Exercise Reduces Effects of Creatine on Lung

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Key words
- allergy
- asthma
- creatine supplementation
- treadmill training
- Th2 response
- IL-10

Abstract

We recently demonstrated that creatine supplementation increased some features of lung allergic sensitization in mice. On the other hand, other studies have shown that aerobic exercise inhibited allergic airway inflammation and remodeling. We hypothesized that aerobic exercise may decrease the exacerbatory effects of the creatine supplementation in a murine model of asthma. Balb/c mice were divided into six groups: Control, Creatine (Cr), Low Intensity Exercise+Creatine (Low+Cr), Ovalbumin (OVA), Ovalbumin+Creatine (OVA+Cr) and Ovalbumin+Creatine+Low Intensity Exercise (OVA+Cr+Low). OVA-sensitized groups were sensitized with OVA intraperitoneal injections (days 0, 14, 28, and 42). Aerosol challenge (OVA 1%) and Cr treatment (0.5 g/kg/day) were initiated on Day 21 until Day 53. Low intensity exercise began on day 22 and was sustained until day 50. Low intensity exercise in the presence of creatine supplementation in sensitized mice resulted in a decreased number of eosinophils in BALF ($p<0.001$) and in the airways ($p<0.001$), and a decreased density of inflammatory cells positive to IL-4 ($p<0.001$) and IL-5 ($p<0.001$), airway collagen ($p<0.001$) and elastic fibers ($p<0.001$) content, airway smooth muscle thickness ($p<0.001$) and bronchoconstriction index ($p<0.05$) when compared with OVA+Cr group. These results suggest that aerobic exercise reduces the exacerbatory effects of creatine supplementation in chronically sensitized mice.

Introduction

Asthma is a chronic inflammatory disease that mainly affects the airways, but also lung vessels and parenchyma [27,29]. The Th_2 immune response in asthma leads to persistent airway inflammation and remodeling, resulting in decreased lung function [27]. Several inflammatory mediators such as leukotrienes, prostaglandins, cytokines, neuropeptides and complement degradation products are involved in the initiation and maintenance of airway inflammation [8]. It has been proposed that purines, mainly adenosine triphosphate (ATP], have a key role in allergen-driven lung inflammation, leading to increased mast cells activation, release of inflammatory cytokines and leukocytes chemotaxis [8,25]. Idkzo et al. [8] demonstrated that increased levels of ATP in the airways are associated with the development of many characteristics of asthma, such as bronchial hyperresponsiveness, eosinophilic airway inflammation, and Th_2 cytokine production. ATP also acts as a mediator of intercellular communication on leukocytes and dendritic cells through its purinergic receptors P2XR and P2YR [8].

Creatine (Cr) is a nutritional supplement synthesized from arginine, glycine and methionine. It represents a high energy buffering system that provides constant ATP supply to the cells [9]. Both short and long term Cr supplementation result in increased tissue content of both Cr and ATP [9,10]. Although Cr supplementation has been used mainly by athletes to improve performance [1], creatine has also been shown to have a beneficial role on chronic diseases such as chronic obstructive pulmonary disease [7], heart diseases [23] and neuromuscular disorders [4]. Since asthma prevalence is high among athletes and creatine is the main nutritional supplement used by sportsmen [12,22], our group recently investigated the role of Cr supplementation in a murine model of asthma [28]. We observed that creatine exacerbated all features associated with chronic allergic lung inflammation, such as airway eosinophilic inflammation, increased expres-

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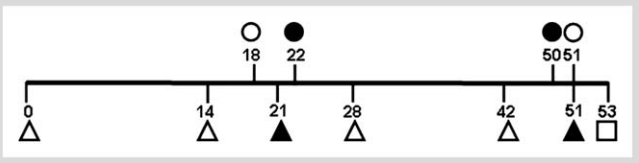

Fig. 1 Time line of the experimental protocol. Mice received intraperitoneal injections of either ovalbumin solution (10 µg per animal) or vehicle on days 0, 14, 28 and 42 (open triangles). Aerosol challenges with either ovalbumin 1 % solution or vehicle were performed three times per week starting on day 21 (closed triangles) until day 51. Creatine administration began on day 21 (closed triangles). Maximal exercise capacity test was performed on days 18 and 51 (open circles). Physical training (five times per week) was started on day 22 (closed circle) and performed until day 50. End of creatine administration and animals sacrifice on day 53 (open square).

sion of Th_2 cytokines, airway remodeling and hyperresponsiveness, suggesting that creatine might have adverse effects on individuals with asthma [28].

There is increasing evidence demonstrating that asthmatic patients benefit from aerobic exercise, with an improvement in ventilatory capacity, a reduction of asthma-related symptoms, improvement of physical fitness and work capacity, and a decrease in dyspnea, exercise-induced bronchospasm, as well as peak expiratory flow variability [5,21]. Experimental studies using murine models of asthma have attempted to elucidate the mechanisms underlying these effects [17,27]. We recently demonstrated that mice with chronic allergic lung inflammation that were subject to both low and moderate intensity aerobic exercise presented a decrease in eosinophilic lung inflammation and Th_2 cytokine expression, as well as a decrease in airway remodeling; these effects were partially mediated by increased IL-10 expression and decreased NF-κβ expression [27,29].

Since Cr supplementation exacerbates the Th_2 immune response in sensitized mice [28] and aerobic exercise decreases this response [17,27], and since the main consumers of Cr supplementation are sportsmen who are often also asthmatic [1,3], we aimed to investigate if aerobic exercise could reduce the exacerbatory effects of Cr supplementation in mice with chronic allergic lung inflammation.

Materials and Methods

This study was approved by the University of Sao Paulo School of Medicine review board for human and animal studies, process number 503/05.

Experimental groups

Male Balb/c mice (20–25 g) were divided into six groups, Control (n = 7), Creatine (Cr, n = 8), Low Intensity Aerobic Exercise + Creatine (Low + Cr, n = 10), Ovalbumin (OVA, n = 8), Ovalbumin + Creatine (OVA + Cr, n = 8) and Ovalbumin + Creatine + Low Intensity Aerobic Exercise (OVA + Cr + Low, n = 8). The study was performed using the same animals as in previous published studies [27,28].

Induction of chronic allergic lung inflammation

OVA, OVA + Cr and OVA + Cr + Low groups were sensitized by an intraperitoneal (i. p.) injection of OVA (10 µg) diluted using three milliliters of sterile saline and two milliliters of alum hydroxy solution (200 µl/mouse) on days 0, 14, 28 and 42. From day 21, OVA, OVA + Cr and OVA + Cr + AE groups received OVA aerosol (1 g OVA diluted in 100 ml of sterile saline) three times per week (30 min/day) up to day 53 [27–29]. Control, Cr and Low + Cr groups were subject to the same protocol using sterile saline [28] instead.

Creatine treatment

The oral creatine supplementation (0.5 g/kg – five times per week) was diluted in sterile water (total volume 50 µl per animal), and animals were given the creatine from day 21 up to day 53. Control and OVA groups were subject to the same protocol using sterile saline [28] instead. All experimental procedures are represented in ○ **Fig. 1**.

Aerobic exercise treadmill test and training

Animals were initially adapted to the treadmill for three days (15 min, 25 % inclination, 0.2 km/h). After that, a maximal exercise capacity test was performed with a five-minute 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, i. e., until they were not able to run even after 10 mechanical stimuli [27]. The test was repeated 72 h before sacrificing the mice. Maximal aerobic exercise capacity (100 %) was established as the speed reached by each animal. Mice were trained to perform low intensity aerobic exercise (50 % of maximal speed) for 60 min a day, five days a week [27]. Aerobic conditioning began on the first day following OVA or saline inhalation and creatine administration [27].

Bronchoalveolar Lavage Fluid (BALF)

Seventy-two hours following the final OVA or saline inhalation and aerobic exercise test, animals were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (40 mg/kg), tracheostomized and cannulated for BALF collection. BALF samples (1 ml) were collected after washing the lungs with 1.5 ml of sterile saline. BALF samples were centrifuged at 800 rpm for ten minutes at 5 °C, supernatant was stored at −70 °C, and the cell pellet was resuspended in sterile saline. Total cell counts were performed using the Laser Blood Cell Counter, (Model JXJ-402 Automatic; Shanghai Sichuan Instrument Co., LTDA, Odin Scientific). Differential cell count was performed by microscopic examination of BALF samples prepared in cytocentrifuge slides, stained with May-Grünwald-Giemsa, and 300 cells were counted per slide [27,28].

Immunohistochemistry and morphometry

Four-micrometer (µm) thick slides of lung tissue were stained with hematoxylin and eosin for routine histological analysis and with Luna staining for eosinophils [27,28]. For the immunohistochemistry analysis, sections were deparaffinized and a 0.5 % peroxidase in methanol solution was applied for ten minutes to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 min. Sections were incubated with anti-IL4 (Santa Cruz®, California, USA, 1:500), anti-IL-5 (Santa Cruz®, California, USA, 1:800), anti-IL-2 (Santa Cruz®, California, USA, 1:400), anti-IFN-γ (Santa Cruz®, California, USA, 1:800) and anti-IL-10 (Santa Cruz®, California, USA, 1:300), and left overnight at 4 °C. An ABC Vectastin Kit (Vector Elite PK-6105) was used as secondary antibody and 3,3 Diaminobenzidine (Sigma Chemical Co, St Louis, MO, USA) was used as chromogen. The sections were counterstained with Harris hematoxylin

Table 1 Aerobic Exercise Treadmill Test.

Groups	Increase in Test Time (min)
control	1.90 ± 4.24
Cr	2.13 ± 2.40
low + Cr	10.10 ± 5.49*
OVA	1.93 ± 3.66
OVA + Cr	2.09 ± 2.87
OVA + Cr + Low	10.75 ± 8.92*

Cr = creatine; Low = Low Intensity Aerobic Exercise; OVA = ovalbumin; * = significantly different from other groups ($p < 0.001$)

(Merck, Darmstadt, Germany). For negative controls, the primary antibody was replaced with PBS during the staining process.

Conventional morphometry was used to determine the peribronchial density of eosinophils and inflammatory cells expressing IL-4, IL-5, IL-2, IFN-γ and IL-10. Using a 100-point grid with a known area (10 000 μm^2 at a 1 000x magnification) attached to the microscope ocular, the number of points hitting the outer area of the airway wall (located between the external limit of smooth muscle layer and the adventitia) was counted. The airway area in each field was calculated using the number of points hitting the airway as a proportion of the total grid area. The number of eosinophils and IL-4, IL-5, IL-2, IFN-γ and IL-10 positive inflammatory cells within that airway wall area was counted. For each cell type, cell density was determined as the number of positive cells in each field divided by tissue area. Measurements were expressed as cells/mm^2. Counting was performed in five airways per animal and five fields per airway, at a 1 000x magnification [27,28].

Airway remodeling and bronchoconstriction

The volume proportion of collagen and elastic fibers in airway walls was calculated as the proportion of the number of points hitting collagen or elastic fibers out of the total number of points hitting the airway wall. Measurements were performed in five airways per animal and five fields per airway, at a 1 000x magnification, and the results were expressed as a percentage (%). The airway smooth muscle thickness index was assessed as the number of points hitting smooth muscle divided by the number of intercepts between the lines of the grid and the basal membrane. The airway bronchoconstriction index was assessed as the number of points hitting the airway lumen divided by the root square of the number of intercepts between the lines of the grid and the airway basal membrane. Measurements were performed in five airways from each animal at a 400x magnification [27,28].

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc test (parametric data) and one-way analysis of variance on Ranks (ANOVA on Ranks) followed by Dunn's post-hoc test (non-parametric data) were used for comparison of the different parameters among groups. Values were expressed as mean ± SD for parametric data and as median (variance) for non-parametric data. The level of significance was set at $p < 0.05$.

Results

Aerobic exercise treadmill test

Results are summarized in **Table 1**. Both groups that had been subjected to low intensity aerobic exercise (Low + Cr and OVA + Cr + Low) presented an increase in exercise capacity as compared to non-trained groups ($p < 0.001$).

Total and differential cell counts in Bronchoalveolar Lavage Fluid (BALF)

All groups that had undergone OVA sensitization (OVA, OVA + Cr and OVA + Cr + Low) presented an increased number of total cells as compared to non-sensitized groups ($p < 0.001$) (**Fig. 2A**). **Fig. 2B** shows that OVA and OVA + Cr groups presented a significantly increased number of eosinophils as compared to all non-sensitized groups ($p < 0.001$). Exercise significantly reduced eosinophil number in OVA + Cr + Low group as compared to OVA + Cr group ($p < 0.001$). **Fig. 2C** and **E** respectively show that only OVA + Cr + Low group presented an increase in the number of neutrophils and lymphocytes as compared to the other groups ($p < 0.05$). **Fig. 2D** shows that OVA and OVA + Cr + Low presented an increase in macrophage number as compared to all groups ($p < 0.001$).

Eosinophils in the peribronchial compartment

Table 2 shows the peribronchial density of eosinophils in all groups. Cr, OVA and OVA + Cr groups presented an increased density of eosinophils as compared to controls ($p < 0.01$). Exercised groups (Low + Cr and OVA + Cr + Low) presented significantly reduced peribronchial eosinophil density as compared to non-exercised groups (Cr and OVA + Cr, respectively, $p < 0.01$).

Peribronchial density of cytokines expression

Fig. 3A–E respectively show the peribronchial density of inflammatory cells positive for Th_2 (IL-4 and IL-5), Th_1 (IL-2 and IFN-γ) and anti-inflammatory (IL-10) cytokines. **Fig. 3A** shows that OVA and OVA + Cr groups presented an increased expression of IL-4 as compared to the other groups ($p < 0.05$) and that IL-4 expression in OVA + Cr group was significantly higher than in the OVA group ($p < 0.001$). Exercise (OVA + Cr + Low group) reduced IL-4 expression to control levels. **Fig. 3B** shows that Cr, OVA and OVA + Cr groups presented an increased expression of IL-5 as compared to the other groups ($p < 0.05$) and that IL-5 expression in OVA + Cr group was significantly higher than in Cr and OVA groups ($p < 0.001$). Exercise (OVA + Cr + Low group) reduced IL-5 expression to control levels. **Fig. 3C** shows that Low + Cr and OVA + Cr + Low groups presented an increased expression of IL-2 as compared to the other groups ($p < 0.05$). The expression of IL-2 in OVA + Cr + Low group was higher than in Low + Cr group ($p < 0.01$). **Fig. 3D, E** show that OVA + Cr + Low presented increased expression of IFN-γ and IL-10, respectively, when compared with all other groups ($p < 0.01$). **Fig. 4** shows the representative photomicrographs of peribronchial density of IL-4 positive cells in the six studied groups.

Airway remodeling and bronchoconstriction

Fig. 5A–D respectively show the bronchoconstriction index, the airway smooth muscle thickness index and volume proportion of airway collagen and elastic fibers. **Fig. 5A** shows an increased bronchoconstriction index in OVA and OVA + Cr groups when compared with control group ($p < 0.05$). Exercised groups (Low + Cr and OVA + Cr + Low) presented a significantly reduced

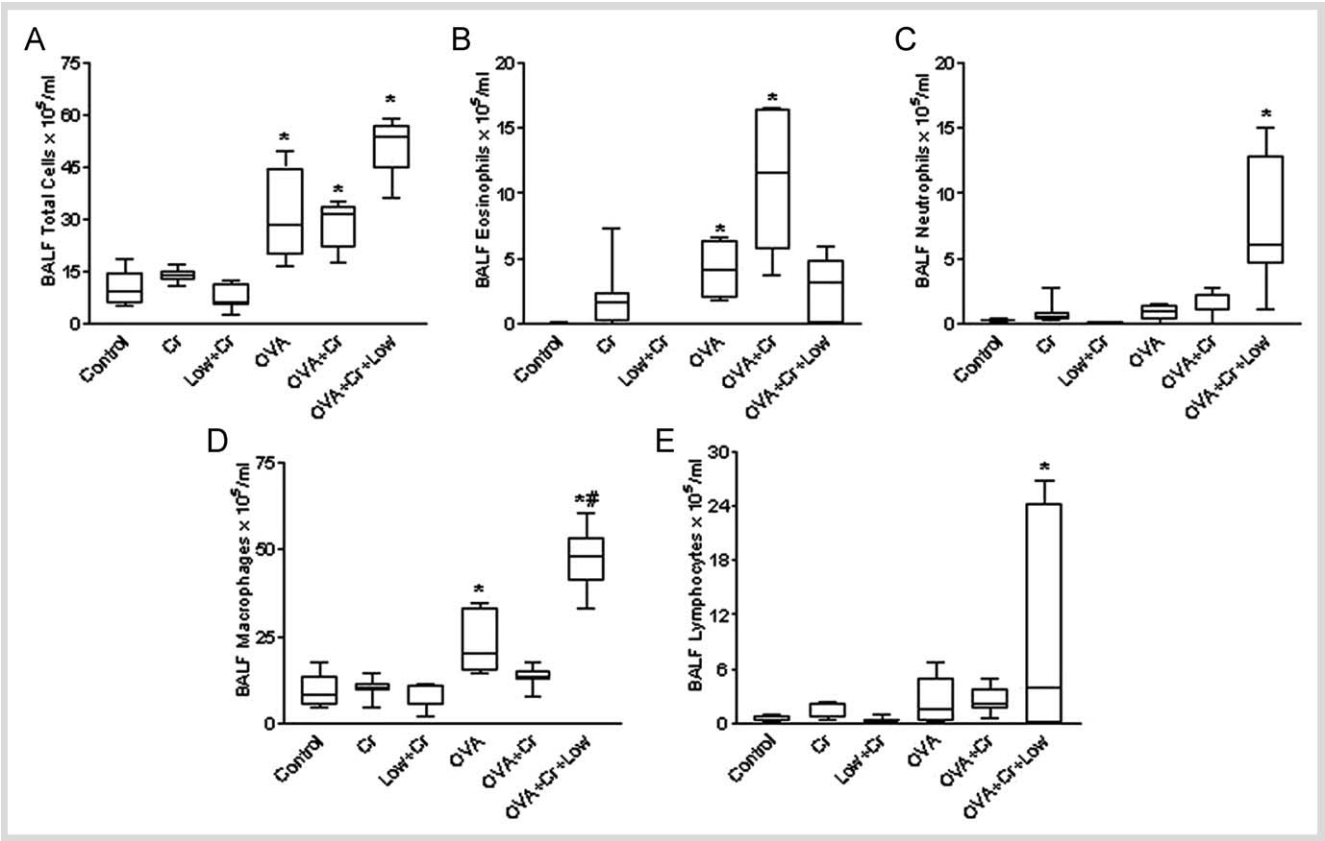

Fig. 2 Box plots of total cells (**A**), eosinophils (**B**), neutrophils (**C**), macrophages (**D**) and lymphocytes (**E**) in bronchoalveolar lavage fluid. Boxes show interquartile range, whiskers show range, and horizontal lines represent median values. In (**A**) * = significantly different from Control, Cr and Low + Cr groups ($p < 0.001$). In (**B**) * = significantly different from Control, Cr, Low + Cr and OVA + Cr + Low groups ($p < 0.001$). In (**C**) and (**E**) * = significantly different from all groups ($p < 0.05$). In (**D**) * = significantly different from other groups ($p < 0.001$) and # = significantly different from OVA group ($p < 0.01$).

Table 2 Peribronchial Eosinophilic Inflammation.

Groups	Peribronchial Density of Eosinophils (cells/mm²)
control	0.00 ± 0.00
Cr	410.36 ± 297.87*
low + Cr	0.00 ± 0.00
OVA	415.65 ± 145.70*
OVA + Cr	772.96 ± 306.39*
OVA + Cr + Low	102.09 ± 102.46

Cr = creatine; Low = Low Intensity Aerobic Exercise; OVA = ovalbumin; * = significantly different from Control ($p < 0.01$)

bronchoconstriction index when compared with OVA and OVA + Cr groups ($p < 0.05$). **Fig. 5B–D** show that Cr, OVA and OVA + Cr groups, compared to control group presented, respectively, an increase in smooth muscle thickness index, and in airway collagen and elastic content ($p < 0.05$). Exercised animals (Low + Cr and OVA + Cr + Low groups) presented a significant decrease in all three indices ($p < 0.05$).

Discussion

The main objective of our study was to investigate the joint effects of creatine supplementation and aerobic exercise on sensitized mice, to evaluate if the pro-inflammatory effects of creatine or the anti-inflammatory effects of aerobic exercise would be predominant. Our results show for the first time that aerobic exercise abolishes the exacerbatory effects of creatine supplementation in chronically sensitized mice and that this effect is mediated by the decreased expression of IL-4 and IL-5 and increased expression of IL-10.

Creatine supplementation has been used mainly by athletes to increase muscle mass and strength [1,3,16,24,28]. Owing to its muscular anti-inflammatory effects following overtraining and overuse, creatine has also been used in sports medicine practice to improve muscular recovery after training [24]. Some studies have further suggested a beneficial role of creatine supplementation on chronic obstructive pulmonary diseases, improving health status and general well being [7]. In contrast, our group recently demonstrated that Cr supplementation significantly increases all features of allergic asthma in a murine model of chronic allergic lung inflammation, such as eosinophilic inflammation, Th_2 immune response, airway remodeling and hyperresponsiveness [28]. This exacerbatory effect of creatine in a mouse asthma model could possibly be related to increased ATP replacement and/or accumulation. Indeed, Idzko et al. [8] demonstrated that ATP administration to mice led to a Th_2 inflammatory airway response. When ATP levels were reduced using apyrase or P2-receptor antagonist, all cardinal features of asthma were abrogated. The authors concluded that the level of ATP may modulate allergic lung inflammation through activation of the P2X and P2Y receptor [8].

On the other hand, aerobic exercise has been extensively used to improve asthmatic patient health [5,21]. Aerobic exercise improves physical fitness, work and ventilatory capacity, and decreases symptoms, dyspnea, exercise-induced bronchospasm,

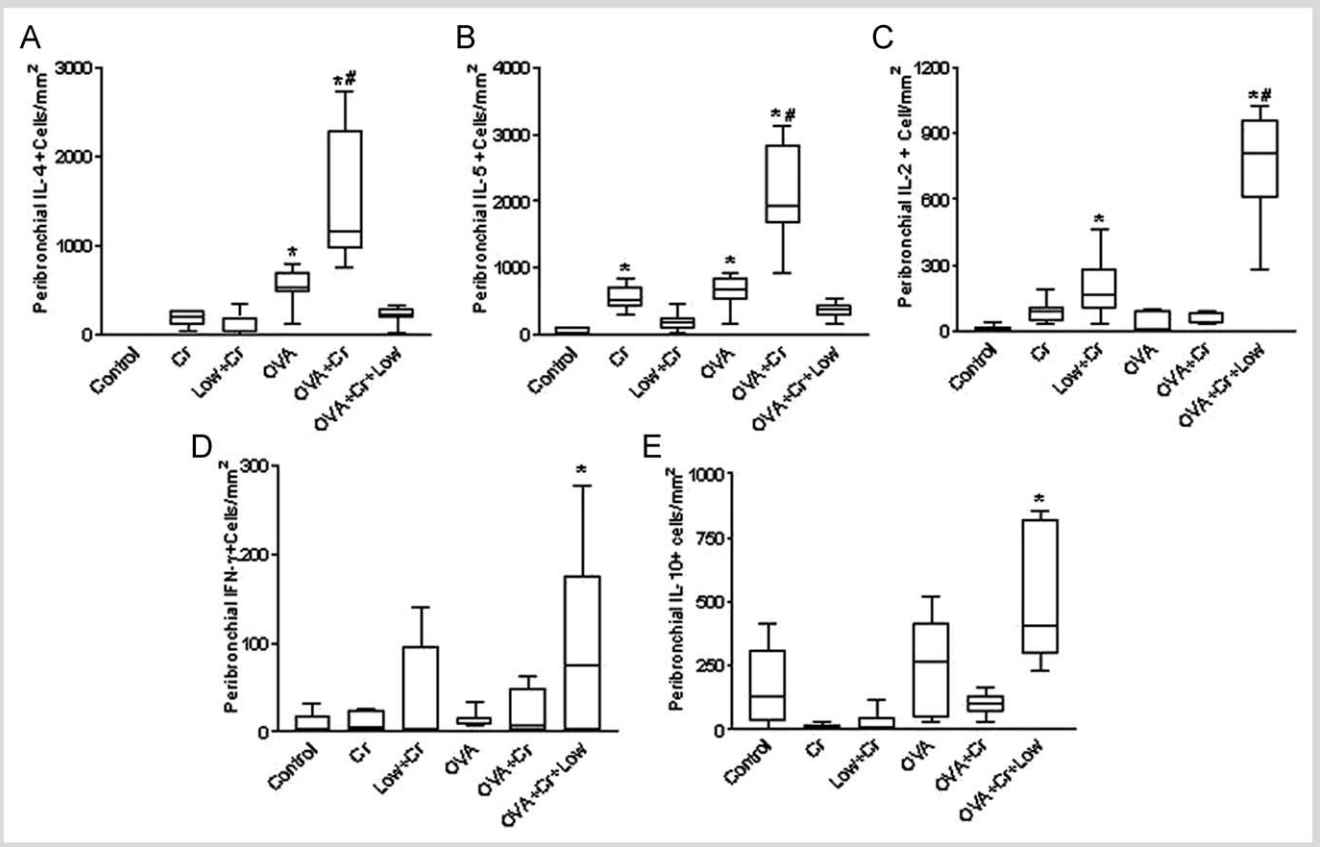

Fig. 3 Box plots of the peribronchial density of positive cells to IL-4 (**A**), IL-5 (**B**), IL-2 (**C**), IFN-G (**D**) and IL-10 (**E**). Boxes show interquartile range, whiskers show range, and horizontal lines represent median values. In (**A**), * = values significantly different from Control, Cr, Low + Cr and OVA + Cr + Low groups ($p < 0.05$) and # = values significantly different from OVA group ($p < 0.001$). In (**B**), * = values significantly different from Control, Low + Cr and OVA + Cr + Low groups ($p < 0.05$) and # = values significantly different from OVA group ($p < 0.001$). In (**C**), * = significantly different from Control, Cr, OVA, OVA + Cr groups ($p < 0.05$) and # = significantly different from Low + Cr group ($p < 0.01$). In (**D**) and (**E**) * = significantly different from other groups ($p < 0.01$).

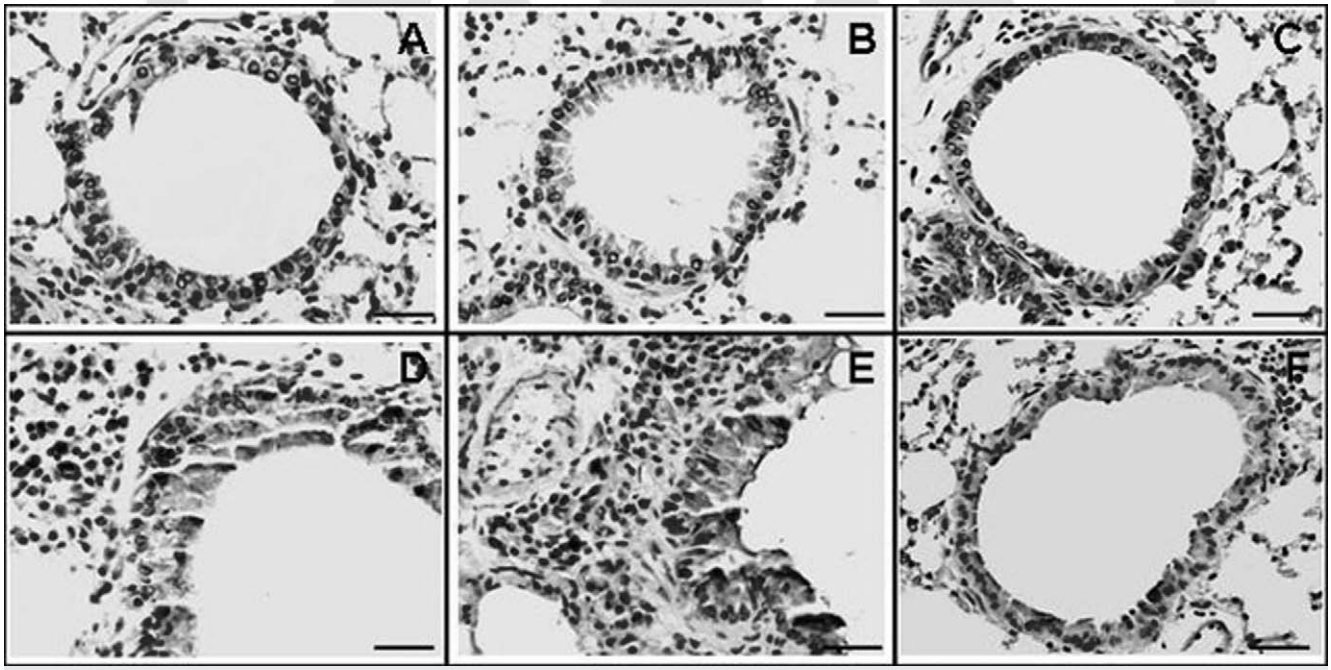

Fig. 4 Photomicrographs of peribronchial density of IL-4 positive cells in Control (**A**), Creatine (**B**), Low + Cr (**C**), OVA (**D**), OVA + Cr (**E**) and OVA + Cr + Low (**F**) groups. Scale bar = 25 µm.

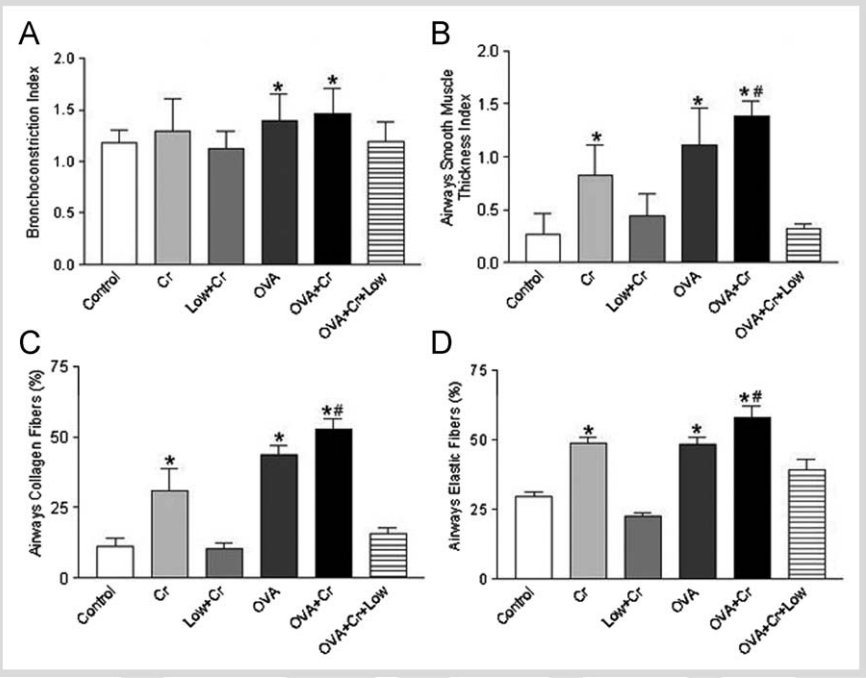

Fig. 5 Figures represent mean ± SD of bronchoconstriction index (**A**), airway smooth muscle thickness index (**B**), volume proportion of collagen in airway wall (**C**) and volume proportion of elastic fibers in airway wall (**D**). In (**A**), * = significantly different from all other groups ($p < 0.05$). In (**B, C** and **D**), * = significantly different from all other groups ($p < 0.05$) and # = significantly different from Cr group ($p < 0.05$).

peak expiratory flow variability and the necessity of corticosteroids [5,21]. So far, only four studies have evaluated the effects of aerobic exercise using experimental models of allergic asthma [17,18,27,29]. Pastva et al [17]. demonstrated that aerobic exercise decreases leukocyte infiltration in the airways, BAL levels of IL-4, and serum IgE in sensitized mice [17]. It was then suggested that these anti-inflammatory effects of aerobic exercise could be partially mediated by increased production of endogenous corticosteroids [18]. We recently demonstrated that besides inhibiting the eosinophilic inflammation and IL-4 expression, aerobic exercise also inhibits IL-5 expression and airway remodeling, and increases the expression of the anti-inflammatory cytokine IL-10 in sensitized mice [27]. Recently, our group also demonstrated that these anti-inflammatory effects of aerobic exercise also are present in pulmonary vessels and parenchyma and also involve the reduction in the NF-κβ expression [29]. IL-4 and IL-5 are responsible for the development and maintenance of many characteristics of allergic asthma, as differentiation, survival, attraction and activation of effectors inflammatory cells, airway remodeling and also hyperresponsiveness [2,11,30].

In the present study, we demonstrated that aerobic exercise reduced all exacerbatory effects of creatine supplementation in sensitized mice, i.e., IL-4 and IL-5 expression by inflammatory cells and airway remodeling. We suggest that these effects were at least partially mediated by exercise-induced increase in IL-10 expression. It has been clearly demonstrated that aerobic training at low and moderate intensities increases the expression of IL-10 [13,19,26]. Previous studies also show that IL-10 release is one of the main mechanisms that mediates aerobic exercise-induced modulation of the immune system [13,19,26]. The anti-inflammatory effects of IL-10 have been demonstrated in cardiovascular diseases, type 2 diabetes, and also in experimental models of chronic allergic lung inflammation [6,14].

Another possible mechanism for the beneficial effects of aerobic exercise over the deleterious effects of creatine could be the high rate of creatine consumption by active skeletal muscle during exercise [31], which could result in a decreased delivery of creatine to leukocytes and dendritic cells during allergen sensitization. This decreased availability of creatine could then result in lower inflammatory cell survival and therefore in less intensive inflammation [20]. Since the level of ATP consumption modulates the expression of purinergic receptors P2X and P2Y, this could be an additional mechanism mediating exercise-induced decreased allergic lung inflammation [8].

We conclude that aerobic exercise decreases the pro-inflammatory and remodeling effects of creatine supplementation in a mouse model of allergic lung inflammation, and that this beneficial effect of exercise is mediated by a decreased expression of IL-4 and IL-5 and by an increased expression of IL-10. Additional studies investigating the role of ATP and purinergic receptors in the modulation of allergic inflammation by exercise and creatine use should be performed.

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