

Aerobic exercise attenuates pulmonary injury induced by exposure to cigarette smoke

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Running title: Exercise training attenuates COPD development

Abstract

Background: It has recently been suggested that regular exercise reduces lung function decline and risk of COPD among active smokers, however the mechanisms involved in this effect remains poorly understood.

Objective: The present study evaluated the effects of regular exercise training in an experimental mouse model of chronic cigarette smoke (CS) exposure.

Methods: Male C57BL/6 mice were divided into four groups: control, exercise, smoke and smoke + exercise for 24 weeks. We measured respiratory mechanics, mean linear intercept (Lm), inflammatory cells and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BAL), collagen deposition in alveolar walls, and the expression of anti-oxidant enzymes, MMP9, TIMP1, IL10 and 8-isoprostane in alveolar walls.

Results: Exercise attenuated the decrease in pulmonary elastance ($P < 0.01$) and the increase in Lm ($P < 0.003$) induced by CS exposure. Exercise substantially inhibited the increase in ROS in BAL and 8-isoprostane expression in lung tissue induced by CS. In addition, exercise significantly inhibited the decreases in IL10, TIMP1 and CuZnSOD induced by exposure to CS. Exercise also increased the number of cells expressing GPx.

Conclusion: Our results suggest that regular aerobic physical training of moderate intensity attenuates the development of pulmonary disease induced by cigarette smoke exposure.

Key Words: aerobic exercise, chronic obstructive pulmonary disease, cigarette smoke, emphysema, oxidative stress.

INTRODUCTION

Although the majority of patients with COPD (80%) are cigarette smokers, only a small proportion of cigarette smokers (15–20%) develop COPD (1), suggesting that there are genetic and/or environmental susceptibility factors involved in the development of this syndrome. Recently, Garcia-Aymerich *et al.* (2) observed that active smokers who practiced moderate or high levels of regular physical activity had a reduced risk of developing COPD compared with the low physical activity group. Previous studies have shown that physical exercise increases the resistance against oxidative stress in skeletal muscle (3). Some epidemiological studies suggest that regular physical exercise reduces the incidence of diseases associated with oxidative stress (4). This phenomenon is considered a result of adaptation induced by exercise. This process involves the activation of the antioxidant system and redox reactions, and it influences transcription and thus gene expression and assembly of proteins (5).

Chronic obstructive pulmonary disease (COPD) is an important cause of morbidity, mortality, and health care costs worldwide (6). Its prevalence is around 9–10% among individuals over 40 years old but can reach up to 20% in some areas of the world (1). Tobacco smoking is the most important risk factor for COPD. Although the pathogenesis of COPD remains incompletely understood, the importance of oxidative stress in lungs and systemic circulation is well established (7). In the gas phase of cigarette smoke there are high concentrations of many oxidants/free radicals ($> 10^{15}$ molecules per puff) (8). In addition, there are also endogenous sources of reactive oxidant species (ROS), such as those produced by activated leukocytes recruited to the lungs in response to cigarette smoke. ROS derived from both cigarette smoke and endogenous sources can damage lipids, proteins, and nucleic acids and, in some cases, induce the formation of more stable reactive molecules that can prolong the oxidant-mediated damage (9).

The purpose of our study was to evaluate the effects of regular physical training during the establishment of pulmonary alterations induced by chronic (24 weeks) exposure to cigarette smoke in mice. Our hypothesis was that aerobic conditioning could attenuate the development of pulmonary disease induced by cigarette smoke exposure. Since there are strong evidences that an impairment in oxidant-antioxidant balance is important in the pathogenesis in pulmonary effects of cigarette smoke, we evaluated the effects of cigarette smoke, exercise training and both on the pulmonary oxidant-antioxidant balance in the lungs.

METHODS

Experimental groups. In all experiments, the Helsinki convention for use and care of animals was followed. Our protocol was approved by the institutional review board. Male C57BL/6 mice (6–8 weeks old) were divided at random into four groups (n = 25–32 per group): a) Control; b) Exercise (submitted to treadmill training); c) Smoke (exposed to cigarette smoke) and d) Smoke+Exercise (submitted to treadmill training and exposed to cigarette smoke). Animals were submitted to exercise training and exposed to cigarette smoke for 60 and 30 minutes/day, respectively, 5 days/week, for 24 weeks. The Smoke+Exercise group was initially submitted to the exercise protocol, the mice were allowed to rest for at least 60 minutes, and the exposure to cigarette smoke was started.

Cigarette smoke exposure protocol. Smoke exposure was performed in an inhalation chamber (28 L), with two inlets, (for air and smoke), one outlet and a fan, for enhancing air and smoke mixture inside the box. Synthetic airflow in the first inlet was set at 2 L/min, and the second inlet received a synthetic airflow that passed through a Venturi System connected to a lit cigarette, suctioning the cigarette smoke and conducting it to inside the box. The airflow rate in this second inlet could be changed, providing more or

less smoke mixture to the box. After several measurements of CO (carbon monoxide) concentration inside the box, we set this flow rate at 1.5 L/min, which produced CO levels ranging from 250 to 350 ppm (parts per million). Carboxyhemoglobin concentration in smoke-exposed mice was kept at 10% (± 1.3). Animals were exposed to 12 (± 1) commercially filtered cigarettes (0.8 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide per cigarette) and total particulate matter (TPM) concentration $354.8 \pm 50.3 \mu\text{g}/\text{m}^3$ per day. Mice allocated to cigarette smoke exposure were kept in the box maintaining these CO levels for 30 minutes a day, 5 days a week, for 24 weeks. Control mice were exposed to room air.

Treadmill aerobic training and test. Mice were initially adapted to the treadmill for 3 days (15 minutes/day, 25% inclination and 0.2 km/h). After that, a maximal exercise capacity test was performed with a 5-minute warm-up (25% inclination and 0.2 km/h) followed by an increase in treadmill speed (0.1 km/h every 2.5 min) until animal exhaustion, that is, until they were not able to run even after 10 gentle mechanical stimuli (10). The test was repeated four times: initial, 8, 16 and 24 weeks after the beginning of the exposure to either cigarette smoke or room air. Body weight, speed and total test time reached for each mouse were registered. Maximal aerobic capacity (100%) was established as the speed reached by each animal. Mice were trained at a moderate intensity of exercise (50% of maximal speed) for 60 minutes/day, 5 days/week for 24 weeks. Aerobic conditioning began at the same day of the beginning of cigarette smoke exposure and continued until the end of the 24th week. Both protocols were performed in the morning. The Smoke/Exercise group was initially submitted to the exercise protocol, then mice were allowed to rest for at least 60 minutes to allow respiratory frequency to return to basal (pre-exercise) values and the exposure to cigarette smoke was started.

Respiratory mechanics evaluation. Twenty-four hours after the end of the exposure protocol, mice were anesthetized, tracheostomized, and mechanically ventilated (n = 10–15 per group) using a FlexiVent ventilator (Scireq, Montreal, Canada). Using the force oscillatory technique and a constant phase model (11), airway resistance (Raw), tissue damping (Gtis) and tissue elastance (Htis) parameters were obtained. Gtis and Htis were normalized to body weight (12).

Total Cells, Macrophages and ROS measurements in Bronchoalveolar Lavage (BAL). Immediately after lung mechanics measurements, mice were killed by exsanguinations, and BAL samples were collected after washing the lungs with 0.5 ml of sterile saline. This procedure was repeated 3 times. Four hundred microliters of cell suspension was added to 10 µl of 5 mM luminol prepared in dimethyl sulfoxide (DMSO) (Sigma). ROS production was measured by a chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co, St Louis, MO) as the probe and a luminometer (MicroBeta TriLux, version 4.7; Perkin Elmer Life Sciences, Turku, Finland) for 15 minutes (13). The results were expressed as 10⁴ counted photons per minute (ppm) per total cells in BAL. The fluid BAL collected was centrifuged at 900G, for 8 minutes, on 5°C, and the cell pellet was resuspended in 1 mL of physiological saline. Total cells were counted using a Neubauer hemocytometer chamber and differential cells (300 cells/slide) were evaluated by microscopic examination of BAL samples prepared in cytocentrifuge slides, stained with Diff Quick (14).

Lung Histology and Immunohistochemistry. Lungs were fixed at a constant pressure (20 cmH₂O), and 5-µm-thick sections of lung tissue were stained with H&E to measure mean linear intercept (Lm), an indicator of mean diameter of airspaces as described previously (15). Sirius red (16) staining was performed to evaluate collagen fibers by image analysis. Lung tissue was also immunostained with the following

antibodies: anti-GPx (goat polyclonal, 1:150), anti-CuZnSOD (goat polyclonal, 1:400), anti-MnSOD (goat polyclonal, 1:200), anti-MMP9 (goat polyclonal, 1:400), anti-TIMP1 (rabbit polyclonal, 1:250), anti-IL10 (rabbit polyclonal, 1:200), anti-ECSOD (goat polyclonal, 1:400) (Santa Cruz Biotechnology, CA, USA) and anti-8-isoprostane (goat polyclonal, 1:500) (Oxford Biomedical, USA) using the biotin-streptavidin peroxidase method. Secondary antibody (Vector ABC Kit Vectastain Anti-rabbit or Vector ABC Kit Vectastain Anti-Goat, both diluted 1:500) were used for reactions. These secondary antibodies were also applied without primary antibody in positive and negative tissue samples, serving as control. Positive cells immunostaining to GPx, CuZnSOD, MnSOD, MMP9, TIMP1 and IL10 in the alveolar parenchyma were assessed using a 100-point grid with a known area (62.500 μm^2 at a x400 magnification) attached to the microscope ocular. We counted the number of points hitting alveolar tissue in each field. The alveolar tissue area in each field was calculated according to the number of points hitting alveolar tissue, as a proportion of the total grid area. We then counted the number of positive cells within that alveolar tissue area and expressed the results as cells/106 μm^2 (17). We also evaluated the volume proportion of 8-isoprostane or SOD-EC positive area expressed as the percentage (%) of the positively immunostained area in relation to the total area of alveolar tissue. We clarify that the area of airways and large vessels were excluded of the total area counted for analysis.

Statistical Analysis. Statistical analysis was performed using Sigma Stat 10 software (Systat Software, Inc., San Jose, CA, USA). Comparisons among groups were carried out by analysis of variance, followed by Holm-Sidak method for parametric data or by analysis of variance on ranks followed by Dunn's method for nonparametric data. Differences were considered significant at $P < 0.05$.

RESULTS

Physical test (time and speed). Figure 1 shows that after 8 weeks of training, the time that the mice could exercise on the treadmill at maximal speed was significantly greater in the groups Exercise and Smoke+Exercise compared to the groups that were not trained (Control and Smoke) ($P<0.001$). Similar results were observed after 16 and 24 weeks of training (Figure 1A). Exposure to cigarette smoke did not influence the results of the exercise tests. In fact, there were no significant differences between Control and Smoke groups or between Exercise and Smoke+Exercise groups. Similar results were observed when maximal speed reached by the mice during the treadmill test was examined (Figure 1B). **Respiratory mechanics evaluation.** Airway resistance (R_{aw}), tissue damping (tissue resistance, G_{tis}) and tissue elastance (H_{tis}) were computed (Figures 2A–C). The values of H_{tis} and G_{tis} were normalized to body weight because the experimental groups that were trained presented greater body mass at the end of the experimental protocol ($p<0.01$ compared to groups Control and Smoke, data not shown) (14). We did not observe significant differences in R_{aw} values when the four experimental groups were compared (Figure 2A). Exposure to cigarette smoke resulted in lower values of G_{tis} (Figure 2B) ($P<0.01$ compared to the two groups that were not exposed to smoke). Physical conditioning did not influence this response. In contrast, pulmonary elastance values (Figure 2C) were lower in the group exposed to cigarette smoke for 24 weeks ($P<0.01$ compared to the other three groups) and this decrease was not observed in Smoke+Exercise group.

Emphysema development and pulmonary parenchyma remodeling . Exposure to cigarette smoke for 6 months resulted in an increase in airspace size and rupture of alveolar septa, hallmarks of emphysema. We observed an increase in mean linear intercept

(Lm) in the two lung compartments in the group of mice exposed to cigarette smoke only (Smoke group) ($P=0.003$ and $P=0.002$ compared to the other three groups, respectively for central and peripheral airspaces) (Figures 3A-E). The group of mice exposed to cigarette smoke and submitted to the aerobic conditioning protocol (Smoke+Exercise group) did not show the increase in Lm compared to Control and Exercise groups.

Exposure to cigarette smoke resulted in the remodeling of alveolar wall (Figures 3F-J) There was a significant increase in collagen fibers in the lungs of mice that were exposed to cigarette smoke ($P<0.001$ compared to the groups not exposed to smoke). Physical training did not influence this response to cigarette smoke.

Leukocytes and reactive oxygen species (ROS) in bronchoalveolar lavage fluid (BAL). Exposure to cigarette smoke resulted in an increase in the number of macrophages in BAL (Figure 4A, $P<0.01$ compared to the groups that were not exposed to smoke). Exercise training did not influence this response. There were no significant differences in the numbers of neutrophils or lymphocytes in BAL among the four experimental groups (data not shown). Figure 4B shows the concentrations of ROS in BAL. Exposure to cigarette smoke increased the production of ROS ($P=0.029$ compared to Control), and aerobic training significantly reduced ROS production ($P=0.001$ compared to Control). The group of mice that were submitted to both exercise and cigarette smoke showed values of ROS similar to the group that exercised but was not exposed to smoke ($P<0.001$ compared to Smoke group).

Immunohistochemical studies. The Smoke group had a lower density of interleukin 10 (IL10)-positive cells than both groups that exercised (Figure 4C, $P=0.013$ and $P=0.009$ vs. Exercise and Smoke+Exercise groups, respectively). Exposure to cigarette smoke resulted in an increase in the amount of 8-isoprostane in lung parenchyma, and this increase was not observed in the Smoke+Exercise group (Figure 4D-H). In fact,

expression levels of 8-isoprostane in lung parenchyma were significantly greater in the Smoke group compared to the other three groups ($P<0.01$).

Exercise training resulted in an increase in the density of cells expressing glutathione peroxidase (GPx) in alveolar walls ($P<0.001$, Exercise vs. Control and Smoke groups, Figures 5A-E). Smoke+Exercise group showed intermediate values of cells expressing GPx ($P<0.001$, $P=0.008$, and $P=0.046$ compared to Exercise, Smoke, and Control groups, respectively). Exposure to cigarette smoke reduced the density of cells in alveolar walls positive for superoxide dismutase CuZn (CuZnSOD), and exercise training inhibited this reduction (Figure 5F-J, $P<0.01$, Smoke group vs. the other three groups). Exposure to cigarette smoke and aerobic physical training did not change the density of alveolar wall cells positive for MnSOD and extracellular superoxide dismutase (EC-SOD) (Figures 5K and L).

Smoke exposure reduced the density of alveolar wall cells positive for tissue inhibitor of metalloproteinase 1 (TIMP1), and physical training inhibited this reduction (Figure 6A-E, $P<0.02$, Smoke vs. other three groups). Smoke exposure and aerobic physical training did not significantly change the density of cells in alveolar walls positive for matrix metalloproteinase 9 (MMP9) (Figure 6F).

DISCUSSION

In this study, we showed that aerobic physical training of moderate intensity reduces oxidative stress and protects against emphysema development induced by cigarette smoke exposure in mice. Exercise training inhibited the increase in mean linear intercept and the decrease in pulmonary elastance induced by cigarette smoke but did not affect

collagen fiber remodeling. In addition, we observed a reduction in the production of reactive oxygen species in BAL, lower accumulation of 8-isoprosane in pulmonary tissue, and an increase in the expression of anti-oxidant enzymes GPx and CuZnSOD induced by exercise training. These results suggest that aerobic training has a protective effect on the imbalance between oxidants and antioxidants involved in the pathogenesis of cigarette smoke induced pulmonary disease.

There are many previous studies evaluating the effects of exercise training or cigarette smoke exposure on the oxidant-antioxidant balance. However, only few studies aimed to evaluate the combined effects of exercise training and smoke. To our knowledge, our study was the first one to evaluate the effects of exercise training on the development of emphysema during long-term (24 weeks) cigarette smoke exposure and to evaluate the possible mechanisms of the protective effects of long-term regular exercise, including the expression of enzymes involved in the oxidant-antioxidant balance in the lungs.

The experimental model used in our study shared several features with human COPD, such as exposure to cigarette smoke being a risk factor, a reduction in lung elastance and the development of pulmonary emphysema (18).

It is well established that several months of exposure to cigarette smoke are required for mice to develop pulmonary emphysema (usually in the order of 24 weeks) (18). The susceptibility of mice to this disease is dependent on the mouse strain (19). C57BL/6 mice exposed to cigarette smoke for 24 weeks show enlargement of airspaces and a pulmonary histology similar to human emphysema (20). A previous study showed a partial improvement in pulmonary alterations in C57BL/6 mice exposed to cigarette smoke for 2 months and subjected to swimming (21).

Interestingly, in our study, mice exposed to cigarette smoke did not perform worse in the physical tests when compared to mice not exposed to smoke. This probably occurred

because the physiological changes present in experimental models of COPD induced by exposure to cigarette smoke mimic a mild form of the disease in humans (22).

We used the mean linear intercept (Lm) as a marker of the degree of alveolar distension, and our results are consistent with the development of emphysema, similar to previous studies (21, 23). We observed enlargement of airspaces in the two lung compartments, defined by the distance from the pleura to the airways, suggesting that the lesion that develops in the lung in this experimental model is diffuse.

In our protocol, the effects of cigarette smoke exposure were more intense in pulmonary parenchyma than in the airways. In fact, we did not observe a significant difference in airway resistance (Raw) values between control group and mice exposed to CS and we did not observe an increase in the density of inflammatory cells in the airways of mice exposed to CS (data not shown). In contrast, we found a decrease in lung elastance (Htis), suggesting a decrease of elastic recoil capacity, compatible with the functional profile of human pulmonary emphysema, and a decrease in tissue damping (tissue resistance, Gtis). Our protocol of aerobic physical training protected the mice from the decrease in lung elastance (Htis) observed in mice exposed to cigarette smoke, which was probably secondary to the inhibition of alveolar wall destruction by exercise. In contrast, we did not observe an effect of aerobic training in tissue damping (Gtis). Tissue resistance may be influenced by the amount of collagen fibers in alveolar walls, and aerobic conditioning did not have an effect on the amount of alveolar wall collagen. Collagen fibers are the most abundant fibers in alveolar walls and perhaps most critical for the mechanical properties of lung parenchyma (24). Interestingly, in a protocol of short-term (2-month) exposure to cigarette smoke, Menegali *et al.* did not observe an effect of daily swimming on the amount of proline in the lungs of C57BL/6 mice (21).

Although the pathogenesis of COPD remains incompletely understood, oxidant–antioxidant and protease–antiprotease imbalances and pulmonary inflammation are central processes associated with the development of this disease, but the participation of each of these mechanisms and how they interact are still unclear (7). Previous studies have shown that chronic exercise training induces the activity of some antioxidant enzymes (25) and anti-inflammatory mediators (26).

We observed an increase in the total number of inflammatory cells and macrophages in BAL of mice exposed to cigarette smoke, corroborating previous studies (27). Exercise training did not modify the quantities of these cells in BAL of mice exposed to cigarette smoke. However, exercise training substantially inhibited ROS production in BAL of both control mice and mice exposed to cigarette smoke. In addition, the density of 8-isoprostane in lung parenchyma was increased in mice exposed to cigarette smoke, and exercise training inhibited this increase. Isoprostanes are prostaglandin-like compounds that are formed from the peroxidation of arachidonic acid and are considered accurate markers of oxidative stress *in vivo*. 8-Isoprostanes are increased in the urine of COPD patients (28).

It is possible that the decrease in ROS production in BAL and in the expression of 8-isoprostanes in pulmonary tissue were due to an increase in the activity of antioxidant enzymes. In fact, we observed an increase in the density of cells expressing the antioxidant enzyme GPX in the lungs of mice submitted to the exercise training protocol. Exercise training also reversed the decrease in the density of lung cells expressing CuZnSOD induced by cigarette smoke. Glutathione peroxidase (GPX) has a role in the protection against lipid peroxidation due to decomposition of lipid hydroperoxides (29), and superoxide dismutases (SOD) are the primary class of enzymes that initiate the process of

detoxifying superoxide anion by converting it into hydrogen peroxide. It was previously shown that both GPX and SOD increase in skeletal muscle after exercise training (25).

In our study, exposure to cigarette smoke did not change the expression of GPX in the lung. Other studies did not show any appreciable changes in the GPX activity in lung tissue obtained from chronic smokers (30). In contrast, in an experimental model of exposure to cigarette smoke, GPX activity was increased (31).

Aerobic exercise inhibited the reduction of superoxide dismutase copper zinc (CuZnSOD) found in mice exposed to smoke, but the expression of extracellular and manganese SODs was not different in the four experimental groups. There are three mammalian SODs. SOD1, copper zinc SOD, is located mainly in the cytosol of cells; mitochondrial manganese SOD (MnSOD), or SOD2, is predominantly located in the mitochondria and the cytosol; and SOD3, or extracellular SOD (ECSOD), is present outside the cells, where it is bound to matrix proteins such as collagen (32). Other studies showed a reduction in the total antioxidant activity of CuZnSOD in the airway epithelium of asthmatics (33) and an increase in the production of superoxide anion in these patients (34). Despite the fact that excessive production of reactive oxygen species, particularly superoxide anion, is implicated in the pathophysiology of chronic obstructive pulmonary disease (7), we are not aware of previous reports showing the expression of CuZnSOD in this disease.

Rush *et al.* (2003) observed an elevation of CuZnSOD in the aortic endothelium of trained guinea pigs (35), and, although the protein levels of manganese superoxide dismutase were not altered, the levels of p67phox, a subunit of the enzyme pro-oxidant NADPH oxidase, was reduced by exercise.

Previous studies have shown that aerobic exercise can produce anti-inflammatory effects against allergic lung inflammation, and part of the anti-inflammatory effects of

exercise are mediated by the release of anti-inflammatory cytokines IL10 and IL1ra, and also by its direct regulation of the release of IL1 β and TNF- α (10, 36). The expression of IL10, a potent anti-inflammatory cytokine, was reduced in our mice exposed to cigarette smoke, in contrast to the results of Guerassimov *et al.* (19). In our study, this smoke-induced reduction was inhibited by exercise training. IL10 modulates the expression of some cytokines, soluble mediators and cell surface molecules by cells of myeloid origin, influencing the ability to activate and sustain immune and inflammatory responses (37).

Inhalation of cigarette smoke can also lead to the secretion of MMP2, MMP9, MMP12, neutrophil elastase and cathepsins K, L and S by macrophages (Abboud). Some studies suggest that pro-inflammatory stimuli induced by smoking may regulate the activity of MMP9 in COPD (38). However, in our mice the expression of MMP9 was not increased by exposure to smoke, but we found a decrease in TIMP1 in mice exposed to cigarette smoke that was reversed by aerobic exercise. A decrease in the release of TIMPs by alveolar macrophages in COPD has also been seen by Hirano *et al.* (39). Some studies have shown that IL10 suppresses the release of MMP9 from monocytes of patients with COPD and, at the same time, stimulates the release of its major endogenous inhibitor, TIMP1 (40). It is likely that the imbalance between proteases and their macrophage antiproteases have a pathogenic role in our experimental model of emphysema.

Our study has some limitations: we did not evaluate the time course of the development of pulmonary effects of cigarette smoke and the influence of exercise on the oxidant-antioxidant balance during the emphysema development. In addition, we did not evaluate the effects of both cigarette smoke and aerobic training on the skeletal muscles. Future studies should be performed to clarify these points.

Many benefits of exercise training in COPD patients have been previously shown. Exercise alleviates symptoms, improve quality of life, decrease exacerbation

episodes and the risk of respiratory mortality in COPD. Our study suggests that exercise training may be important not only in pulmonary rehabilitation in COPD but also as an adjuvant in the prevention and progression of pulmonary disease due to cigarette smoking.

In summary, we have shown that regular aerobic physical training of moderate intensity reduces oxidative stress and protects against the development of emphysema in mice. Exercise training inhibited the emphysema development by a significant decrease in oxidative stress, with reductions in reactive oxygen species production in BAL and in 8-isoprostane expression in lung parenchyma, and with increases in antioxidant enzymes (GPX and CuZnSOD), TIMP1, and IL10 in the inflammatory cells of the alveolar wall. These results support the hypothesis that the antioxidant effect of exercise combats cellular oxidative stress, which is induced by cigarette smoke and has an important role in COPD development.

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FIGURE LEGENDS

Figure 1. Results of the tests performed on the treadmill to evaluate the intensity of aerobic conditioning of the studied mice. Tests were performed before the beginning of the exposure to cigarette smoke (time 0) and after 8, 16 and 24 weeks of the protocol. (A) Time that the mice could exercise in the treadmill; (B) Maximal speed reached by each mouse among the four experimental groups. Values are means and SD. Control, mice that were not trained and not exposed to cigarette smoke; Smoke, mice exposed to cigarette smoke and not trained; Exercise, mice submitted to the protocol of aerobic conditioning; Smoke+Exercise, mice exposed to cigarette smoke and trained. * $P < 0.001$ comparing Control and Smoke to Exercise and Smoke+Exercise.

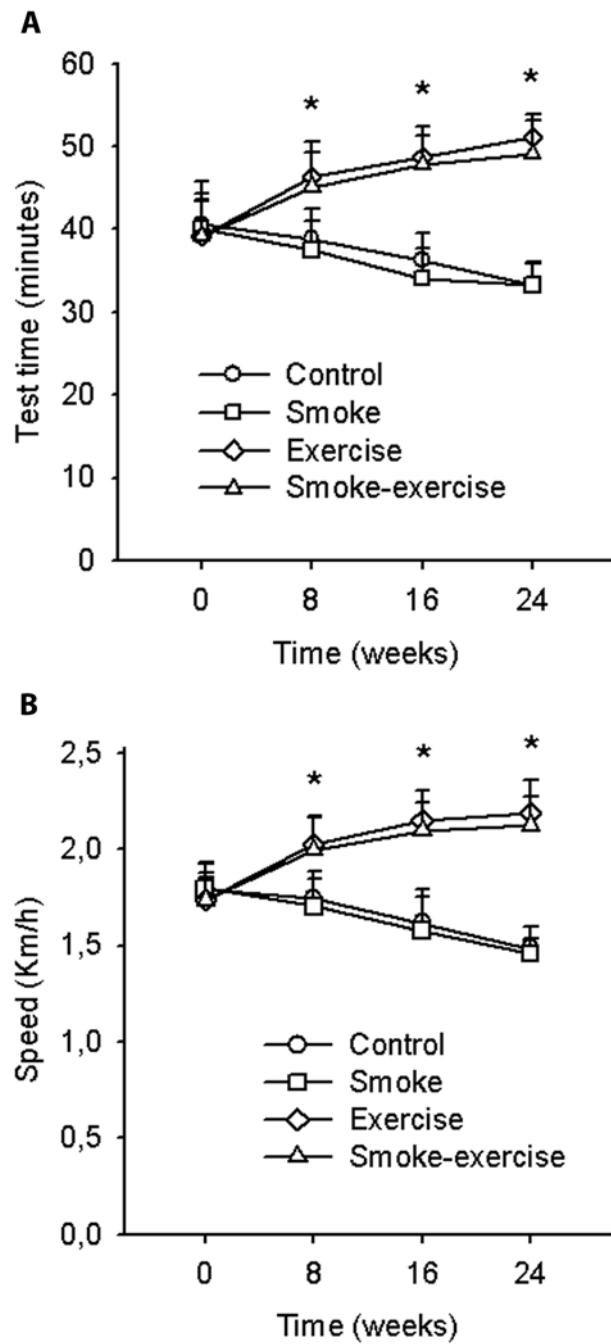


Figure 2. (A) Airway resistance (Raw), (B) pulmonary elastance (Htis) and (C) tissue damping (tissue resistance, Gtis) obtained in the four experimental groups, after 24 weeks of the experimental protocol. Values are means and SD. The values of Htis and Gtis were normalized to body weight. * $P < 0.01$ compared to the two groups that were not exposed to smoke. ** $P < 0.01$ compared to the other three groups.

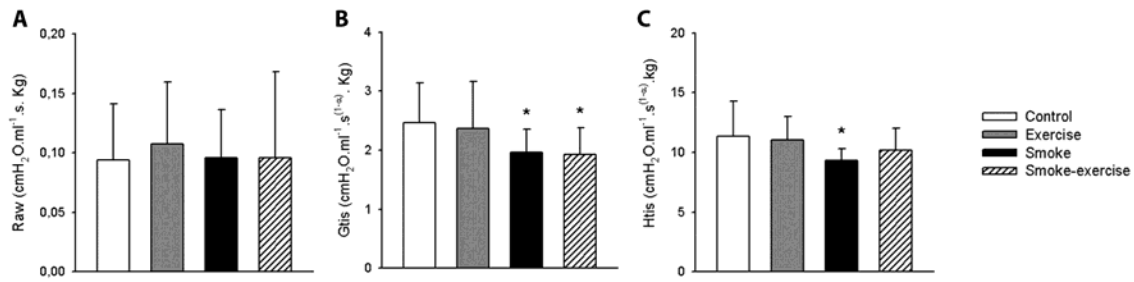


Figure 3. (A-D) Representative photomicrographs of hematoxylin and eosin-stained pulmonary parenchyma of Control, Exercise, Smoke and Smoke+Exercise groups, respectively. Scale bars, 50 μm . (E) Mean (and SD) values of mean linear intercept (Lm) measured in central and peripheral areas of the lungs. $*P=0.003$ and $**P=0.002$ compared to the other three groups. (F-I) Representative photomicrographs of slices of lung parenchyma from the Control, Exercise, Smoke and Smoke+Exercise groups, respectively, stained with Sirius red for collagen fibers (*arrows*). Scale bars, 50 μm . (J) Percentage of the alveolar wall with collagen fibers (means and SD). $*P<0.001$ compared to the groups not exposed to cigarette smoke.

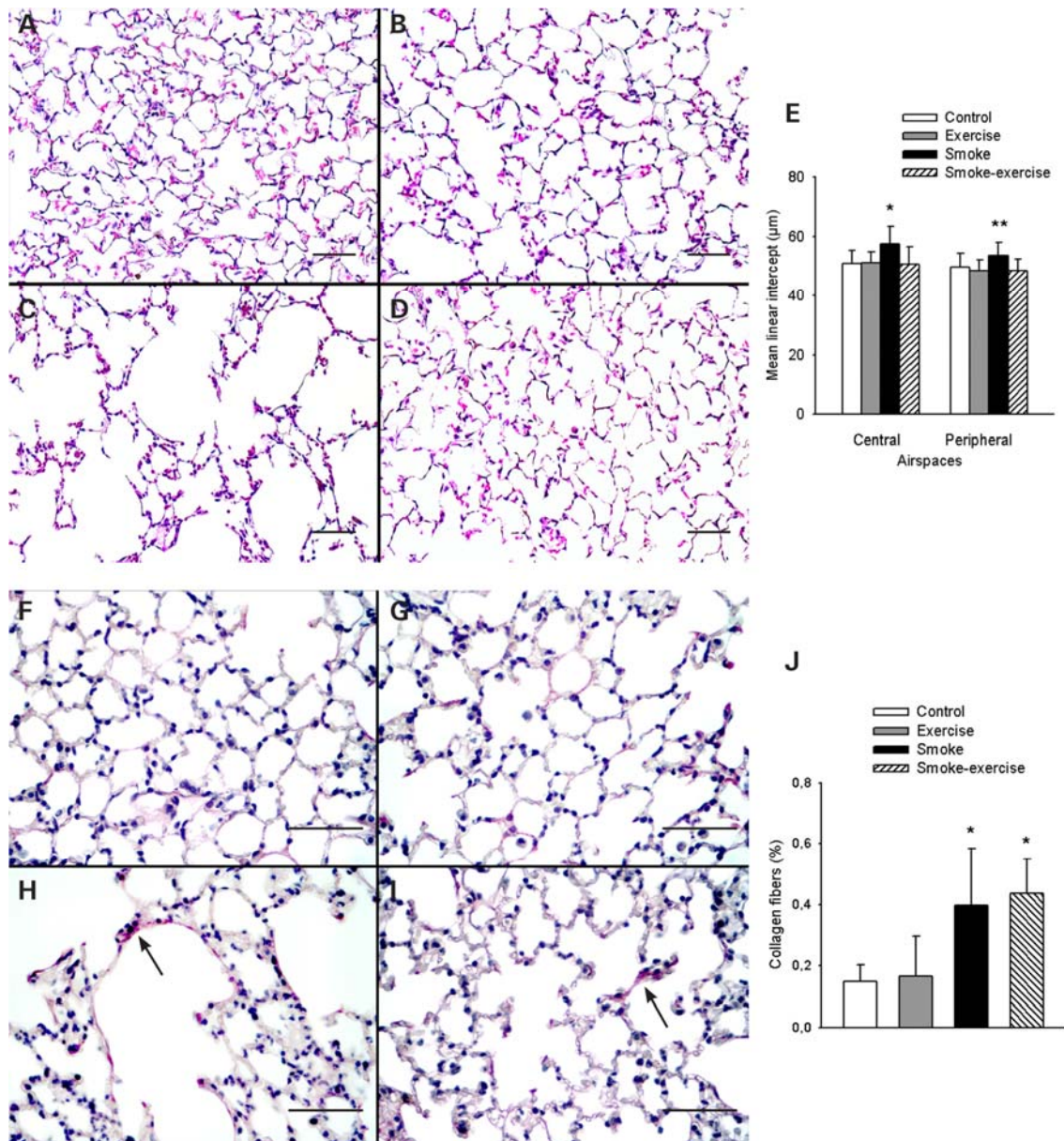


Figure 4. (A) Number of macrophages in bronchoalveolar lavage fluid (BAL). Values are means and SD. * $P < 0.01$ compared to the groups not exposed to cigarette smoke (Control and Exercise). (B) Concentration of reactive oxygen species (ROS) in BAL. * $P = 0.029$ compared to Control group; ** $P = 0.001$ compared to Control group; *** $P < 0.001$ compared to Smoke group. (C) Density of cells in alveolar walls positive for IL10. * $P = 0.013$ and $P = 0.009$ vs. Exercise and Smoke+Exercise groups, respectively (D-G) Representative photomicrographs of slices of lungs from the Control, Exercise, Smoke and

Smoke+Exercise groups, respectively, immunostained with anti-8-isoprostane (*arrows*).

Scale bars, 50 μm . (H) Density of alveolar tissue immunostained with antibody against 8-

isoprostane. * $P < 0.01$ compared to the other three experimental groups.

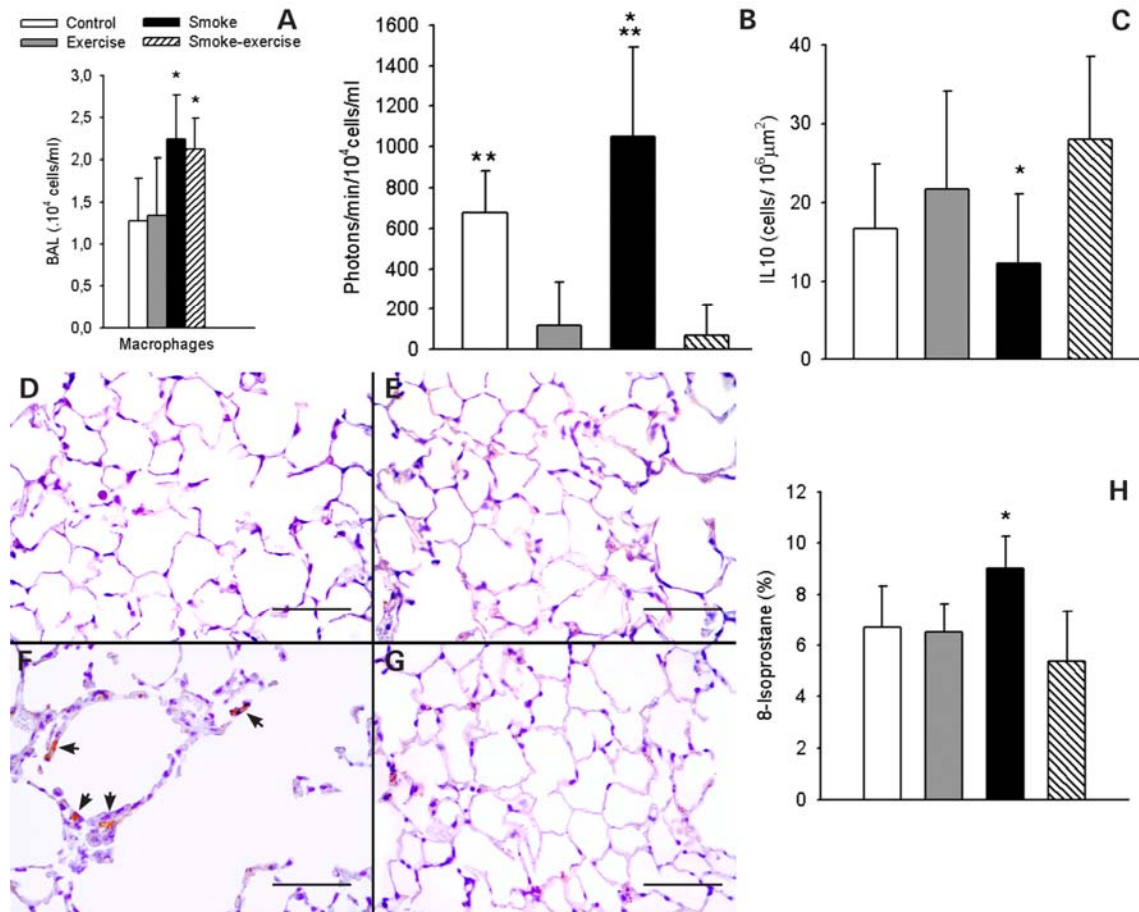


Figure 5. (A-D) Representative photomicrographs of slices of lungs from Control, Exercise, Smoke and Smoke+Exercise groups, respectively, immunostained with anti-GPx. (*arrows*) (magnification $\times 400$). Scale bars, 50 μm . (E) Density of cells expressing glutathione peroxidase (GPx) in alveolar walls. * $P < 0.001$ compared to Control and Smoke groups. Mean and SD; ** $P < 0.001$, $P = 0.008$ and $P = 0.046$ compared to Exercise, Smoke and Control groups, respectively. (F-I) Representative photomicrographs of slices of lungs from the four experimental groups immunostained with anti- superoxide dismutase CuZn (CuZnSOD). Scale bars, 50 μm . (J) Density of cells in alveolar walls positive for

CuZnSOD $*P < 0.01$ compared to the other three groups. (K) Density of cells in alveolar walls positive for MnSOD. (L) Density of cells in alveolar walls positive for extracellular superoxide dismutase (EC-SOD).

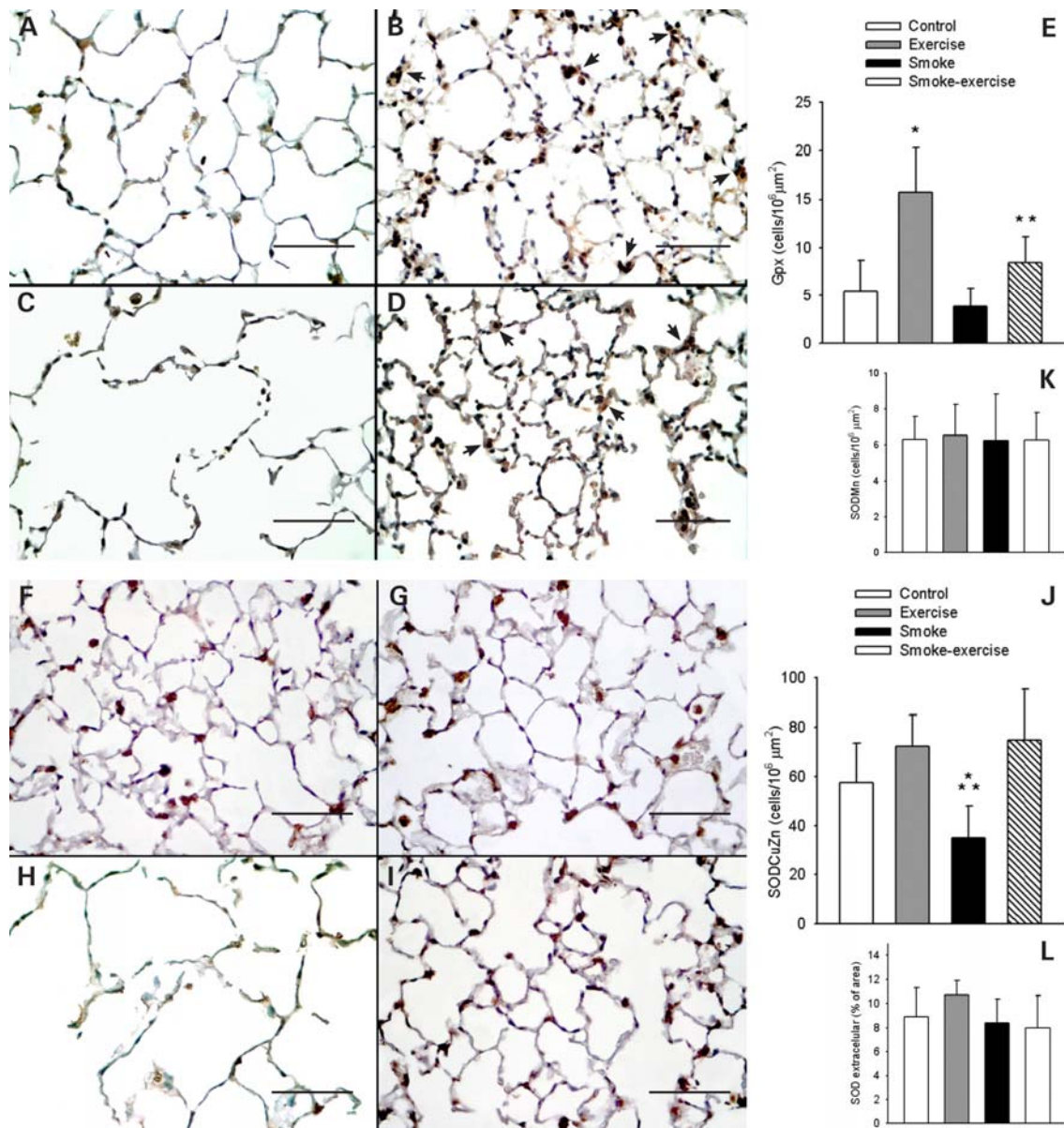


Figure 6. (A-D) Representative photomicrographs of slices of lungs from the Control, Exercise, Smoke and Smoke+Exercise groups, respectively, immunostained with anti-TIMP1. Scale bars, 50 μm. (E) Density of cells in alveolar walls positive for tissue inhibitor of metalloproteinase 1 (TIMP1). $*P < 0.02$ compared to other three groups. (F) Density of cells in alveolar walls positive for matrix metalloproteinase 9

(MMP9).

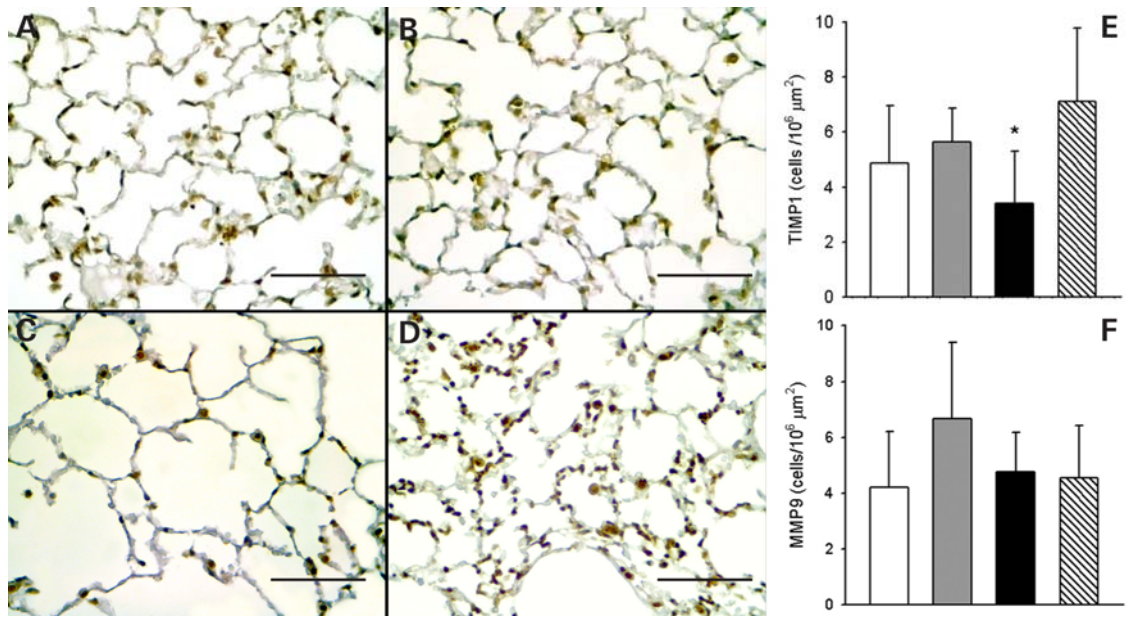


Figure 7

