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Time-course effects of aerobic physical training in the prevention of cigarette smoke-induced COPD

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Running head: Exercise training attenuates COPD development

Abstract

A previous study by our group showed that regular exercise training (ET) attenuated pulmonary injury in an experimental model of chronic exposure to cigarette smoke (CS) in mice, but the time-course effects of the mechanisms involved in this protection remain poorly understood. We evaluated the temporal effects of regular ET in an experimental model of chronic CS exposure. MaleC57BL/6 mice were divided into four groups: Control (sedentary+air), Exercise (aerobic training+air), Smoke (sedentary+smoke) and Smoke+Exercise (aerobic training+smoke). Mice were exposed to CS and ET for 4, 8 or 12 weeks. Exercise protected mice exposed to CS from emphysema and reductions in tissue damping and tissue elastance after 12 weeks (P<0.01). The total number of inflammatory cells in the BAL increased in the Smoke group, mainly due to the recruitment of macrophages after 4 weeks, neutrophils and lymphocytes after 8 weeks and lymphocytes and macrophages after 12 weeks (P<0.01). Exercise attenuated this increase in mice exposed to CS. The protection conferred by exercise was mainly observed after exercise adaptation. Exercise increased IL-6 and IL-10 in the quadriceps and lungs (P<0.05) after 12 weeks. Total antioxidant capacity and SOD was increased and TNF-α and oxidants decreased in lungs of mice exposed to CS after 12 weeks (P<0.05). The protective effects of exercise against lung injury induced by cigarette smoke exposure suggests that anti-inflammatory mediators and anti-oxidant enzymes play important roles in COPD development mainly after the exercise adaptation.

Keywords: cigarette smoke; emphysema; aerobic exercise; anti-inflammatory; oxidative stress.

New & Noteworthy:

These experiments investigated for the first time the temporal effects of regular moderate exercise training in the cigarette smoke-induced chronic obstructive pulmonary disease. We demonstrate that aerobic conditioning had a protective effect in emphysema development induced by cigarette smoke exposure. This effect was most likely secondary to an effect of exercise on oxidant-antioxidant balance and anti-inflammatory mediators.

INTRODUCTION

Regular aerobic exercise plays a protective role in the pathogenesis of several lung diseases, such as asthma (22,47), pulmonary infection (29), air pollution exposure (47) and cigarette smoke (CS)-induced chronic obstructive pulmonary disease (COPD)(25, 45). Garcia-Aymerich et al. (2007) showed that moderate or high levels of regular physical activity reduce the risk of developing COPD in active smokers (15). We and others have also demonstrated that moderate aerobic exercise performed regularly attenuates smoking-induced airway inflammation, changes in pulmonary mechanics, and oxidative damage and protects mice from emphysema (25, 45).

The innate and adaptive inflammatory immune responses are the primary host defence mechanisms of the lungs against exposure to thousands of reactive chemicals and particles and trillions of free radicals contained in cigarette smoke (6). Macrophages, which are activated by oxidants present in CS, secrete inflammatory cytokines such as TNF-α and IL-6, attracting neutrophils and cells from acquired immunity (51). The adaptive immune response is dependent on lymphocytes following tissue injury, suggesting that these inflammatory cells are responsible for lung injury caused by cigarette smoke (8). Oxidative stress also induces the generation of cytokines, stress response gene expression and production of antioxidative enzymes such as MnSOD and thioredoxin (50).

Exercise exerts anti-inflammatory and anti-oxidant effects in part mediated by myokines such as IL-6 and IL-10 released during muscle fibers contraction (19). We hypothesized that the protection against the emphysema development induced by exercise training would be observed after aerobic conditioning.

Understanding the mechanisms mediating favourable changes in the lungs before the progression of emphysema is critical for the development of novel therapies and protective strategies. However, the time-course effects of exercise on the mechanisms involved in the protection of emphysema development remain poorly understood. Therefore, the purpose of our study was to investigate the time course of the potential mechanisms (oxidative stress, inflammation and antioxidant activity) through which aerobic exercise protects mice from changes induced by CS exposure.

METHODS

Experimental groups. Male six- to eight-week-old C57BL/6J mice were grouped with free access to food and water and were maintained at constant temperature on a 12:12-h light: dark cycle. Animals were divided at random into four groups (n=24 per group): a) Control (sedentary+air); Exercise (submitted to treadmill training +air), Smoke (sedentary+smoke) and Smoke+Exercise (submitted to treadmill training + exposed to CS). In all experiments, the Declaration of Helsinki was followed for the use and care of the animals. The protocol was approved by the Ethical Committee of the University of Sao Paulo (CAPpesq/025/10). Animals were submitted to exercise training/testing and cigarette smoke exposure as previously described (12, 47). The experiments were repeated three times using 10 animals per group in each set. These animals were divided among morphological, functional and molecular analyses.

Aerobic Exercise Training and Testing. Briefly, the treadmill training sessions consisted of 30 minutes/session/day, twice a day, 5 days/week for four, eight or

twelve weeks. Mice were trained at 50% of maximal speed (moderate intensity) reached in the maximal aerobic capacity testing and 25% inclination (45, 47). Maximal aerobic capacity testing was performed with five minutes warm-up (0.2 km/h) followed by an increase in treadmill speed (0.1 km/h every 2.5 min) until animal exhaustion, as described previously (47). Daily exercise sessions were performed with four hours in between. After the second session of exercise mice remained resting for at least 60 minutes before exposure to cigarette smoke. The aerobic capacity test was repeated at four, eight and 12 weeks after the beginning of the exposure to either cigarette smoke or room air.

Cigarette smoke exposure. Twenty-four commercially filtered cigarettes per day were used in this study (10 mg tar, 0.8 mg nicotine and 10 mg CO per cigarette). CS exposure occurred 30 minutes/session/day, twice a day, 5 days/week for four, eight or 12 weeks by using a custom-made smoking machine (45).

Twenty-four hours after the end of the exposure/training protocols animals from each group (n=24/group) were divided into three groups of eight animals per group to avoid interference in the following measurements: pulmonary mechanics and bronchoalveolar lavage (n=8/group); lung histology (n=8/group) and molecular assays (n=8/group).

Pulmonary mechanics evaluation. Mice (n=8/group) were deeply anaesthetised by an intraperitoneal injection of 50 mg/kg of sodium pentobarbital, tracheotomised and mechanically ventilated with a tidal volume of 10 mL/kg, respiratory rate of 120 cycles/min and sinusoidal inspiratory flow curve using a ventilator for small animals (FlexiVent, SCIREQ, Scientific Respiratory Equipment, Montreal, Quebec). To abolish their breathing effort, the animals received pancuronium (0.2

mg/kg intraperitoneal). The respiratory system input impedance (Zrs) was measured by applying 16 s of oscillatory airflow perturbation (frequencies from 0.25 to 9.125 Hz), with the exhalation valve kept closed. The pressure values were generated, and the impedance was calculated as a function of different frequencies produced. To calculate the respiratory mechanics parameters of airway resistance (Raw), tissue damping (Gtis) and tissue elastance (Htis) from Zrs data, we used the constant phase model described by Hantos et al.(16):$Z(f) = Raw + i(2\pi f)Iaw + \frac{[Gtis - iHtis]}{(2\pi f)^{\alpha}}$

In this model, Z(f) is the impedance as a function of frequency, i is the imaginary component, f is the frequency, Iaw is the inertance of the airways, and $\alpha = 2/\pi * \arctan(Htis/Gtis)$.

Bronchoalveolar lavage (BAL). Immediately after respiratory mechanics measurements, mice were euthanized and BAL samples were collected after washing the lungs with 3 x 0.5 mL of sterile saline. The fluid was centrifuged at 900xg for 8 minutes at 5°C, and the cell pellet was resuspended in 1 mL of physiological saline. Total cells were counted using a Neubauer haemocytometer chamber, and differential cells (300 cells/slide) were evaluated by microscopic examination of BAL samples prepared on cytocentrifuge slides and stained with Diff Quick (Medion Diagnostics, Dundingen, Switzerland)(31).

Lung histology. In a different group of mice euthanized after anaesthesia (n=8/group) lungs were removed and fixed at a constant pressure (20 cmH_2O) (45). Five-µm-thick sections of lung tissue were stained with H&E to measure

mean linear intercept (Lm), an indicator of the mean diameter of airspaces, as described previously (42). The Lm were analyzed in a blinded fashion and 15 non-overlapping fields were assessed for each slide (n=9 per group).

Enzyme-linked immunosorbent assay (ELISA). Mice were euthanized after anaesthesia (n=8/group). Tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 10 (IL-10) were quantified in lung and skeletal muscle (quadriceps) homogenates samples 24 hours after the last session using ELISA kits from eBioscience, (San Diego, CA, USA) and a microplate reader (BioTek, Biosystems). The readings were taken at a wavelength of 450 nm, and the results are expressed in pg/mL (22).

Measurement of tert-butyl hydroperoxide-initiated CL. Tissues were homogenised in an Ultraturrax homogeniser containing 10 mg/mL of tissue in 30 mMKH_2PO_4/K_2HPO_4 buffer and 120 mMKCl at pH 7.4. The total homogenate was used for tert-butyl hydroperoxide–stimulated chemiluminescence. A supernatant obtained from the total homogenate after centrifugation at 11,000xg was used for antioxidant assays. Reaction mixtures were placed in 2-mL luminescence tubes containing the following: lungs homogenate (8.75 mg/mL), 10 mMKH_2PO_4/K_2HPO_4 buffer (with 120 mM KCl, pH 7.4), and 6 Mm tert-butyl hydroperoxide in a final volume of 1 mL. The tert-butyl hydroperoxide-initiated CL reaction was assessed using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA), with a response range of 300–650 nm. The tubes were kept in the dark until the assay was conducted in a room at 33°C (17, 29).For each animal, a 40-min curve in which each point represented the differential smoothing of 600 readings was obtained by interpolation. The results are expressed in relative light units per gram

of tissue (RLU/g tissue). After the final calculation, the area under the curve, extracted by integrating the curve for each animal, was used to determine the amount of lipid hydroperoxide present in the sample (44).

Total antioxidant capacity (TRAP). The total antioxidant capacity of lungs was measured by CL in a reaction mixture containing 20 μM 2-azo-bis-(2-amidinopropane) and 200 μM luminol. After maximal emission was attained, 70 μL of tissue supernatant or Trolox was added to the reaction mixture. The total quenching time was compared with Trolox quenching, and the results were expressed in μM Trolox (26).

Superoxide dismutase activity. Superoxide dismutase (SOD) activity was determined according to previous studies based on the inhibition of pyrogallol autoxidation in an aqueous solution of SOD (24). This oxidation was accompanied by yellow colour formation in the reaction mixture, which was monitored at 420 nm. Aliquots of lungs supernatant, diluted in Tris buffer with 1N HCl and 5 mM EDTA (pH 8.0), were added to pyrogallol. The reaction was monitored continuously for 5 min. The autoxidation of pyrogallol alone was used as control. The amount of SOD that inhibited 50% of pyrogallol autoxidation was defined as an enzymatic activity unit (U). The final SOD results are expressed as units per milligram of protein (36).

Glutathione assay. The levels of total glutathione GST (GSH-GSSG) were determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid), evidenced by a yellow colour formation. Oxidised glutathione (GSSG) was measured in the same manner in the supernatant that was previously incubated with 4-vinylpyridinefor 60 min at room temperature, according to the method described previously. Supernatant volumes were adjusted for the assay to contain 50 mg tissue/mL

KH_2PO_4.The results are expressed in micromoles per milligram of protein (14). The GSH/GSSG ratio was also calculated to establish the level of oxidative stress that influenced the glutathione system.

Statistical analysis. Statistical analysis was performed using Sigma Stat 10 software (Systat Software**,** Inc**.,** San Jose**,** CA, USA). A two-way (time × group) repeated-measures ANOVA was performed on body weight, velocity and time spending running during the maximal exercise testing since these parameters were evaluated in the same group of animals across the time. To the other parameters the comparisons among groups were carried out by one way analysis of variance followed by the 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

Exercise capacity tests and body weight. The treadmill physical tests evaluated the intensity of aerobic conditioning in mice after four, eight and 12 weeks. There was a significant interaction between group and time ($P<0.001$) for the velocity reached by mice during the maximal exercise testing and the time spending running during the test. The Exercise and Smoke+Exercise groups displayed increased treadmill maximal speed and time test after eight and 12 weeks of training compared to the groups that were not trained (Control and Smoke) (Figure 1A and 1B; $P<0.001$). The Exercise group showed increased time test results compared to the Smoke+Exercise group after 12 weeks (Figure 1B; $P<0.05$). The aerobic conditioning was increased from four to 12 weeks in the Exercise group

(P<0.001) and after eight and 12 weeks in the Smoke+Exercise (P<0.001) compared to baseline. Control and Smoke groups presented a deconditioning after eight and 12 weeks compared to baseline and four weeks (P<0.001). There was a significant interaction between group and time (P<0.001) for body weight. Animals submitted to exercise training increased body weight after 8 and 12 weeks compared to Control group (Figure 1C; P<0.01). Exercise group also increased body weight after 8 and 12 weeks compared to Smoke group (Figure 1C; P<0.01). After eight and 12 weeks the body weight was increased in animals submitted to exercise training (Exercise and Smoke+Exercise groups) compared to four weeks (P<0.001). The body weight in the Control group increased after 12 weeks compared to four weeks (P<0.001). There was no change in the body weight in the Smoke group across the time.

Pulmonary mechanics evaluation. After four weeks, the airway resistance (Raw) values increased in the Smoke group (*P*<0.001 versus Control and Exercise groups) and in the Smoke+Exercise group (*P*<0.001 versus Control group, Figure 2A). There were no significant differences in Raw values among the groups after eight or 12 weeks. After 12 weeks physical conditioning protected the animals exposed to CS against the decline in tissue damping (Gtis) and tissue elastance (Htis) (*P*<0.01, Figure 2B and *P*<0.001, Figure2C, respectively).

BAL cellular profile. The total number of inflammatory cells in the BAL increased after eight and 12 weeks in mice exposed to CS compared to the other groups (Figure 3A; *P*<0.001). This increase was due to the recruitment of macrophages, neutrophils and lymphocytes. Macrophages increased after four, eight and 12 weeks in mice exposed to CS (Figure 3B), and exercise training protected against

this increase only after 12 weeks. The numbers of macrophages, neutrophils and lymphocytes (Figures 3B-D, respectively) increased after eight and 12 weeks in mice exposed to CS (Fig. 3B: $P<0.05$, Smoke group compared to Exercise group and $P<0.001$ Smoke group compared to Control group; Fig. 3C:$P<0.05$, Smoke group compared to Control group and $P<0.001$ Smoke group compared to the other groups, Fig. 3D: $P<0.001$, Smoke group versus the other groups). After 8 weeks of exercise training, fewer macrophages, neutrophils and lymphocytes were found in the BAL of mice exposed to CS, and after 12 weeks of exercise training, fewer lymphocytes and neutrophils were found in mice exposed to CS.

Emphysema development. In a different group of mice, the mean linear intercept (Lm), a hallmark of lung emphysema, showed an increase after 12 weeks of CS exposure compared to the other groups ($P<0.001$; Figure 4). Aerobic conditioning protected the animals from emphysema development only after exercise adaptation.

Inflammatory mediators*.* TNF-α was increased in lungs of mice exposed to CS after 12 weeks (Figure 5A; $P<0.01$ versus Exercise and Control groups). The Smoke+Exercise group showed no differences in TNF-α compared to the other groups. IL-6 and IL-10 were increased in the lungs of mice in the Smoke+Exercise group after 12 weeks compared to the Control group ($P<0,01$; Figure 5B), and the Smoke group ($P<0.05$; Figure 5C). There was an increase of TNF-α levels in the skeletal muscle of mice exposed to CS after eight and 12 weeks (Figure 5D; $P<0.05$ versus the other groups). IL-6 increased in the skeletal muscle of Exercise and Smoke+Exercise groups after 12 weeks compared to the Control group ($P<0,05$; Figure 5E). There was an increase of IL-10 levels in the skeletal muscle

of mice of Exercise and Smoke+Exercise groups after 12 weeks compared to the Smoke group (P<0,01; Figure 5F).

Oxidative stress and antioxidants. Tert-butyl hydroperoxide-initiated chemiluminescence (TBHP) was used to analyse the integrity of non-enzymatic antioxidant defences and the levels of lipoperoxides in lung tissue (17-23). Exposure to CS increased TBHP after eight and 12 weeks (Figure 6A; P<0.05 versus the Control group and P<0.001 versus the other groups) and reduced the total antioxidant capacity (TRAP) after 12 weeks (Figure 6B; P<0.001 Smoke versus the other groups). After 12 weeks of exercise training, mice were protected from this response. After 8 weeks, GSH was increased in the Smoke group compared to the Control and Exercise groups (P<0.01). Additionally, after 12 weeks, the GSH/GSSG ratio was reduced in lung tissue of mice exposed to CS (Figure 6C; P<0.05 versus control group and P<0.001 versus the other groups). Curiously, when mice were exposed simultaneously to CS and exercise, the GSH/GSSG ratio showed a transient decrease after 4 weeks of training (P<0.01 compared to Control group). CS exposure increased superoxide dismutase enzyme (SOD) activity in lung tissue after 4 weeks (Figure 6D; P<0.01 versus Control, Exercise and Smoke+Exercise groups) and 8 weeks (Figure 6D; P<0.01 versus control and exercise groups). Exercise training increased SOD in lung tissue of mice after 12 weeks (Figure 6D; P<0.001 versus Smoke and Control groups).

DISCUSSION

For the first time, we have shown the time-course effects of chronic, regular moderate-intensity treadmill training in the protection against CS-induced COPD. In response to four weeks of CS exposure, we observed an infiltration of macrophages and an increase in airway resistance before the exercise conditioning (adaptation) phase. In this period, exercise was not able to reduce the alterations induced by CS exposure. After establishing consistent aerobic conditioning by 8 weeks of exercise, the numbers of inflammatory cells in the BAL, including macrophages, neutrophils and lymphocytes, were reduced. However, the most evident effect of exercise was observed after 12 weeks: exercise increased IL-6, IL-10, total GSH and SOD activity and decreased pulmonary inflammation and TNF-α in mice exposed to CS. In addition, exercise inhibited the establishment of COPD and improved lung mechanics by decreasing lung elastance and resistance in mice exposed to CS. The benefits caused by exercise training in animals exposed to CS compared with their non-smoker counter parts seem to be mediated by the inhibition of inflammatory mediators and oxidative stress after the period of exercise adaptation by increasing antioxidant defence and anti-inflammatory mediator release.

The features observed in our experimental model of CS-induced COPD, including respiratory mechanics impairments, pulmonary emphysema and inflammation, are similar to patients diagnosed with COPD (52).The severity of COPD in humans is currently classified according to the Global Initiative on Chronic Obstructive Lung Disease (GOLD)/WHO (World Health Organization) by a 1–4 scale based on the deterioration of lung function. Experimental models of COPD in rodents lead from mild to severe emphysema that is compatible with GOLD I-IV (46). In the current

study the decrease in pulmonary function (tissue damping and tissue elastance) in the CS-exposed group after 12 weeks could be equivalent to moderate emphysema. We previously reported the establishment of emphysema after 6 months of CS exposure (45). However, this model is time-consuming and expensive. In the current study, we adjusted the CS exposure to twice a day instead of once a day, reducing by half the length of experimentation; with this model, we observed enlargement of airspaces after twelve weeks of exposure to CS, as reported by others (25). CS exposure leads to bronchial reactivity even in individuals with normal lung function (11). We found a transient increase in the airway resistance (Raw) after 4 weeks of CS exposure, and exercise training had no effects on this change. These results suggest that in this experimental model, there was an initial inflammatory process near the airways involving the posterior destruction of the alveolar walls (2, 7, 9).

Beyond morphological changes (i.e., enlargement of alveolar space) and lung mechanics impairments (i.e., increased lung tissue resistance and elastance), emphysema is characterised by Th1-type inflammation (7, 35). In our study, mice exposed to CS showed increased numbers of total inflammatory cells, mainly due to the infiltration of macrophages, which remained elevated from four to 12 weeks. Macrophages releaseTh1 inflammatory mediators, including TNF-α and reactive oxygen species (ROS), as observed in our study by increased lipid peroxidation in lung homogenates after 12 weeks of CS exposure.

Aerobic exercise significantly reduced the accumulation of macrophages after 8 and 12 weeks of CS exposure. The accumulation and activation of macrophages in the lungs contribute to CS-induced emphysema (7, 9, 35). In the present study, after 8

and 12 weeks, there was an increase in the accumulation of neutrophils and lymphocytes in the lungs; these cells are also involved in the cellular mechanisms underlying COPD (23, 38). For instance, increased numbers of neutrophils in bronchial biopsies and in induced sputum are correlated with the severity of COPD and the rate of decline in lung function (10, 39). In the present study, our CS-induced COPD model also had more neutrophils and lymphocytes after 8 and 12 weeks of CS exposure. Again, aerobic exercise reduced neutrophil and lymphocyte accumulation in the lungs, reinforcing the anti-inflammatory effects of exercise.

Physical exercise training plays an important role in orchestrating the anti-inflammatory response involving the activation of macrophages and TNF-α, particularly by increasing IL-10, as demonstrated in our previous study (45). Regular exercise training offers protection against all-cause mortality, primarily by protecting against atherosclerosis, diabetes, and breast and colon cancers (20). Additionally, physical training is an effective non-pharmacological treatment for patients with chronic diseases such as chronic obstructive pulmonary disease (16). For instance, Garcia-Aymerich et al. showed that active smokers who practiced moderate or high levels of regular physical activity had a reduced risk of developing COPD (15). Additionally, we and others have shown that moderate aerobic exercise performed regularly attenuates smoking-induced airway inflammation, airway mechanics changes, and oxidative damage and protects mice from developing emphysema (25,45).

Thus, we can suggest that inflammatory mediators such as cytokines play a central role in the pathogenesis of COPD (7, 8, 19, 35, 40, 45, 50). Several pro-inflammatory cytokines (i.e., IL-1β, IL-6, IL-8, IL-17, IL-18, IL-32, TNF-α and IFN-γ)

are increased both in the lungs and systemically in COPD patients (3). Additionally, the anti-inflammatory cytokine IL-10 is decreased in COPD compared with non-COPD individuals, due to impaired T-regulatory cell functions (41, 43). Similarly, we observed up-regulation of the pro-inflammatory cytokine TNF-α in CS-exposed mice as well as a reduction in IL-10. The present study showed for the first time the time-course of IL-6, IL-10 and TNF-alpha expression by skeletal muscle and lungs in the context of experimental COPD and exercise training. IL-6 is a myokine produced during skeletal muscle contraction independent of TNF-α (19). Notably, aerobic exercise training reduced the CS-induced up-regulation of TNF-α and increased IL-6 and IL-10, conferring strong protection against CS-induced COPD. Of particular interest, IL-6 is a controversial cytokine because some authors have claimed it is pro-inflammatory while others have claimed it is immune regulatory and anti-inflammatory (31, 31, 34). We found that exercise in CS-exposed mice significantly increased pulmonary IL-6. This finding adds new information to the topic of exercise as an immune regulator and to the skeletal muscle as an endocrine organ, demonstrating that exercise may increase IL-6 not only systemically but also specifically in the lungs. The secreted molecules, IL-6 and IL-10, during exercise could mediate these inter-organ communications and have a protective effect in the emphysema development after exercise adaptation.

The major constituents of the gaseous phase of cigarette smoke are oxidants and aldehydes that mediate oxidative stress, which has been implicated in the pathogenesis of smoking-related diseases (11). Some other compounds, such as acrolein and crotonaldehyde, which are also abundant in the gaseous phase of CS, are the major mediators of macrophage activation (24).

In the present study, we showed that oxidative stress, as reflected by increased lipid peroxidation in lung homogenates, was increased in our experimental model after eight and 12 weeks of CS exposure. The increase of oxidative stress, even after 12 weeks of CS exposure, was followed by an increased number of macrophages not only after 12 weeks of CS exposure but in all periods. A previous study has shown increased 8-isoprostane in patients with COPD compared with healthy non-smokers (2). Normally, beyond increased ROS, COPD patients also present a reduced antioxidant capacity (14). This is true even for patients presenting α-1-anti-trypsin deficiency (14, 42). We showed that CS-exposed mice presented increase oxidative stress and reduced antioxidant capacity when emphysema was already established. Decrease in the ratio of reduced GSH to oxidised GSH (GSSG) is one of the most sensitive marker for evaluating oxidative stress and disease (12). It is interesting to note that GSH:GSSG ratio was not only decreased after 12 weeks of CS exposure, but also at four weeks of CS exposure before exercise adaptation (Smoke+Exercise group). The explanation for this phenomenon can reside on two main facts: (1) oxidative stress is a dynamic phenomenon, that can change during time under the same challenge. In the CS group, the consumption of reduced glutathione at 12 weeks of training (as demonstrated by GSH:GSSG ratio) is coincident with TRAP decreasing and also lipid peroxidation increasing, without enhancement of enzymatic antioxidant systems, here represented by SOD. Apparently, when there is no additional stimulus to antioxidant increasing, antioxidant consumption can be induced after a primary lipid peroxidation stimulus without endogenous response, demonstrating a worsening of lung injury; (2) on the contrary, when exercise is

applied in early stages of a disease, a precocious stimulus to the tissue promotes positive adaptation, preventing the worsening of tissue injury. This is one of the principles of adaptation of skeletal muscle during exercise (12), and can be applied for other tissues. The absence of lipid peroxidation at 8 and 12 weeks on the smoke-exercise group reinforces this theory. Additionally, exogenous administration of catalytic antioxidants exerts a protective effect against smoke-induced lung injury in preclinical models (21). Recent evidence suggests that regular exercise elevates the expression of antioxidant enzymes in the lungs (48). The present study, highlights the importance of regular exercise training in managing COPD.

The potential limitation of the current study was use of exclusively male mice. In fact, there are a body of evidences indicating the existence of sex or gender-related differences regarding the susceptibility to deleterious effects of cigarette smoking and clinical characteristics of COPD (4, 32, 38). However, one difficult to perform experiments with female is the need to adjust the estral cycle in order to avoid or exclude the interference of sexual female hormones. This is particularly important in our case since the pulmonary inflammatory response as well as the effects of exercise can be modulated differently by gender and specially by female sexual hormones (1, 5, 35). This issue should be addressed in future studies.

In summary, we have shown that after reaching physical conditioning animals exposed to CS presented a protection against the emphysema development. Particularly, exercise inhibited local oxidative stress and pro-inflammatory cytokine release while increasing the release of the anti-inflammatory cytokine such as IL-10 by skeletal muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.C.T., R.P.V., C.J.L. and M.A.M. conception and design of research; A.C.T., R.P.V., F.A.G., C.L.S., A.C.N., C.R.O., P.M.M. A., F.M.A., F.D.Q.S.L., E.M.C.R., and R.C. performed experiments, analyzed data and interpreted results of experiments; C.L.S., A.C.N., C.R.O., P.M.M. A., F.M.A., F.D.Q.S.L., E.M.C.R. and R.C.prepared figures; A.C.T., R.P.V., F.A.G., C.J.L. and M.A.M. drafted the manuscript; critical revision and approved final version of manuscript—all authors.

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Figure Legend

Figure 1. Aerobic performance reached in the maximal exercise test on the treadmill at baseline and after four, eight and 12 weeks of aerobic training and/or cigarette smoke exposure. A and B) ***P*<0.01 Exercise and Smoke+Exercise versus to Control and Smoke groups, #*P*<0.05 Exercise versus Smoke+Exercise; &P<0.05 Exercise versus baseline; *P<0.01 the four experimental group versus baseline; n=10/group C) **P*<0.05 versus Control group; **P<0.05 versus Smoke group; #*P*<0.05 versus 4 weeks; n=9/group. Values are means and SD.

Figure 2. Exposure to CS increased airway resistance (A) after four weeks; exposure to CS decreased pulmonary elastance (Htis) (B) and tissue damping (C) (Gtis) after 12 weeks of the experimental protocol, and exercise inhibited this change (n=7-9/group). (A) ***P*<0.001 Smoke and Smoke+Exercise versus Control and Exercise groups; (B) **P*<0.01 and (C) ***P*<0.001 Exercise and Smoke+Exercise versus Control and Smoke groups. Values are means and SD.

***Figure 3*.** Bronchoalveolar cellular profile. The data show the numbers of total inflammatory cells (A), macrophages (B), neutrophils (C) and lymphocytes (D) in the bronchoalveolar lavage fluid (n=7-10/group). ***P*<0.001, Smoke vs the other groups; $^{\#}$*P*<0.01 Smoke and Smoke+Exercisevs Control and Exercise;**P*<0.05 Smoke vs Exercise group;$^{\&}$*P*<0.05, Smoke vs Control group. Values are means and SD.

Figure 4. Photomicrographs of lung histological sections stained with hematoxylin and eosin,obtained from the Control group(A), Smoke group (B), the Exercise group (C), and Smoke+Exercise group (D) after 12 weeks. Scale bars: 50 µm. Emphysema shown by the mean linear intercept after four, eight and 12 weeks of CS exposure (n=7-10/group);**P<0.001 for Smoke versus Control, Exercise and Smoke+Exercise (E).

669 **Figure 5.** TNF-α (A), IL-6 (B) and IL-10 (C) levels in lung homogenates of the mice
670 (n=6-10/group). (A) ***P*<0.01, Smoke versus Exercise and Control groups; (B)
671 **P*<0.01, Smoke vs Control group; and (C) **P*<0.05, Smoke+Exercise versus
672 Smoke group. TNF-α (D), IL-6 (E) and IL-10 (F) levels in skeletal muscle
673 (quadriceps) homogenates of the mice (n=5/group). (D) # *P*<0.05, Smoke vs the
674 other groups; (E) **P*<0.05, Smoke vs Control group; and (F) ***P*<0.05,
675 Smoke+Exercise versus Smoke group. Values are means and SE.
676
677 **Figure 6.** Tert-butyl hydroperoxide-initiated chemiluminescence (A); total
678 antioxidant capacity (B); ratio of reduced glutathione (GSH) to oxidized glutathione
679 (GSSG) (C) and superoxide dismutase activity (D) in lung homogenates of mice
680 (n=6-9/group). **P<0.001 versus the other groups; *P<0.01 versus the Control
681 group; #P<0.001 versus the Control and Smoke groups. Values are means and
682 SD.
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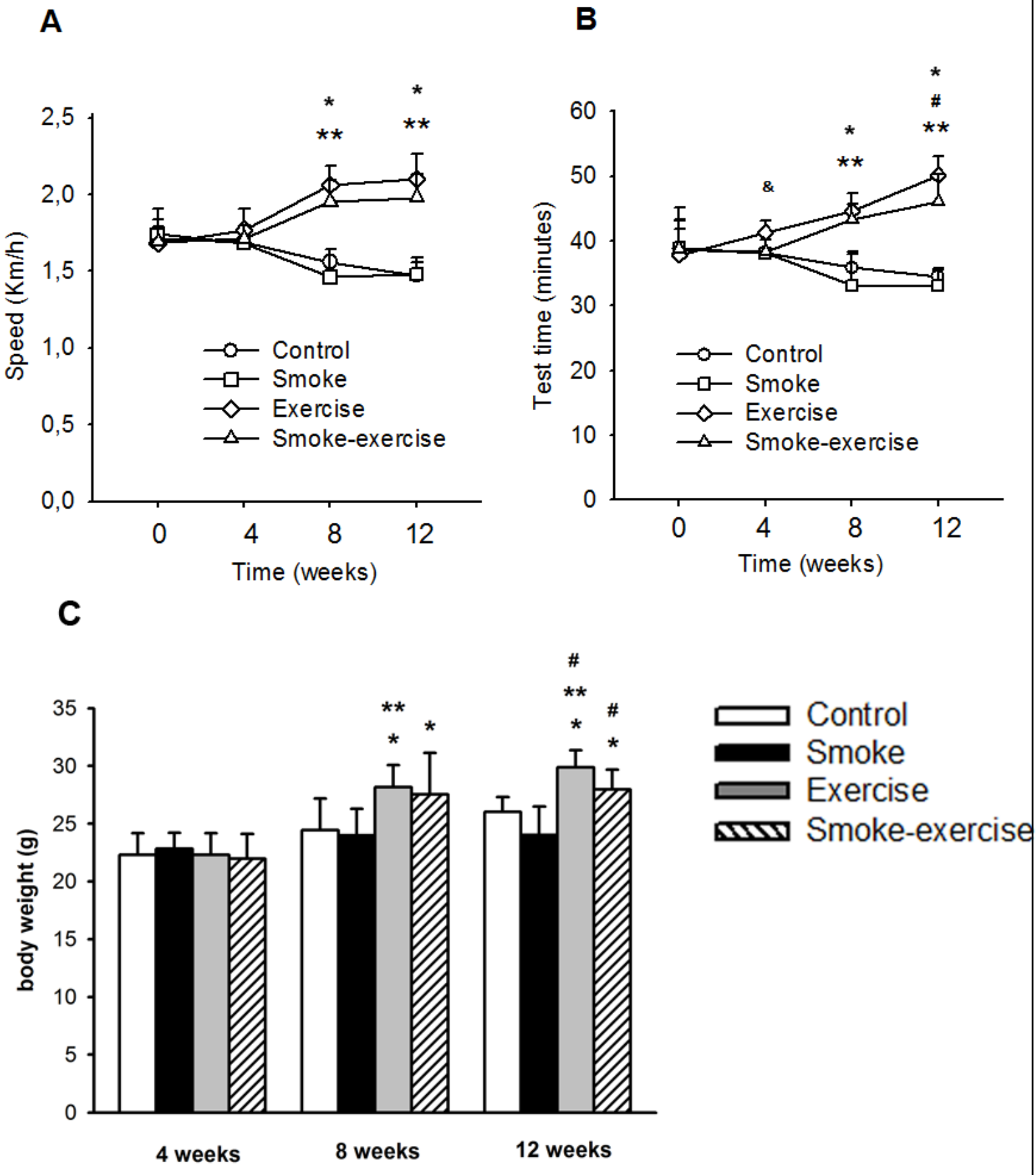

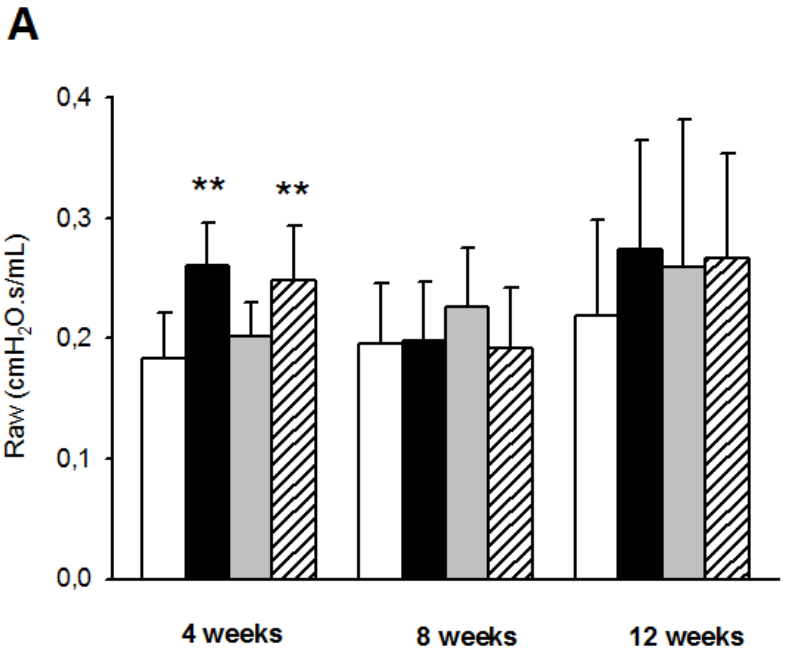

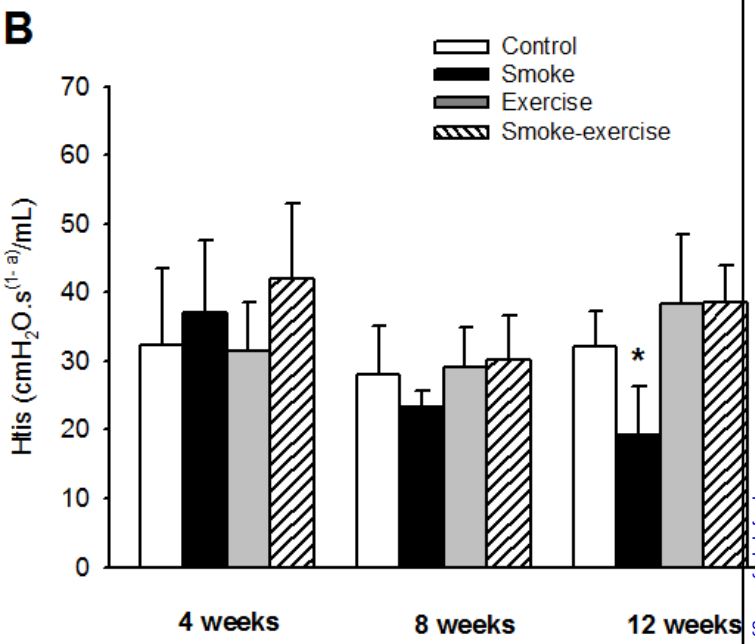

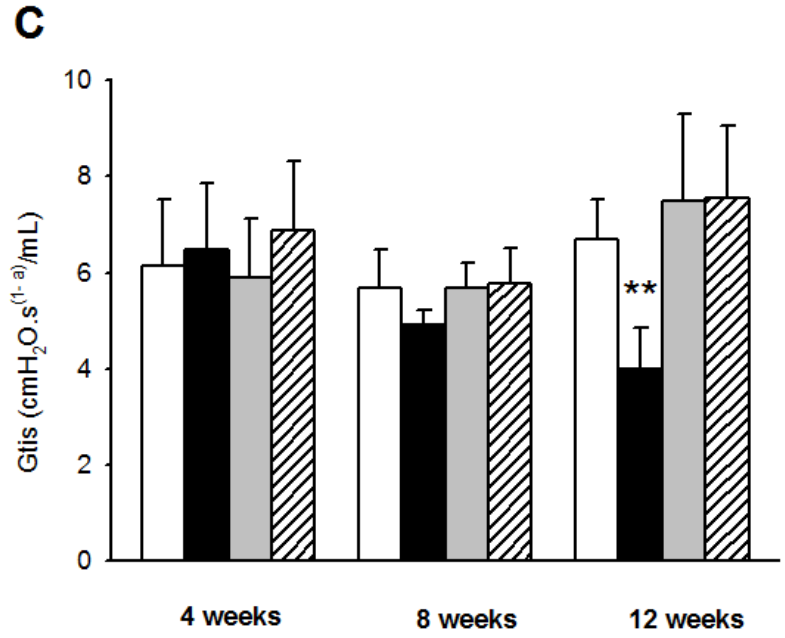

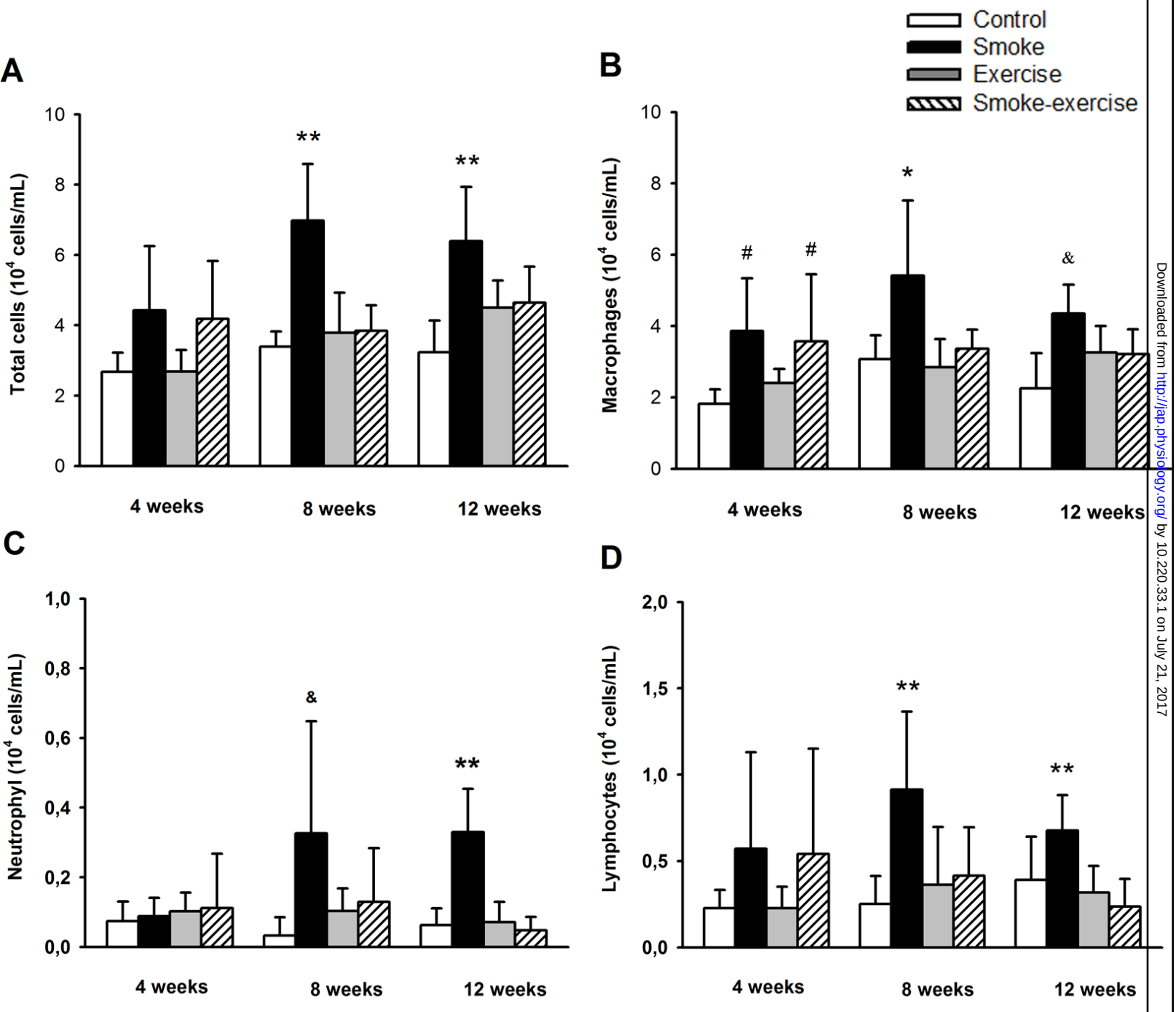

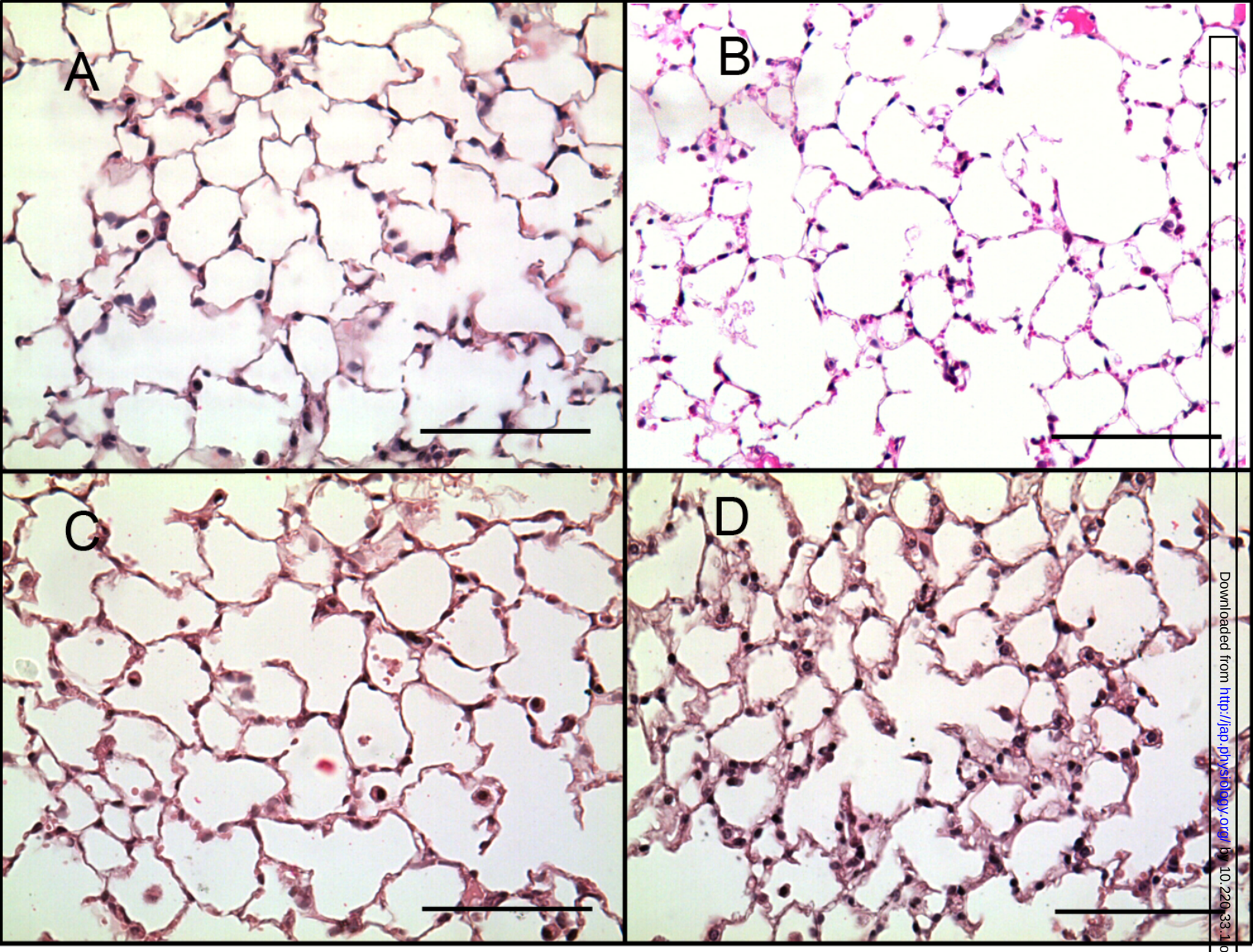

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