < 0.001$ ) and DEP ( $F = 15.031$ ,  $P = 0.001$ ) presented a significant effect on the exercise capacity, but no interaction effects between exercise and DEP was observed ( $F = 3.19$ ,  $P = 0.086$ ).

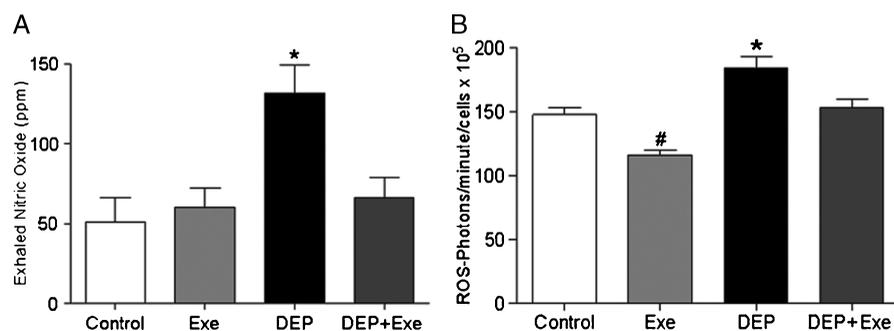
**Effects of aerobic exercise and DEP exposure on eNO and ROS release.** Figure 1A shows that DEP instillation increased the release of eNO compared with all groups ( $P < 0.01$ ). Exercised mice exposed to DEP (Exe + DEP) showed a significantly lower production of eNO compared with the DEP group without exercise ( $P < 0.01$ ). The multivariate ANOVA demonstrated that exercise alone did not result in significant effect on eNO release ( $F = 3.03$ ,

TABLE 1. Effects of aerobic training and DEP on exercise capacity.

Groups	Initial Test	Final Test	Final – Initial Test
Control	23.6 ± 2.3	30.1 ± 3.4	6.4 ± 3.1
Exe	21.8 ± 2.5	38.6 ± 3.1	16.79 ± 4.0*
DEP	25.2 ± 3.5	28.6 ± 4.6	3.4 ± 2.9
DEP + Exe	28.1 ± 4.2	36.8 ± 4.2	8.4 ± 4.1

Results are expressed as mean ± SD.

\*  $P < 0.01$  compared with all other groups.



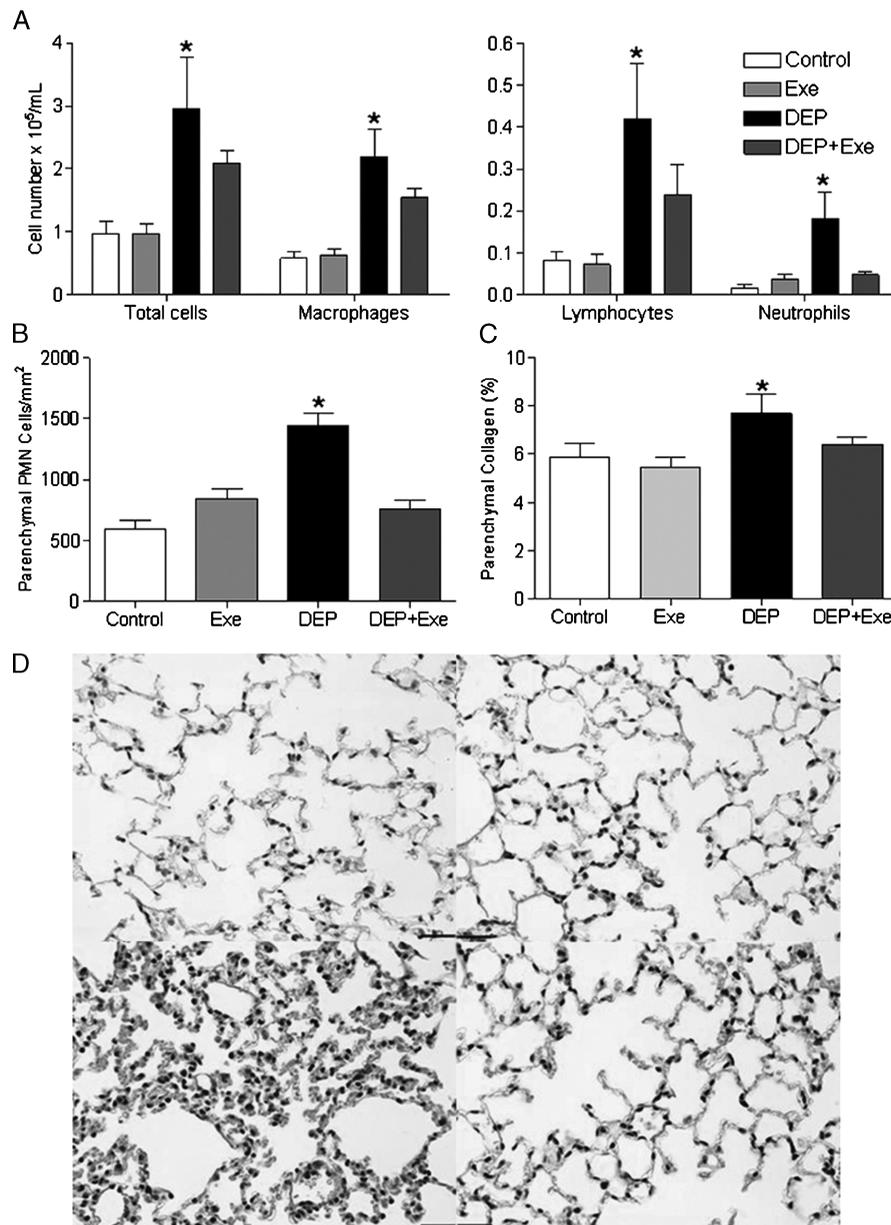
**FIGURE 1**—Effects of aerobic exercise and DEP on the levels of eNO and the levels of ROS in BALF. **A**, eNO: bars represent the mean  $\pm$  SD of eight animals from each group. Asterisk indicates treatments that are significantly different at  $P < 0.01$  compared with all of the other groups. **B**, ROS: bars represent mean  $\pm$  SD. Asterisk indicates treatments that are significantly different at  $P < 0.05$  compared with all of the groups. Pound sign indicates treatments that are significantly different at  $P < 0.01$  compared with all of the groups.

$P = 0.094$ ), DEP instillation resulted in a significant effect ( $F = 9.77$ ,  $P = 0.004$ ), and there was an interaction effect between DEP and Exe ( $F = 7.20$ ,  $P = 0.013$ ). Figure 1B shows that DEP significantly increased the ROS levels in BALF compared with all the other groups ( $P < 0.05$ ). Aerobic exercise (Exe) reduced the ROS levels compared with all other groups ( $P < 0.01$ ). We also observed that DEP + Exe animals presented lower levels of ROS production compared with the DEP group ( $P < 0.05$ ). The multivariate ANOVA confirmed the effects of exercise ( $F = 23.20$ ,  $P < 0.001$ ) and DEP instillation ( $F = 32.69$ ,  $P < 0.001$ ) on ROS release, but no interaction between DEP and exercise was observed ( $F = 0.001$ ,  $P = 0.971$ ).

**Effects of aerobic exercise and DEP exposure on lung inflammation and remodeling.** Data presented in Figure 2A demonstrate that DEP exposure induced a significant increase in the number of total cells ( $P < 0.05$ ), neutrophils ( $P < 0.01$ ), macrophages ( $P < 0.05$ ), and lymphocytes ( $P < 0.05$ ) in BALF compared with the control and Exe groups. We also observed that aerobic exercise (DEP + Exe) inhibited the pulmonary accumulation of neutrophils ( $P < 0.01$ ) and lymphocytes ( $P < 0.05$ ) compared with the DEP group. The multivariate ANOVA revealed that only DEP alone resulted in a significant effect on the number of total cells ( $F = 16.22$ ), lymphocytes ( $F = 10.44$ ), and macrophages in BALF ( $F = 25.35$ ,  $P \leq 0.004$ ), whereas no effects of exercise alone ( $P \geq 0.175$ ) or DEP interaction with exercise ( $P \geq 0.175$ ) were observed. The multivariate ANOVA showed a significant effect of DEP on the number of neutrophils in BALF ( $F = 8.54$ ,  $P = 0.007$ ) as well as an interaction effect between DEP and exercise ( $F = 6.72$ ,  $P = 0.015$ ). DEP induced an increase in the density of PMN cells in the lung parenchyma compared with all other groups ( $P < 0.001$ ). Aerobic exercise in animals instilled with DEP (DEP + Exe) significantly reduced the density of PNM cells in the lung parenchyma compared with the DEP group ( $P < 0.001$ ; Fig. 2B). The multivariate ANOVA showed an effect of both exercise ( $F = 6.71$ ,  $P = 0.016$ ) and DEP ( $F = 21.88$ ,  $P < 0.001$ ), and also an interaction effect between DEP and exercise ( $F = 32.14$ ,  $P < 0.001$ ) on the density of PMN cells

in the lung parenchyma. Figure 2C shows that DEP induced an increase in the volume proportion of collagen fibers in the lung parenchyma when compared with all of the other groups ( $P < 0.05$ ). Aerobic exercise significantly inhibited DEP-induced collagen accumulation in the lung parenchyma ( $P < 0.05$ ). The multivariate ANOVA showed that DEP significantly affected the volume proportion of collagen fibers in the lung parenchyma ( $F = 5.54$ ,  $P = 0.029$ ), whereas exercise alone did not present any effect ( $F = 3.68$ ,  $P = 0.069$ ). No interaction between DEP and exercise ( $F = 2.07$ ,  $P = 0.166$ ) was observed. Figure 2D shows representative photomicrographs of slides stained with hematoxylin and eosin from all experimental groups, demonstrating the level of inflammation in the lung parenchyma.

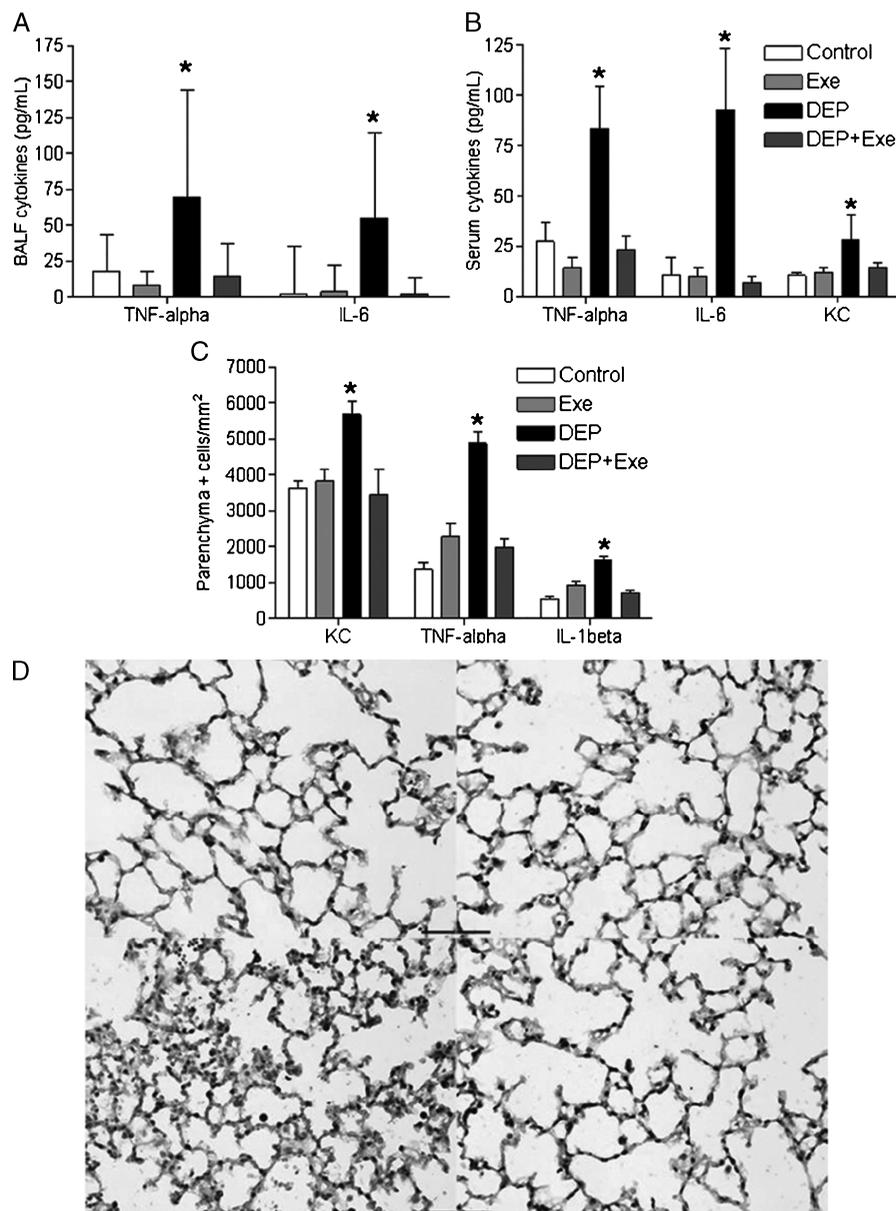
**Effects of aerobic exercise and DEP exposure on cytokine production.** Data presented in Figure 3A demonstrate that DEP instillation increased the TNF- $\alpha$  and IL-6 levels in BALF compared with all of the other groups ( $P < 0.05$ ) and that aerobic exercise in DEP-instilled animals significantly decreased the levels of these cytokines compared with the DEP group ( $P < 0.05$ ). The multivariate ANOVA also demonstrated that exercise ( $P \leq 0.027$ ) and DEP ( $P < 0.040$ ) presented a significant effect on the TNF- $\alpha$  (Exe,  $F = 5.57$ ; DEP,  $F = 5.97$ ) and IL-6 levels (Exe,  $F = 6.57$ ; DEP,  $F = 4.69$ ), but no interaction effect was observed ( $P \geq 0.06$ ). Data presented in Figure 3B demonstrate that DEP induced the increase in TNF- $\alpha$ , IL-6, and KC levels in serum compared with all other groups ( $P < 0.05$ ). Again, the results demonstrated that aerobic exercise in DEP-instilled animals significantly decreased the levels of these cytokines compared with the DEP group ( $P < 0.05$ ). The multivariate ANOVA showed that exercise ( $F = 8.96$ ,  $P = 0.006$ ) and DEP ( $F = 8.36$ ,  $P = 0.007$ ) presented a significant effect on serum levels of TNF- $\alpha$ , whereas no interaction effect between DEP and exercise was observed ( $F = 3.48$ ,  $P = 0.073$ ). There were an effect of exercise ( $F = 7.29$ ,  $P = 0.012$ ) and DEP ( $F = 6.45$ ,  $P = 0.018$ ) and an interaction effect of DEP and exercise ( $F = 7.47$ ,  $P = 0.012$ ) on the IL-6 levels in serum. Further, a significant effect on serum levels of KC was observed for DEP ( $F = 6.35$ ,  $P = 0.018$ ), as well as an



**FIGURE 2—Effects of aerobic exercise and DEP on lung inflammation and remodeling. Bars represent the mean  $\pm$  SD of eight animals from each group. A, Asterisk indicates treatments that are significantly different at  $P < 0.05$  (total cells and macrophages) compared with the control and Exe groups. For neutrophils, asterisk indicates treatments that are significantly different at  $P < 0.01$ . For lymphocytes, asterisk indicates treatments that are significantly different at  $P < 0.05$  compared with all of the other groups. B, Asterisk indicates treatments that are significantly different at  $P < 0.001$  compared with all of the other groups. C, Asterisk indicates treatments that are significantly different at  $P < 0.05$  compared with all of the other groups. D, Representative photomicrographs of hematoxylin-eosin–stained slides from the control (right upper panel), Exe (left upper panel), DEP (right bottom panel), and DEP + Exe (left bottom panel). Scale bar = 50  $\mu$ m.**

interaction effect between DEP and exercise ( $F = 4.84$ ,  $P = 0.036$ ). In addition, we observed that DEP induced the expression of KC ( $P < 0.01$ ), TNF- $\alpha$  ( $P < 0.001$ ), and IL-1 $\beta$  ( $P < 0.001$ ) by leukocytes in the lung parenchyma compared with the control group (Fig. 3C). Similarly, aerobic exercise in DEP-instilled animals (DEP + Exe group) significantly reduced the expression of KC ( $P < 0.01$ ), TNF- $\alpha$  ( $P < 0.001$ ), and IL-1 $\beta$  ( $P < 0.001$ ) by leukocytes compared with DEP instillation without exercise (DEP group) (Fig. 3C). The multivariate ANOVA showed an effect of Exe ( $F = 9.86$ ,

$P = 0.004$ ) and an interaction effect between DEP and exercise ( $F = 13.42$ ,  $P = 0.001$ ) on the expression of KC in lung tissue, whereas no effect of DEP alone was observed ( $F = 2.94$ ,  $P = 0.099$ ). The multivariate ANOVA also showed an effect of exercise ( $P \leq 0.003$ ) and DEP ( $P < 0.001$ ) and an interaction effect between DEP + Exe ( $P < 0.001$ ) on the expression of TNF- $\alpha$  (Exe,  $F = 12.77$ ; DEP,  $F = 33.68$ ; DEP + Exe,  $F = 46.78$ ) and IL-1 $\beta$  (Exe,  $F = 10.89$ ; DEP,  $F = 34.06$ ; DEP + Exe,  $F = 74.05$ ) in lung tissue. Figure 3D shows representative photomicrographs of TNF- $\alpha$ –stained



**FIGURE 3**—Effects of aerobic exercise and DEP on TNF- $\alpha$ , IL-6, and KC levels in BALF and serum and the expression of KC, TNF- $\alpha$ , and IL-1 $\beta$  by leukocytes in the lung parenchyma. **A**, Bars show the median + interquartile range of eight animals from each group for the BALF levels of TNF- $\alpha$  and IL-6. **B** and **C**, Bars represent the mean  $\pm$  SD of eight animals from each group, for TNF- $\alpha$ , IL-6, and KC levels in serum and the expression of KC, TNF- $\alpha$ , and IL-1 $\beta$  by peribronchial leukocytes, respectively. In **A** and **B**, asterisk indicates treatments that are significantly different at  $P < 0.05$  when compared with all of the groups. In **C**, asterisk indicates treatments that are significantly different at  $P < 0.01$  (KC) and asterisk indicates treatments that are significantly different at  $P < 0.001$  (TNF- $\alpha$  and IL-1 $\beta$ ). **D**, Representative photomicrographs of TNF- $\alpha$ -stained slides from the control (right upper panel), Exe (left upper panel), DEP (right bottom panel), and DEP + Exe (left bottom panel). Scale bar = 50  $\mu$ m.

slides from the control (right upper panel), Exe (left upper panel), DEP (right bottom panel), and DEP + Exe (left bottom panel).

## DISCUSSION

In the present study, we demonstrated for the first time that aerobic exercise inhibits lung inflammation, pulmonary and systemic proinflammatory cytokine release, and the pulmonary levels of oxidative and nitrosative stress in an

experimental model of chronic DEP-induced pulmonary inflammation.

Several experimental and human studies have demonstrated that increased levels of air pollution boost the release of free radicals, notably reactive oxygen and nitrogen species, either systemically or locally in different organs (4,14,19,21,42,43). The release of reactive species is closely associated with pulmonary inflammation (42,43). Moreover, the use of antioxidant therapeutic strategies in animals exposed to particulate matter decreases the levels of several inflammatory parameters (42,43). In agreement with these previous studies,

we showed that long-term exposure to DEP increased the release of pulmonary ROS and RNS, specifically eNO, reinforcing the concept that exposure to air pollution is associated with increased production of free radicals. Furthermore, we demonstrated that the long-term and regular practice of low-intensity aerobic exercise was protective from the effects of DEP exposure by inhibiting the pulmonary production of ROS and nitric oxide. We also showed that DEP induced a significant increase in the number of total cells, neutrophils, macrophages, and lymphocytes in the BALF and in the density of PMN cells in the lung parenchyma. This DEP-induced inflammatory response was also inhibited by aerobic exercise. Our results suggest that the reduction in oxidative stress is a primary event linked to the anti-inflammatory effects of long-term exercise.

The increase in oxidative and nitrosative stress induced by particulate matter is also associated with the release of proinflammatory cytokines in humans and mice and in isolated cell cultures (6,23,42,43). Zhao et al. (43) demonstrated that DEP increased the production of ROS and nitric oxide by alveolar macrophages, and DEP increased production of the proinflammatory cytokine IL-12. Similarly, den Hartigh et al. (6) demonstrated that some components of particulate matter are involved in the activation of endothelial cells, which resulted in the further coactivation of circulating monocytes. In addition, it was recently demonstrated that DEP induced the systemic release of IL-6, a cytokine considered to be a reliable biomarker of the inflammatory process, in mice (2,9,23). In this study, exposure to DEP induced increased levels of IL-6 in both serum and BALF. Other proinflammatory cytokines, such as IL-1 $\beta$ , TNF- $\alpha$ , and KC, were also increased in BALF and lung parenchyma after DEP instillation. By using quantitative immunohistochemistry analysis, we also demonstrated that leukocytes in the lung parenchyma are involved in the release of these cytokines and that low-intensity aerobic exercise inhibited both the total amount of proinflammatory cytokines and the activation of parenchymal leukocytes.

By evaluating lung collagen content through a quantitative histomorphometric technique, we observed that exposure to DEP increased parenchymal remodeling. Lung remodeling is likely a consequence of unresolved chronic inflamma-

tion and is associated with functional changes in airway and interstitial pulmonary diseases (7,17,21). We demonstrated that aerobic exercise reduced the DEP-induced lung parenchymal remodeling, indicating the potential benefit of long-term aerobic exercise in preventing structural changes in the lung parenchyma associated with chronic inflammatory conditions.

The pulmonary and systemic effects of a short-term exercise session performed in a highly polluted area were recently evaluated (12). The authors observed that, immediately after the exercise session, the numbers of blood neutrophils increased significantly, but no other important parameters of inflammation, such as exhaled nitric oxide, IL-6 levels, platelet function, and serum levels of Clara cell protein, were changed. The authors concluded that healthy people should be discouraged from performing exercise in areas of heavy traffic with concentrated air pollution (12). Because the authors reached this conclusion based on a short-term experiment, long-term experiments evaluating populations performing daily exercise in air-polluted areas are urgently required. Therefore, our experiment was designed to mimic this situation in a laboratory setting, aiming to provide initial evidence concerning the long-term effects of aerobic exercise on systemic and pulmonary inflammation in populations living in polluted areas. Taken together, our results indicate that the decreased inflammatory status achieved by long-term aerobic exercise has a protective effect on DEP-exposed mice, decreasing the proinflammatory effects of long-term exposure to air pollution.

We conclude that low-intensity aerobic exercise presents protective effects from DEP-induced lung inflammation, remodeling, and pulmonary oxidative stress.

This work was supported by the Programa Institutos Nacionais de Ciência e Tecnologia do Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq/MCT, Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), and Laboratórios de Investigação Médica da Faculdade de Medicina da Universidade de São Paulo (LIMHC-FMUSP). Rodolfo P. Vieira holds a European Respiratory Society/Marie Curie Postdoctoral Fellowship 2010/2011. The authors declare no conflicts of interest.

The results of this study do not constitute endorsement by the American College of Sports Medicine.

## REFERENCES

1. Athayde KS, Cocuzza M, Agarwal A, et al. Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *J Androl*. 2007;28(4):613–20.
2. Bozza FA, Salluh JI, Japiassu AM, et al. Cytokine profiles as markers of disease severity in sepsis: a multiplex analysis. *Crit Care*. 2007;11(2):R49.
3. Campbell ME, Li Q, Gingrich SE, Macfarlane RG, Cheng S. Should people be physically active outdoors on smog alert days? *Can J Public Health*. 2005;96(1):24–8.
4. Cao D, Tal TL, Graves LM, et al. Diesel exhaust particulate-induced activation of Stat3 requires activities of EGFR and Src in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2007;292(2):L422–9.
5. Daigle CC, Chalupa DC, Gibb FR, et al. Ultrafine particle deposition in humans during rest and exercise. *Inhal Toxicol*. 2003;15(6):539–52.
6. den Hartigh LJ, Lamé MW, Ham W, Kleeman MJ, Tablin F, Wilson DW. Endotoxin and polycyclic aromatic hydrocarbons in ambient fine particulate matter from Fresno, California initiate human monocyte inflammatory responses mediated by reactive oxygen species. *Toxicol In Vitro*. 2010;24(7):1993–2002.
7. Dolnikoff M, da Silva LF, de Araujo BB, et al. The outer wall of small airways is a major site of remodeling in fatal asthma. *J Allergy Clin Immunol*. 2009;123(5):1090–7.
8. Donaldson K, Tran L, Jimenez LA, et al. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part Fibre Toxicol*. 2005;2:10.

9. Gimeno D, Delclos GL, Ferrie JE, et al. Association of CRP and IL-6 with lung function in a middle-aged population initially free from self-reported respiratory problems: the Whitehall II study. *Eur J Epidemiol.* 2011;26(2):135–44.
10. Halonen JI, Lanki T, Yli-Tuomi T, Kulmala M, Tiittanen P, Pekkanen J. Urban air pollution, and asthma and COPD hospital emergency room visits. *Thorax.* 2008;63(7):635–41.
11. Inoue K, Takano H, Yanagisawa R, et al. The role of toll-like receptor 4 in airway inflammation induced by diesel exhaust particles. *Arch Toxicol.* 2006;80(5):275–9.
12. Jacobs L, Nawrot TS, de Geus B, et al. Subclinical responses in healthy cyclists briefly exposed to traffic-related air pollution: an intervention study. *Environ Health.* 2010;9:64.
13. Ji LL. Modulation of skeletal muscle antioxidant defense by exercise: role of redox signaling. *Free Radic Biol Med.* 2008;44(2):142–52.
14. Kodavanti UP, Thomas R, Ledbetter AD, et al. Vascular and cardiac impairments in rats inhaling ozone and diesel exhaust particles. *Environ Health Perspect.* 2011;119(3):312–8.
15. Kokkinos P, Myers J. Exercise and physical activity: clinical outcomes and applications. *Circulation.* 2010;122(16):1637–48.
16. Laks D, de Oliveira RC, de André PA, et al. Composition of diesel particles influences acute pulmonary toxicity: an experimental study in mice. *Inhal Toxicol.* 2008;20(11):1037–42.
17. Leigh R, Ellis R, Wattie JN, et al. Type 2 cytokines in the pathogenesis of sustained dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med.* 2004;169(7):860–7.
18. Maier KL, Alessandrini F, Beck-Speier I, Hofer TP, Diabaté S, Bitterle E. Health effects of ambient particulate matter—biological mechanisms and inflammatory responses to *in vitro* and *in vivo* particle exposures. *Inhal Toxicol.* 2008;20(3):319–37.
19. Martin S, Fernandez-Alanis E, Delfosse V, et al. Low doses of urban air particles from Buenos Aires promote oxidative stress and apoptosis in mice lungs. *Inhal Toxicol.* 2010;22(13):1064–71.
20. Menegali BT, Nesi RT, Souza PS, et al. The effects of physical exercise on the cigarette smoke-induced pulmonary oxidative response. *Pulm Pharmacol Ther.* 2009;22(6):567–73.
21. Morales MM, Pires-Neto RC, Inforsato N, et al. Small airway remodeling in acute respiratory distress syndrome: a study in autopsy lung tissue. *Crit Care.* 2011;15(1):R4.
22. Mussi RK, Camargo EA, Ferreira T, et al. Exercise training reduces pulmonary ischaemia-reperfusion-induced inflammatory responses. *Eur Respir J.* 2008;31(3):645–9.
23. Nemmar A, Al-Salam S, Zia S, Dhanasekaran S, Shudadevi M, Ali BH. Time-course effects of systemically administered diesel exhaust particles in rats. *Toxicol Lett.* 2010;194(3):58–65.
24. Paffenbarger RS Jr, Hyde RT, Wing AL, Hsieh CC. Physical activity, all-cause mortality, and longevity of college alumni. *N Engl J Med.* 1986;314(10):605–13.
25. Pastva A, Estell K, Schoeb TR, Atkinson TP, Schwiebert LM. Aerobic exercise attenuates airway inflammatory responses in a mouse model of atopic asthma. *J Immunol.* 2004;172(7):4520–6.
26. Patel H, Eo S, Kwon S. Effects of diesel particulate matters on inflammatory responses in static and dynamic culture of human alveolar epithelial cells. *Toxicol Lett.* 2011;200(1–2):124–31.
27. Patel MM, Chillrud SN, Correa JC, et al. Traffic-related particulate matter and acute respiratory symptoms among New York City area adolescents. *Environ Health Perspect.* 2010;118(9):1338–43.
28. Provoost S, Maes T, Willart MA, Joos GF, Lambrecht BN, Tournoy KG. Diesel exhaust particles stimulate adaptive immunity by acting on pulmonary dendritic cells. *J Immunol.* 2010;184(1):426–32.
29. Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med.* 2008;44(2):153–9.
30. Ramos DS, Olivo CR, Quirino Santos Lopes FD, et al. Low-intensity swimming training partially inhibits lipopolysaccharide-induced acute lung injury. *Med Sci Sports Exerc.* 2010;42(1):113–9.
31. Rao KM, Ma JY, Meighan T, Barger MW, Pack D, Vallyathan V. Time course of gene expression of inflammatory mediators in rat lung after diesel exhaust particle exposure. *Environ Health Perspect.* 2005;113(5):612–7.
32. Sharman JE. Clinicians prescribing exercise: is air pollution a hazard? *Med J Aust.* 2005;182(12):606–7.
33. Sharman JE, Cockcroft JR, Coombes JS. Cardiovascular implications of exposure to traffic air pollution during exercise. *QJM.* 2004;97(10):637–43.
34. Strak M, Boogaard H, Meliefste K, et al. Respiratory health effects of ultrafine and fine particle exposure in cyclists. *Occup Environ Med.* 2010;67(2):118–24.
35. Takano H, Yanagisawa R, Ichinose T, et al. Diesel exhaust particles enhance lung injury related to bacterial endotoxin through expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1. *Am J Respir Crit Care Med.* 2002;165(9):1329–35.
36. Toledo AC, Magalhaes RM, Hizume DC, et al. Aerobic exercise attenuates pulmonary injury induced by chronic exposure to cigarette smoke in mice. *Eur Respir J.* 2012;39(2):254–64.
37. Vieira RP, Claudino RC, Duarte AC, et al. Aerobic exercise decreases chronic allergic lung inflammation and airway remodeling in mice. *Am J Respir Crit Care Med.* 2007;176(9):871–7.
38. Vieira RP, de Andrade VF, Duarte AC, et al. Aerobic conditioning and allergic pulmonary inflammation in mice. II. Effects on lung vascular and parenchymal inflammation and remodeling. *Am J Physiol Lung Cell Mol Physiol.* 2008;295(4):L670–9.
39. Vieira RP, Duarte AC, Santos AB, et al. Exercise reduces effects of creatine on lung. *Int J Sports Med.* 2009;30(9):684–90.
40. Vieira RP, Toledo AC, Ferreira SC, et al. Airway epithelium mediates the anti-inflammatory effects of exercise on asthma. *Respir Physiol Neurobiol.* 2011;175(3):383–9.
41. Yoshizaki K, Brito JM, Toledo AC, et al. Subchronic effects of nasally instilled diesel exhaust particulates on the nasal and airway epithelia in mice. *Inhal Toxicol.* 2010;22(7):610–7.
42. Zhao H, Barger MW, Ma JK, Castranova V, Ma JY. Cooperation of the inducible nitric oxide synthase and cytochrome P450 1A1 in mediating lung inflammation and mutagenicity induced by diesel exhaust particles. *Environ Health Perspect.* 2006;114(8):1253–8.
43. Zhao H, Ma JK, Barger MW, et al. Reactive oxygen species- and nitric oxide-mediated lung inflammation and mitochondrial dysfunction in wild-type and iNOS-deficient mice exposed to diesel exhaust particles. *J Toxicol Environ Health A.* 2009;72(8):560–70.