

Exercise Inhibits Allergic Lung Inflammation

Authors

A. C. D. Silva¹, R. P. Vieira², M. Nisiyama¹, A. B. G. Santos³, A. Perini⁴, T. Mauad³, M. Dolhnikoff⁵, M. A. Martins⁴, C. R. Carvalho¹

Affiliations

¹Physical Therapy, University of São Paulo, São Paulo, Brazil

²Post Graduate Program in Biophotonics Applied to Health Sciences, Nove de Julho University, São Paulo, SP, Brazil

³Pathology (LIM 05), University of São Paulo, São Paulo, Brazil

⁴Clinical Medicine (LIM 20), University of São Paulo, São Paulo, Brazil

⁵School of Medicine, Pathology, São Paulo University, São Paulo, Brazil

Key words

- asthma
- cytokines
- lungs
- aerobic conditioning
- adhesion molecules
- growth factors

Abstract

Aerobic conditioning (AC) performed either during or after sensitization reduces allergic inflammation in mice; however, the effects of AC performed before and during allergic sensitization on airway inflammation are unknown. Mice were divided into Control, AC, OVA, and AC+OVA groups. Mice were trained in a treadmill followed by either ovalbumin (OVA) sensitization or saline administration. Peribronchial inflammation, OVA-specific IgE and IgG1 titers, the expression of Th1 and Th2 cytokines, and airway remodeling were evaluated, as well as the expression

of Eotaxin, RANTES, ICAM-1, VCAM-1, TGF- β and VEGF. Aerobic conditioning performed before and during allergic sensitization displayed an inhibitory effect on the OVA-induced migration of eosinophils and lymphocytes to the airways, a reduction of IgE and IgG1 titers and an inhibition of the expression of Th2 cytokines. The AC+OVA group also demonstrated reduced expression of ICAM-1, VCAM-1, RANTES, TGF- β and VEGF, as well as decreased airway remodeling ($p < 0.05$). The effects of AC before and during the sensitization process inhibit allergic airway inflammation and reduce the production of Th2 cytokines and allergen-specific IgE and IgG1.

Introduction

Allergic disorders classically represent an acute response to allergens mediated by IgE and a late response related to the migration of eosinophils and lymphocytes to the inflammatory site. The influx of inflammatory cells in the late response appears to be orchestrated by the production of Th2-type cytokines (e.g., IL-4, IL-5 and IL-13) in experimental animal models [15,35] and also in patients with allergic diseases [30]. In addition, the recruitment and activation of inflammatory cells depend on the expression of many classes of inflammatory mediators, such as cytokines (IL-4, IL-5 and IL-13), chemokines (eotaxin, RANTES) and adhesion molecules (ICAM-1 and VCAM-1) [15,31,35]. Chronically, non-resolving allergic airway inflammation leads to remodeling, a process associated with an imbalance between the synthesis and degradation of extracellular matrix components [10,41]. The remodeling process in asthma is also related to an increased release of growth factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor (TGF- β), that is thought to play a pivotal role in inflammation and remodeling in allergic airway diseases [12].

Exercise can have both positive and negative effects on immune function [6]. For example, the relationship between exercise and susceptibility to infection has been modeled in the form of a “J” shaped curve [17,20,22] and suggests that exercise training of moderate intensity increases the anti-microbial capacity of neutrophils via an increase in the Th1 cytokines [26]. On the other hand, strenuous exercise can result in increased risk for infections of the respiratory tract [17,22,26,28]. Although the effect of acute and chronic exercise on the incidence of infection has been widely studied, the effect of acute and chronic exercise on different allergic diseases has not.

Allergic diseases most likely have a multifactorial etiology, and there is a general agreement that a westernized lifestyle contributes to its increased prevalence; reduced physical fitness seems to represent an important contributor [2,32]. On the other hand, a recent study from our group demonstrated for the first time that asthmatic patients subjected to aerobic exercise training had decreases in the number of eosinophils in induced sputum [16]. In addition, another interesting study has demonstrated that aerobic exercise in asthmatic children reduces the levels of

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Bibliography

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Correspondence

Dr. Celso Ricardo Fernandes Carvalho

University of São Paulo
Physical Therapy
Av. Dr. Arnaldo 455 room 1210
São Paulo
01246-903
Brazil
Tel.: +55-11-8415-3234
Fax: +55-11-3061-7317
ccscarval@usp.br

total and specific IgE, corroborating with the findings of our study [18]. In corroboration with these findings in asthmatic patients, several studies have demonstrated that low-to-moderate intensity aerobic exercise demonstrates anti-inflammatory effects in animal models of allergic airway inflammation. However, all of these studies were performed with the airway inflammation preceding any exercise intervention [8,34,37–40]. To our knowledge, the effect of aerobic conditioning beginning prior to the development of an experimental allergic response has never been investigated.

In the present study, we hypothesized that an improvement in aerobic conditioning performed before and during allergen sensitization could result in reduced allergic airway inflammation. Additionally, we investigated whether the improvement of the aerobic conditioning could affect the Th1/Th2 cytokine balance.

Materials and Methods

This study was approved by the review board for human and animal studies of the School of Medicine of the University of Sao Paulo (protocol 317/06). All animals received humane care in compliance with the "Guide for care and use of laboratory animals" (NIH publication 85-23, revised 1996). In addition, the study also meets the ethical standards of the journal [7].

Animals and experimental groups

32 male BALB/c mice (20–25 g), 8–10 weeks old were divided into 4 groups (n=8): Control (non-trained and non-sensitized); AC (aerobic conditioning and non-sensitized); OVA (non-trained and OVA-sensitized); and OVA+AC (aerobic conditioning and OVA-sensitized). Animals were exposed to a 12 h/12 h light/darkness cycle and received water and food *ad libitum*.

Exercise test and aerobic training

Animals were initially adapted to the treadmill for 5 days (15 min, 25% inclination and 0.2 km/h). After that, a maximal exercise capacity test was performed with a 5 min warm-up (25% inclination, 0.2 km/h) followed by an increase in treadmill speed (0.1 km/h every 2.5 min) until animal exhaustion defined as the inability to continue to run, even after 10 gentle mechanical stimuli [34,37–40]. Maximal aerobic capacity was established as the maximum speed reached by each animal. Mice were trained at low exercise intensity (50% of maximum speed) [34,37–40]. Exercise training was performed for 60 min per day, 5 days a week, for 8 weeks. After this initial 8 week period of exercise training, OVA sensitization or saline administration began and exercise training continued until animals were euthanized.

OVA sensitization

BALB/c mice were sensitized with 2 intraperitoneal injections of OVA (20 µg) adsorbed with aluminum hydroxide [days 56 and 70 of the experimental protocol] followed by 4 inhalations of OVA (1%) or saline solution on days 77, 79, 81 and 83.

Anesthesia and euthanasia of animals

48 h after the last OVA inhalation, animals were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (40 mg/kg), tracheostomized, cannulated and exsanguinated.

Total and differential cell counting in bronchoalveolar lavage fluid (BALF)

The lungs were gently washed with 1.5 ml of saline (3×0.5 ml) via the tracheal cannula. Total cell counts were performed automatically (laser cell counter, JXJ-402; Shanghai Odin Science and Technology, Shanghai, China). Differential counting was performed with a Grün-Wald-Giemsa stain (300 cells per lamina) [34,37–40].

Passive cutaneous anaphylaxis (PCA) to OVA-specific IgE and IgG₁

PCA was performed in Wistar rats and BALB/c mice for measurements of titers of OVA-specific IgE and IgG₁, respectively [19,24,34,37]. The backs of the animals were shaved and injected intradermally with different dilutions of blood plasma. Animals were challenged intravenously with 0.5 mg of OVA in 0.5% Evans Blue solution after a sensitization period of 18–24 h in Wistar rats for IgE or 2 h in BALB/c mice for IgG₁ titration. PCA titer was expressed as the reciprocal of the highest dilution that yielded a lesion greater than 5 mm in diameter in duplicate tests. The detection threshold of this technique was established at a 1:5 dilution.

Lung histology, immunohistochemistry and morphometric analysis

The lungs were fixed in 10% formalin and embedded in paraffin. 5 micrometer-thick sections were stained with hematoxylin and eosin for lung structure analysis and with Luna staining for the detection of eosinophils [13,34,37,38]. The sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 min to inhibit endogenous peroxidase activity. Additionally, an avidin/biotin blocking system (Dako Deutschland, Hamburg, GE) was used to avoid nonspecific antibody binding. Antigen retrieval was performed with citrate solution for 30 min. The sections were incubated with anti-IL-4, anti-IL-5, anti-IL-13, anti-IFN-γ, anti-IL-2, anti-eotaxin, anti-RANTES, anti-ICAM-1, anti-VCAM-1 and anti-TGF-β antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) by the biotin-streptavidin-peroxidase method. Specific IgG-positive sera were used as negative controls in correspondence with the source species of the primary antibodies.

With a 50-line and 100-point grid connected to the ocular lens of a microscope with a known area (10000 µm² at 1000× magnification), we assessed the peribronchial density of eosinophils, mononuclear cells, and cells positive for IL-4, IL-5, IL-13, IFN-γ, IL-2, eotaxin, RANTES, ICAM-1, VCAM-1, TGF-β and VEGF using a point-counting technique [37]. Measurements were performed in 5 airways per animal, and the area of interest was determined as the number of points hitting the outer area of the airway wall [located between the external limit of smooth muscle layer and adventitia]. The density of positive inflammatory cells was determined as the number of positive cells in each field divided by the tissue area. Measurements are expressed as cells/mm² [3,11,37].

Collagen and elastic fibers

Using an Eclipse E-200 microscope (Nikon®, Tokyo, Japan), a Cool Snap digital camera (Photometrics®, Tokyo, Japan) and the image analysis software Image Proplus 4.5 (Carlsbad, CA, USA), we evaluated the deposition of collagen and elastic fibers. 5 airways at 400x magnification were evaluated for each animal

Table 1 Effects of aerobic exercise on physical performance and on immunoglobulins levels.

Groups	Exercise Test (min)			Immunoglobulins (Log)	
	Initial	Pre-Sensitization	Post-Sensitization	IgE	IgG1
Control	38.8 ± 3.6	39.2 ± 2.0	40.1 ± 1.8	1.32 ± 0	1.32 ± 0
AC	39.9 ± 2.8	48.0 ± 4.1 *	50.2 ± 4.6 *	1.32 ± 0	1.32 ± 0
OVA	39.9 ± 2.8	40.9 ± 2.8	39.7 ± 3.1	5.32 ± 2.6 *	5.76 ± 2.1 *
OVA+AC	38.9 ± 3.2	48.5 ± 5.8 *	49.5 ± 4.0 *	2.82 ± 1.2	3.48 ± 1.4

Data are expressed as mean ± SD of aerobic treadmill performance (minutes on the treadmill) and titers of OVA-specific IgE and * $p < 0.05$ compared with intra-group pre-sensitization test

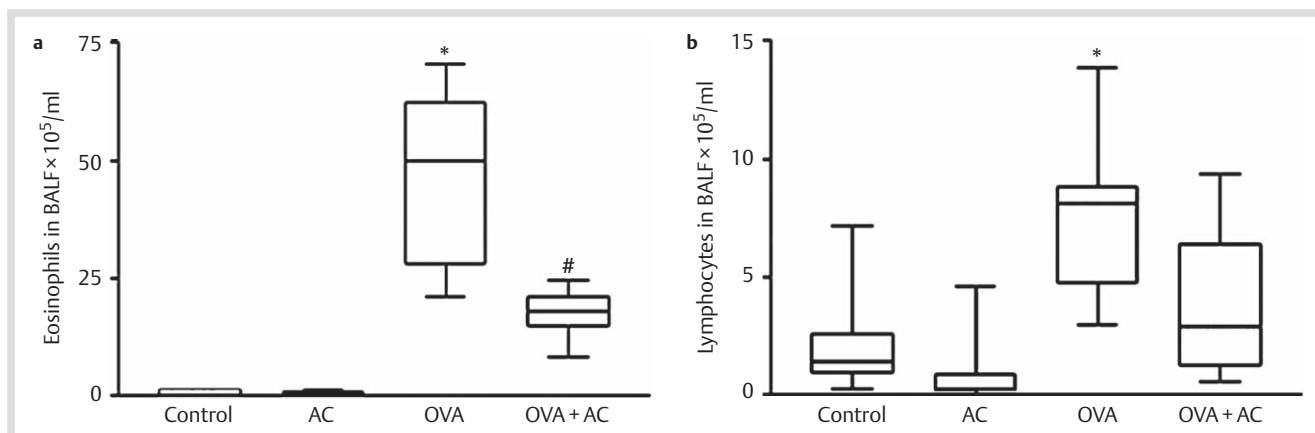


Fig. 1 Box plots of eosinophil **a** and lymphocyte **b** counts in the bronchoalveolar lavage fluid [BALF]. Horizontal lines represent median values, boxes represent the interquartile range [25–75%] and whiskers represent the 5–95% range. In Figures **a** and **b**, * $p < 0.01$ when compared with all groups and # $p < 0.01$ when compared with the OVA group.

[34, 37, 39]. The airway deposition of collagen and elastic fibers was quantified in the area between the airway epithelial basal membrane and the adventitia. The positive areas of collagen and elastic fibers were expressed as percentages of total airway wall areas [1, 37].

Statistical analysis

Parametric data were expressed as the means ± SD or medians ± 95% confidence interval (95%CI). Comparisons between groups were performed by 2-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test (parametric data; • **Table 1**) or by 1-way analysis of variance on ranks followed by Dunn's post-hoc test (non-parametric data; • **Fig. 1–5**); significance level was adjusted to 5% ($p < 0.05$).

Results

Experimental protocol

Aerobic physical conditioning was performed for 8 weeks before the beginning of OVA sensitization and was continued thereafter. OVA sensitization and aerosol exposure were performed during weeks 9–12. Euthanasia was performed 48 h after the last aerosol exposure.

Aerobic exercise treadmill tests

Pre-sensitization and post-sensitization aerobic exercise treadmill tests are presented in • **Table 1**. There was no significant difference between any groups in the initial test; however, animals subjected to aerobic conditioning in non-sensitized (AC)

and sensitized (OVA + AC) groups improved their exercise capacity either in pre-sensitization or post-sensitization tests as compared with the initial test ($p < 0.05$).

Passive cutaneous anaphylaxis to OVA-specific IgE and IgG₁

Aerobic conditioning in non-sensitized animals did not change the levels of IgE or IgG₁ as compared to the Control group. OVA sensitization increased levels of IgE and IgG₁ when compared with non-sensitized groups ($p < 0.01$). AC prior to OVA sensitization (OVA + AC group) inhibited the OVA-induced increases in the IgE and IgG₁ levels (• **Table 1**; $p < 0.01$).

Inflammatory cells in bronchoalveolar lavage fluid (BALF)

AC did not change the influx of inflammatory cells into the airways in non-sensitized animals, and the number of inflammatory cells was similar to the Control group (• **Fig. 1a, b**, respectively). OVA sensitization increased the numbers of eosinophils and lymphocytes when compared with the Control and AC groups ($p < 0.01$). AC in sensitized mice reduced the eosinophil and lymphocyte counts in the BALF as compared with the OVA group ($p < 0.01$) (• **Fig. 1a, b**, respectively).

Peribronchial density of inflammatory cells

Eosinophils and mononuclear cells were also counted in the peribronchial compartment. AC did not change the number of eosinophils or mononuclear cells in the non-sensitized animals. OVA sensitization increased peribronchial eosinophil (from 24.2 ± 27.4 to 570.4 ± 110.1 cells/mm²; $p < 0.001$) and mononu-

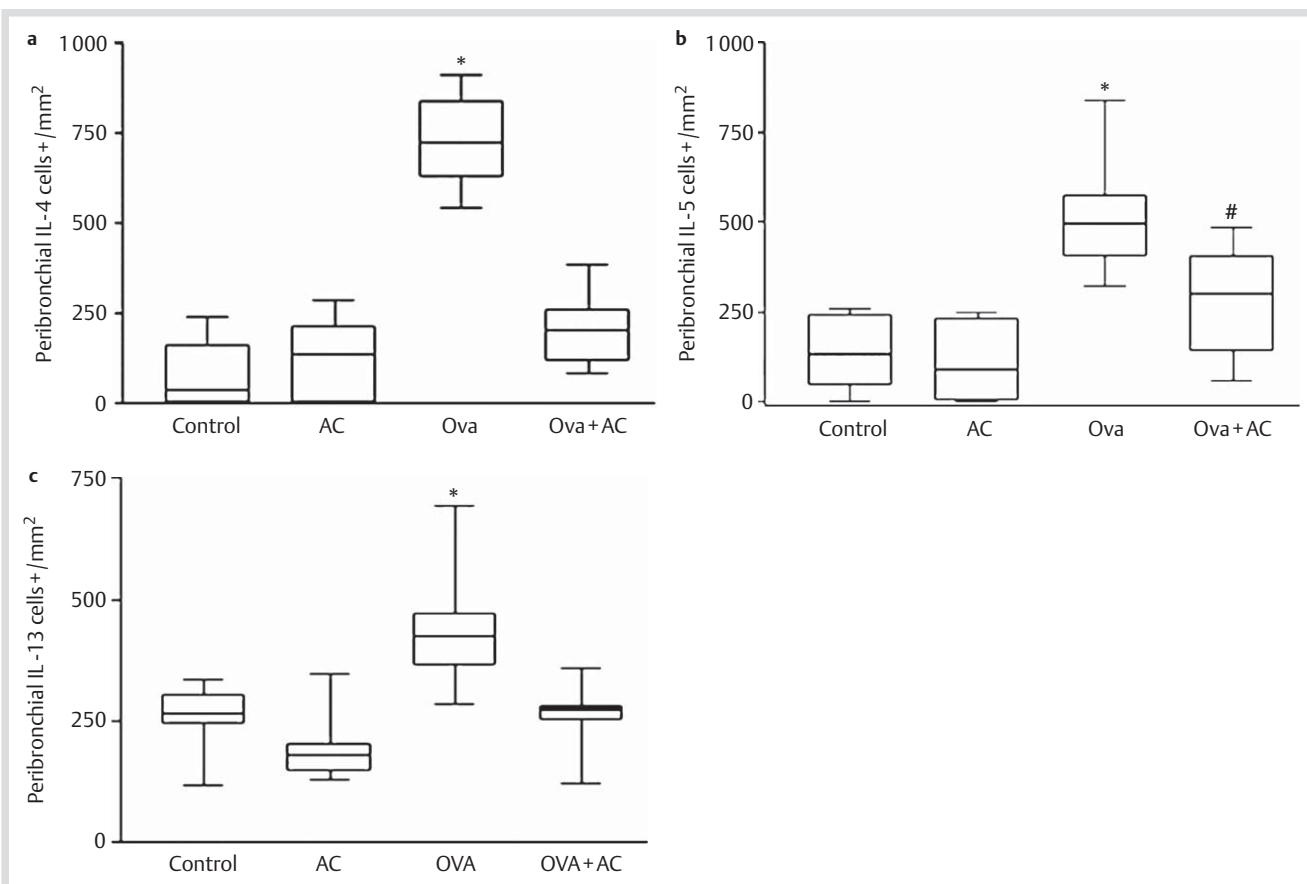


Fig. 2 Box plot of the density of peribronchial inflammatory cells expressing IL-4 **a**, IL-5 **b** and IL-13 **c**. In **a**, * $p < 0.01$ when compared with all groups; # $p < 0.05$ when compared with the Control and AC groups.

clear cell (from 318.3 ± 138.9 to 718.8 ± 165.5 cells/mm²; $p < 0.001$) counts as compared to the non-sensitized groups. AC in sensitized animals (OVA+AC group) substantially inhibited eosinophilic infiltration (364.2 ± 123.6 cells/mm²; $p < 0.01$) but did not affect mononuclear cell migration ($p > 0.05$).

Expression of Th2 Cytokine by inflammatory cells

The expression of IL-4, IL-5 and IL-13 by inflammatory cells was analyzed in the peribronchial compartment (Fig. 2a–c, respectively). AC did not change the peribronchial density of cells positive for IL-4, IL-5 or IL-13 in non-sensitized animals. OVA sensitization increased the peribronchial density of cells positive for the Th2 cytokines IL-4 (7-fold), IL-5 (5-fold) and IL-13 (around 50%) as compared to the non-sensitized groups ($p < 0.01$). The OVA+AC group demonstrated a lower expression of all Th2 cytokines evaluated when compared with the OVA group ($p < 0.01$) (Fig. 2a–c, respectively). Fig. 3 shows representative photomicrographs of IL-4 immunostaining for the Control (A), OVA (B), AC (C) and AC+OVA (D) groups.

Expression of chemokines and adhesion molecules by inflammatory cells

Neither AC nor OVA sensitization modified the expression of eotaxin by inflammatory cells in the peribronchial compartment ($p > 0.05$) (Fig. 4a). OVA sensitization increased the expression of RANTES by inflammatory cells as compared to non-sensitized groups ($p < 0.01$) (Fig. 4b). In addition, the OVA+AC group displayed a lower expression of RANTES when compared with the

OVA group ($p < 0.05$). The expression of VCAM-1 and ICAM-1 was not modified by the AC in non-sensitized animals (Fig. 4c, d, respectively). OVA sensitization increased the expression of VCAM-1 (around 50%) and ICAM-1 (2.5-fold) by inflammatory cells when compared with non-sensitized groups ($p < 0.05$). The OVA+AC group showed lower expression levels of both VCAM-1 and ICAM-1 in comparison with the OVA group ($p < 0.05$) (Fig. 4c, d, respectively).

Airway remodeling and expression of TGF- β and VEGF

AC did not change either the deposition of collagen and elastic fibers or the expression of TGF- β and VEGF in non-sensitized animals ($p > 0.05$). OVA sensitization increased the proportion of collagen and elastic fibers. Improvement of AC in sensitized animals (OVA+AC group) inhibited the deposition of collagen and elastic fibers when compared with the OVA group (Fig. 5a, b, respectively) ($p < 0.05$). The expression of TGF- β and VEGF was also enhanced in the OVA group ($p < 0.01$) and significantly inhibited by aerobic conditioning (OVA+AC group) (Fig. 5c, d, respectively).

Expression of Th1 cytokines

One possible mechanism of inhibition of Th2 cytokines was evaluated: modulation of Th1 cytokines (IL-2 and IFN- γ) (Fig. 6a, b, respectively). Our results show that neither AC nor OVA sensitization modified the expression of IL-2 or IFN- γ by inflammatory cells in the peribronchial compartment.

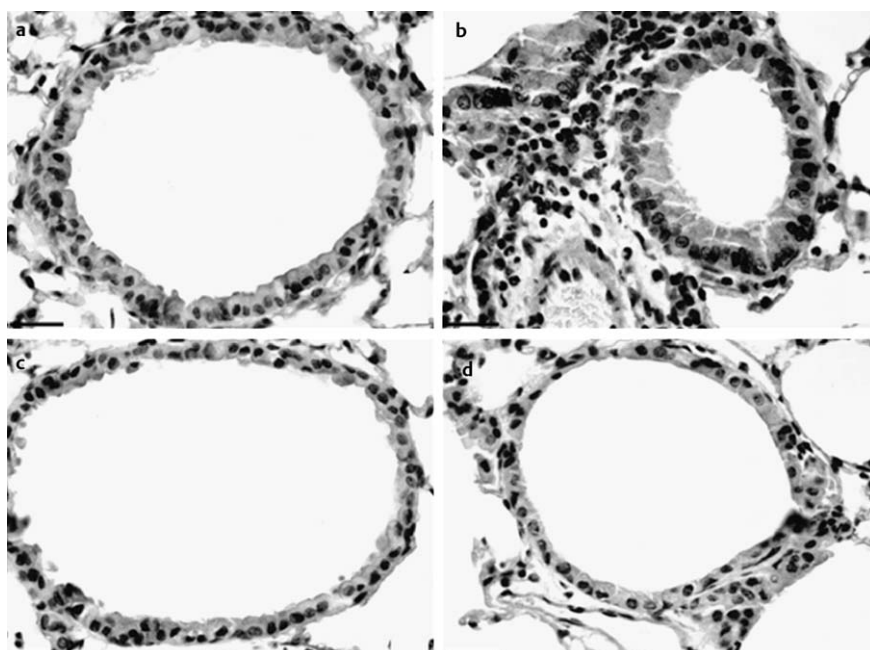


Fig. 3 Panels **a** through **d** show representative photomicrographs of airways stained with anti-IL-4 in the Control, OVA, AC and OVA + AC groups, respectively. Note inflammatory cells positive for IL-4 in the peribronchial compartment. Bars = 100 μ m.

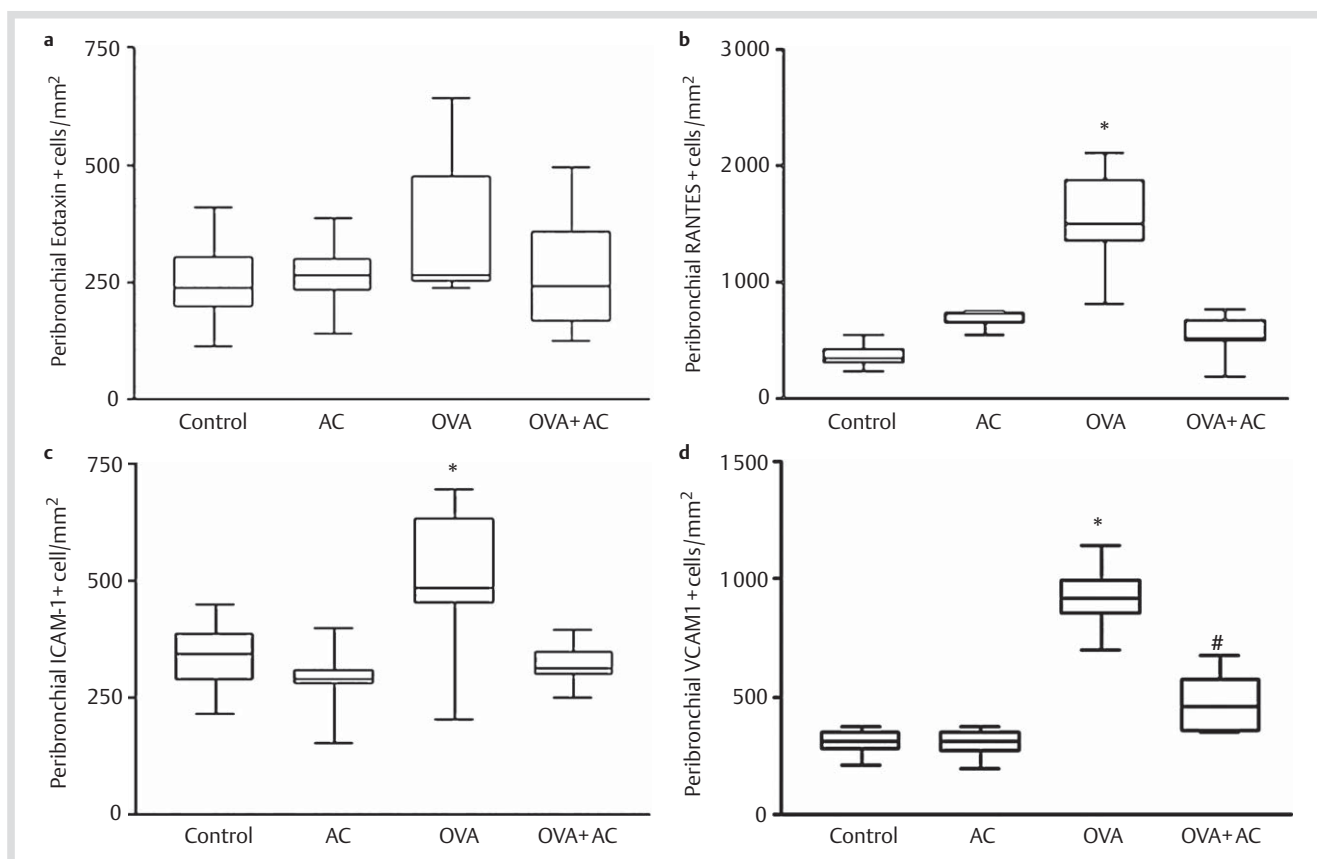


Fig. 4 Box plots representing the density of inflammatory cells in the peribronchial compartment positive for Eotaxin **a**, RANTES **b**, ICAM-1 **c** and VCAM-1 **d**; * $p < 0.01$ when compared with all groups and # $p < 0.01$ when compared with the Control and AC groups. No significant difference was observed in **Fig. 3a**.

Discussion

We demonstrate for the first time that aerobic conditioning beginning prior to sensitization and continued during a sensitization and allergen challenge period inhibited the development of allergic sensitization and inflammation as demonstrated by

the reduction of the following: 1) production of OVA-specific allergic immunoglobulins; 2) migration of eosinophils and lymphocytes into the airways; 3) expression of Th2 cytokines by inflammatory cells; 4) expression of adhesion molecules; 5) expression of chemokines; and 6) remodeling of airways.

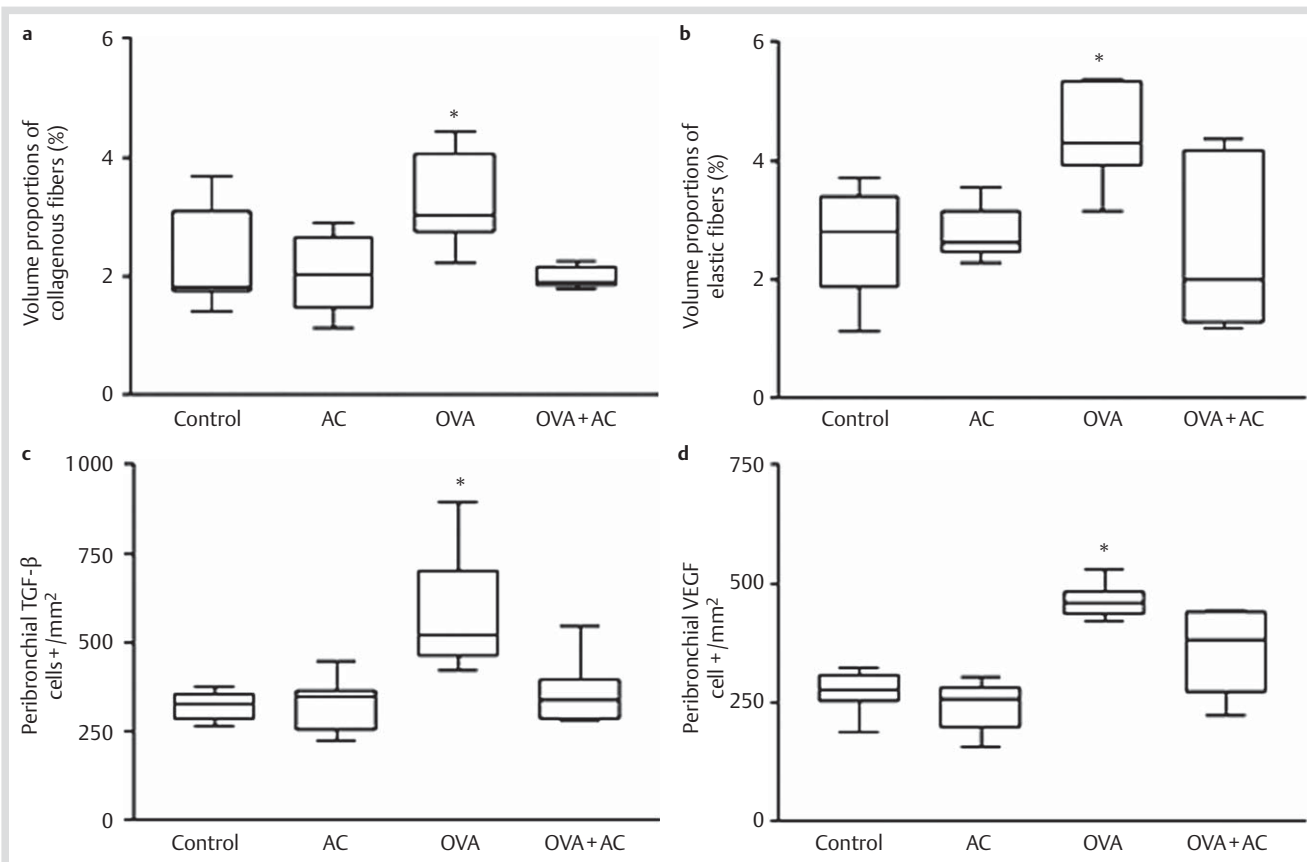


Fig. 5 Box plots representing volume proportion [%] of collagen **a** and elastic **b** fibers in the airways and density of inflammatory cells expressing TGF- β **c** and VEGF **d**. * $p < 0.05$ when compared with all groups.

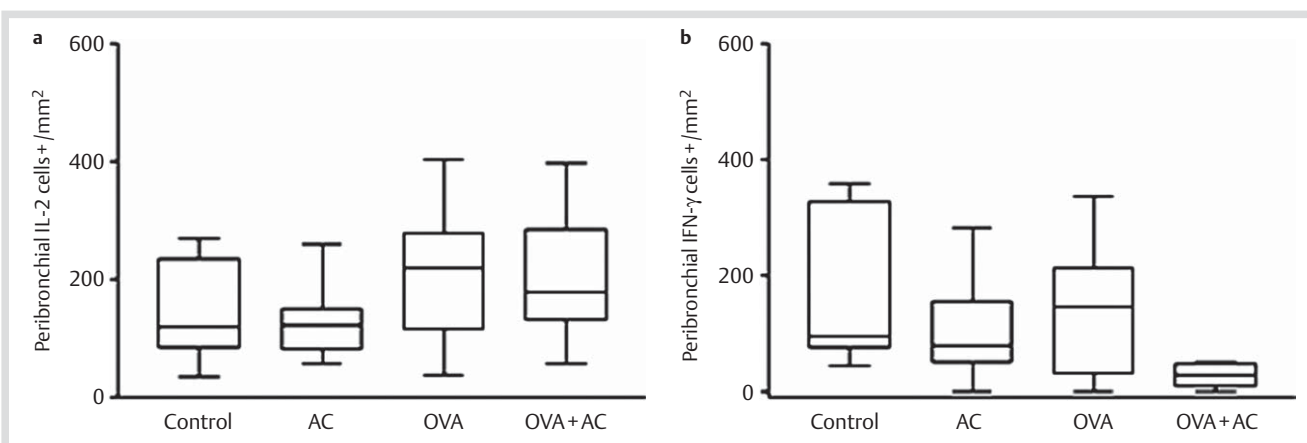


Fig. 6 Box plot of density of peribronchial inflammatory cells expressing IL-2 **a**; **b** IFN- γ . No differences were found in **a** or **b**.

In our study, aerobic conditioning was performed before the sensitization protocol, and the production of specific OVA-induced IgE and IgG₁ was inhibited. While previous studies have investigated the effects of aerobic conditioning on the production of immunoglobulins by using animal models of asthma, the data are controversial, and all of the studies evaluated the effects of aerobic conditioning during or after sensitization. Vieira and colleagues evaluated the effects of aerobic conditioning beginning after the first OVA aerosol challenge, but they did not observe any changes in the levels of OVA-specific IgE or IgG₁ [37]. On the contrary, Pastva and colleagues also evaluated the effects of aerobic conditioning on IgE levels during the sensitization

process, and they demonstrated a reduction in the OVA-specific IgE levels without changes of total IgE levels [25]. The discrepancies between these studies could be at least partially explained by the experimental protocols used because Vieira et al. began the physical training after the sensitization protocol; in Pastva's study, the physical conditioning began simultaneously with OVA sensitization. In any case, our study showed that improvement in aerobic conditioning before and during sensitization reduces the production of specific immunoglobulins; however, the mechanisms underlying such effects remain to be elucidated. The immune regulation of allergic responses seems to involve homeostasis of Th1/Th2 activity [17,29,30]. We observed an

inhibition of the expression of Th2 cytokines when animals were subjected to aerobic conditioning. This inhibitory effect of aerobic conditioning on the Th2 response was not followed by any changes in the expression of Th1 cytokines. These results reinforce the notion that the immune mechanisms underlying allergic diseases might be more complex than a simple Th1/Th2 dichotomy. Cross-sectional and longitudinal studies have demonstrated a strong association between an up-regulated Th2 immune response and atopic diseases; however, the relationship between Th1-mediated immune responses and atopic diseases is controversial [20]. Based on previous studies showing that aerobic exercise might increase Th1 responses [17,22,27,28,36] and to better understand the mechanisms involved in allergic inflammation, we hypothesized that aerobic conditioning could increase Th1 responses and thus cause a decrease in the expression of Th2 cytokines in this model of allergic airway inflammation.

However, our results show that aerobic conditioning did not modify the expression of Th1 cytokines (IL-2 and IFN- γ) by inflammatory cells. Although we did not observe any change in the expression of IFN- γ and IL-2 by inflammatory cells, we cannot exclude the possible role of exercise-induced expression of Th1 cytokines other than those tested here (e.g., IFN- α) that may be involved in the Th1/Th2 imbalance in asthma [9]. This hypothesis needs to be investigated in the near future.

Some studies showed that IL-4 and IL-5 act both directly and indirectly to promote the differentiation, proliferation, recruitment and survival of inflammatory cells at allergic sites [21,26]. In our animal model, large increases in the expression levels of IL-4 and IL-5 by inflammatory cells in peribronchial lung compartments were observed. IL-4 is involved in the synthesis of immunoglobulins by B lymphocytes, and this finding may suggest that aerobic conditioning could have at least partially inhibited IgE and IgG₁ production by reducing IL-4 expression. In this study, we observed that exercise strongly reduces the expression of Th2 cytokines (IL-4, IL-5 and IL-13) by inflammatory cells, while decreasing eosinophil migration to the airways. IL-13 also promotes the recruitment of eosinophils by promoting VCAM-1 expression in vascular endothelium. Based on our results, we can then hypothesize that aerobic exercise reduced the eosinophilic inflammation by reducing Th2 cytokine expression and the expression of adhesion molecules (ICAM-1 and VCAM-1).

The OVA sensitization protocol employed in this study induced airway remodeling characterized by the increased deposition of collagen and elastic fibers. Airway remodeling has been generally considered as a consequence of persistent inflammation because it can continue even after the effective treatment of airway inflammation. Adhesion molecules (e.g., ICAM-1 and VCAM-1) and growth factors (e.g., TGF- β and VEGF) have important roles in airway remodeling. VCAM-1 and ICAM-1 contribute to airway eosinophilic inflammation [42,43], and during chronic allergic lung inflammation, TGF- β promotes airway remodeling [4,23]. VEGF can also mediate airway inflammation and remodeling in allergic airway disease. Our results show that aerobic exercise inhibited the expression of ICAM-1 and VCAM-1 as well as eosinophil migration and expression of TGF- β and VEGF. Taken together, our results demonstrate that improvement in aerobic conditioning inhibits airway inflammation and consequent remodeling in our model of allergic airway inflammation, probably by modulation of VCAM-1, ICAM-1, VEGF and TGF- β . Our data suggest for the first time that aerobic conditioning performed before and during the sensitization period has a preven-

tive role in the development of allergic inflammation. In addition, these changes were followed by inhibition of the expression of Th2 cytokines, eosinophilic and lymphocytic airway inflammation and remodeling and also by decreased IgE and IgG₁ production. Allergic reactions might occur with a higher intensity near the 2 ends of human life (i.e., childhood and old-age) when the immune system is biased toward Th2-like responses [5,33]. Interestingly, it is also near these 2 endpoints of life that humans are likely to be more physically deconditioned [14].

We conclude that the improvement in physical conditioning before and during the sensitization phase seems to have a preventive role in allergic airway inflammation and remodeling that involves an inhibition of OVA-specific IgE and IgG₁ production and Th2 responses in mice.

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