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ORIGINAL ARTICLE



Pharmacological modulation of reactive oxygen species (ROS) improves the airway hyperresponsiveness by shifting the Th1 response in allergic inflammation induced by ovalbumin

Renata Tiscoski Nesi^a , Marina Valente Barroso^b , Valdirene de Souza Muniz^a , Ana Carolina de Arantes^c , Marco Aurélio Martins^c , Lylia de Brito Gitirana^a , Josiane Sabbadini Neves^a , Cláudia Farias Benjamim^a , Manuella Lanzetti^a  and Samuel Santos Valença^a 

^aFederal University of Rio de Janeiro, Institute of Biomedical Sciences, Rio de Janeiro, Brazil; ^bFederal University of Rio de Janeiro, Institute of Microbiology Paulo Góes, Rio de Janeiro, Brazil; ^cLaboratory of Inflammation, Fundacao Oswaldo Cruz, Oswaldo Cruz Institute, Rio de Janeiro, Brazil

ABSTRACT

Asthma is an allergic inflammation driven by the Th2 immune response with release of cytokines such as IL-4 and IL-13, which contribute to the airflow limitations and airway hyperresponsiveness (AHR). The involvement of oxidative stress in this process is well-established, but the specific role of the superoxide anion and nitric oxide in asthma are poorly understood. Thus, the aim of this study was to investigate the mechanisms underlying the superoxide anion/nitric oxide production and detoxification in a murine asthma model. BALB/c male mice were sensitised and challenged with ovalbumin (OVA). Pretreatments with either apocynin (14 mg/kg) or allopurinol (25 mg/kg) (superoxide anion synthesis inhibitors), aminoguanidine (50 mg/kg) (nitric oxide synthesis inhibitor) or diethyldithiocarbamate (100 mg/kg) (superoxide dismutase inhibitor) were performed 1 h before the challenge. Our data showed that apocynin and allopurinol ameliorated AHR and reduced eosinophil peroxidase, as well as IL-4 and IL-13 levels. Apocynin also abrogated leukocyte peribronchiolar infiltrate and increased IL-1 β secretion. Aminoguanidine preserved lung function and shifted the Th2 to the Th1 response with a reduction of IL-4 and IL-13 and increase in IL-1 β production. Diethyldithiocarbamate prevented neither allergen-induced AHR nor eosinophil peroxidase (EPO) generation. All treatments protected against oxidative damage observed by a reduction in TBARS levels. Taken together, these results suggest that AHR in an asthma model can be avoided by the down-regulation of superoxide anion and nitric oxide synthesis in a mechanism that is independent of a redox response. This down-regulation is also associated with a transition in the typical immunological Th2 response toward the Th1 profile.

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Introduction

Asthma is an exacerbated eosinophilic response raised against allergic stimuli and caused by a complex interaction of immune cells and other cell factors that are mediated under the strong influence of reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively [1]. Although asthma is a complex disease that has inflammatory, oxidative and nitrosative components, the immune response during the allergic process has important features such as the Th2 response (release of IL-4, IL-5, and IL-13), the increased production of IgE and mast cell activation, as well as eosinophils. All these factors contribute to airway hyperresponsiveness (AHR) [2]. Inflammatory and resident cells from the airways of asthma patients release

mediators such as cytokines, histamine and leukotrienes, which are associated with recurrent episodes of hyperresponsiveness, bronchoconstriction and hypersecretion [3–5].

In inflammatory conditions, oxidants, such as superoxide anion and nitric oxide, react rapidly to produce peroxynitrite, an extremely powerful oxidant, which contributes to tissue injury, lipid peroxidation and nitration of tyrosine residues [6]. These changes in asthma may be responsible for apoptosis of epithelial cells [7,8], increased vascular permeability [9,10] and inactivation of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [11]. Even with the same content of SOD between healthy subjects and asthmatic patients, the antioxidant activity of this enzyme is

CONTACT Manuella Lanzetti  manuella.lanzetti@gmail.com  CCS, Federal University of Rio de Janeiro (UFRJ), Institute of Biomedical Sciences, Bloco J, Av Carlos Chagas Filho, 373, Sala 25, Rio de Janeiro, Brazil

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reduced in asthma [12]. Both copper–zinc SOD (CuZnSOD) and mitochondrial manganese SOD (MnSOD) are sensitive to redox modifications, and their inactivation in asthmatic lungs has been related to the airway hyperresponsiveness and airflow limitations [11,13,14]. Moreover, eosinophil peroxidase (EPO), released by activated eosinophils during asthma, contributes to nitration of protein-bound tyrosine residues, which usually leads to inactivation of proteins [15–17]. Brennan et al. have shown that EPO produces up to 70% of nitrotyrosine in the murine asthma model [18,19].

Nitrosative damage, ROS production, decline of antioxidant activity and the deterioration of lung function have been implicated in asthma. Many efforts have been performed to evaluate if the control of ROS/RNS production can ameliorate these symptoms, but the findings are controversial. The NADPH oxidase inhibitor apocynin, for instance, was shown in distinct studies to prolong [20] and to attenuate [21] allergen-induced inflammation and asthma changes in mice. Treatment with aminoguanidine also presented controversial effects depending on the administration scheme [22]. A single dose of aminoguanidine before the last challenge suppressed airway inflammation caused by allergen provocation, while subsequent repeated administrations of this drug before each allergen exposure potentiated the airway inflammation. It is believed that the detrimental effect is probably caused by an extensive reduction of nitric oxide (NO) and the down-regulation of SOD.

However, no study has attempted to screen the critical points of superoxide anion metabolism via pharmacological interferences at different levels of this pathway. Therefore, this study investigated the superoxide anion regulation by different pharmacological interventions in a murine model of ovalbumin-induced asthma.

Materials and methods

Ethics statement

Male BALB/c mice (8–10 weeks) were obtained from Oswaldo Cruz Foundation Breeding Center. Mice were bred and housed in a temperature controlled room with free access to food and water, except when water was replaced by treatment. All procedures were approved by the Animal Ethics Committee of the Federal University of Rio de Janeiro (protocol number IBCCF 106). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Induction of asthma and treatment protocols

Mice were immunised subcutaneously on day 0 and boosted 14 days later with 50 µg ovalbumin (OVA – grade V, intraperitoneal), which was dissolved with 5 mg of Al(OH)₃ into 200 µl of sterile saline (0.9% sodium chloride), as reported previously [23]. OVA challenges (50 µg/25 µl in saline) were performed on days 19, 20, and 21 under halothane volatile anaesthesia (Cristália, São Paulo, Brazil) by intranasal instillation. Sensitised control mice were challenged only with vehicle. This protocol was applied in both phases of the study, as shown in Figure 1.

In the first setting, the euthanasia was performed by cervical displacement, at different time points: 6, 12, 24, 48, and 72 hours after the last challenge, after which a bronchoalveolar lavage (BAL) was performed ($n = 14$ per group). BAL samples were used in the protocols for ROS and EPO measurements. ROS was measured by nitroblue tetrazolium method into the BAL cellular fraction [24] whereas EPO was measured based on methods that were adapted from previously published protocol [24,25]. Briefly, 25 µl of either BAL fraction was added to a reaction buffer (50 mM Tris-HCl, 4 mM H₂O₂, 0.1% Triton X, 10 mM O-phenylenediamide) in the presence or absence of the peroxidase specific inhibitor, 3-amino-1,2,4-triazole (30 mM). The reaction proceeded for 15–30 minutes at 37 °C, after which a “stop solution” (2N H₂SO₄) was added and the plate was read at 490 nm.

In another experiment, several pharmacological treatments targeting superoxide anion or nitric oxide pathways were performed. Sensitised mice received the NADPH oxidase inhibitor, apocynin (14 mg/kg); or the xanthine oxidase inhibitor, allopurinol (25 mg/kg); or SOD inhibitor, diethyldithiocarbamate (100 mg/kg); or iNOS inhibitor, aminoguanidine (50 mg/kg). The administration was by intraperitoneal injection 1 h before the allergen provocation.

Measurement of pulmonary inflammation

Forty-eight hours after the last challenge, mice were euthanised by an anesthetic overdose (sodium pentobarbital, 500 mg/kg intraperitoneal). The cells were recovered from the airways through the procedure of BAL which was carried by two injections of 0.75 ml of PBS containing 10 mM EDTA, *via* a tracheal cannula. BAL fluid was centrifuged (400 × *g*, 10 min, 4 °C). Cell pellets were suspended in 250 µl PBS for further leukocyte enumeration using a light microscope in a Neubauer chamber (BX50; Olympus, Center Valley, PA) after dilution with Türk solution (2% acetic acid).

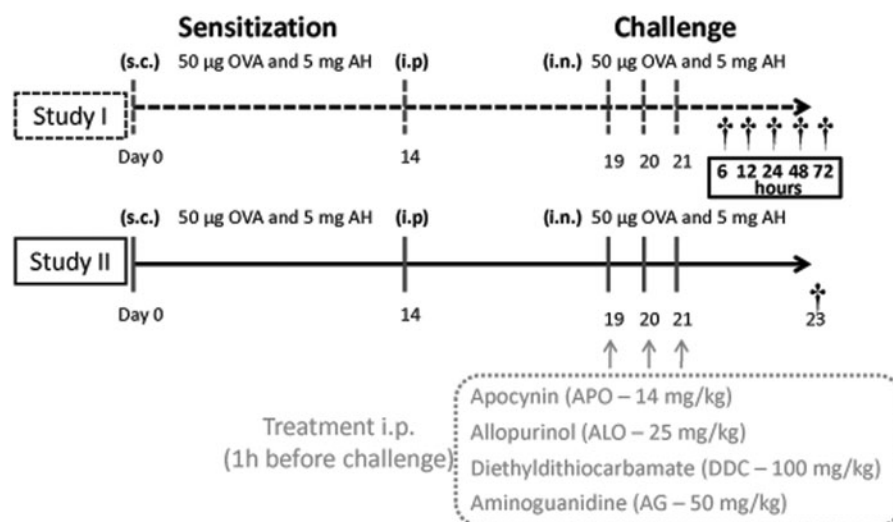


Figure 1. Experimental design of the asthma induction-protocol. Balb/c male mice were immunised subcutaneously (s.c.) on day 0 and boosted by intraperitoneal route (i.p.) 14 days later with 50 µg ovalbumin (OVA) adsorbed to 5 mg of AH into 200 µl of sterile saline. OVA challenges (50 µg/25 µl in saline) were performed on days 19, 20 and 21 by intranasal (i.n.) instillation. Study one evaluated the kinetics of ROS and EPO production in a short period of OVA-challenge. Study two targeted to modulate pathways involved in synthesis of superoxide anion (apocynin and allopurinol) or nitric oxide (aminoguanidine) or to inhibit superoxide dismutase activity (diethyldithiocarbamate). All the treatments were performed 1 hour before challenge by intraperitoneal route and euthanasia (†) was done 48 hours the last challenge.

Lung histology

The left lung was fixed in Millonig buffer solution (pH 7.4) with 4% paraformaldehyde to preserve the pulmonary architecture. Samples were embedded in paraplast media and 4-µm-thick sections were stained with haematoxylin and eosin stain. To observe the eosinophilic infiltration, sections were stained in a solution of chromotrope 2R. Eosinophil infiltrate was evaluated around the airway as well as between the bronchial epithelium and adventitia.

Quantification of cytokines

Commercial enzyme immunosorbent assay (ELISA) kits were used for the measurements of cytokines in the BAL. Levels of IL-1β, IL-4, IL-10, and IL-13 (PeproTech, Rocky Hill, NJ) were detected in the BAL samples, according to manufacturer's instructions, with detection limit of 10 pg/ml. The plate reader FlexStation 3 and the software Softmax Pro 5.3 (Molecular Devices Corporation, Sunnyvale, CA) were used for the detection of optical density.

Redox markers

Lung homogenates were used for biochemical assays. SOD activity was assayed by monitoring the adrenaline auto-oxidation inhibition at 480 nm [26]. Catalase (CAT) activity was measured by the rate of decrease of hydrogen peroxide concentrations monitored at 240 nm [27].

As an index of oxidative damage by lipid peroxidation, the thiobarbituric acid reactive substances (TBARS) method was performed to analyse malondialdehyde (MDA) products during an acid-heating reaction, and then read at 532 nm, as previously described by Draper and Hadley [28]. Immunohistochemistry was performed using labelled streptavidin biotin kit (LSAB, DAKO/Denmark). Sections were incubated overnight with primary antibody against 4-HNE (1:800; Millipore, Darmstadt, Germany), 3-nitrotyrosine (1:50; Santa Cruz Biotechnology, Dallas, TX) and NOX2 (1:200; Abcam, Cambridge, MA). Omission of primary antibody was used as a negative control. The brown colour produced by three, 3-diaminobenzidine chromogen was used to count septal and alveolar positive cells per field in lung slices. Images were acquired by camera DFC 425 coupled to the light microscope DM750 (Leica Microsystems, Heerbrugg, Germany).

Airway responsiveness to methacholine

AHR was determined as changes of airway function after aerosolised methacholine 48 h after the last ovalbumin challenge. Transpulmonary resistance and compliance were assessed in anaesthetised (Nembutal 60 mg/kg intraperitoneal), tracheostomised and mechanically ventilated mice. Airflow and transpulmonary pressure were recorded with a Buxco Pulmonary Mechanics Computer (Buxco Electronics, Sharon, CT). Lung resistance (RL) (cm H₂O/ml/s) and dynamic lung

compliance (ml/cm H₂O) were taken in each breath cycle, at a baseline condition, after aerosolised phosphate-buffered saline (PBS) and at increasing concentrations of aerosolised methacholine (3, 9, and 27 mg/ml). Expressed results comprised the mean absolute values of the responses of lung resistance and dynamic lung compliance collected during 5 min after the administration of methacholine aerosol as previously reported [23].

Statistical analysis

Data analyses were performed with a statistical software package (Prism version 5.0, Graph-Pad Software, San Diego, CA). Data were expressed as the means $\pm$ SEM. All tests were carried out using a one-way ANOVA, except for those concerning the airway hyperreactivity data, which were analysed using two-way ANOVA with a *post hoc* Bonferroni correction. For all the other normally distributed data, analyses were done with an ANOVA followed by the Student–Newman–Keuls test. Statistical differences were considered to be significant if $p < .05$.

Results

Kinetic of ROS and EPO production in ovalbumin-challenged mice

To choose the best time-point to observe increase in the ROS and EPO levels, mice were temporally euthanised for comparison between control and OVA groups. Both ROS (Figure 2(a)) and EPO (Figure 2(b)) values were increased 48 h after the last challenge compared to the control group. No changes were detected in all the other points, except for increased ROS levels noted 6 h postchallenge, leading to the choice of the 48-h time point for the analyses in all the further experiments.

Inflammation and redox markers in ovalbumin-challenged mice

Lung histological analysis showed an intense recruitment of leukocytes in the peribronchiolar area, as well as in the lung parenchyma in the OVA group. This response was accompanied by a thickened airway smooth muscle, both contributing to the reduction of the luminal area. Treatment with allopurinol did not reduce leukocyte recruitment in the peribronchiolar area or lung parenchyma while treatments with apocynin, diethyldithiocarbamate or aminoguanidine clearly reduced allergen-induced leukocyte infiltration in the

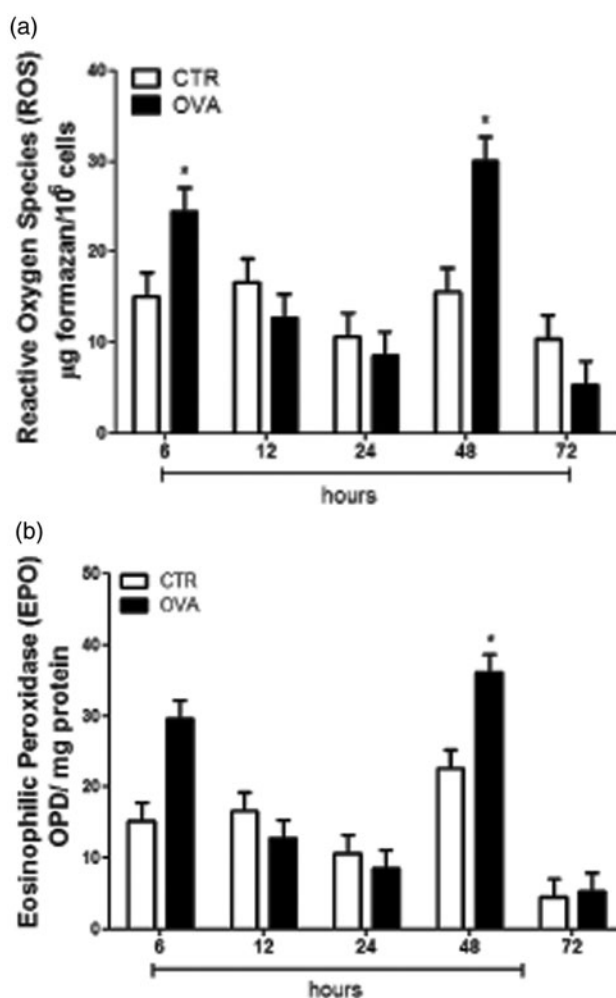


Figure 2. Release of ROS and EPO had a peak at 48 hours after ovalbumin challenge. Mice submitted to asthma protocol by sensitisation and challenge with OVA and euthanised temporally at 6, 12, 24, 48 and 72 hours after last challenge. Detection of reactive oxygen species – ROS (a) and eosinophil peroxidase – EPO (b) in BAL samples. Symbol: * means different of control group when $p < .05$.

lung tissue compared to the control (Figure 3(a)). As shown in Figure 3(b), a similar pattern of interference in the leukocyte accumulation was observed in BAL samples, allopurinol was inactive whereas treatments with apocynin, diethyldithiocarbamate or aminoguanidine were active in preventing the response.

Allergen provocation caused a significant increase in the BAL levels of IL-4, IL13, and IL1 β without modifying the concentration of IL-10 (Figure 4). All treatments, except diethyldithiocarbamate, attenuated IL-4 production (Figure 4(a)). Apocynin and allopurinol treatments partially reduced IL-13 levels under conditions where diethyldithiocarbamate and aminoguanidine almost abolished the production of this cytokine compared to OVA group (Figure 4(b)). On the other hand, allergen-induced increase in IL-1 β was abolished by allopurinol

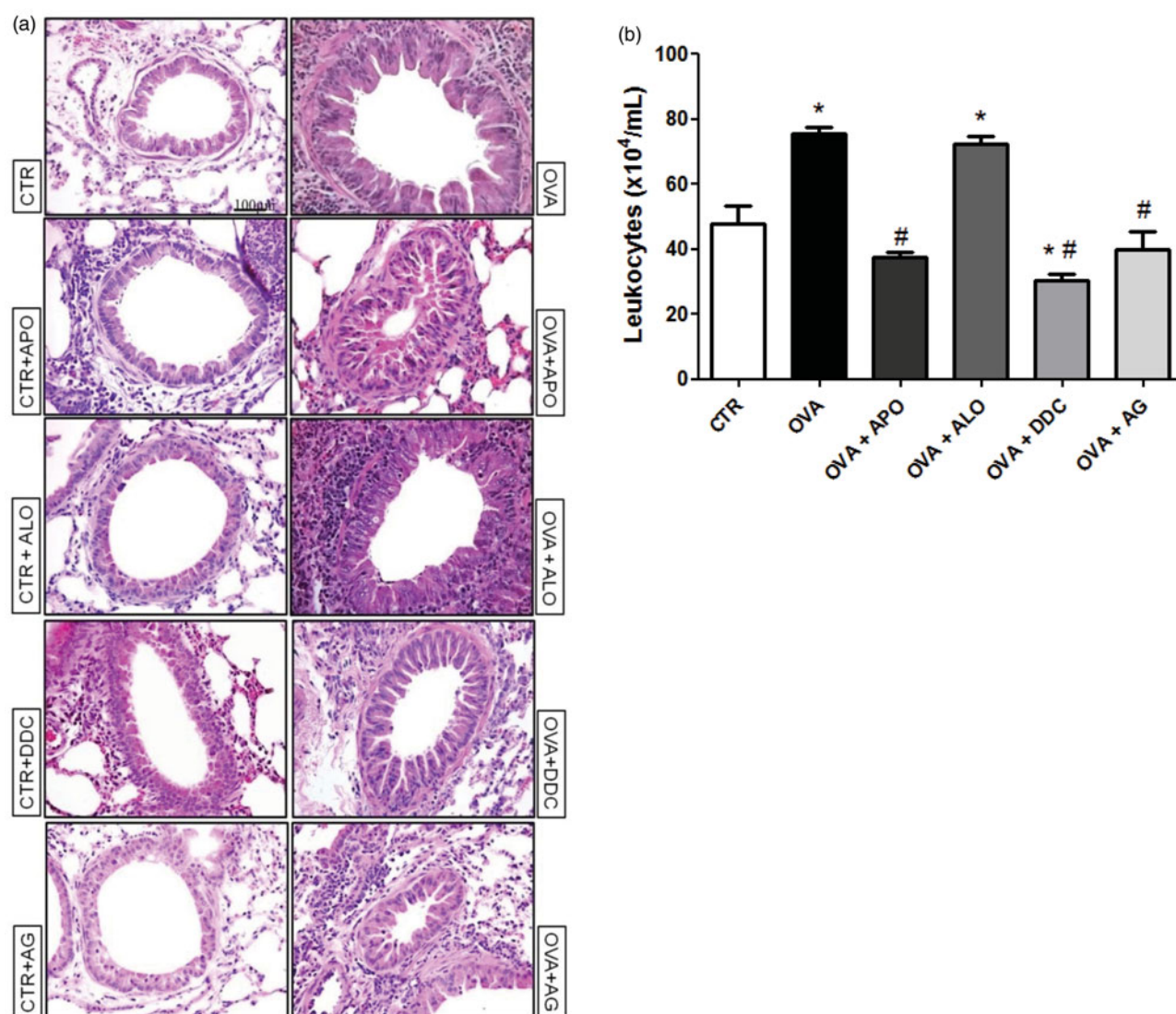


Figure 3. Pharmacological modulation of allergic inflammation induced by ovalbumin. Lung slices stained by H&E focusing on peribronchiolar area; scale bar = 100 μ m (a). Total leukocytes counted in BAL (b). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

and elevated by apocynin (approximately 120%), aminoguanidine (approximately 100%), and diethyldithiocarbamate (approximately 320%) compared to the OVA group (Figure 4(c)). The reduction in baseline levels of IL-10 were noted following diethyldithiocarbamate and aminoguanidine treatments, whereas no changes were noted following apocynin and allopurinol treatments (Figure 4(d)).

Eosinophil recruitment was evaluated in the lungs of challenged mice. Lung slices that were stained by chromotrope 2R show the eosinophil granules in red. The number of positive cells was increased in the OVA group relative to the control. The diethyldithiocarbamate-treated group showed many positive cells distributed in the lung parenchyma, but all the other treated groups showed a reduction in eosinophil influx (Figure

5(a)). These findings were reinforced by quantification of EPO by BAL measurements, which were elevated in OVA and diethyldithiocarbamate-treated groups. The aminoguanidine-treated group also had increased EPO levels in comparison to the control. However, this increase had some modulation, because it was smaller than the observed in OVA group (Figure 5(b)). Apocynin- and allopurinol-treated groups were not different from the control group.

The redox profile was measured by different methods. ROS levels were elevated in OVA group, while all the treated groups were like the control (Figure 6(a)). SOD activity was reduced in all OVA challenged groups when compared to the control group and the reduction was even greater in the apocynin and allopurinol groups. Diethyldithiocarbamate and aminoguanidine-

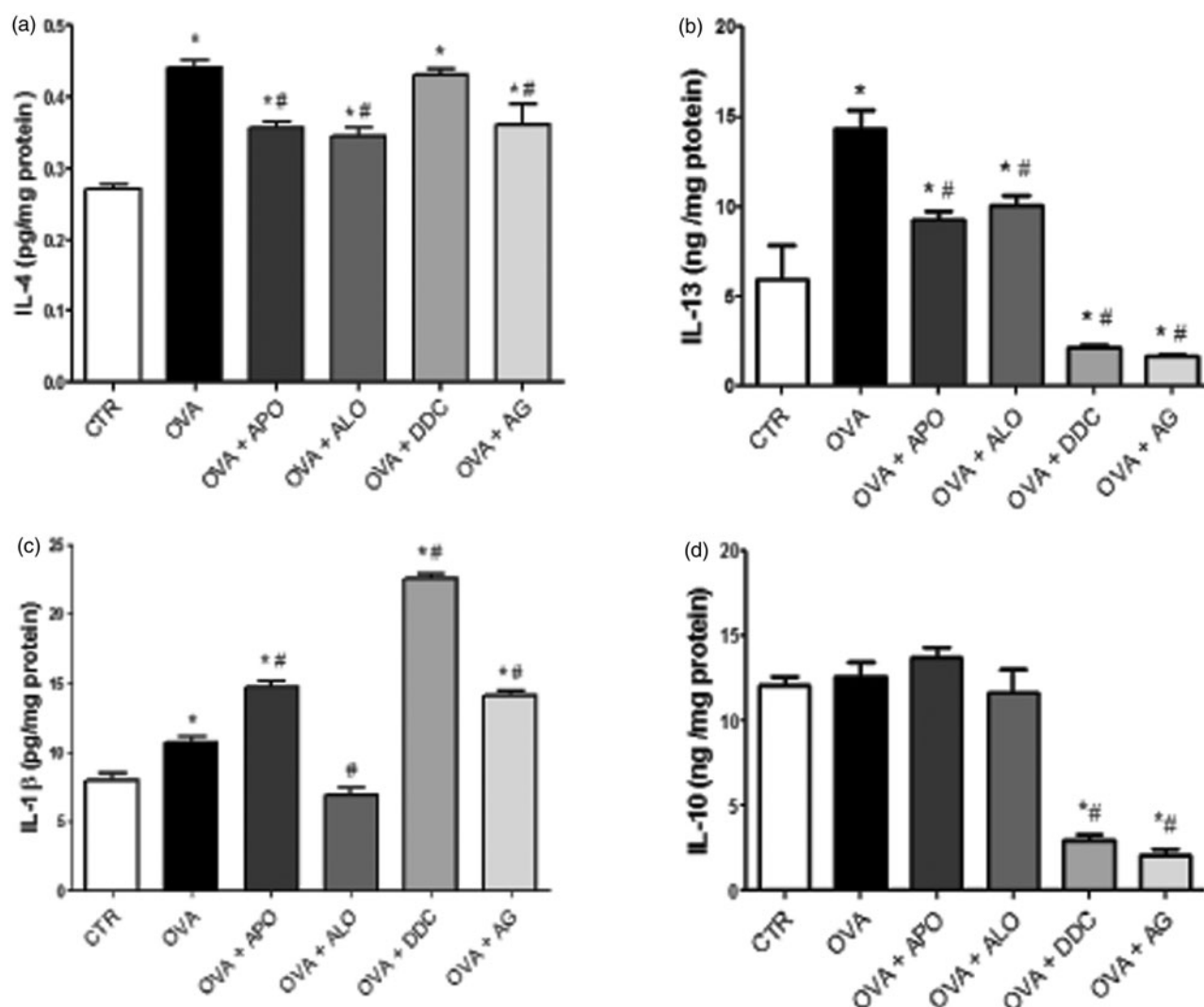


Figure 4. Inhibition of pathways involved in superoxide anion and nitric oxide synthase change cytokine profile. Levels of IL-4 (a), IL-13 (b), IL-1 β (c) and IL-10 (d) detected in BAL sample of mice subjected to asthma protocol induced by ovalbumin with no treatment (OVA group) or treated with apocynin (OVA + APO), allopurinol (OVA + ALO), diethyldithiocarbamate (OVA + DDC) or aminoguanidine (OVA + AG). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

treated groups were higher than OVA group but still reduced in comparison to the control (Figure 6(b)). OVA, apocynin and allopurinol groups showed reduced CAT activity in comparison to the control, while diethyldithiocarbamate and aminoguanidine-treated groups were increased when compared to both control and OVA groups (Figure 6(c)). TBARS levels, a marker of oxidative damage, were increased only in the OVA group, and diethyldithiocarbamate- and aminoguanidine-treated groups showed lower levels than the control (Figure 6(d)).

Immunohistochemistry against 4-HNE also revealed oxidative damage increased in OVA group, by the increased number of 4-HNE⁺-cells in septal tissue and also in alveolar spaces. All the treated groups presented reduction in 4-HNE⁺-septal cells in comparison to the OVA group. In relation to the alveolar spaces, OVA

groups also had increase of 4-HNE⁺-cells. Just apocynin and aminoguanidine treatments were able to reduce it to control level. Allopurinol and diethyldithiocarbamate were similar to OVA group (Figure 7). 3-Nitrotyrosine was also observed by immunohistochemistry as an oxidative damage marker and showed increase in 3-nitrotyrosine⁺-alveolar cells in OVA group. All the treated groups presented reduction of this marker in alveolar cells, but allopurinol group also presented increase in relation to the control group (Figure 8). The expression of NOX-2, was also observed once it is involved in superoxide anion by leukocytes. The OVA group presented increase in NOX-2⁺-alveolar cells when compared to the control. All the treated groups presented reduction of this marker in alveolar cells, but allopurinol group also presented increase in relation to the control group (Figure 9).

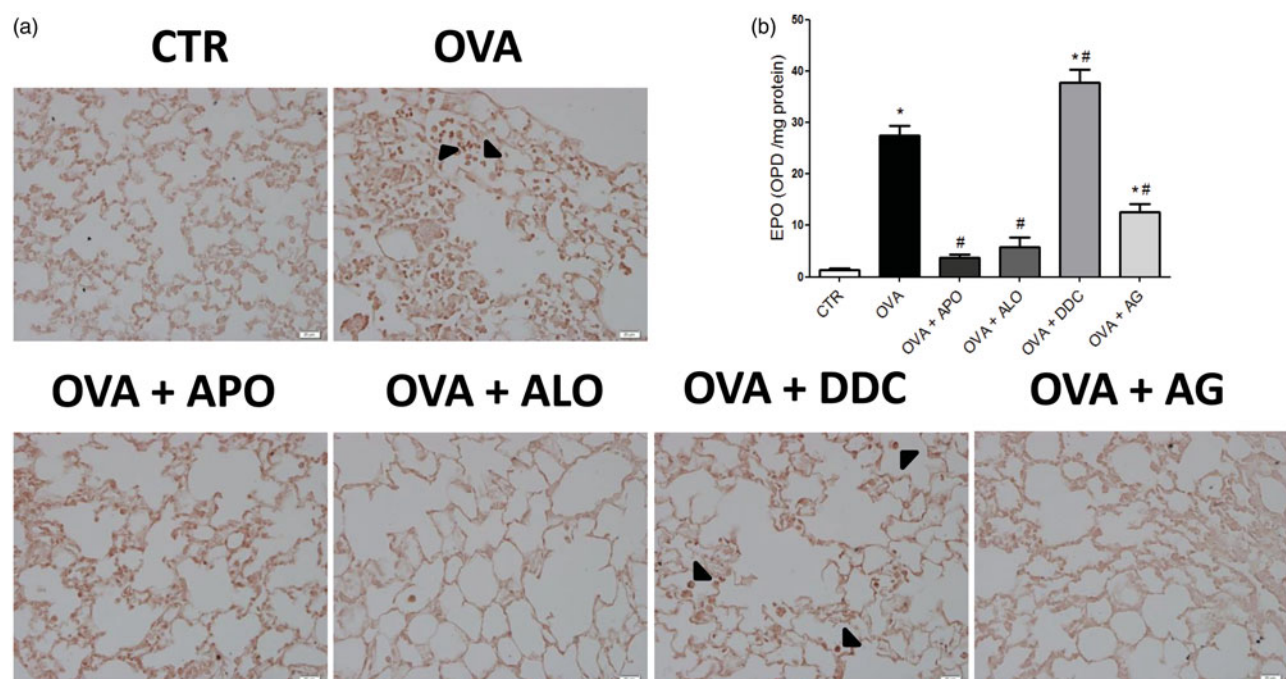


Figure 5. Inhibition of NADPH oxidase, XO and iNOS attenuated eosinophilic inflammation. Lung slices stained with chromatrope 2R to evidenciate eosinophils – head arrow (a). Peroxidase eosinophilic (EPO) levels detected in BAL samples of mice subjected to the asthma protocol induced by ovalbumin with no treatment (OVA group) or treated with apocynin (OVA + APO), allopurinol (OVA + ALO), diethyldithiocarbamate (OVA + DDC) or aminoguanidine (OVA + AG) (b). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

Airway hyperresponsiveness in ovalbumin-challenged mice

As expected, allergen provocation of sensitised mice was followed by a state of airway hyper-reactivity following exposure of increased concentrations of methacholine, as revealed by a decrease in lung compliance (Figure 10(a)), an elevation in lung elastance (Figure 10(b)) and an increase airway resistance (Figure 10(c)). These changes were significantly inhibited by treatments with apocynin, allopurinol or aminoguanidine. In contrast, diethyldithiocarbamate did not alter airway hyperresponsiveness triggered by allergen challenge (Figure 10(a–c)).

Discussion

Asthma is an allergic inflammatory disease of the airways triggered by an allergic process, in which myeloid cells and neutrophils and eosinophils release high superoxide anion (O_2^-) levels from the NADPH oxidase (NOX) system [29–31]. Excessive production of ROS by NOX is commonly thought to be responsible for tissue injury associated with a range of respiratory inflammatory diseases, such as acute respiratory distress syndrome (ARDS), asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [32].

Several studies have attempted to elucidate the role of the oxidative stress in the pathogenesis of asthma [21,29,31]. Understanding the cause/consequence ratio between ROS and the allergic inflammation could reveal interesting targets for future therapeutic approaches. In that direction, the NOX inhibition represents an attractive therapeutic strategy. Sevin et al. showed that mice deficient in gp91phox, a subunit of NOX also known as NOX-2, had an inhibition of the allergic inflammation induced by ovalbumin and they showed a decrease in both ROS production and allergic airway inflammation [30].

However, ROS is essential for many physiological events, what turn the complete inhibition of NOX a risky strategy. Thus, partial negative modulation of NOX activity and others pro-oxidant enzymes seems to be a promising alternative. In this context, this study investigated the role of drugs, which act by different manner to control superoxide anion and nitric oxide levels. Among these drugs, apocynin and allopurinol regulate different upstream sources of superoxide anion production, which elucidates if this type of intervention could protect lung from ovalbumin stimuli. Although there are other sources of superoxide, as mitochondria, endogenous NADPH oxidases are the major source of superoxide in lung [33]. Beyond, NADPH oxidase is responsible for the respiratory burst in immune cells,

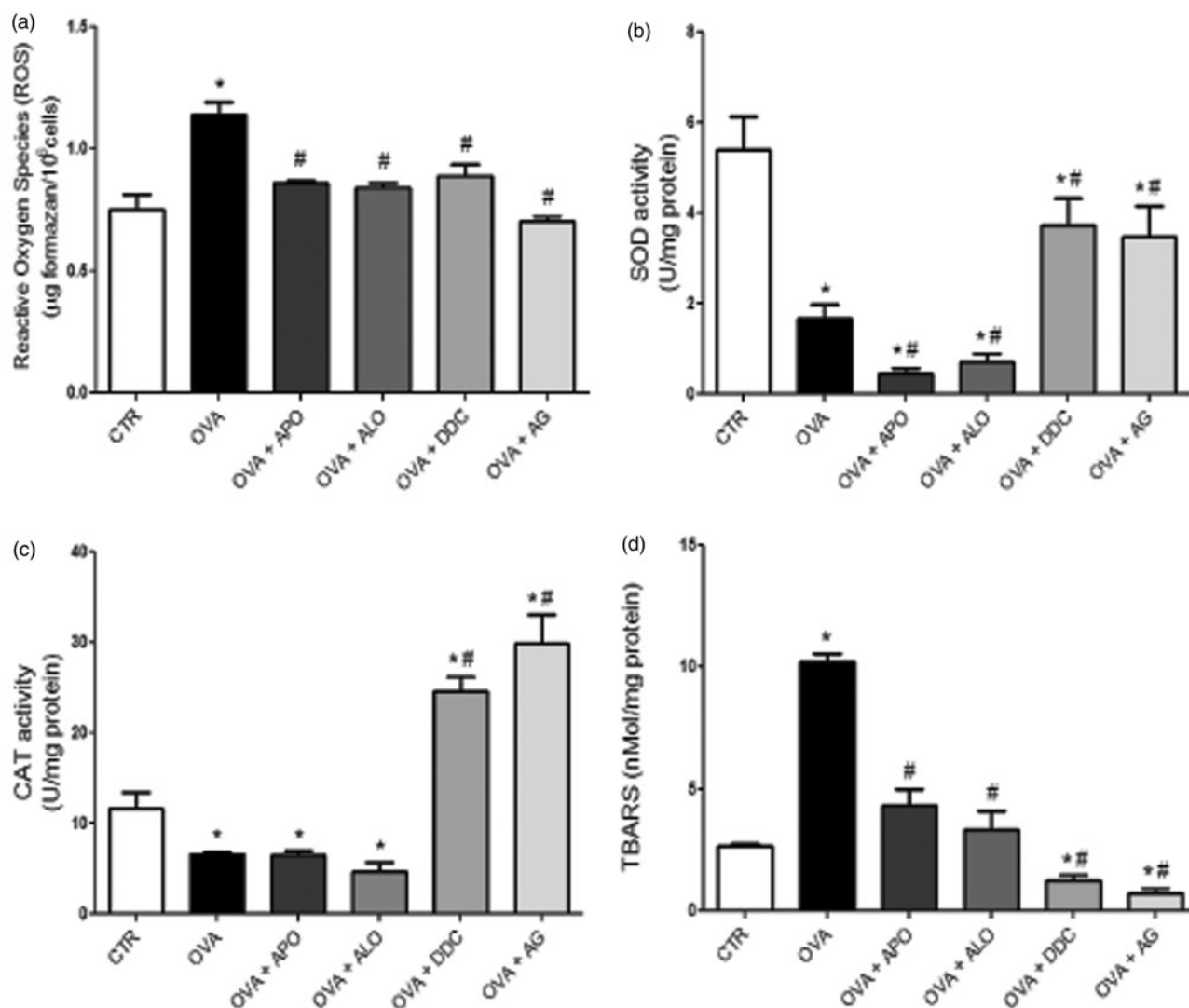


Figure 6. Target treatments abrogated oxidative stress induced by ovalbumin. Detection of reactive oxygen species – ROS – in cells from BAL (a). Detection of superoxide dismutase activity (b); catalase activity (c) and thiobarbituric acid reactive substances as marker of oxidative damage (d) in lung homogenates of mice subjected to asthma protocol induced by ovalbumin with no treatment (OVA group) or treated with apocynin (OVA + APO), allopurinol (OVA + ALO), diethyldithiocarbamate (OVA + DDC) or aminoguanidine (OVA + AG). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

and is implicated in asthma development [34]. Diethyldithiocarbamate, acting late, inhibited SOD activity, which contributed to the increase of superoxide anion. Aminoguanidine, an iNOS-inhibitor, contributed to reduced levels of NO in the inflammatory process.

Apocynin and allopurinol are both inhibitors of superoxide anion production by different mechanisms. Apocynin inhibits NADPH oxidase activity by preventing the translocation of p47phox to the NOX-2 membrane region in leukocytes, monocytes and endothelial cells [35]. Allopurinol inhibits xanthine oxidase, an enzyme that catalyses the oxidation of hypoxanthine to xanthine, and it can further catalyse the oxidation of xanthine to uric acid, involving the steps in ROS production. These inhibitors were the best choice to

prevent the typical signs of asthma in this study. Mice treated with apocynin had no leukocyte infiltration in the peribronchiolar area and the airways, which occurred in parallel with a significant attenuation of the Th2 response (as shown by the reduction in the BAL levels of IL-4 and IL-13). In addition, we observed that apocynin led to an elevation of IL-1 β levels when compared to those in the OVA group, suggesting a transition toward the Th1 immunologic response, despite superoxide anion inhibition from NADPH oxidase. Kim et al. showed the anti-inflammatory effects of apocynin in OVA-induced experimental asthma, with reduction of Th2 cytokines and airway hyperresponsiveness [21]. However, this is the first time that apocynin is shown to antagonise the OVA-induced Th2 response and stimulate

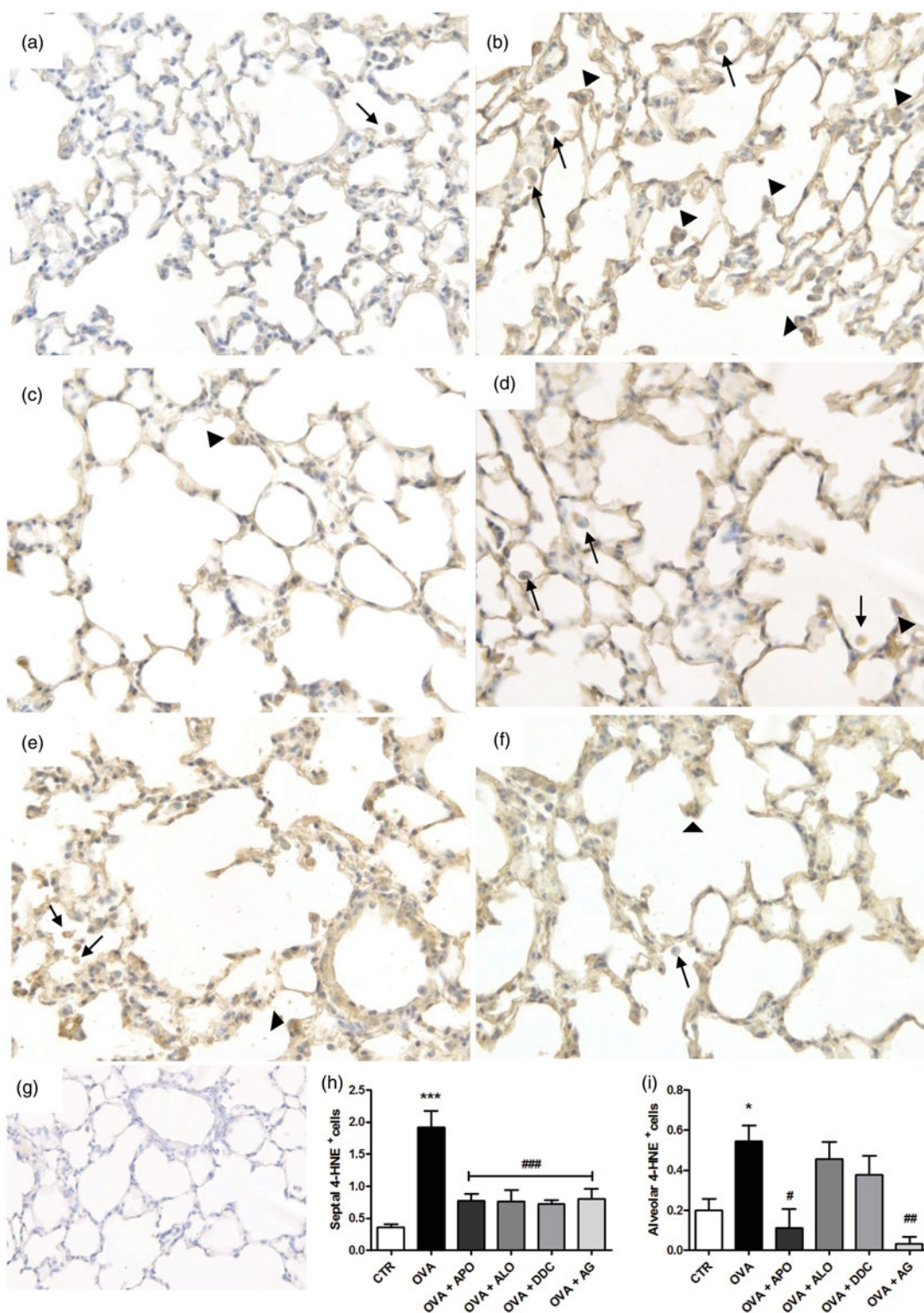


Figure 7. Immunohistochemistry panel showing septal and alveolar 4-HNE positive cells. Control (a), OVA (b), OVA + APO (c), OVA + ALO (d), OVA + DDC (e), OVA + AG (f), negative control for immunostain (g), 4-HNE⁺-septal cells count (h), 4-HNE⁺-alveolar cells count (i). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

the Th1 response as well as improve functional, morphological and inflammatory parameters. It is known that Th1 lymphocytes antagonise the Th2 response, which is positive in the context of asthma [36].

Furthermore, apocynin had an effective antioxidant action. Despite the antioxidant enzymes activities reduced in the apocynin-treated group, the oxidative damage was blocked, by the reduction on TBARS levels,

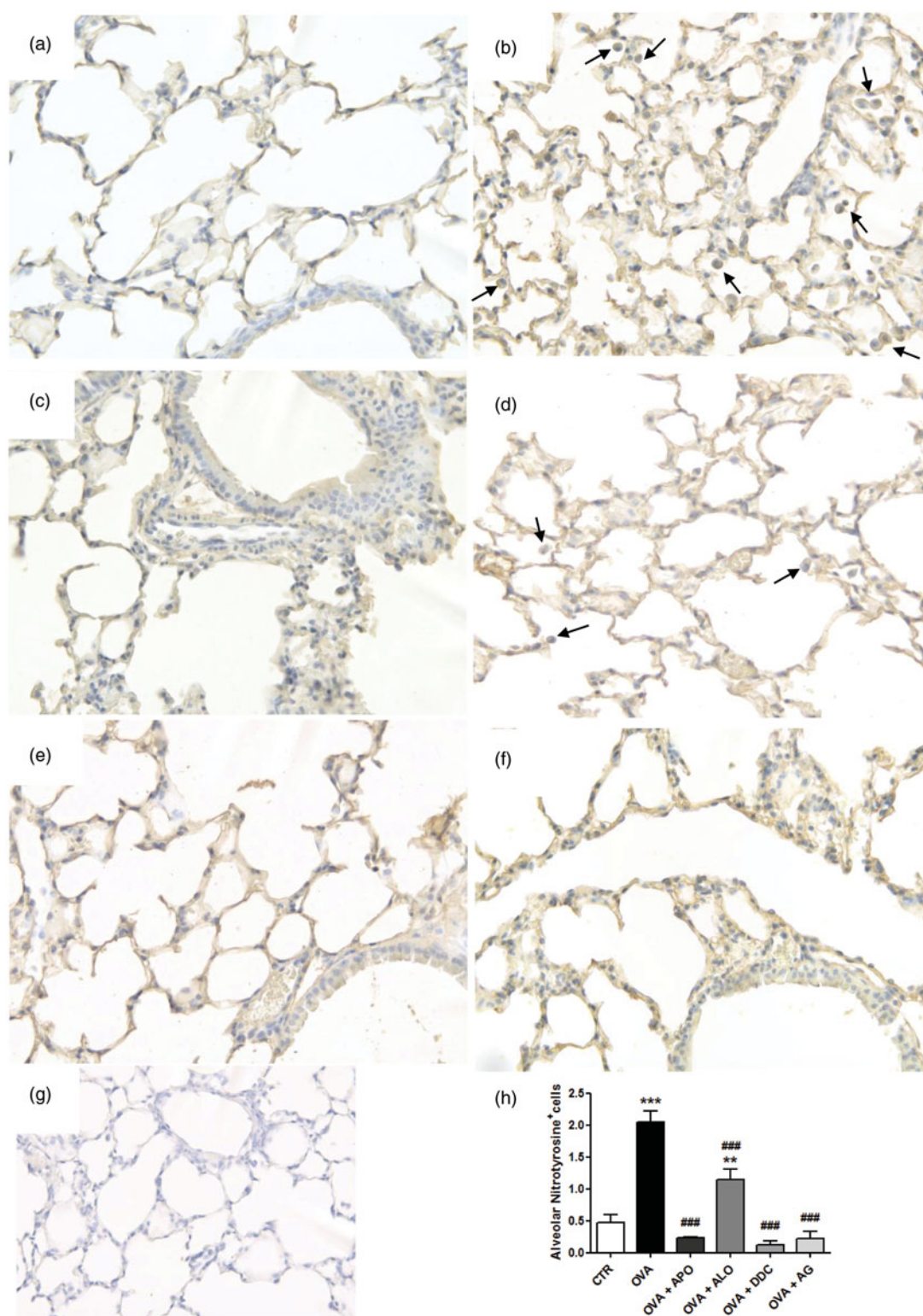


Figure 8. Immunohistochemistry panel showing alveolar 3-nitrotyrosine positive cells. Control (a), OVA (b), OVA + APO (c), OVA + ALO (d), OVA + DDC (e), OVA + AG (f), negative control for immunostain (g), 3-nitrotyrosine⁺-alveolar cells count (i). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

4-HNE⁺ and nitrotyrosine⁺ cells. Apocynin also kept the ROS levels similar to control and reduced the number of NOX-2⁺-alveolar cells. Together, these data suggest that OVA-challenged mice treated with apocynin were

protected against the oxidative damage and reduced ROS to control levels. In addition to the NADPH oxidase inhibitor, apocynin can also act as a scavenger [37], what contributed for reduction in SOD and CAT

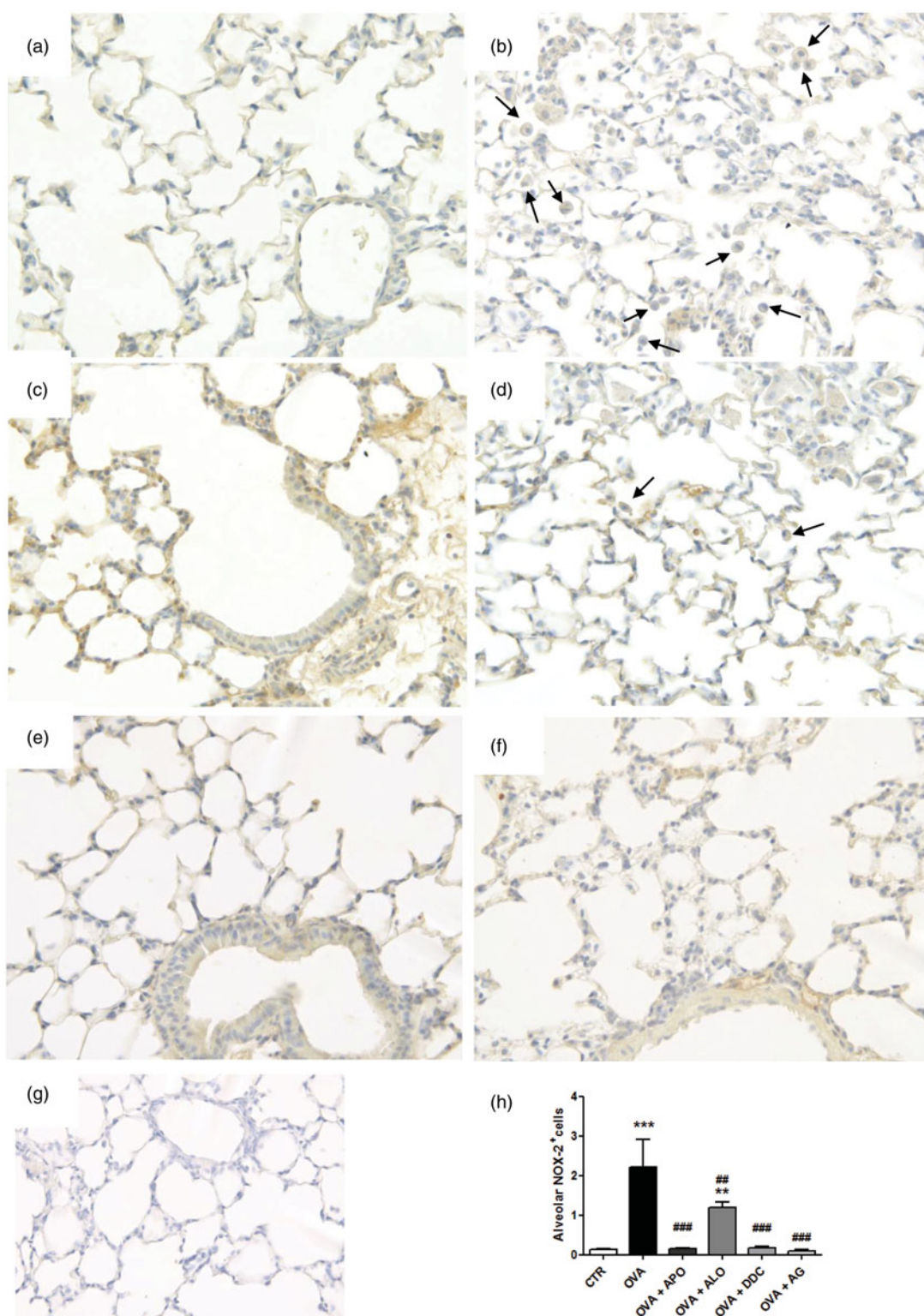


Figure 9. Immunohistochemistry panel showing alveolar NOX-2 positive cells. Control (a), OVA (b), OVA + APO (c), OVA + ALLO (d), OVA + DDC (e), OVA + AG (f), negative control for immunostain (g), NOX-2⁺-alveolar cells count (i). Symbols: * means different of control group; # means different of OVA group when $p < .05$.

activities, making of apocynin an effective antioxidant in OVA-induced asthma.

Surprisingly, the results from the allopurinol-treated group were generally similar to apocynin with the

exception of the massive peribronchiolar infiltrate. The effect of allopurinol was not able to prevent the recruitment of inflammatory cells to the airways, as shown in [Figure 3](#). However, the recruitment of eosinophils was

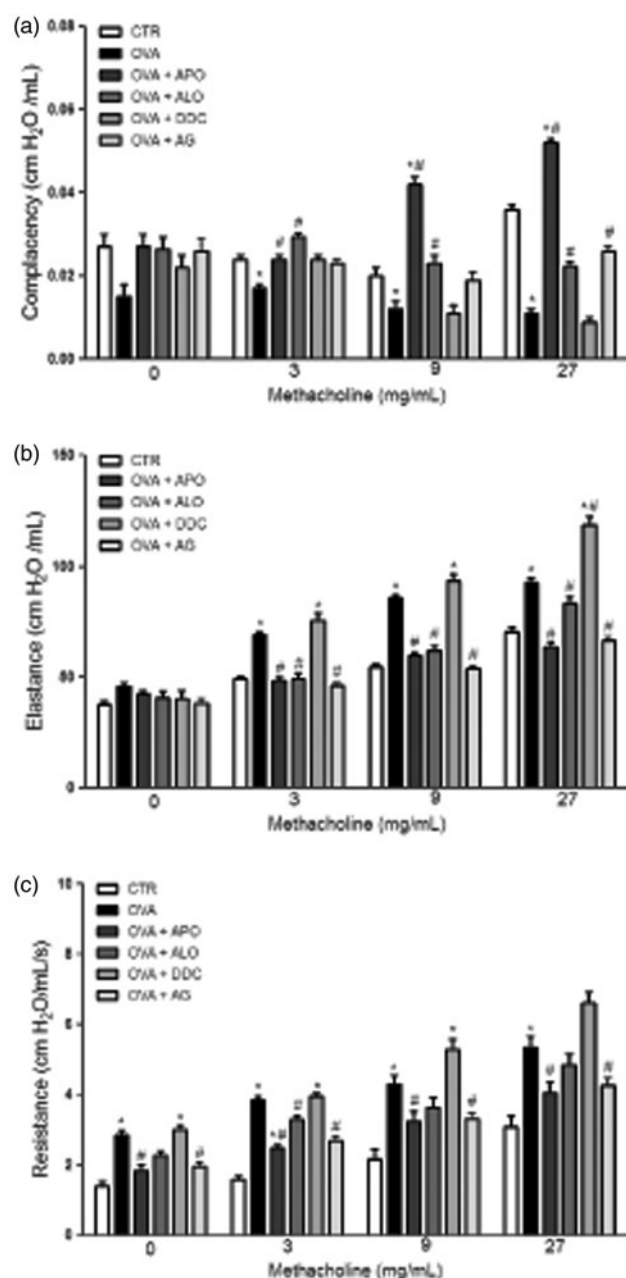


Figure 10. Inhibition of NADPH oxidase, XO and iNOS abrogated airway hyper-reactivity induced by ovalbumin. Assay of bronchial hyper-reactivity front of challenge with methacholine (0, 3, 9 e 27 mg/kg) in mice subjected to asthma protocol induced by ovalbumin with no treatment (OVA group) or treated with apocynin (OVA + APO), allopurinol (OVA + ALO), diethyldithiocarbamate (OVA + DDC) or aminoguanidine (OVA + AG). Lung complacency (a), elastance (b) and airway resistance (c) parameters were collected by BUXCO system. Symbols: * means different of control group; # means different of OVA group when $p < .05$.

reduced after this treatment, as shown in Figure 5. The inhibition of xanthine oxidase did not prevent peribronchiolar infiltrate. However, it attenuated release of the Th2 cytokines, protected against both oxidative stress and AHR, as well as preserved lung compliance,

elastance and airway resistance. Allopurinol treatment was effective to reduced 4-HNE⁺-septal cells, but these 4-HNE⁺-cells were elevated in alveolar spaces, similar to OVA group. These data were corroborated with 3-nitro-tyrosine and NOX-2 markers, once OVA + ALO group presented increased positive cells in comparison to the control, but yet smaller than OVA group. This observed increase make sense because of the massive peribronchiolar infiltrate in this group. Taken together, these data suggest that inhibition of xanthine oxidase changed the phenotype of cellular infiltrate by recruiting cells with a proresolutive profile, such as macrophages, mast cells and dendritic cells. These recruitment, even with a proresolutive profile, brought to the lung a worst antioxidant response than the observed by apocynin treatment.

These data suggest that the source of superoxide anion is important in driving the recruitment of inflammatory cells for the allergic inflammatory response induced by OVA. Modulation of upstream superoxide anion production seems to effectively block changes induced by OVA, especially because it can regulate the inflammatory cell recruitment and the profile of immunological response. In addition, the inhibition of superoxide anion synthesis protected against the eosinophilic AHR, once the main granule release from eosinophils during allergic inflammation is the EPO. It works as a key molecule on oxidative and nitrosative processes, and it was reduced by apocynin and allopurinol treatments.

In support of this notion, diethyldithiocarbamate group presented the worst response as a therapeutic strategy against asthma. Mice treated with DDC showed a worsening of the parameters evaluated, becoming similar to OVA group. The diethyldithiocarbamate-treated group did not show peribronchiolar infiltrate, but eosinophils increased in the remaining cells, which was similar to the OVA group (as shown by high EPO levels (Figure 5)). This group presented a typical Th2 response with high IL-4 levels and the associated pulmonary function impairment, as shown by an exacerbated airway resistance and elastance, similar to the OVA group. However, by the immunohistochemistry analysis, OVA + DDC group did not present any investigated marker increased. The conflicting result of reduced NOX-2, mediated by this SOD inhibitor, is observed due to the cellular infiltrate blockage induced by DDC. The data suggest that once few cells were recruited, the ROS production was smaller than in the OVA group, what was accompanied by a slight reduction in SOD activity.

These data are in accordance with Nishida et al. who showed that pretreatment with diethyldithiocarbamate

increased the severity and duration of the asthma attack [38]. Diethyldithiocarbamate is a copper chelator, which decreases the activity of Cu, Zn superoxide dismutase what can contribute to increases superoxide anion [39]. However, contrary to the expected result, the ROS levels measured in diethyldithiocarbamate-treated group were equal to the OVA group. This finding may be observed because of the low leukocyte recruitment into the peribronchiolar area found in this group, although the eosinophil recruitment was very strong and selective, as shown by EPO (Figure 5). These data suggest a difficulty of the leukocytes to get into the airways, with exception of the eosinophils, which were selectively attracted. It is known that activated Th2 lymphocytes recruited to the lungs orchestrate the inflammation releasing several mediators, among them IL-4 and IL-13, promoting more activation of B lymphocytes, which activate mast cells to secrete mediators chemoattractants to eosinophils [40].

However, the products released from mast cells led to leukocyte recruitment due to the induction of expression of adhesion molecules at the surface of endothelial cells [41]. Nevertheless, Wei et al. showed that tetrathiomolybdate, another copper chelator, can inhibit vascular inflammation in a mouse model of atherosclerosis [42]. The mechanisms of arterial monocyte recruitment and retention are characterised by the expression of cellular adhesion molecules and chemokines, such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1), by vascular endothelial cells [43]. They showed that copper deficiency, induced by tetrathiomolybdate, reduced the levels of soluble VCAM-1 and ICAM-1, as well as the gene expressions of VCAM-1, ICAM-1, MCP-1 and proinflammatory cytokines [42]. As these adhesion molecules are required for leukocytes recruitment, we suggest that diethyldithiocarbamate, a copper chelator could reduce the leukocyte influx to the inflammatory site by this mechanism. However, we have not examined it. Despite this leukocyte blockage, the diethyldithiocarbamate-treated group presented AHR, with increased elastance and resistance.

Regarding iNOS inhibition by aminoguanidine, mice challenged with OVA and treated with aminoguanidine showed protection against the asthmatic response. The aminoguanidine-treated group presented a slight increase in IL-4 levels, a reduction in IL-13 and an increase in IL-1 β , suggesting a transition from the Th2 to the Th1 response. Additionally, no peribronchiolar infiltrate was observed, and the increase in EPO was attenuated in comparison to OVA group. Furthermore, this group showed an effective antioxidant response with an increase in SOD and CAT activity in comparison

to OVA group. Other than that, aminoguanidine also showed effectiveness to preserve lung function, reduce elastance and resistance. It is known that protein nitration is detected in sputum samples of asthmatic patients [44]. Chan et al. used another asthma-induction model, and they also showed increased protein damage through elevation in 3-nitrotyrosine marker induced by house dust mite [45]. In the present study, aminoguanidine treatment reduced the number of 3-nitrotyrosine⁺ cells in alveolar spaces induced by OVA, showing a protection against protein damage. The present data suggest that down-regulating nitric oxide synthesis mediated by iNOS reduction, a better antioxidant response could be achieved by aminoguanidine treatment. As purposed by Ogino et al., serum arginase I could be observed as biomarker in asthma [46]. Considering that arginase I can be up-regulated by NOS inhibition, due to elevation in L-arginine levels, this parallel pathway could also be mobilised after aminoguanidine treatment, however this hypothesis was not evaluated. A previous study of our group showed an incomplete inhibition of iNOS by 1% aminoguanidine treatment, in a model of pulmonary emphysema induced by porcine pancreatic elastase, because there was a remaining iNOS expression. But even with this result, there was a reduction in 3-nitrotyrosine marker [47]. Beyond, some inhibition was also detected in eNOS isoform, corroborating what Laszlo et al. stated that aminoguanidine is a dual constitutive and inducible nitric oxide synthase inhibitor [48].

In conclusion, these data suggest that NOX, xanthine oxidase (XO) and iNOS inhibition attenuated allergic inflammation and shifted the Th1 immunological response and avoided AHR by improving airway resistance, lung compliance and elastance parameters. Apocynin and allopurinol were capable of reducing the EPO marker, but the NOX-2 inhibition by apocynin also prevented the peribronchiolar cellular infiltrate. Thus, in different levels, the down-regulation in superoxide synthesis seems to be a good target to treat allergic inflammation.

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ORCID

Renata Tiscoski Nesi  <http://orcid.org/0000-0002-6774-0893>
 Marina Valente Barroso  <http://orcid.org/0000-0001-8135-9100>
 Valdirene de Souza Muniz  <http://orcid.org/0000-0003-0102-2861>
 Ana Carolina de Arantes  <http://orcid.org/0000-0003-0875-3106>
 Marco Aurélio Martins  <https://orcid.org/0000-0002-4708-5705>
 Lycia de Brito Gitirana  <http://orcid.org/0000-0002-5580-6939>
 Josiane Sabbadini Neves  <http://orcid.org/0000-0002-6645-1715>
 Cláudia Farias Benjamim  <http://orcid.org/0000-0001-6247-9596>
 Manuella Lanzetti  <http://orcid.org/0000-0003-3913-608X>
 Samuel Santos Valença  <http://orcid.org/0000-0002-1868-9905>

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