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Copaiba oil suppresses inflammation in asthmatic lungs of BALB/c mice induced with ovalbumin

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ABSTRACT

Asthma is a chronic inflammatory disease that represents high hospitalizations and deaths in world. Copaiba oil (CO) is popularly used for relieving asthma symptoms and has already been shown to be effective in many inflammation models. This study aimed to investigate the immunomodulatory relationship of CO in ovalbumin (OVA)-induced allergic asthma. The composition of CO sample analyzed by GC and GC–MS and the toxicity test was performed in mice at doses of 50 or 100 mg/kg (by gavage). After, the experimental model of allergic asthma was induced with OVA and mice were orally treated with CO in two pre-established doses. The inflammatory infiltrate was evaluated in bronchoalveolar lavage fluid (BALF), while cytokines (IL-4, IL-5, IL-17, IFN-γ, TNF-α), IgE antibody and nitric oxide (NO) production was evaluated in BALF and lung homogenate (LH) of mice, together with the histology and histomorphometry of the lung tissue. CO significantly attenuated the number of inflammatory cells in BALF, suppressing NO production and reducing the response mediated by TH2 and TH17 (T helper) cells in both BALF and LH. Histopathological and histomorphometric analysis confirmed that CO significantly reduced the numbers of inflammatory infiltrate in the lung tissue, including in the parenchyma area. Our results indicate that CO has an effective *in vivo* antiasthmatic effect.

1. Introduction

Asthma is a worldwide disease that affects around 300 million people culminating in an airflow obstruction with different clinical courses [1] that are caused through a variety of mechanisms, mainly by the interaction of TH2, TH17 and TH1 cells and specific production of their interleukins [2].

Many impediments related to the diagnosis of asthma, and the difficulty to access the right treatment and available therapies continue to encourage the search for new medicinal alternatives for the management of this disease [1].

CO, obtained from the trees of the genus *Copaifera* (such as *Copaifera officinalis*, Fabaceae—Caesalpinioideae), is a mixture of terpenes widely used in folk medicine as anti-inflammatory [3]. Previous study showed the *in vitro* immunomodulatory effect of CO on the TH1 and TH17 responses in the multiple sclerosis model, also showing an

Abbreviations: AA, allergic asthma; AHR, bronchial hyperreactivity; ALB, albumin; ALK, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BALF, bronchoalveolar lavage fluid; CBR, Reproduction Biology Center; CO, copaiba oil; CR, creatinine; DEX, dexamethasone; EAE, experimental autoimmune encephalomyelitis; EC, Ethical Committee; GGT, gamma glutamyl transferase; GLU, glucose; HE, hematoxylin and eosin; i.n., intranasally; i.p., intraperitoneally; LH, lung homogenate; LPS, lipopolysaccharide; NC, normal control group; NO, nitric oxide; OVA, Ovalbumin; PC, positive control group; p.o., orally; RNS, reactive nitrogen species; ROS, reactive oxygen species; TH, T helper cells; Treg, T regulatory cells; UFJF, Federal University of Juiz de Fora; UR, urea

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inhibitory action on NO and hydrogen peroxide [4]. Additionally, in human monocytes, THP-1 stimulated with lipopolysaccharide (LPS), CO inhibited IL-1β, TNF-α, IL-6, and nuclear factor kappa β [5]. Likewise, in the *in vivo* anti-inflammatory assay, CO was able to inhibit NO production and reduce the acute inflammation of pleura, induced by zymosan in mice [6].

It was shown that CO inhibited many inflammatory mediators involved in asthma. However, no previous studies of CO effects on asthma have been reported. Believing that this compound may act on asthmatic inflammation in a similar way as previously described, the aim of this work was to analyze for the first time, the *in vivo* the action of CO in models of acute pulmonary inflammation as an alternative to treat asthma.

2. Materials and methods

2.1. Copaiba oil and reagents

CO was purchased from Pharmanostra (São Paulo, SP, Brazil), lot 16E09-B027-009523. Ketamine (10%) were obtained from Syntec® (São Paulo, SP, Brazil) and Xylasin (2%) purchased from Ceva® (São Paulo, SP, Brazil). ELISA assay kits were procured from Peprotech Inc (Ribeirão Preto, SP, Brazil). All other chemicals, including OVA (purity ≥ 98%) and nitrite (purity ≥ 99%) were obtained from Sigma-Aldrich® (São Paulo, SP, Brazil).

2.2. GC and GC–MS analysis

CO sample was dissolved in ethyl ether and analyzed by GC-FID on a Shimadzu GC2010 Plus (Shimadzu Corporation, Kyoto, Japan) gas chromatograph equipped with an AOC-20s autosampler and fitted with FID and a data-handling processor. A Rtx-5, Restek Co. (Bellefonte, PA, USA), fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) was employed. The operation conditions were as follows: column temperature programmed to rise from 60 to 240 °C at 3 °C/min., then held at 240 °C for 5 min. The carrier gas was helium (99.999%) at a flow rate of 1.0 ml/min; injection mode; injection volume, 0.1 μl (split ratio of 1:10); injector and detector temperatures, 240 and 280 °C, respectively. The relative concentrations of the components were obtained by peak area normalization (%). The relative areas were the average of triplicate GC-FID analyses.

The same sample was also analyzed by GC–MS on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column consisted of Rtx-5MS, RestekCo. (Bellefonte, PA, USA), fused silica capillary (30 m × 0.25 mm i.d.; 0.25 μm film thickness). The electron ionization mode was used at 70 eV. Helium (99.999%) was employed as the carrier gas at a constant flow of 1.0 ml/min. The injection volume was 0.1 μl (split ratio of 1:10). The injector and the ion-source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC. Mass spectra were taken with a scan interval of 0.5 s, in the mass range from 40 to 600 Da. The identification of the CO components was based on their retention indices on an Rtx-5MS capillary column under the same operating conditions as in the case of GC, relative to a homologous series of *n*-alkanes (C_8-C_{20}); structures were computer-matched with the Wiley 7, NIST 08, and FFNSC 1.2 spectral libraries [7], and their fragmentation patterns were compared with literature data. Standard compounds were also co-eluted with CO components to confirm the identity of some essential oil components.

2.3. Animals

For the subacute toxicity study, fifteen healthy female mice (*Mus musculus*, 8-week-old) were used, while twenty-five BALB/c female mice (8-week-old) were used for the asthma induction. All animals were obtained from the Reproduction Biology Center (CBR) of the Federal University of Juiz de Fora (UFJF, Juiz de Fora, MG, Brazil) and kept according to the requirements of the Animal Experimentation Ethics Council. Animals were stored in polypropylene cages, with temperature around 22 °C, daylight-enhanced mixed lighting and automatically controlled fluorescent lamps to light at 6 a.m. and turn off at 6 p.m. They were stored in cages on selected but non-sterile shaving beds, a bottle of water and a palletized feed trough, with free access to food and water. All care and experimental procedures were approved by the UFJF / EC n°. 015/2016) and carried out in accordance with the Brazilian Guide to the Production, Maintenance or Use of Animals in Teaching or Scientific Research Activities.

2.4. Subacute toxicity study

Animals were randomly divided into three groups (5 animals/group). The first group was orally (p.o.) treated with the vehicle, while the second received CO at 50 mg/kg p.o. and the third received CO at 100 mg/kg p.o., over 12 days, according to the experimental protocol provided in the OVA-induced bronchial hyper reactivity (AHR) model. On 13th day, all mice were sacrificed by overdose of anesthesia (ketamine/xylazine, 50.0/3.33 mg/kg) and rupture of the diaphragm, and the blood samples were subjected to the biochemical studies. Briefly, whole blood was centrifuged at 1090*g* for 10 min (centrifuge excelsa II, 206BL) to obtain serum, and the following parameters were determined: glucose (GLU), creatinine (CR), urea (UR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase, (GGT), alkaline phosphatase (ALK) and albumin (ALB). Technique based on the previous study of Destryana et al. [8].

2.5. Asthma induction - allergen sensitization, challenge and treatment

Based on the previous study by Reber et al. [9], mice were sensitized intraperitoneally (i.p.) on days 0, 7, and 14 with 50 μg OVA adsorbed on 2 mg aluminum hydroxide in saline. Normal control (NC) animals received i.p. injections of saline only. Mice were challenged intranasally (i.n.) on days 16, 18, 20, 23, 25 and 27 with 10 μg OVA in saline or with saline alone for NC. Intranasal administrations (12.5 μl/nostril) were performed under anesthesia, i.p. (ketamine/xylazine, 50.0/3.33 mg/kg). Animals were sacrificed 24 h after the last challenge (day 28) to characterize CO effects (Fig. 1).

After the induction process, animals were divided into five groups (5 animals/group): (I) NC (saline-induced) treated with diluent (2% Tween 80), by gavage (p.o.); (II) Positive control (PC) (OVA-induced), treated with diluent (2% Tween 80), p.o.; (III) OVA-induced, treated with dexamethasone (DEX), 2 mg/kg, i.p.; (IV) OVA-induced, treated with CO 50 mg/kg, p.o. and (V) OVA-induced, treated with CO 100 mg/kg, p.o.

The treatment was started next to the first day of the last OVA challenge, being continuous until the 27th day. The first dose of the day was administered 2 h after each challenge and at the same time on days when the animals were not challenged. The second dose was always given around the same time, 12 h after the first dose of the day, based on the fact that the plasma concentration of *β*-caryophyllene (BCP), major compound, approaches 0 μg/ml after 12 h of administration [10].

DEX is considered a classic option to suppress asthma inflammation [11] administrated here via i.p. (parenteral) by the superiority in the absorption of this route in relation to the enteral one [12], although i.p. and gavage have liver as a metabolizing target, gavage for CO was chosen here as the treatment investigation route because is more comfortable.

2.6. Collection and management of BALF

The trachea was exposed by midline surgical section under anesthesia (ketamine/xylazine, 50.0/3.33 mg/kg) and lungs and airways

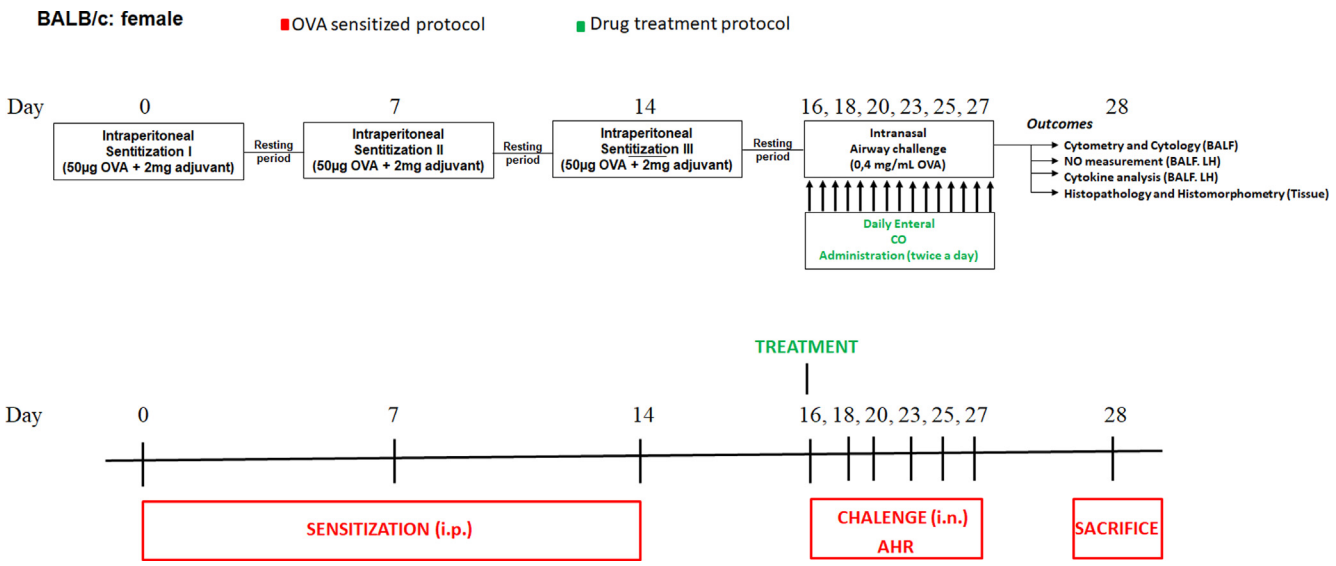

Fig. 1. A schematic overview of OVA induction.

were washed three times with 0.5 ml PBS, pH: 7.2 (final wash volume: 1.2–1.5 ml). Of each BALF, 20 µl were used for cytometry which total countage of cells was performed using a Neubauer chamber. The rest of the contents were centrifuged at 1500 rpm (190*g*) for 10 min at 4 °C (Microcentrifuge NT 800, New Technique, Piracicaba, SP, Brazil), and the supernatant collected for NO measurement and determination of cytokines production. The decanted material was used for the verification of the cellular profile (cytology) using Panoptic stain (Laborclin, Pinhais, PR). Both recognition of inflammatory cells and differential counts were performed on slides by light microscopy (Trinocular Biological Microscope, Physis, 0900082, Medellín, Colombia) (magnitude 1000x). Adapted from Nagato et al. [13].

2.7. LH preparation

After BALF collection, a thoracotomy was performed with ablation of the adjacent anatomical structures for removal of the lungs. The right lungs were sectioned longitudinally, fixed in 4% formalin, and destined for histopathological procedures. The left lungs were homogenized in 1 ml PBS, pH: 7.2, centrifuged at 7500 rpm (4725*g*) for 10 min (Microcentrifuge NT 800, New Technique, Piracicaba, SP, Brazil), and supernatants were collected for NO measurement and determination of cytokines production. Adapted from Nagato et al. [13].

2.8. Nitric oxide measurement

NO production was measured with Griess assay considering an absorbance of 492 nm and using a microplate reader (TP Reader NM; Thermoplate, Nanshan District, Shenzhen, China). The nitrite concentration was calculated using sodium nitrite as standard.

2.9. Determination of cytokines production and IgE

The concentrations of IL-4, IL-5, IL-17, IFN-γ, TNF-α and IgE were measured by ELISA, according to the manufacturer's protocol where Peprotech INC [14] for cytokines and IgE by Ray Biotech, Norcross, GA.

2.10. Histopathological and histomorphometric assessments

The sections of right lungs fixed in 4% formalin followed for histological processing using the tissue processor PT05 TS and the inclusion center CL 2014 (Lupetec, São Carlos, SP). Five-micron-thick right lung tissue sections were stained with hematoxylin and eosin (HE) and examined by Zeiss microscope (Hallbergmoos, Germany) in magnifications of 100x, 200x and 400x. All samples were scanned (Axion Cam ICC 5 computer system, Zeiss, Berlin, Germany), at a magnification of 100x for panoramic view of the sample and, at a magnification of 400x, images were taken from a section of bronchus, a section of terminal bronchiole, two sections of respiratory bronchioles, and two regions of alveolar parenchyma, in each sample. The images captured were submitted to morphometry using the Zen 2012 program (Blue Edition), approved by the National Institute of Health, Bethesda, USA.

In the bronchi and bronchioles, vasodilation and congestion, interstitial or intraluminal edema, bronchial epithelium desquamation, goblet cell hypertrophy, intraluminal mucus secretion, contraction and shortening of smooth muscles, and loss of ciliary function of the epithelial cells were analyzed. The inflammatory infiltrate was descriptively evaluated according to localization (diffuse, perivascular, peribronchial and predominant cell type) and semi-automatic morphometry, inflammatory cells were quantified in their compartments, i.e.: "external zone" of the bronchioles, between the smooth muscle and the region of the alveolar fixation, and "inner zone" of the bronchi, between the smooth muscle and the epithelium [15]. Data were quantified in one section of the bronchus, one section of terminal bronchiole, two sections of respiratory bronchioles of each sample and expressed in simple arithmetic mean per group.

Each sample was also submitted to automatic morphometry by the same imaging system to quantify the area of the alveolar parenchyma in five fields. Pulmonary parenchyma area was calculated according to Nagato et al. [13]. The results were expressed in μm^2 and converted in percentage, according to the scale selection and the increase of the digitalization, obtained the simple arithmetic mean by group.

2.11. Statistical analysis

Data were analyzed by bidirectional ANOVA. Significance was calculated by Tukey's test using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. GC and GC–MS analysis

The results of the chemical composition of the studied CO are presented in Table S1. The major compound identified were *β*-

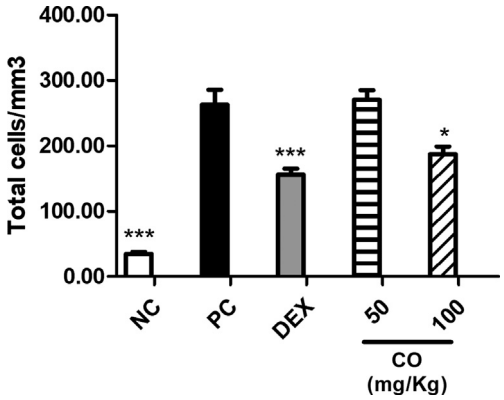

Fig. 2. Effects of oral administration of CO on the contingent of inflammatory cells of BALF . Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

caryophyllene (62.8%), α-humulene (9.1%), α-copaene (5.1%) and bergamotene (4.4%).

3.2. Subacute toxicity study

To assess the *in vivo* effect of CO in the described model, we first evaluated the possible toxic effects on glucose levels and renal and hepatic animal functions due to prolonged use of 12 days. As in shown in Table S2, the administration of CO, by gavage, at 50 and 100 mg/kg, in the expected period, did not show any significant changes in the evaluated parameters.

3.3. Cellular profiles of BALF after ovalbumin-induced airway inflammation and CO treatment

Mice induced to OVA sensitization and challenge (PC group) increased significantly the inflammatory cells when compared to the NC ($p < 0.001$) (Fig. 2). The cellular profile analysis showed that PC presented a significant increase of eosinophils ($p < 0.001$) (Fig. 3, A), neutrophils ($p < 0.001$) (Fig. 3, B), monocytes ($p < 0.01$) (Fig. 3, C) and lymphocytes ($p < 0.001$) (Fig. 3, D).

At 100 mg/Kg, CO inhibited the number of total inflammatory cells in mice ($p < 0.05$) (Fig. 2) and regarding the different cellular profiles, reduced number of eosinophils ($p < 0.001$) (Fig. 3, A) and neutrophils ($p < 0.001$) (Fig. 3, B). At the same time, this concentration caused an increased in the number of lymphocytes (Fig. 3, D) ($p < 0.01$). At 50 mg/kg, CO was able to cause a statistically significant reduction only on the number of neutrophils ($p < 0.01$) (Fig. 3, B).

3.4. Nitric oxide measurement after ovalbumin-induced airway inflammation and CO treatment

After OVA-induced pulmonary inflammation, there was an increased NO production in the PC group, for both BALF ($p < 0.001$) (Fig. 4, A) and LH ($p < 0.01$) (Fig. 4, B). The treatment with CO at both dosages had a significant effect in BALF ($p < 0.001$) (Fig. 4, A), while no significant differences was observed in LH ($p > 0.05$) (Fig. 4, B).

3.5. Cytokines and IgE production after ovalbumin-induced airway inflammation and CO treatment

As shown in Figs. 5 and 6, all animals that received OVA had a significant increase in all cytokines and IgE levels, both in BALF and LH, except in INF-γ levels. Treatment with CO (at both doses) was able to significantly reduce all increased cytokines and IgE antibody in BALF ($p < 0.001$) (Fig. 5). In LH, CO was able to cause a significant reduction on cytokines, including IFN-γ, and IgE only at 100 mg/Kg (Fig. 6). At 50 mg/Kg, CO was able to reduce TNF-α ($p < 0.001$, Fig. 6, D), and IgE ($p < 0.05$, Fig. 6, F). The action on IgE was also dose dependent ($p < 0.001$, between CO groups).

3.6. Histopathological and histomorphometric of lung tissue following ovalbumin-induced airway inflammation and CO treatment

The semi-automatic morphometric analyses, performed on the lung tissue, showed in PC a significant increase in the total inflammatory cells ($p < 0.001$) (Fig. 7). Equally, the histopathological examination revealed an increase of polymorph cells in the lung extension of PC (Fig. 8, B), and also hyperplasia and hypertrophy of the smooth muscle (Fig. 9, PC-widescreen and bronchioles), increased vascular congestion (Fig. 9, PC-widescreen), as well as thickening of the alveolar wall (Fig. 9, PC-parenchyma). There was thickening of the parenchyma area in PC (55% of parenchyma area/microscopic area) compared to 37% in the NC ($p < 0.01$) (Fig. 10).

Lungs of groups treated with CO presented an anti-inflammatory effect, since it was possible to note a recovery in lung tissues (Fig. 9) together with a reduced inflammatory infiltrate (Fig. 7). CO treated groups also presented a reduced area in the parenchyma ($p < 0.01$, 50 mg/Kg and $p < 0.05$, 100 mg/Kg) in comparison to the PC (Fig. 10). In contrast, DEX was not able to reduce significantly the parenchyma area, although it also did not present significant differences in comparison with CO (at both doses) ($p > 0.05$) (Fig. 10).

4. Discussion

First, the identification of compounds in CO by CG-MS and CG-FID was performed by comparison of their mass spectra with those of the spectral libraries, as well as by comparison of their retention indexes with those in the literature. All compounds presented in CO have also been previously identified in other CO samples [6,16], and BCP was identified as the major compound in our CO.

However, as CO may have different chemical composition [3], before using CO for *in vivo* treatment, its toxicological profile on renal, hepatic and glucose rate should be evaluated. Aiming to verify any possible biochemical alterations in mice after administration of CO, our preliminary toxicological studies showed that CO was not able to cause any biochemical changes in the evaluated parameters under the pre-established conditions, which suggest a safety of CO in this model by these criteria. It is important to point out that no similar toxicological studies with CO were previously published, involving the same parameters analyzed.

Next, BALB/c mice were sensitized with OVA in order to induce the allergic asthma model. It is reported that BALB/c mice is the most common lineage used in asthma study [17], mainly for their ability to produce high levels of IgE against OVA with strong TH2 response in the lungs [18], and in particular female animals, which tend to have a more pronounced inflammatory response than male [19].

According to Radermecker et al. [20], OVA-induced asthma model simulates an allergic state when an increase in the eosinophilic profile is characteristic of the allergic asthma (AA), in which a high neutrophil count may also be observed.

Considering the induction of AA, it was observed that our adopted AA model was successfully established in the animals, since an increase of TH2 and TH17 responses, perceived by the increase of IL-4, IL-5, immunoglobulins type E (IgE) and IL-17 levels in both BALF and LH was observed, mainly in animals of PC group, which are in agreement with profiles described for this model in the literature [2,15].

In addition, after the induction of AA model, a significant increase in NO and TNF-α production was observed in both BALF and LH. NO production was also noted by Kandhare et al. [21] using Wistar rats. As described, reactive nitrogen species (RNS), such as NO, can damage lipids, proteins, and nucleic acids, which contributes to the asthma

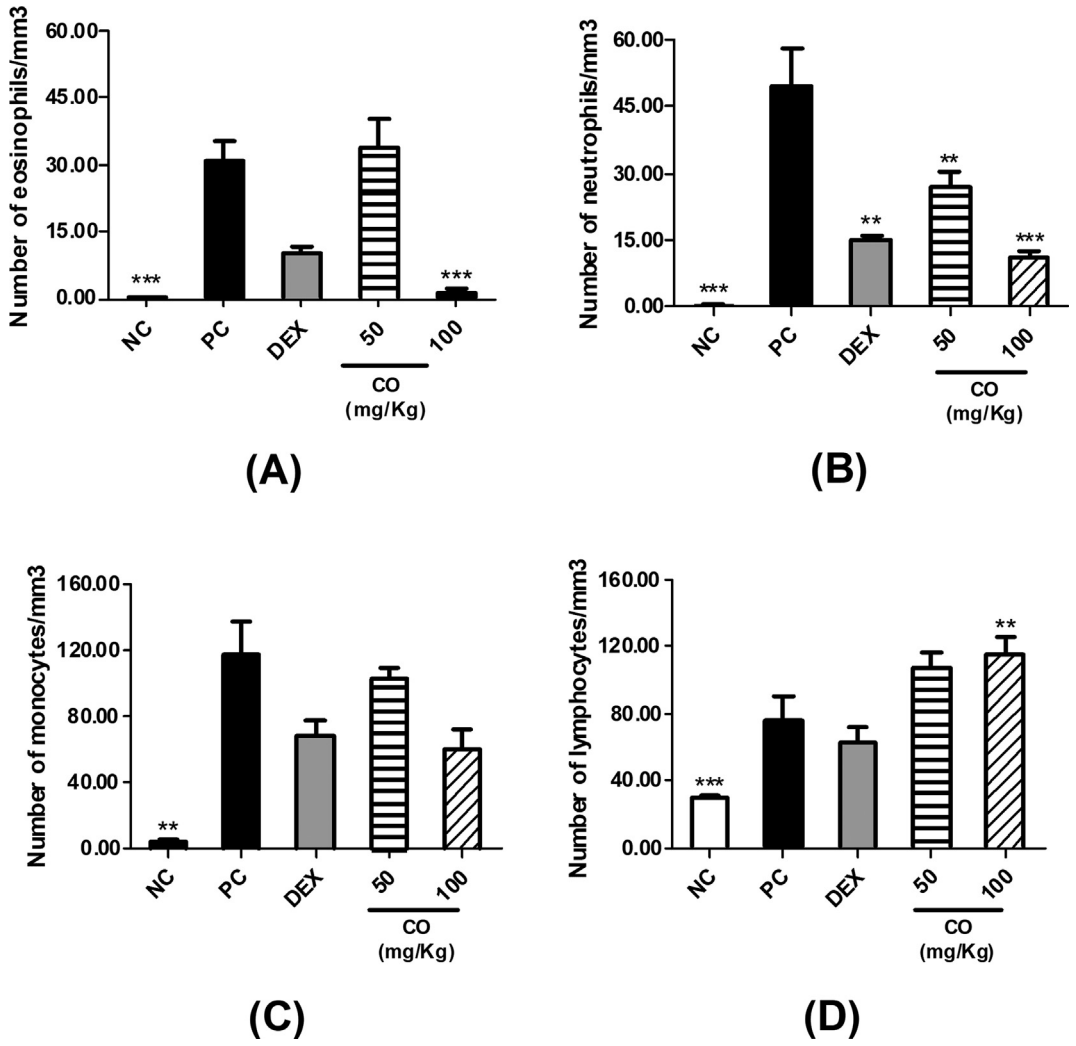

Fig. 3. Effects of oral administration of CO and under the different cell profiles: eosinophils (A), neutrophils (B), monocytes (C) and lymphocytes (D). Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

severity [22].

As previously reported by Manni et al. [23], it is noteworthy that the TH2 response, plus the increase of NO and TNF-α production, are described to severe asthma by synergistic effect with IL-17A, which influence the influx of neutrophils to the lungs, remodeling of the airways. Then, as expected, mice from PC group were marked by high cellular levels of eosinophils and neutrophils, especially in the BALF. Also, observed an increase of 30% in the influx of neutrophils in BALF. As reported, neutrophils are one of the first cells recruited into the lungs during specific asthma-related events endowed, with many

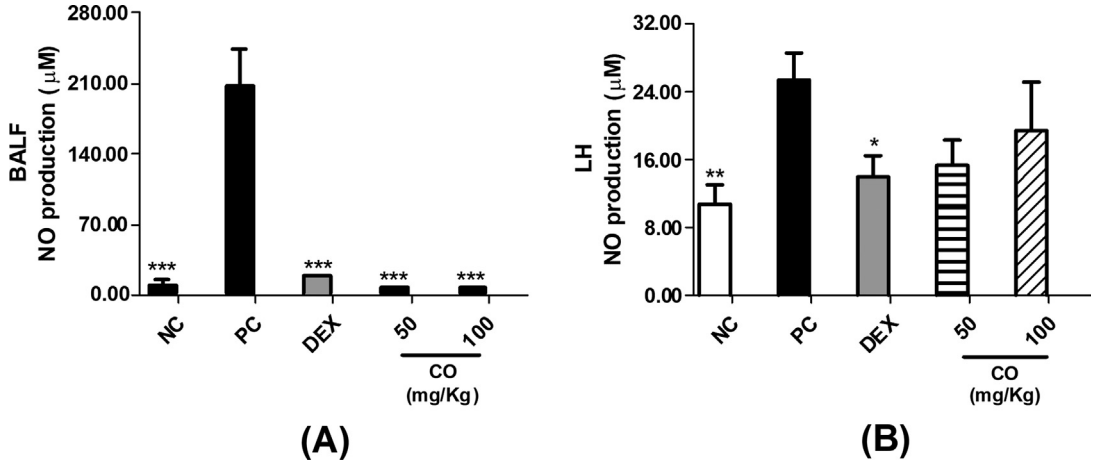

Fig. 4. Effects of oral administration of CO on NO production on BALF (A) and LH (B) samples. Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

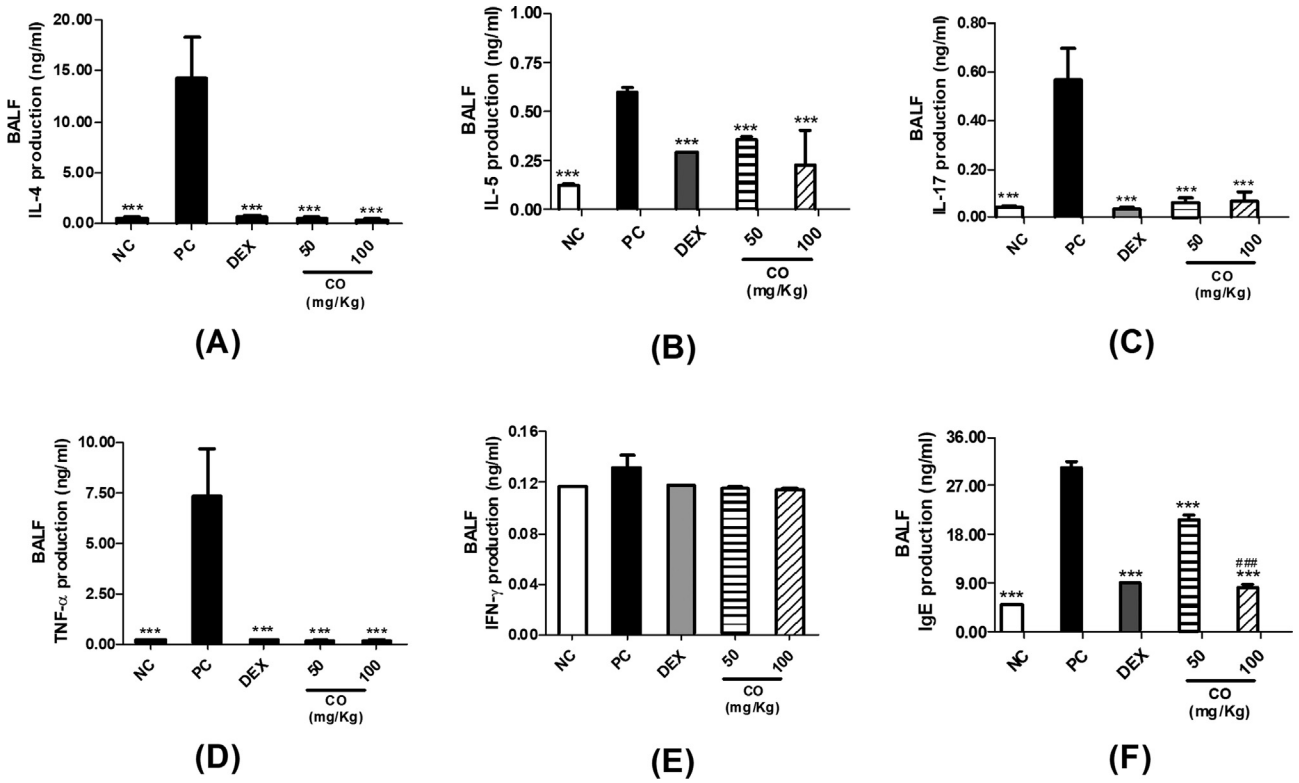

Fig. 5. Effects of oral administration of CO on the production of IL-4 (A), IL-5 (B), IL-17 (C), TNF-α (D), IFN-γ (E) and IgE (F) on BALF. Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group. $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. the CO 50 mg/Kg group.

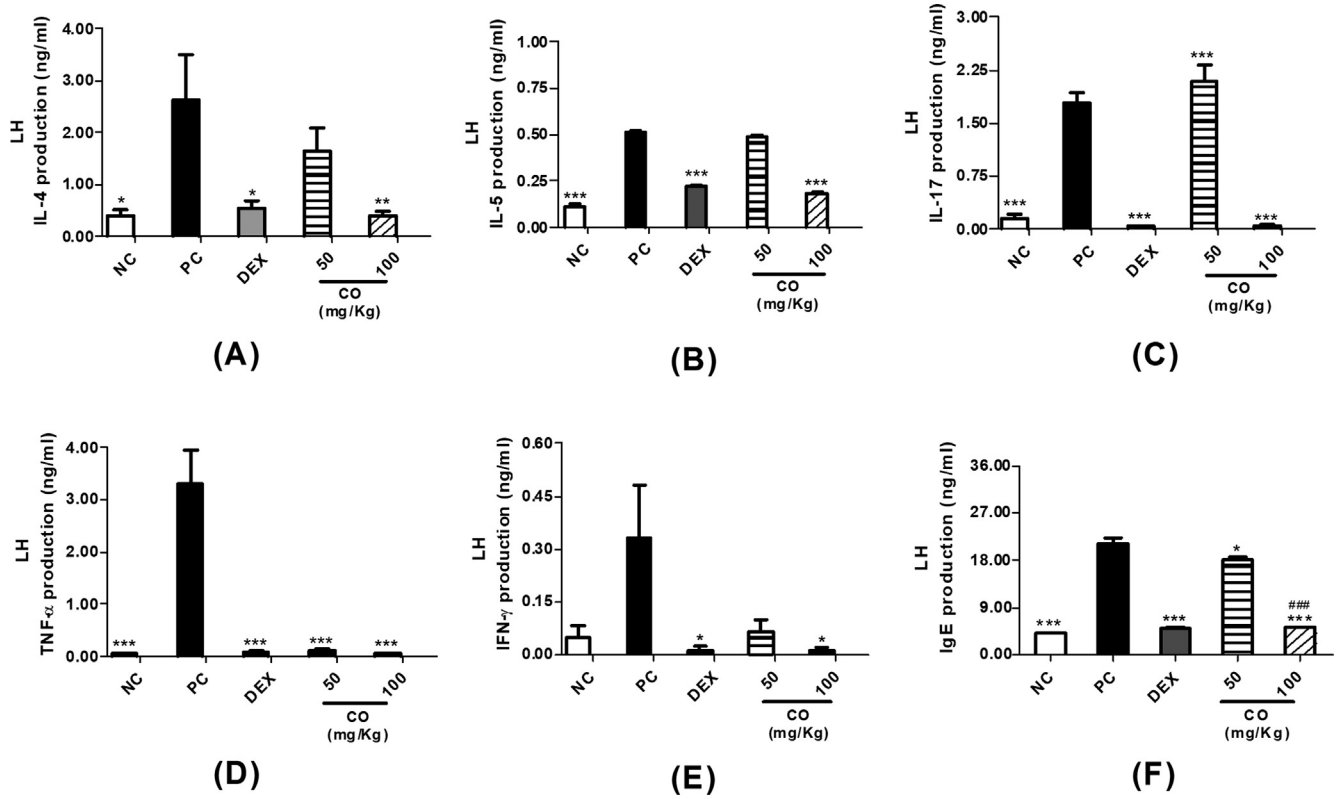

Fig. 6. Effects of oral administration of CO on the production of IL-4 (A), IL-5 (B), IL-17 (C), TNF-α (D), IFN-γ (E) and IgE (F) on LH. Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group. $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. the CO 50 mg/Kg group.

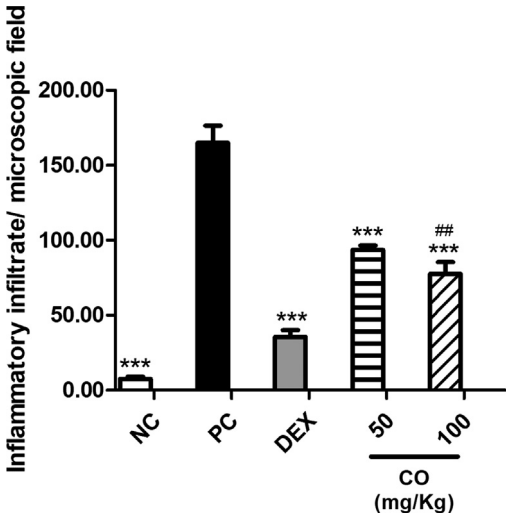

Fig. 7. Quantitative semi-automatic morphometry of peritubular inflammatory infiltrates (bronchi and bronchioles) in right lung sections of BALB/c mice. Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group. # $p < 0.05$; ## $p < 0.01$, ### $p < 0.001$ vs. the CO 50 mg/Kg group.

immunoregulatory properties [20].

According to the methodology employed to induce AA, different cellular profile may be found in BALF of animals. A mixed profile of 20% eosinophils and 50% neutrophils from BALF of BALB/c mice with asthma induced with OVA and low dose of LPS have been previously reported by Yu; Chen [2]. In our adopted model, based on Kumar; Herbert; Foster [24] previous work, we induced an acute AA by increasing the exposure with sensitizing agents (OVA), which lead to a more robust inflammatory response.

Also, in our adopted model, it was also observed a significant increase of monocytes and lymphocytes in BALF of mice. Regarding monocytes, they are present in larger numbers in this AA model probably due to the fact that these cells have a large capacity to migrate into the lung tissue [25]. In addition, in animal models of AA, it has been established that the local microenvironment plays an essential role in monocyte regulation, leading not only to migration, but also to differentiation of macrophages with inflammatory properties, contributing to the generation of reactive oxygen species (ROS) and RNS [25,26] and promoting the TH2-mediated immune response in asthma.

Next, after induction of AA model in BALB/c mice the *in vivo* immunomodulatory effects of CO in allergic asthma was investigated. It was verified the *in vivo* anti-inflammatory effects of CO on the number and profile of cells from BALF of mice. Our results showed that CO treatments (especially at 100 mg/kg, gavage) were able to reduce the total inflammatory cells in lung, mainly in the number of eosinophils and neutrophils, which are the markers of, respectively, the allergic inflammation and the severity of inflammatory process [2,15,27,28].

However, CO (at 100 mg/kg) promotes an increase of lymphocytes in comparison with PC group, which was not expected, since literature has been previously reported that CO is capable of causing a leukocyte suppression in Swiss mice with pleurisy [6]. Nevertheless, differences in the species and in the disease model employed, as well as in the experimental protocol used in the treatment may have modified the cellular effects of CO in reducing number of lymphocytes in asthma.

In addition, CO also showed noticeable *in vivo* immunomodulatory effects on production of NO, TNF-α, IFN-γ, IL-17 IL-4, IL-5, and IgE antibody in LH and BALF of AA induced-mice. Previous studies have shown that CO can improve diseases mediated by inflammatory processes, such as gastrointestinal and pulmonary diseases [3]. In addition, it has also been reported that CO is capable of inhibiting NF-κB

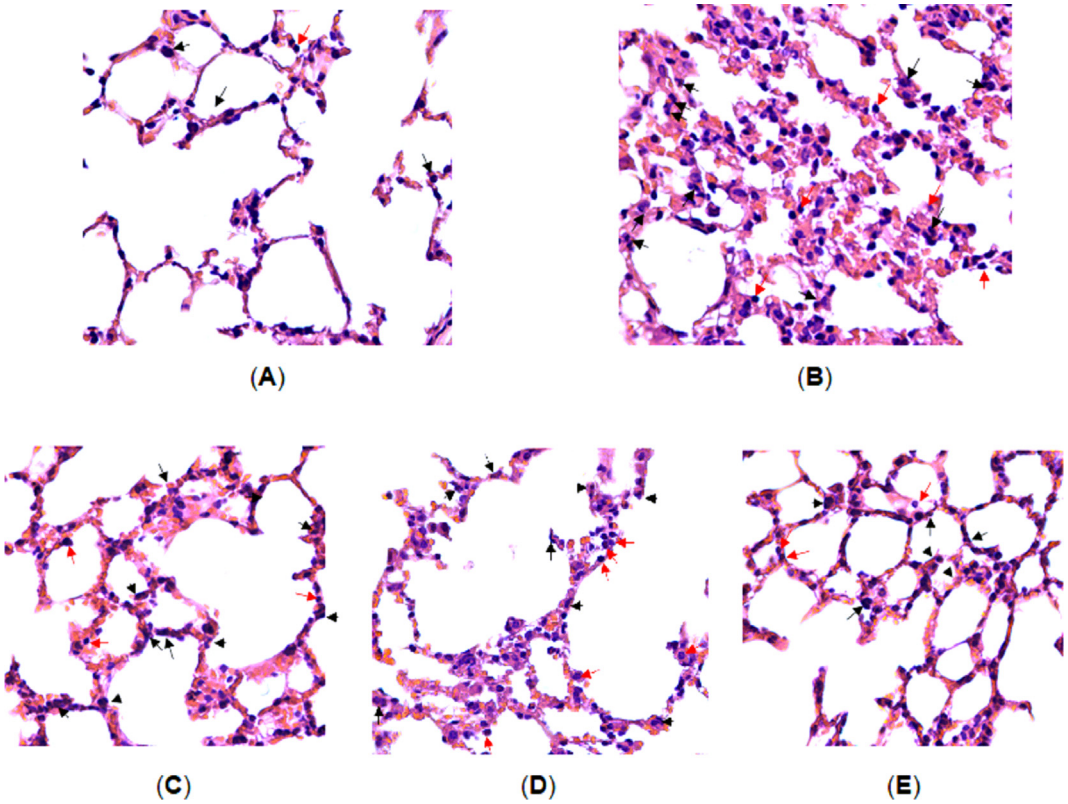

Fig. 8. Samples of BALB/c lung: visualization of the parenchyma and accumulation of inflammatory cells with and without the treatment. Healthy lung (A); Asthmatic lung (B); DEX treated parenchyma (C); CO 50 mg/Kg treated parenchyma (D); CO 100 mg/Kg treated parenchyma (E). HE coloration. 400× magnification. The red arrows indicate the monomorphonuclear cells and the black arrows the polymorphonuclear cells.

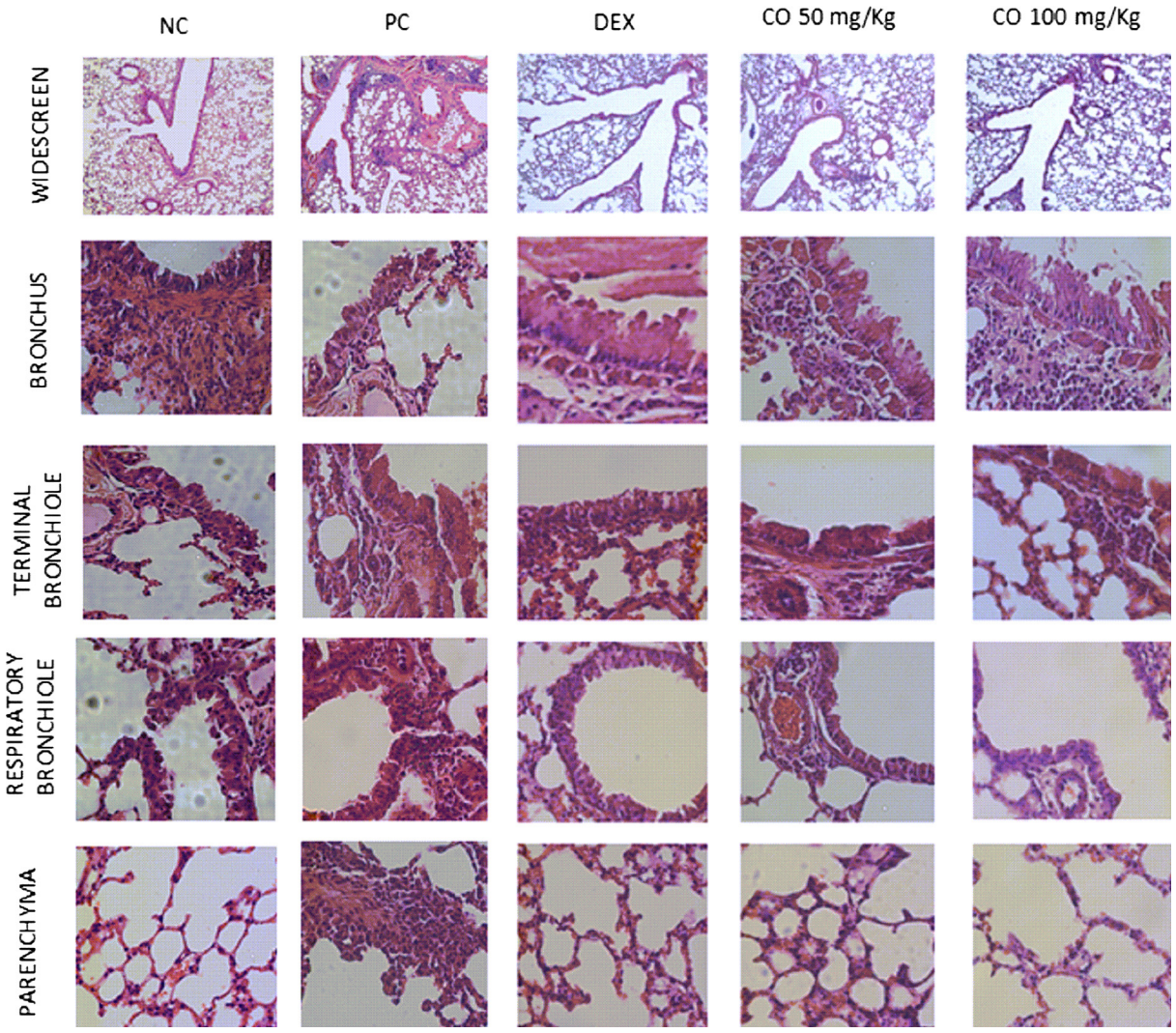

Fig. 9. Effects of oral administration of CO throughout the asthmatic lung extension of BALB/c mice. HE coloration. Widescreen: 100× magnification; Bronchus, terminal bronchiole , respiratory bronchiole and parenchyma: 400× magnification.

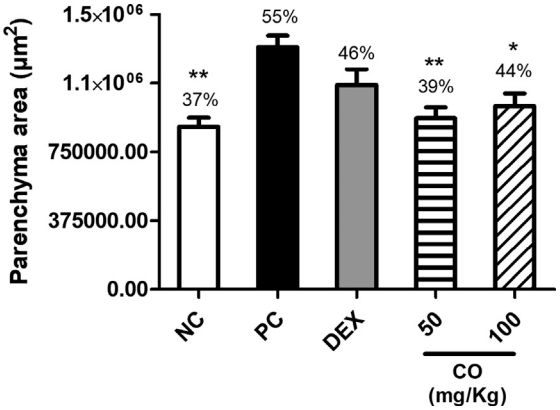

Fig. 10. Automatic morphometry in right pulmonary sections of BALB/c mice. Evaluation of the parenchyma area. Data are presented in mean values ± SD. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ vs. the PC group.

translocation and, consequently, inhibit the secretion of some pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α [5,29], and IL-17, as well as the production of NO [4].

ROS play an important role in the pathogenesis of several airway diseases, such as cystic fibrosis, chronic obstructive pulmonary disorder, and asthma [2,30]. For the first time, we provided evidence for the *in vivo* NO suppression of CO in the AA model. In our study, the treatments with CO showed an efficacy effect in suppressing NO production, mainly in BALF. This finding is of the great importance, since an increased amount of NO leads to oxidative damage to cells [22]. Regarding the effects on TH cells response, CO reduced IL-4, IL-5, IL-17, TNF-α, and IgE antibody in BALF, confirming its immunomodulatory effects on TH2 and TH17 profiles, being the first report that CO is able to decrease TH2 response in asthma.

In LH samples, CO was effective in reducing IL-4, IL-5, IL-17, TNF-α, and IgE and also able to decrease the concentration of IFN-γ, evidencing the contribution of this oil in inhibiting TH1 mediators on a model that has the tendency to increase IFN-γ. Apparently, the predominant effect of CO on TNF-α is favored by its ability in inhibiting the translocation of NF-κB [4], which is a protein complex increased in lung tissue in the OVA induction model that controls transcription of DNA, cytokine production and cell survival [31,32].

Considering the exposure of allergen in the airways, IL-4 is produced by T cells and, the subsequent activation of B cells occurs, leading to the increased of IgE production, which is a central pathway of allergen-specific immune response in asthma [30]. Also, IL-5 is directly and significantly involved in eosinophil proliferation, differentiation, recruitment and prolonged survival. Eosinophil is believed to be an important effector cell that plays a central role in chronic

inflammation of the asthmatic airways [2,30].

We also believed that this study may be the first one that describes the *in vivo* action of CO in IL-5, IL-17, IFN-γ and TNF-α using gavage in asthma inflammation models. Until now, evidences in the effect of CO in IL-17, TNF-α and IFN-γ [4] was only reported for the *in vitro* experimental autoimmune encephalomyelitis (EAE) model, for multiple sclerosis, and in TNF-α on human THP-1 monocytes model [5].

Additionally, histopathological investigations showed that CO treatment markedly attenuated the inflammatory cell infiltration into lungs of AA induced-mice. In our adopted AA model, the main asthma-related manifestations were observed by the histopathological examination, such as increasing of polymorph cells, hyperplasia, hypertrophy of the smooth muscle, increased vascular congestion, and thickening of the alveolar wall. All these clinical manifestations were previously described in other AA experiments [33,34]. Our results from the semi-automatic morphometry showed that the CO administration was able to reduce both the inflammatory infiltrate and the incidence of histopathological changes in the lung tissue, similarly to the DEX effects.

Complementary analysis of the peripheral region of the lung tissue, including the parenchyma area, was considered necessary in this study, since these distal airways have a larger volume and surface airway than the great ones. Also, the presence of edema and inflammation lead to the thickening of this tissue, affecting the transmission of elastic recoil forces in order to isolate the airway from the effects of pulmonary elastic recoil and deep inspiration [35]. In agreement with this scenario previous described, the present study verified the thickening of the parenchyma area in the PC animals. In contrast, these scenarios were not observed in lungs of animals treated with CO, where the presented pulmonary architecture were similar to the healthy animals. Also, in the CO treated groups a reduction on parenchyma area was observed, indicating that CO was also able to reduce the tissue inflammation. On the other hand, DEX was not significantly able to reduce the parenchyma area. Interestingly, DEX group did not present any significant differences when compared with the CO treated groups.

The beneficial effect of CO, as described here, can be attributed to its ability to act on NF-κB. However, it must be taken to account, that BCP was the major identified compound in CO, which is in agreement with previous chromatographic results that depicted BCP as the main compound found in CO samples [6,16]. In fact, literature has attributed the highest anti-inflammatory potential of CO to the presence of BCP [36].

It has recently been reported that BCP decreased the inflammatory levels of some mediators (such as IL-1β, TNF-α, PGE_2, *iNOS*, NO, and ROS) and increase the levels of some anti-inflammatory mediators (such as IL-10), also polarizing microglia to anti-inflammatory phenotype [37]. In EAE animal model, induced in C57BL/6 mice, BCP was able to reduce NO, IFN-γ, IL-17 and TNF-α productions without toxicity [38,39].

Also, it has been demonstrated that BCP downregulated the activation of NF-κB[40] and is also capable of increasing the anti-inflammatory profile cytokine, IL-10, present in the Treg (T regulatory cells) response [40,41]. Askari; Shafiee-Nick [37] reported that BCP is effective in treating EAE, being able to reduce the clinical and pathological scores. In addition, BCP action occurred through the modulating of the innate and adaptive immune systems of the inflammatory state (TH1/TH17/M1), towards the anti-inflammatory and healing states (Treg).

Askari; Shafiee-Nick [37] demonstrated the ability of BCP in inhibiting the expression of intracellular messengers T-bet and Ror- γT, and positively regulating Foxp3 expression in a regulatory (suppressor) T response. Also, an activation of Treg response ultimately led to the high production of markedly anti-inflammatory TGF-β1 and IL-10 cytokines.

Interestingly, in our study, CO treatment was able to reduce the neutrophils and eosinophils infiltrations. We also observed that CO increased the lymphocyte population. Based on these results and comparing them with that previously described we hypothesized that BCP may act in the same way. Firstly, the inhibition of TH2 responses improves signals related to the IL-4, IL-5, and IgE production, which reduce the production of IL-17 (TH17 related to severe asthma) and TNF-α that are linked to neutrophil migration. In addition, as the lymphocyte population increases in the animals treated with CO, we hypothesized that these results may link to a Treg response, since BCP has the ability to, positively, modulate Foxp3 expression [39]. This modulation may direct IL-10 and TGF- β1 production, which are suppressive, and may acts of the anti-inflammatory manner.

Therefore, in addition to the reported direct effect of CO on NF-κB and, considering all the reported evidences, BCP may be one of the active compounds related to the immunomodulatory activity of CO.

We can conclude that CO was able to reduce the total number of cells and the eosinophils and neutrophils of BALF. CO was also able to reduce the production of cytokines, markedly IL-4, IL-5 and IL-17 cytokines and IgE antibody, linked to TH2 and TH17 response patterns and associated with eosinophil and neutrophil migrations. Moreover, we shown the strong effect of CO on NO and TNF-α, which are mediators directly involved in the AA model. In addition, the histomorphometric analysis showed that CO treatment reduced the parenchyma area and diminished the tissue inflammation of AA induced-mice.

Finally, even with the clear results presented, it should be considered that CO is a mixture of several compounds and may have synergistic or additive effects between BCP and other chemical constituents presented in this oil, and that the immunomodulatory activity of CO may be related to the combination of these compounds.

In summary, we have demonstrated, for the first time, the anti-inflammatory and antioxidant effects of CO on the acute pulmonary inflammation model employed in the investigation of AA, mainly at 100 mg/kg, through the reduction of inflammatory cells and other mediators of allergic reactions in the airways with similar effects of dexamethasone.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary material

Chemical composition of the CO (GC and GC-MS) and the effects of oral administration of CO, over 12 days, on glucose production, kidney and liver function are available as Supporting Information.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.106177.

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