

Comparison of the Effects of Aerobic Conditioning Before and After Pulmonary Allergic Inflammation

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Abstract—The aim of this study is to compare the effects of aerobic conditioning (AC) before (ACBS) and after (ACAS) allergic sensitization. BALB/c mice were divided into two main groups: ACBS and ACAS. Each group was divided into subgroups: control (nonsensitized/nontrained), AC (nonsensitized/trained), ovalbumin (OVA) (sensitized/nontrained), AC + OVA (trained/sensitized), and OVA + AC (sensitized/trained). Sensitization was induced using OVA and AC performed in treadmill (moderate intensity). We examined IgE and IgG₁ levels, eosinophil counting, expression of Th1 (interleukin (IL)-2, IFN- α) and Th2 cytokines (IL-4, IL-5, IL-13), IL-10, vascular endothelial growth factor (VEGF), and airway remodeling. IgE and IgG₁ were decreased only when exercise was performed before sensitization (ACBS); however, there was a decrease of eosinophils, Th2 cytokines, VEGF, and airway remodeling and increase in IL-10 in either ACBS or ACAS groups. Our results demonstrate that aerobic conditioning reduces Th2 response before and after sensitization by increasing IL-10 while the production of anaphylactic antibodies is reduced only when exercise is performed before sensitization.

KEY WORDS: exercise; cytokine; allergy; immune system; remodeling.

INTRODUCTION

Studies have shown that improvements in physical fitness reduce low-grade systemic inflammation [1–3], suggesting that aerobic conditioning (AC) either at low or at moderate intensity can trigger anti-inflammatory effects in nonallergic diseases [4, 5]. Allergic diseases are distinct from low-grade inflammation, and the anti-inflammatory effect of AC on diseases such as asthma remains less understood. Allergic diseases are characterized by a predominant Th2 response [6, 7], and the increase in its prevalence has been associated with a western lifestyle (e.g., sedentary lifestyles and indoor entertainment), suggesting

an imbalance in the immune response (Th1/Th2 pathways), a supposition referred to as the “hygiene hypothesis” [8, 9]. This hypothesis is reinforced by evidence showing that more physically active subjects have fewer respiratory symptoms [10] and that regular exercise is important in reducing allergic inflammation [11].

The benefits induced by AC in patients with allergic disease such as asthma were initially described by Clark and Cochrane [39] suggesting an increase in the ventilatory threshold. Later on, Pastva *et al.* [40] and Vieira *et al.* [13] showed that AC could reduce the airway inflammation in a murine asthma model, demonstrating that improvements in physical fitness can also reduce Th2 inflammation in allergic diseases such as asthma. More recently, a study also demonstrated that improvements in AC can reduce exhaled nitric oxide and eosinophil counting and in the sputum of asthmatic patients [12]. Although these anti-inflammatory effects of AC on allergic diseases have been suggested, the mechanisms remain poorly understood.

In previous studies, we showed that AC reduces the Th2 response by decreasing the expression of the interleukin (IL)-4, IL-5, and IL-13 without altering the expression

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of the Th1 cytokines (interferon gamma (IFN- γ) and IL-2); in addition, AC reduced airway remodeling [13, 14]. However, in these studies, AC was performed after the allergic inflammation has been established, and little is known about the effect of AC before the induction of allergic airway injury. Our goal was to compare mechanisms induced by AC in airway inflammation before and after allergic sensitization aiming to understand what occurs in real life when individuals are more physically active before allergen exposure (before sensitization) with those observed by asthmatic after improving aerobic fitness (after sensitization). Then, in the present study, we compared the effects of AC both before and after the allergic airway inflammation protocols.

METHODS

This study was approved by the review board for human and animal studies of the School of Medicine of the University of São Paulo (São Paulo, Brazil). All of the animals in the study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NHI publication 85–23, revised 1996). The experiments were approved by the committee of ethics of the Medicine School, University of São Paulo (USP).

Animals

We used 64 mice (BALB/c males) at 6–8 weeks of age. The animals were kept in the Central Animal FMUSP with a temperature-controlled (22–25 °C) room, a 12-h light/dark cycle at 70 % humidity, and access to food and water *ad libitum* (Purina, Brazil).

Experimental Groups

Sixty-four BALB/c mice were divided into two groups: aerobic conditioning before (ACBS) or after (ACAS) sensitization (32 each). In the ACBS, mice were divided into four subgroups (eight each): control (nonsensitized and nontrained), aerobic exercise conditioning (AC) (nonsensitized and trained), ovalbumin (OVA) (nontrained and sensitized), and 8 weeks aerobic exercise conditioning followed by OVA expositions (AC + OVA). In the ACAS, mice were divided into four subgroups (eight each): control (nonsensitized and nontrained), aerobic exercise conditioning (AC) (nonsensitized and trained), OVA (sensitized and nontrained), and OVA followed by aerobic exercise conditioning (OVA + AC) (sensitized and trained).

Protocols of AC and Pulmonary Allergic Inflammation

ACBS group. Animals were assigned to one of the four groups as follows: control (CT), AT, OVA, and AT + OVA. First, all of the animals were initially adapted to the treadmill for 5 days (15 min, 25 % incline, and 0.2 km/h). After that, a maximal exercise capacity test was performed with a 5-min warm-up (25 % incline and 0.2 km/h) followed by an increase in the treadmill speed (0.1 km/h every 2.5 min) until animal exhaustion defined as the inability to continue to run, even after ten gentle mechanical stimulations. The maximal aerobic capacity was defined as the maximum speed reached by each animal. The mice were trained at a low exercise intensity (50 % of maximum speed) [14]. The 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 (OVA and AC + OVA subgroups) or saline administration (CT and AT subgroups) began and the exercise training continued until the animals were euthanized. The BALB/c mice were sensitized with two intraperitoneal injections of OVA (intraperitoneal (i.p.), 20 μ g/mouse on days 56 and 70) adsorbed with aluminum hydroxide (OVA + Al(OH)₃) followed by four inhalations of OVA (1 %) or saline solution on days 77, 79, 81, and 83 (Fig. 1).

ACAS group. Animals were assigned to one of four groups ($n=8$ in each group) as follows: CT, AT, OVA, and OVA + AT. The OVA groups received OVA (i.p., 10 mg/mouse) diluted in aluminum hydroxide on days 0, 14, 28, and 42. After day 21, the mice were exposed to an OVA aerosol (1 %) for 30 min per session for three sessions a week until day 54. The CT and AT groups were exposed to a saline solution. On days 21 to 23, the mice were placed on the treadmill for 15 min at a speed of 0.2 km/h and at a 25° incline. On days 25 and 53, a maximal exercise test was performed. The treadmill training (50 % of maximal exercise capacity reached in the physical exercise test) began on day 28 and was performed over 4 weeks, 60 min per session for five sessions a week. In both moments, the nonsensitized groups (CT and AT) received only i.p. aerosol with saline.

Anesthesia and Blood Collection

The euthanasia of the ACBS groups occurred on day 86, and for the ACAS groups, it occurred on day 56. The animals were anesthetized with sodium thiopental (50 mg/kg, i.p.) (Cristalia, Brazil), tracheotomized with a polyethylene tube, and shaved aseptically in the abdominal region

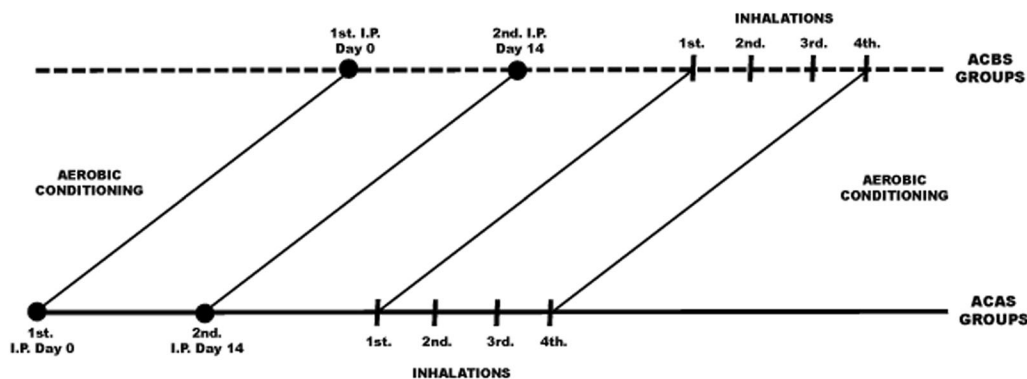


Fig. 1. Design of study protocol. ACBS—aerobic conditioning (AC) followed by ovalbumin (OVA) sensitization and inhalations. ACAS—OVA sensitization and inhalations followed by AC (OVA + AC). This schematic figure demonstrates that both protocols have a significant similarity in the chronic allergic sensitization and inflammation; *i.p.* intraperitoneal injections; *OVA* ovalbumin; *1st*, *2nd*, *3rd*, and *4th* number of OVA inhalations.

using povidone iodine (Polvidini, Brazil) before performing the incision, and then, 1 mL of blood was collected from the inferior vena cava with a syringe previously prepared with 0.03 mL of sodium heparin (Hipolabor, Sao Paulo, Brazil). After the blood collection, while still under anesthesia, the animals were sacrificed by severing the vena cava. Then, the blood samples were centrifuged at 5 °C for 15 min at 1000 rpm, and the plasma was collected and aliquoted (50 μ l) and stored at –80 °C for future analysis [13–15].

Collection of Lungs and Immunohistochemistry

The chest was opened and the lungs were removed. The right lung was then removed, pre-fixed with 0.30 mL of 10 % formalin, and immersed in 4 % formalin for 12 h. After this period, the lungs were submitted to routine histological paraffin embedding [16]. Sections of 5- μ m thickness were stained with (i) Luna to evaluate the density of the peribronchial eosinophils [17], (ii) picrosirius to analyze the content of collagen fibers in the airway walls, and (iii) resorcin-fuchsin to evaluate the elastic fibers. Blank slides were silanized for immunohistochemistry [13, 14]. The immunohistochemistry was performed on histological sections of lung tissue. Briefly, the sections were deparaffinized and hydrated. After blocking the endogenous peroxidases, an antigen retrieval step was performed using either high-temperature citrate buffer (pH=6) or trypsin. The following primary antibodies were used: IL-4 (1:100), IL-5 (1:300), IL-13 (1:350), IL-10 (1:600), IFN- γ (1:100), IL-2 (1:100), and vascular endothelial growth factor (VEGF) (1:3000) (Santa Cruz, CA, USA).

The secondary antibodies were also applied without primary antibody in positive and negative tissue samples, serving as CTs. The counting of peribronchial eosinophils was performed by counting the number of Luna-stained positive cells using a Weibel reticle. The inflammatory infiltrate between the bronchus and the adjacent artery divided by the number of points corresponded to the total area of tissue (magnification of $\times 1000$). On each slide, five airways were analyzed [17–19].

Analyses of IgE and IgG₁ Titers for PCA

A passive cutaneous anaphylaxis (PCA) reaction was used to detect and estimate the levels of anaphylactic IgE and IgG₁ OVA-specific antibodies. The title of the antibody class IgE anti-OVA was determined by passive cutaneous anaphylaxis reaction in Wistar rats. These animals were used because only this class of anaphylactic antibodies (IgE) mice is able to sensitize the skin of rats. For IgG₁ measurement, the serum was heated for 3 h at 56 °C to inactivate IgE activity; the heated serum was injected for PCA after a short latency period [20]. The title of the antibody class IgG₁ anti-OVA was determined using the BALB/c mice. The backs of the animals were shaved and injected intradermally with different dilutions (from 1:5 to 1:1280) 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 [20].

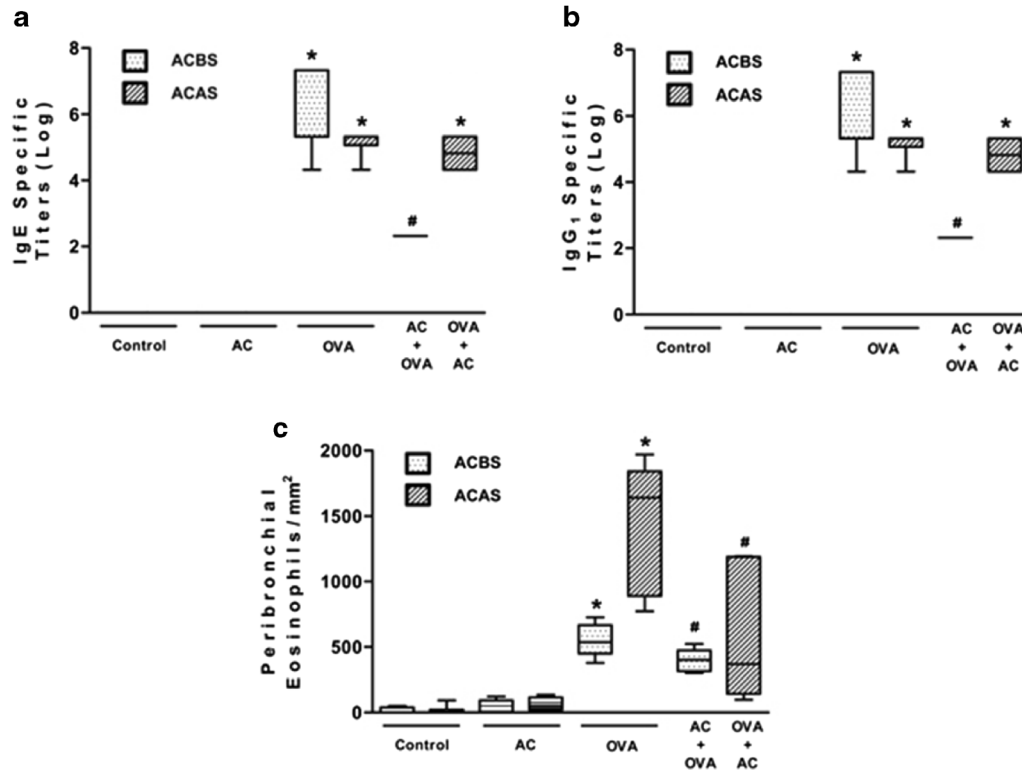


Fig. 2. IgE (a) and IgG₁ (b) levels: * $P < 0.001$ when comparing the OVA groups with the CT groups. # $P < 0.05$ when comparing the OVA groups with the AC + OVA group (ACBS). Peribronchial eosinophils (c): * $P < 0.05$ when comparing the OVA groups with the CT and AC groups. # $P < 0.05$ when comparing the AC + OVA and OVA + AC groups with the OVA groups and the AC groups, respectively.

Evaluation of Remodeling by Image Analysis

The airway remodeling study was done by the analysis of collagen and elastic fiber deposition; these features were evaluated by image analysis using an Eclipse E-200 (Nikon, Japan) microscope, a Cool Snap digital camera (Photometrics, Japan), and image analysis software (Image Pro Plus 4.5, CA, USA). Five airways from each animal were evaluated at $\times 400$ magnification. The deposition of collagen and elastic fibers in the airways was assessed in the area between the epithelial basement membrane and the adventitia of the airway. The positive areas were expressed as a percentage of the total area of the airway walls, and the results are expressed as a percentage [14].

Statistical Analysis

The parametric data were analyzed by two-way ANOVA followed by the Student–Newman–Keuls post hoc test, and the nonparametric data were analyzed by two-way ANOVA followed by the Holm–Sidak post hoc

test. The data are presented as the mean $\pm$ SD for the parametric data and as the median (variance) for the nonparametric data. The significance level was adjusted to 5 %.

RESULTS

Sensitization and Eosinophilic Airway Inflammation

The levels of the specific IgE and IgG₁ immunoglobulins were increased in all of the sensitized groups (OVA, AC + OVA, and OVA + AC) when compared with the nonsensitized groups (CT and AC; $P < 0.05$). Exercise only reduced the IgE and IgG₁ levels in the AC + OVA groups (ACBS) ($P < 0.05$). Peribronchial eosinophils were increased in all of the sensitized groups when compared with the nonsensitized groups ($P < 0.05$), and exercise reduced eosinophil migration when the exercise was performed either before or after the sensitization (Fig. 2; $P < 0.05$).

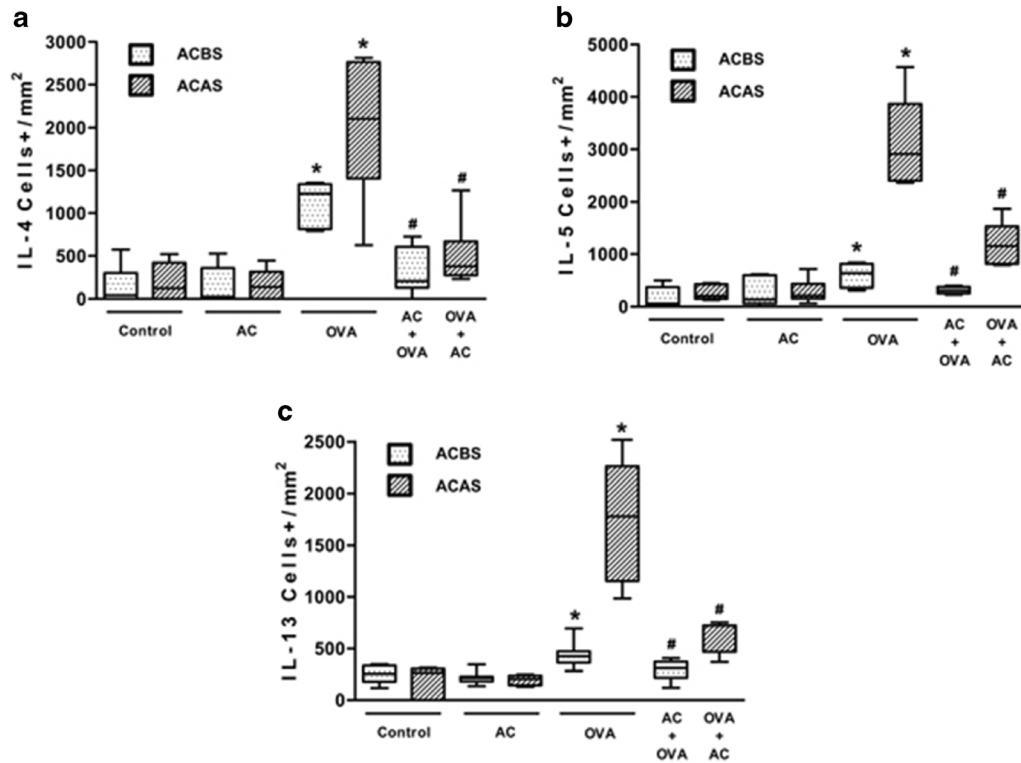


Fig. 3. The production of IL-4 (a), IL-5 (b), and IL-13 (c) by positive peribronchial cells. * $P < 0.05$ when comparing the OVA groups with the CT groups. # $P < 0.05$ when comparing the AC + OVA and OVA + AC groups with the OVA groups and the AC groups, respectively.

Th2 Response

In the present study, the Th2 response was evaluated by the expression of IL-4, IL-5, and IL-13, and our results show an increase in the levels of these cytokines in the sensitized groups (OVA) when compared with the non-sensitized animals (CT and AC; $P < 0.05$). The sensitized + AC and AC groups presented reduced levels of IL-4, IL-5, and IL-13 when compared with the OVA groups when the exercise was performed either before or after the sensitization, respectively (Fig. 3; ACBS and ACAS groups; $P < 0.05$).

Analyses of Remodeling Features

We also analyzed the remodeling features in the airways. The results show an increase in the collagen and elastic fibers deposited in all of the sensitized groups (OVA) when compared with the non-sensitized animals (CT and AC; $P < 0.05$). The sensitized + AC and AC animals had reduced collagen and elastic fiber deposition when compared with the OVA groups when the exercise was performed either before or after the sensitization,

respectively (ACBS and ACAS groups; $P < 0.05$). The production of VEGF was also increased in all of the sensitized groups (OVA) when compared with the non-sensitized animals (CT and AC; $P < 0.05$). The sensitized + AC and AC animals had reduced VEGF production when compared with the OVA groups when the exercise was performed either before or after the sensitization, respectively (Fig. 4; ACBS and ACAS groups; $P < 0.05$).

Mechanisms of Protection and Anti-Inflammatory Effects

The Th1 response was evaluated by immunohistochemistry. The results did not change in any of the groups when the production of IL-2 and IFN- γ ($P > 0.05$) was evaluated. The production of the anti-inflammatory cytokine IL-10 did not increase in the OVA groups (ACBS and ACAS, $P > 0.05$); however, the sensitized + AC and AC animals had increased IL-10 levels when compared with the OVA groups when the exercise was performed either before or after the sensitization, respectively (Fig. 5; ACBS and ACAS groups; $P < 0.05$).

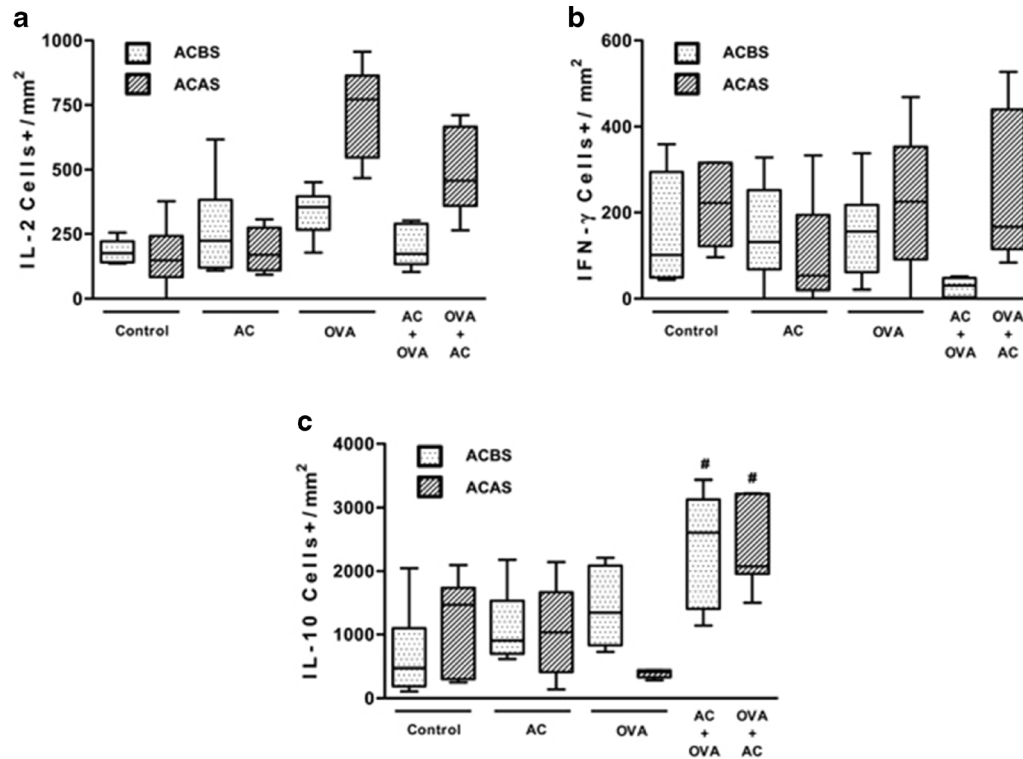


Fig. 4. The production of IL-2 (a), IFN- γ (b), and IL-10 (c) by positive peribronchial cells. # $P < 0.05$ when comparing the AC + OVA and OVA + AC groups with the OVA groups and the AC groups, respectively.

DISCUSSION

Our results show that aerobic conditioning (AC) exercise decreased the production of specific immunoglobulins (Ig) E and IgG₁ only when it was performed before the sensitization, while the peribronchial eosinophilic inflammation, airway remodeling, and the production of Th2 cytokines (IL-4, IL-5, and IL-13 cytokines) were observed when the AC was performed either before (ACBS) or after the sensitization (ACAS). We also showed that the decrease in the allergic response promoted by the AC in both protocols increased the expression of IL-10, suggesting that this cytokine mediates the anti-inflammatory response.

Despite the fact that regular exercise reduces low-grade inflammation in diseases such as obesity [21] and diabetes [22], the effects of AC on the allergic inflammatory diseases are poorly understood. Our group has evaluated the effects of AC in asthma animal models [14, 15, 23, 24] and in asthmatic subjects [12], and we have shown that aerobic exercise conditioning can reduce airway inflammation when performed after sensitization. In the present study, we added information to the literature showing that

AC is also able to reduce eosinophil migration when the exercise was performed either before (ACBS group) or after (ACAS group) the onset of allergic inflammation. These results suggest that an improvement in exercise capacity might reduce the onset of allergic disease or that it might reduce the effects of ongoing allergic disease.

In our opinion, the benefits promoted by AC either before or after sensitization allergic could be attributed to three possible mechanisms: (i) changes in the immunoglobulin E (IgE) and IgG₁ expression, (ii) imbalance in the Th1/Th2 response (hygiene hypothesis), (iii) changes in the expression of anti-inflammatory cytokines. All of these mechanisms were evaluated in the present study, and our results demonstrate that AC reduces the expression of IgE and IgG₁ when the exercise is performed before but not after the sensitization. Our data do not corroborate the findings of Pastva *et al.* [40] that showed a reduction in the OVA-specific IgE levels when the AC was performed after the sensitization. On contrary, studies in mice [13–15] and in guinea pigs [24] corroborate our findings, reinforcing the observation that the AC does not modify the levels of Igs when performed after the mice have been exposed to

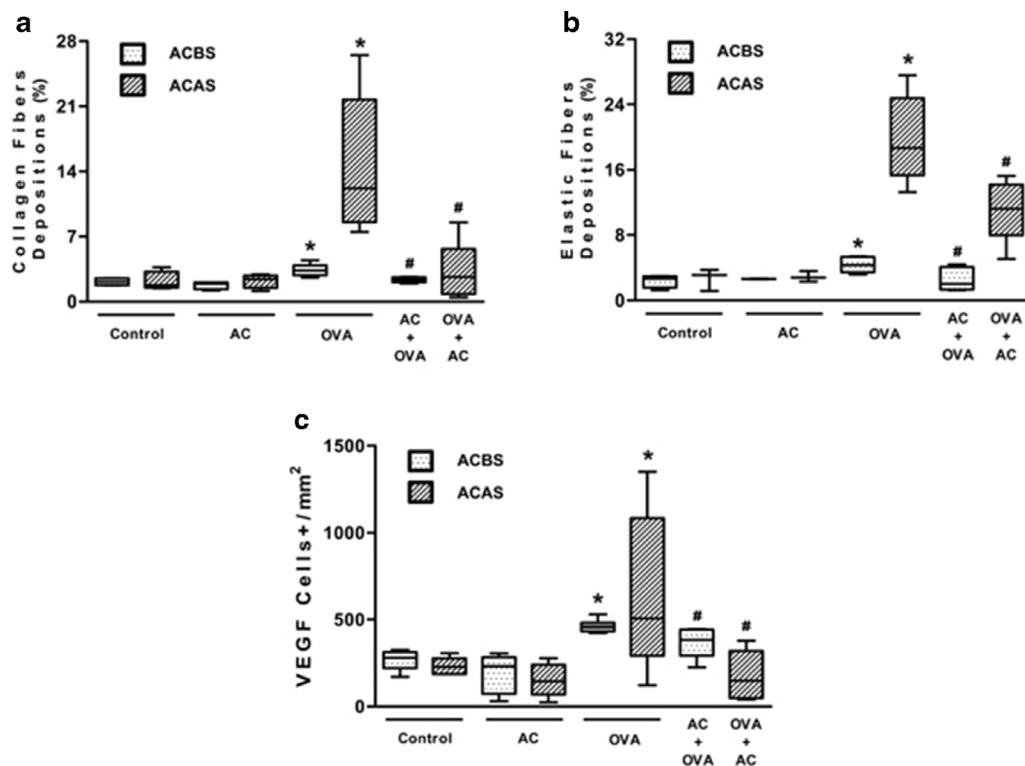


Fig. 5. Collagen (a) and elastic fiber (b) deposition. The production of VEGF (c) in positive cells. * $P < 0.05$ when comparing the OVA groups with the CT groups. # $P < 0.05$ when comparing the AC + OVA and OVA + AC groups with the OVA groups and the AC groups, respectively.

the allergic sensitization. These findings demonstrate that AC prior to allergen exposure significantly reduces the expression of anaphylactic antibodies. Although purely speculative, we can hypothesize that the increase in aerobic capacity reduces the expression of IL-4 and IL-5, attenuating Th2-mediated responses and subsequent humoral responses, such as the production of allergen-specific antibodies. This hypothesis is based on previous findings reported in the literature. First, a study showed that exercise can modulate the capacity of macrophages to present antigen to B lymphocytes through a pathway that modulates the production of Ig antibodies [41]. Second, current and previous findings from our group demonstrated that exercise, when performed during or after sensitization, reduces the production of IL-4 and IL-5 [13, 24]. It is important to reinforce that Pastva and colleagues [40] were the first to suggest this hypothesis.

We also tested whether AC could modify the Th1/Th2 balance by quantifying the Th1 (IL-2 and IFN- γ) and Th2 cytokines (IL-4, IL-5, and IL-13), and our results show that aerobic exercise reduces the expression of the Th2 but not of the Th1 cytokines independently of whether the increase

in exercise capacity occurs before or after the sensitization. Interestingly, the hygiene hypothesis postulates that the increase in the prevalence of allergy associated with a westernized lifestyle (for instance, obesity and a sedentary lifestyle) decreases the Th1 response leading to an increase in the Th2 response (Th1/Th2 imbalance). However, based on our results, we could suggest that the reduction in the allergic response induced by exercise occurred directly by reducing the Th2 pathway. There are very few studies evaluating the effects of AC before sensitization; however, our findings on the reduction of the Th2 response induced by AC are corroborated by previous studies independent of the chronic allergic exposure or the animal model [14, 19, 23–25].

Previous studies have shown that AC can modulate the expression of anti-inflammatory cytokines in such nonallergic chronic disease such as atherosclerosis [26], cancer [27], and type 2 diabetes [28]. Based on these findings, we investigated whether AC could modulate the expression of anti-inflammatory cytokines such as IL-10 before and after allergic sensitization, and our results demonstrate that IL-10 is

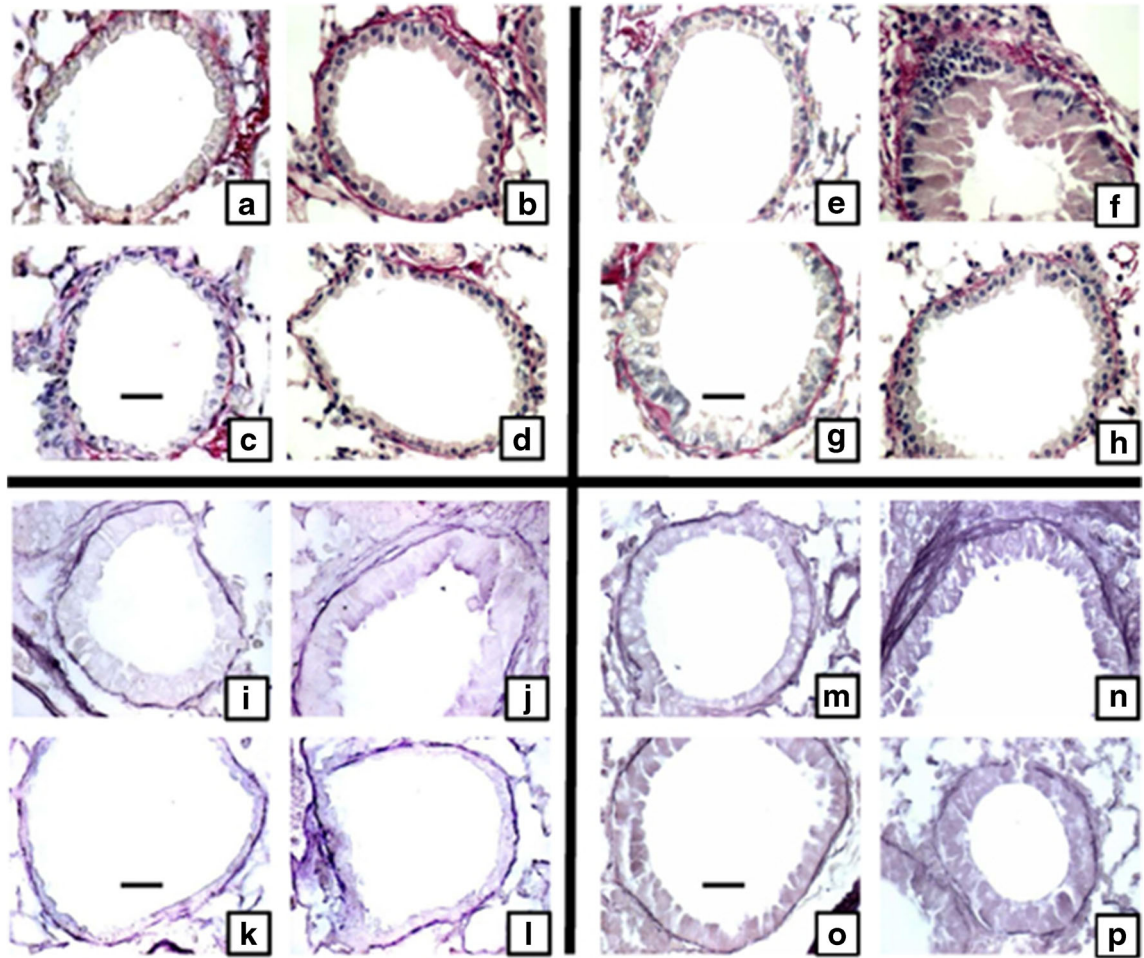


Fig. 6. Representative photomicrographs of collagen and elastic fibers. Scale bars = 25 μ m. ACBS groups: a, i control; c, k AC; b, j OVA; d, l AC + OVA. ACAS groups: e, m control; g, o AC; f, n OVA; h, p OVA + AC.

increased when exercise is performed either before or after exercise. These data are quite relevant because they show, for the first time, that the increase in aerobic capacity can alter the functionality of the immune system against future allergic exposures through IL-10.

Importantly, the improvements in the AC performed either before or after allergic sensitization promoted a reduction in the Th2 response (IL-4, IL-5, and IL-13), in the migration of eosinophils to the airways and in the airway remodeling (reducing collagen and elastic fiber deposition (Fig. 6)). Since eosinophils seem to contribute to the maintenance of the airway inflammation [13, 14, 25, 29] and remodeling [30, 31], our results suggest that the reduction in the eosinophil migration may have been the main reason for the reduced airway remodeling. However,

based on our data, we can also suggest that reduction in the airway remodeling could be a result of a decrease in the levels of VEGF, which has been previously shown to promote the formation of blood vessels, to mediate vascular and extravascular remodeling, and to promote inflammation in the lung [30–33]. Taken together, our results demonstrate that an improvement in AC inhibits airway inflammation and remodeling either before or after the allergic sensitization due to the reduction in the Th2 response, VEGF production, and eosinophil migration.

Limitations

The present study has some limitations. First, the benefits of AC (before and after allergen sensitization) were evaluated in experimental models of pulmonary allergic

disease that simulate asthma pathophysiology in mice but do not necessarily represent what occurs in asthmatic patients. For instance, mice present parenchymal and vascular inflammation, which is not seen in human asthmatics [34]. On the other hand, the murine model has been considered to be the most representative of human asthma and presents all features of chronic airway inflammation [35]. Second, the time course of sensitization and aerobic training in both animal models used in the present study was not exactly identical; however, this does not seem to impact the results, which were proposed based on two previous assumptions reported in the literature: First, we used asthma murine models that mimic airway inflammation to that which occurs in asthma pathophysiology [35, 36]. Second, the exercise intensity (50 % of maximal exercise capacity) [13, 37] and exercise conditioning duration [23, 38] were previously shown to have anti-inflammatory effects.

CONCLUSION

Our results demonstrate that improvement in the aerobic capacity also performed before or after allergic sensitization promotes anti-inflammatory effect by increasing the expression of IL-10. In addition, a reduction in the expression of allergic Igs occurs only when exercise is performed before sensitization.

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