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Aerobic training reverses airway inflammation and remodeling in asthma murine model

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Short Title: Aerobic training decreases airway inflammation and remodeling

ABSTRACT

Aerobic training (AT) decreases dyspnea, exercise-induced bronchospasm and improves aerobic capacity and quality of life, however the mechanisms for such benefits remains poorly understood. The aim of the present study was to evaluate the AT effects in a chronic model of allergic lung inflammation in mice after the establishment of airway inflammation and remodeling.

Mice were divided in Control, AT, OVA, and OVA+AT groups exposed to saline or Ovalbumin. AT began on the 28th day(60min/5x-wk/4-wks). Respiratory mechanics, specific IgE and IgG_1, collagen and elastic fibers deposition, smooth muscle thickness, epithelial mucus, peribronchial density of eosinophils, CD3+ and CD4+,IL-4,IL-5,IL-13,IFN-γ,IL-2,IL-1ra,IL-10, NF-κB, and Foxp3 were evaluated.

OVA group presented increase of IgE and IgG_1, eosinophils, CD3+,CD4+,IL-4,IL-5,IL-13,NF-κB, collagen and elastic, mucus synthesis, smooth muscle thickness and lung tissue resistance and elastance. OVA+AT presented increase of IgE and IgG_1, and reduction of eosinophils, CD3+,CD4+,IL-4,IL-5,IL-13,NF-κB, airway remodeling, mucus synthesis, smooth muscle thickness and tissue resistance and elastance compared with OVA group ($P<0.05$). OVA+AT also shown increased of IL-10 and IL-1ra ($P<0.05$), independently of Foxp3.

AT reversed airway inflammation and remodeling, Th2 response and improves respiratory mechanics. These results seem to occur by increase in the expression of IL-10 and IL-1*ra* and a decrease of NF-κB.

Total Words: 2885

Keywords: aerobic training; airway inflammation; airway remodeling; asthma; exercise; rehabilitation.

INTRODUCTION

Asthma is characterized by chronic airway inflammation and remodeling, hyperresponsiveness and increased levels of Th2 cytokines[1]. Asthmatic airways present an increase in eosinophils, subgroups of CD3 T helper cells, mainly CD4+ T cells and mast cells [1,2], mucus hypersecretion, smooth muscle hypertrophy and hyperplasia, increased sub-epithelial deposition of collagen and elastic fibers, and increased epithelial thickness resulting in airway obstruction[3]. The chronic inflammation in asthma is mediated by Th2 cytokines, mainly IL-4, IL-5 and IL-13, which play an important role in the maintenance of inflammation and airway remodeling[4]. Th1 cytokines, particularly IL-2 and IFN-γ are also present in asthmatics[5], however its roles in the inflammation remains controversial. Some studies demonstrate that Th1 cytokines reduce asthma features[6], although others suggest that Th1 cytokines exacerbate Th2 asthmatic response[5].

Aerobic training (AT) has been used as an important part of rehabilitation program of asthmatic patients[7]. AT decreases dyspnea, airway hyperresponsiveness, exercise-induced bronchospasm, use of corticosteroids, and improves aerobic capacity and quality of life[7]. The benefits of AT are attributed to an increase in the ventilatory threshold thereby lowering the minute ventilation during mild and moderate exercise[8]. Consequently, breathlessness and the likelihood of provoking exercise induced asthma could be reduced or abolished. AT may also decrease the perception of breathlessness through other mechanisms including respiratory muscles strengthening[9]. However, possible effects of AT mediated through immune system have been poorly evaluated.

In healthy individuals, AT modulates the immune system by increasing Th1 response that seems to suppress Th2[10]. For instance, IFN-γ expressed by Th1 lymphocytes of aerobic trained

individuals suppresses proliferation of Th2 lymphocytes[11] and this response can be amplified by IL-12 produced by Th1 lymphocytes and antigen presenting cells that enhances IFN-γ expression[12]. At this moment, few studies have investigated the effects of AT on Th2 immune response. Pastva, et al.[13] and Vieira, et al.[14,15] showed that AT decreases leukocyte migration to the airway as well as IL-4 and IL-5 expression, IgE levels and NF-κB expression in asthma animal models. However, a main criticism in those studies is that AT was initiated concomitant to the challenges in airways, that were devoid of significant inflammation and structural changes. This situation may not represent the clinical status of the majority of asthmatics that are submitted to pulmonary rehabilitation.

Therefore, the present study aimed to evaluate the effects of AT performed after the establishment of airway inflammation and remodeling using a chronic model of allergic airway inflammation and investigated a possible role of NF-κB, Foxp3, Th1 and anti-inflammatory cytokines mediating these effects. The effects of AT were evaluated on respiratory mechanics, IgE and IgG_1 antigen-specific levels, airway eosinophilia and CD3+ and CD4+ lymphocitary inflammation. The expression of Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL5 and IL-13) cytokines as well as regulatory/anti-inflammatory cytokines (IL-10,L-1*ra*) and the transcription factors NF-κB and Foxp3 were also evaluated.

MATERIAL AND METHODS

Animals

Twenty-eight male BALB/c mice (4-wk-old,28±2g) were maintained under standard laboratory conditions with temperature (22°C±1) and relative humidity (40–60%) controlled, on a 12-h light/dark cycle, provided with food and water ad libitum (Labina, Purina®, Brazil). The experimental protocol was approved by the Ethical Committee of the School of Medicine of the University of Sao Paulo. All cares and experimental procedures followed the international recommendations of the Helsinki convention for the use and care of animals.

Protocol of Chronic Allergic Lung Inflammation

The mice were assigned in 4 groups (n=7 each): Control, Aerobic Training (AT), Ovalbumin (OVA) and OVA+Aerobic Training (OVA+AT). The OVA groups received OVA (i.p.;10μg/mouse) diluted in aluminum hydroxide on days 0, 14, 28, and 42 as model previously described[14,15,16]. After the 21st day, mice were exposure to OVA aerosol (1%), 30 minutes by session, 3x/week until the 54th day. Control and AT groups were exposed to saline solution.

Physical Test and Exercise Training Protocol

On days 21 to 23 mice were adapted to the treadmill (Inbramed®, Brazil) for 15 minutes, at 0.2 km/h, 25º inclination. On days 25 and 53 the maximal exercise test was performed as previously described[14,15,16]. Treadmill training (50% of maximal exercise capacity reached in the physical exercise test) began on day 28 and it was performed during 4 weeks, 5x/week for 60 minutes (Figure 1).

Evaluation of Respiratory Mechanics

Mice were anesthetized (thiopental/50mg/Kg;i.p.), tracheostomized and connected to a ventilator for rodents (FlexiVent, Scireq, Canada) with the tidal volume and frequency set at 10 mL/Kg and 2 Hz, respectively. Oscillatory lung mechanics was performed by producing flow oscillations at different prime frequencies (from 0.25 to 19.625 Hz), for 16 seconds[17]. Pressure and flow data were obtained and airway impedance was calculated at each frequency[18]. Tissue impedance (Gtis) and elastance (Htis) were obtained by applying the constant-phase model.

IgE and IgG_1 anti-OVA antibody titration by PCA

PCA was performed in Wistar Furth rats and in BALB/c mice for anti OVA IgE and IgG_1, respectively. The back of the animals was shaved and injected intradermally with different serum dilutions. The animals were challenged intravenously with 0.5 mg of OVA in 0.25% Evans Blue solution, after a sensitization period of 18-24h in rats for IgE, and 2h in mice for IgG_1 titration. The PCA titer was expressed as the reciprocal of the highest dilution that gave an intradermic allergic reaction greater than 5mm in diameter in duplicate of tests. The detection of threshold of the technique was established at 1:5 dilution[19].

Lung Histology

Five-μm thick sections of the lungs were stained with H/E to the analyses of the lung architecture and quantification of airway smooth muscle thickness[14,20]. Picrosirius was used for bronchial collagen fibers quantification[14,21], Weigert's Resorcin Fuchsin with Oxidation for elastic fibers **[**22**]** and Luna for detection of peribronchial eosinophils[14,23]. Periodic Acid-Schiff (PAS)-Alcian blue was used to evaluate mucus synthesis [14,24]. The slides were coded and the researcher who performed the measurements was unaware of the study groups. Using a

microscope Eclipse E-200 (Nikon, Japan), a digital camera Cool Snap (Photometrics, Japan) and the image analyses software Image Proplus 4.5 (Media Cybernetics, USA), we evaluated airway smooth muscle thickness, collagen and elastic fibers deposition on the airway wall and epithelial mucus production. Five airways at 400xmagnification were evaluated for each animal. The area of the smooth muscle layer was quantified by delineating its inner to outer borders. Airway collagen and elastic fibers deposition were evaluated in the area compressed between epithelial basal membranes until airway adventitia. The positive area of collagen and elastic fibers was expressed as percentage of total airway wall area. Mucus production was evaluated counting the epithelial area PAS/AB-positive and the results were expressed in percentages[24].

Immunohistochemistry

Five-μm thickness sections of the paraffin embedded specimens were used to determinate the peribronchial count of positive inflammatory cells expressing Foxp3,IL-4,IL-5,IL-13,IFN-γ, IL-2,NF-κB,IL-10,IL-1*ra,* and also to CD3+ and CD4+ lymphocytes, by immunohistochemistry technique [14]. Briefly, sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 minutes to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 minutes. Sections were incubated with anti-Foxp3(1:550), anti–IL-4(1:600), anti–IL-5(1:700), anti–IL-13(1:300), anti–IFN-γ (1:400), anti-IL-2(1:500), anti-NF-κB(1:600), anti-IL-10(1:700) and anti-IL-1*ra*(1:750), anti-CD3+(1:500), anti-CD4+(1:400) (Santa Cruz, USA) and left overnight at 48^{o}C. ABC Vectastain Kit (Vector Elite PK-6105; Vector Laboratories, USA) was used as secondary antibody and 3,3 Diaminobenzidine (Sigma Chemical Co., USA) was used as chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). All primary and secondary antibodies were applied

to negative and positive controls. The slides were coded and the researcher who performed the morphometrical analyses was unaware of the study groups**.** The peribronchial count of positive inflammatory cells for the antibodies described above and also to CD3+ and CD4+ T lymphocytes and eosinophils was analyzed by using a reticulated ocular of 50 lines and 100 points (1,000 x magnification; area of 10,000 μm^2)[25]. Cell density was determined as the number of positive marked cells in each field divided by tissue area and expressed as cells/μm^2. The results were then transformed to cells/mm^2 by adjusting the units[25,28].

Statistical Analysis

The parametric data were analyzed by Two-way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls post-hoc test and non-parametric data by Two-way (ANOVA) followed by Holm-Sidak post-hoc test. The data were showed as means ± SD for parametric data and as median (variance) for nonparametric data. The significance level was adjusted to 5%. Parametric data are presented with bars (Figures 2 and 7) and non-parametric data as box plots (Figures 3, 4 and 6).

RESULTS

Exercise Capacity: Control and OVA groups did not present changes in the maximal exercise capacity when we compared the initial and final tests ($P>0.05$; Table I). AT and OVA+AT groups presented improved exercise capacity when the initial and final tests were compared ($P<0.01$) and also when compared with non trained groups (Control and OVA; $P<0.01$).

OVA-specific IgE and IgG_1: OVA sensitization increased IgE and IgG_1 titers when compared with non-sensitized groups ($P<0.01$; Table 1) and AT in sensitized animals did not change the IgE and IgG_1 titers when compared with OVA group ($P>0.05$).

Respiratory Mechanics: AT in non-sensitized mice did not change tissue resistance (Gtis) and elastance (Htis) when compared with Control group ($P>0.05$; Figure 2). OVA exposure increased Gtis and Htis compared with Control and AT groups ($P<0.05$). AT performed in sensitized animals (OVA+AT group) resulted in a decrease of Gtis and Htis to control levels ($P<0.05$) (Figures 2A-B, respectively).

Peribronchial Eosinophils, CD3+ and CD4+ T cells: AT in non-sensitized animals (AT group) did not change the peribronchial density of eosinophils and CD3+ and CD4+ T lymphocytes compared with Control group ($P>0.05$;Figure 3). Chronic OVA exposure increased the peribronchial density of eosinophils and CD3+ and CD4+ T lymphocytes when compared with non-sensitized group ($P<0.05$). AT in sensitized animals (OVA+AT group) significantly decreased peribronchial eosinophils, and CD3+ and CD4+ lymphocytes when compared with OVA group ($P<0.05$) and increased when compared with non-sensitized groups ($P<0.001$; Figures 3A-C, respectively).

Peribronchial Expression of Th2 Cytokines: AT in non-sensitized animals did not change the peribronchial expression of IL-4, IL-5 and IL-13 when compared with Control group ($P>0.05$; Figure 4). OVA sensitization increased the peribronchial expression of IL-4, IL-5 and IL-13 when compared with non-sensitized group ($P<0.05$). OVA+AT group presented a significant decrease in the peribronchial expression of IL-4, IL-5 and IL-13 ($P<0.05$) when compared with OVA group (Figures 4A-C, respectively). Figures 5A-D are representative photomicrographs of peribronchial expression of IL-5 in all groups.

Peribronchial Expression of Th1, Regulatory/Anti-inflammatory Cytokines (IL-10 and IL-1*ra*) and NF-κB and Foxp3: The expression of IL-2, IFN-γ, Foxp3, IL-10, IL-1*ra* and NF-κB was not changed in the AT as compared with Control group ($P>0.05$; Figures 6A-F). OVA group increased the peribronchial expression of NF-κB when compared with Control group($P<0.05$). However, OVA sensitization did not change the peribronchial expression of IL-2, IFN-γ, IL-10 and IL-1*ra* ($P>0.05$). OVA+AT group presented a decrease in the expression of NF-κB ($P<0.05$; Figure 6C), and increased the expression of IL-10 and IL-1*ra* when compared with OVA group ($P<0.05$). OVA+AT group did not change the peribronchial expression of IL-2 and IFN-γ. The expression of Foxp3 was modified neither by OVA-induced allergic inflammation nor by AT in sensitized animals ($p>0.05$).

Airway Remodeling: AT group did not change airway collagen and elastic fibers deposition, smooth muscle thickness and mucus production when compared with Control group ($P>0.05$; Figures 7A-D, respectively). OVA sensitization increased airway collagen and elastic fibers deposition and smooth muscle thickness and epithelial mucus production when compared with Control group. OVA+AT group presented a reduction of airway collagen deposition, smooth

muscle thickness, and epithelial mucus production when compared with OVA group ($P<0.05$), however no changes were observed in elastic fibers ($p>0.05$).

DISCUSSION

The present study demonstrates that aerobic training reverses OVA-induced peribronchial accumulation of eosinophils, CD3+ and CD4+ lymphocytes, peribronchial expression of IL-4,IL-5,IL-13,NF-κB, collagen fibers deposition, increase in airway smooth muscle thickness and epithelial mucus production as well as reduces tissue resistance and elastance. Our results also suggest that these effects are associated to increased expression of anti-inflammatory cytokines IL-10 and IL-1*ra*.

Asthmatics subjects submitted to an AT program present a reduction in corticosteroids consumption, dyspnea and exercise-induced bronchospasm as well as an improvement in the aerobic capacity and health related quality of life features[7,9]. Previous experimental studies investigated the hypothesis that such effects in asthmatic patients could be associated to anti-inflammatory effects of exercise training[13,14,15] Pastva, et al.,[13] demonstrated that AT decreases neutrophil infiltration into the airways, IL-4 levels in BAL fluid and the expression of NF-κB, effects that seem mediated at least partly by endogenous corticosteroids. Vieira, et al[14,15] demonstrated that also low or moderate AT intensity decreases eosinophilic inflammation and IL-4 and IL-5 expression, and these effects are, at least partially, mediated by an increase in the anti-inflammatory cytokine IL-10. Despite the fact that these studies innovate by describing such anti-inflammatory effects in lung allergic inflammation, both models have as a major criticism the fact that exercise training was initiated simultaneously to the OVA challenges and hence prior to the development of airways inflammation and structural changes.

In the present study, we used a chronic animal model of allergic lung inflammation and the exercise training started after the establishment of airway inflammation and remodeling and our results demonstrated that AT reverses airway eosinophilic (Figure 3A) and lymphocyte migration (CD3+ and CD4+; Figures 3B-C). These results are highly relevant as CD3+ and CD4+ T cells, are related with IgE specific synthesis and have a direct effect on the airways through the recruitment of eosinophils[26] and also as a source of pro-inflammatory Th2 cytokines (IL-4,IL-5 and IL-13), which are correlated with the development and maintenance of airway inflammation and remodeling[4].

For instance, IL-4 seems to perpetuate mast cell activation, which is responsible for IgE and IgG_1 release, eosinophils activation and recruitment, and mucus production and play role in the Th2 maintenance[1,2,27]. Interleukin-5(IL-5) stimulates the proliferation, differentiation, migration, survival and activation of eosinophils in the bone marrow and is a chemoattractant to eosinophils[4,28] reducing the inhibitory effects of IL-2 on eosinophils migration[29]. IL-13 causes increased levels of mucus production by airway epithelial cells [4,24]. Our study showed that AT decreased the peribronchial expression of IL-4, IL-5 and IL-13 by inflammatory cells, predominantly by mononuclear cells (Figures 4 A-C). These data reinforces that AT is capable to reverse Th2 immune response in a murine model of asthma.

Airway remodeling is defined as the presence of structural changes in the airways with increased deposition of collagen and elastic fibers and other extracellular matrix proteins, smooth muscle hyperplasia and hypertrophy, goblet cells hyperplasia followed by excessive mucus secretion[3]. Previous studies using animal models have shown that after 30 days exposition to OVA-inhalation mice present airway remodeling[30,31,32]. In the present study, the AT begun after 28 days of OVA inhalation and our results demonstrate that AT reduces OVA-induced airway remodeling, including collagen fibers deposition, smooth muscle thickness and mucus production

in this murine model of asthma(see Figures 7 A-D). These data are relevant since no previous studies have demonstrated that AT can revert airway inflammation and remodeling. Our murine model of asthma also resulted in an increased tissue resistance and elastance, which was completely reversed by AT (Figure 2 A-B). Our results reinforce previous findings by showing that changes in lung mechanics in sensitized mice seems related to airway inflammation and remodeling[31,32,33].

We evaluated three possible mechanisms through which exercise could modulate the observed effects: (i) by increasing Th1 response (IL-2 and IFN-γ)[34]**;** (ii) by changing the expression of modulatory cytokines (IL-10 and IL-1ra)[35]**;** and (iii) through modulating nuclear factor (NF-κB and Foxp3[36,37]. We observed that AT did not change the expression IL-2 and IFN-γ either in non-sensitized or in sensitized mice (see Figures 5A-B), suggesting that the beneficial effects of AT in our murine model of asthma are not mediated by an improvement of Th1 response. We also observed that AT in sensitized animals reduced the peribronchial expression of NF-κB by inflammatory cells suggesting that it could be involved in both anti-inflammatory and anti-fibrotic effects induced by AT. In addition, we also showed that AT increased the peribronchial expression of expression of anti-inflammatory cytokines IL-10 and IL-1*ra*. We showed that Foxp3 levels were unchanged either after OVA inflammation or by exercise, suggesting that the increase in IL-10 in our animal model seems to occur independently of Foxp3 regularion.

The clinical benefits induced by AT in asthmatics subjects [7,39] have been explained by the reduction in ventilatory threshold[8]. The present study is the first to evaluate the effect to AT after several OVA inhalations to simulate airway inflammation as occur in asthmatics patients and our results might suggest that improvement in AT can modulate airway allergic inflammation. Interesting, a recent study showed that asthmatic women with higher physical

fitness present less episodes of asthma exacerbations. The authors hypothesized that increase in physical fitness should be used as adjuvant treatment in asthma [40], what is supported by our findings.

Conclusion: We conclude that aerobic training reverses airway inflammation and remodeling improves respiratory mechanics and reduces Th2 immune response in a murine model of asthma. These effects seem to occur in response to a decreased expression of NF-κB and increased expression of anti-inflammatory cytokines IL-10 and IL-1*ra*. Our data give fully support to the "emerging concept" that physical fitness should be used as adjuvant treatment in asthma.

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Table I – Animals weight, physical test performance and titers of immunoglobulins

Groups	Weight (g)		Physical Test (min)		Specific OVA-antibodies (Log)	
	Initial	Final	Initial	Final	IgE Titer	IgG_1 Titer
Control	28.6	29.0	36.0	33.1	0.0	0.0
	(1.3)	(1.7)	(5.5)	(7.2)	(0)	(0)
AT	28.5	29.1	35.8	44.8**	0.0	0.0
	(1.5)	(1.3)	(4.9)	(4.4)	(0)	(0)
OVA	26.8	27.9	35.3	29.8	5.1*	7.3*
	(1.6)	(0.6)	(2.8)	(2.3)	(0.4)	(0)
OVA+AT	28.1	28.3	31.8	39.8**	4.8*	7.8*
	(1.7)	(1.8)	(3.5)	(3.4)	(0.5)	(0.7)

Control Group=non-sensitized and non-trained; AT Group=non-sensitized and Aerobic Trained; OVA Group=sensitized and non-trained; OVA+AT=sensitized and aerobic trained (Control and OVA); g=grams; min=minute; Log: logarithm. **P<0.01 compared to non-trained groups (Control and AT); *P<0.001 compared to non-sensitized groups (Control and AT). Data are expressed as mean ± standard deviation (SD) in parenthesis.

Figure 1

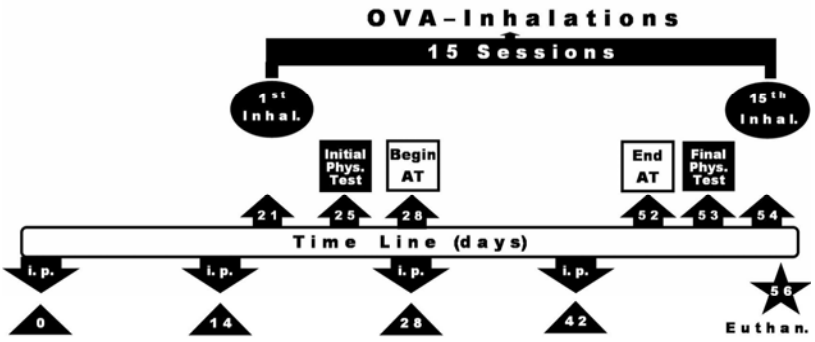

Figure 2

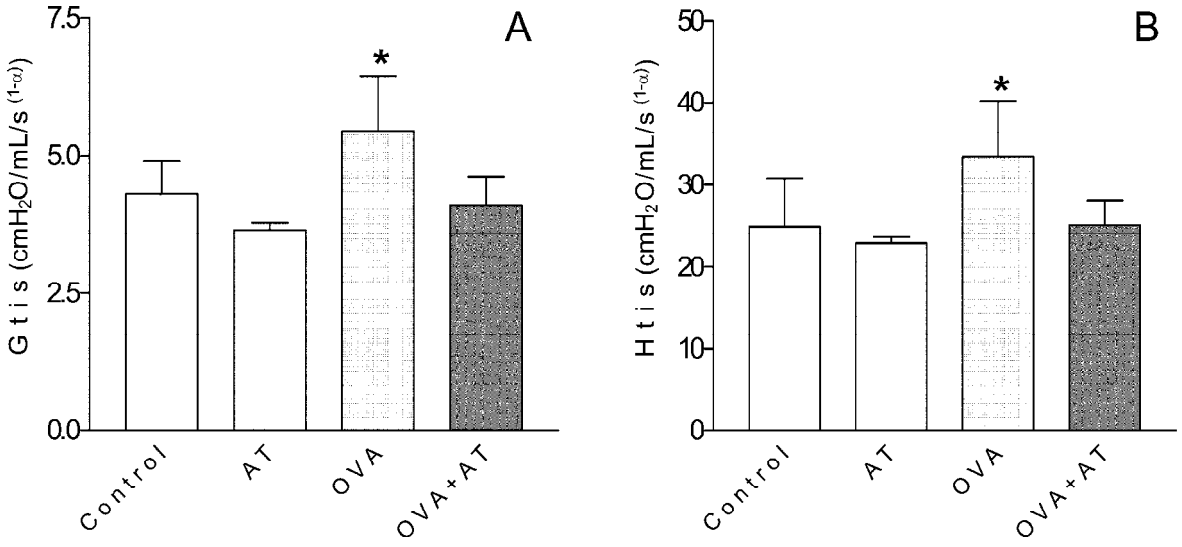

Figure 3

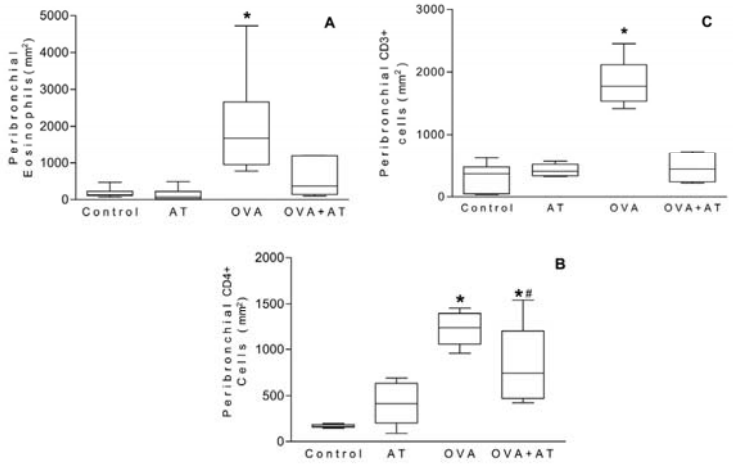

Figure 4

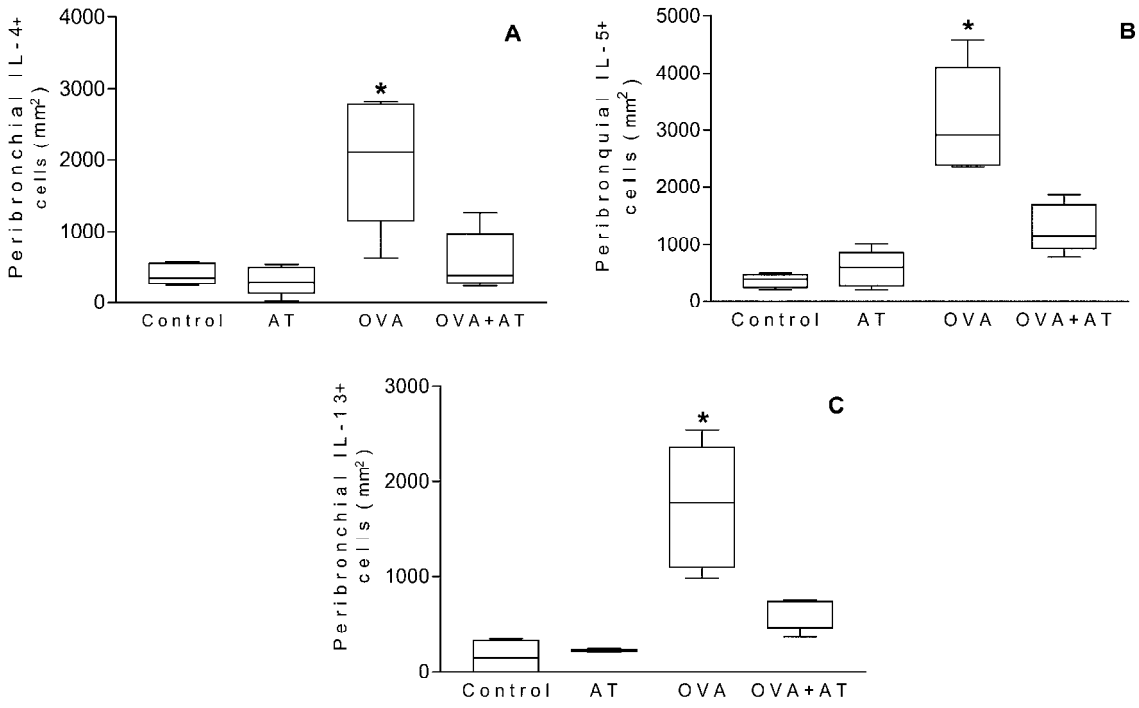

Figure 5

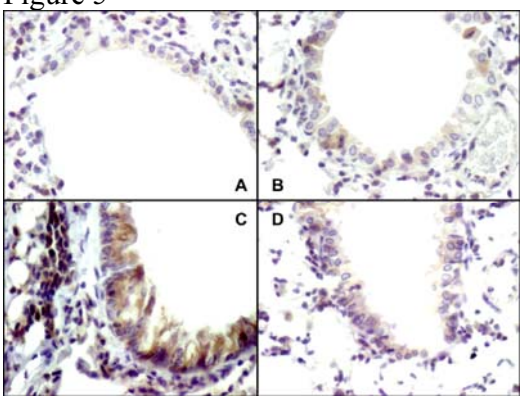

Figure 6

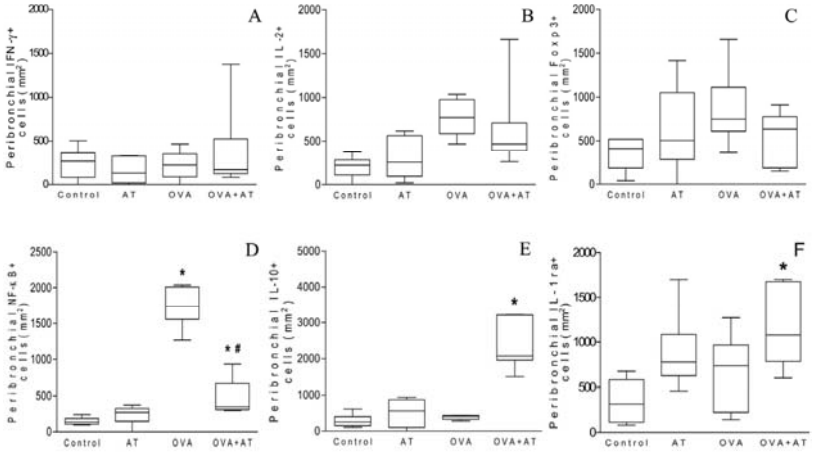

Figure 7