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Propolis reversed cigarette smoke-induced emphysema through macrophage alternative activation independent of Nrf2

Running Title: Propolis reversed emphysema by macrophage alternative activation

Marina Valente Barroso[a], Isabella Cattani-Cavalieri[b], Lyciade Brito-Gitirana[b], Alain Fautrel[c], Vincent Lagente[d], Martina Schmidt[eg], Luís Cristóvão Porto[f], Bruna Romana-Souza[f], Samuel Santos Valença[b], Manuella Lanzetti[b]

[a] Programa de Pós-graduação em Imunologia e Inflamação, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

[b] Instituto de Ciências Biomédicas, UFRJ, Rio de Janeiro, Brazil

[c] Laboratoire d'Anatomie Pathologique, Faculté de Médecine, Université de Rennes, Rennes, France

[d] Faculté des Sciences pharmaceutiques et biologiques (UMR U991 Inserm), Université de Rennes, Rennes, France

[e] University of Groningen, Department of Molecular Pharmacology, Groningen, The Netherlands

[f] Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

[g] GRIAC Research Institute, University of Groningen, University Medical Center Groningen, The Netherlands

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Keywords: propolis, emphysema, lung repair, M2 macrophage, Nrf2 pathway

Abbreviations: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; ROS, reactive oxygen species; Nrf2, NF-E2-related factor 2; Keap1, Kelch-like ECH-associated protein 1; ARE, antioxidant response element; HO-1, heme-oxygenase-1; NQO1 (NAD(P)H:quinone oxidoreductase 1; GCL, γ-glutamate-cysteine ligase; IGF, insulin-like growth factor; BAL, bronchoalveolar lavage; SIRT1, sirtuin 1; TGFβ, transforming growth factor β; MMP, metalloprotease.

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an incurable and progressive disease. Emphysema is the principal manifestation of COPD, and the main cause of this condition is cigarette smoke (CS). Natural products have shown antioxidant and anti-inflammatory properties that can prevent acute lung inflammation and emphysema, but there are few reports in the literature regarding therapeutic approaches to emphysema. We hypothesized that supplementation with natural extracts would repair lung damage in emphysema caused by CS exposure. Mice were exposed to 60 days of CS and then treated or not with three different natural extracts (mate tea, grape and propolis) orally for additional 60 days. Histological analysis revealed significant improvements in lung histoarchiteture, with recovery of alveolar spaces in all groups treated with natural extracts. Propolis was also able to recovery alveolar septa and elastic fibers. Propolis also increased MMP-2 and decreased MMP-12 expression, favoring the process of tissue repair. Additionally, propolis recruited leukocytes, including macrophages, without ROS release. These findings led us to investigate

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the profile of these macrophages, and we showed that propolis could promote macrophage alternative activation, thus increasing the number of arginase-positive cells and IL-10 levels and favoring an anti-inflammatory microenvironment. We further investigated the participation of Nrf2 in lung repair, but no Nrf2 translocation to the nucleus was observed in lung cells. Proteins and enzymes related to Nrf2 were not altered, other than NQO1, which seemed to be activated by propolis in a Nrf2-independent manner. Finally, propolis downregulated IGF1 expression. In conclusion, propolis promoted lung repair in a mouse emphysema model via macrophage polarization from M1 to M2 in parallel to the downregulation of IGF1 expression in a Nrf2-independent manner.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is an incurable and progressive, but preventable respiratory disease that is positioned to become the third leading cause of death worldwide by 2020 [1, 2]. COPD is characterized by chronic inflammation, remodeling of the small airways and destruction of lung parenchyma, leading to emphysema [3]. Cigarette smoke (CS) is the major risk factor for emphysema, and it currently affects approximately 10% of the population over 45 years of age, rising to 50% in heavy smokers [2]. CS contains approximately 5,000 different chemical compounds, as well as a high inhalative environmental burden of different oxidants such as reactive oxygen species (ROS) [4, 5]. CS is also an exogenous source of oxidants; it can increase the number of inflammatory cells in alveoli, releasing more ROS [6]. Inflammatory cells together with ROS can also inactivate important antiproteases and release large quantities of proteases, leading to tissue destruction and intensifying the development of lung emphysema [7]. Both proteolytic and redox imbalances are involved in the pathogenesis of pulmonary emphysema.

One of the most important transcription factors orchestrating the principal cellular response to pro-oxidant insult is Nrf2 (NF-E2-related factor 2). Nrf2 is a member of the basic leucine zipper type transcription factors; it binds to Keap1 (Kelch-like ECH-associated protein 1), a cytoplasmic protein, which retains Nrf2 in the cytoplasm under physiological conditions [8, 9]. However, in response to a stimulus such as oxidative stress, covalent modification of cysteinyl thiols in Keap1 results in Nrf2 release and translocation to the nucleus [10, 11]. When Nrf2 translocates to nucleus, it binds to antioxidant response element (ARE), inducing phase II enzyme and antioxidant gene production [12]. Among the phase II antioxidant enzymes produced by the Nrf2 pathway are HO-1 (heme-oxygenase 1), which

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catalyzes the rate-limiting step in heme catabolism [13], NQO1 (NAD(P)H:quinone oxidoreductase 1), which is involved in the biotransformation of xenobiotics and drugs [14], and GCL (γ-glutamate-cysteine ligase) with catalytic and modifier subunits, which plays a role in glutathione metabolism [15].

Among the inflammatory cells present in the lung, alveolar macrophages reach 90-95% of the cellular content under normal conditions [16]. Macrophages contribute to tissue development through apoptosis, phagocytic clearance of cellular debris associated with tissue remodeling, and as potent effector cells that produce a large variety of trophic factors, for example, insulin-like growth factor (IGF1)-1 [17], stimulating growth, regulating cellular differentiation and promoting angiogenesis [18, 19]. Macrophages have phenotypic plasticity, undergoing distinct activation programs depending on the microenvironment that changes the functions of macrophages according to the acquired profile. Macrophages can act by protecting, repairing or sometimes damaging tissue [20, 21]. Macrophage "M1 polarization" (classical activation) is induced by signals that are generated during the Th1-mediated immune response, such as interferon (IFN)-γ, and by exposure to components of pathogens such as bacteria. However, macrophage "M2 polarization" (alternative activation) is induced upon exposure to the Th2 cytokines IL-4 and IL-13 or immunoregulatory signals such as IL-10, which induce the expression of anti-inflammatory cytokines and molecules associated with tissue remodeling [20-22].

Propolis is a bee-metabolized resinous substance (also known as bee glue) from plant sap and gums. Propolis contains, among others, quercetin, caffeic acid, caffeic phenethyl ester (CAPE) and rutin [23]. It has been shown that flavonoids present in propolis are capable of scavenge free radicals, ROS and inactivating electrophiles [24, 25]. In recent years, studies have shown that propolis is a potential therapeutic product due to its antioxidant [26-28], anti-inflammatory [29-31], anticarcinogenic [27], antibacterial [32] and hepatoprotective [33, 34] properties. Propolis has been studied in patients, and clinically it has demonstrated antioxidant and anti-inflammatory actions that improve ventilatory functions and inflammatory mediators in asthmatic patients [35] and enhance wound closure in diabetic patients [36].

There is currently no treatment for emphysema. First, we decided to investigate whether some natural extracts such as mate tea, grape and propolis extract would be capable of reversing the lung damage caused by cigarette smoke exposure. Our previous studies

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showed that mate tea [37, 38] could prevent the development of acute lung inflammation and emphysema. Extracts of grape [39] and propolis [26] can prevent acute lung inflammation. However, in the course of this work, based on our results, we decided to use only propolis extract. Considering all the positive effects demonstrated by propolis, we hypothesized that post-treatment with propolis, meaning treatment after emphysema is already established, could repair lung damage. Treatment after established emphysema is important because it mimics the patients with established disease who seek treatment. Furthermore, we aimed to investigate whether the Nrf2 pathway participates in lung repair mediated by propolis and if the treatment could alter the leukocyte profile in the lung environment.

2. Materials and Methods

2.1. Animals

Eight-week-old male C57BL/6 mice (18-22 g) were purchased from the Multidisciplinary Center for Biological Investigation on Laboratory Animal Science (CEMIB - UNICAMP, Campinas, Brazil). The mice were fed Purina chow (Nuvilab®, Curitiba, Brazil) and were housed in a room with a controlled environment maintained at 18-22°C, 50-70% relative humidity, and a 12-h light/dark cycle. The mice were allowed to acclimatize for two weeks prior to the experimental procedures. All procedures were performed in accordance with conventional guidelines for experimentation with animals, and the local committee approved the experimental protocol. Commercial samples of roasted Yerba mate were purchase from "Leão Jr SA" (Paraná, Brazil), grape extract was purchase from "Comercial de Alimentos Grazziotini LTDA." (Rio Grande do Sul, Brazil) and propolis extract was purchase from "Orient Mix Fitoterápicos do Brasil LTDA." (Rio de Janeiro, Brazil). All extracts were diluted in mineral water to the following concentrations: 500 mg/kg/day for mate tea and 200 mg/kg/day for grape and propolis [26, 38]. Beverage consumption was measured daily.

2.2. Cigarette smoke exposure

Mice were divided into 5 groups: one group exposed to ambient air for 120 days (CTR group, n=10) and 4 groups exposed to CS for the first 60 days and then to ambient air for the next 60 days (CS, CS+mate, CS+grape and CS+propolis, n=10 each group). All groups were treated orally with mineral water during the first 60 days. The CTR and CS

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groups were then treated with mineral water, while the other groups were treated with their respective natural extract for the subsequent 60 days. This model characterizes a post-treatment since at 60 days, the animals already had emphysema and the treatment was initiated after disease was established. Mice from the CS groups were exposed to 12 commercial filtered cigarettes per day (4 cigarettes in the morning, 4 cigarettes at noon and 4 cigarettes in the afternoon) using a smoking chamber as described previously [40-43]. Briefly, the animals were placed in an inhalation chamber (40 cm long, 30 cm wide, 25 cm high) inside an exhaustion chapel. A cigarette was coupled to a plastic 60-ml syringe so that puffs could be drawn in and subsequently expelled into the exposure camber. One liter of smoke from 1 cigarette was aspirated with this syringe, and then each puff was immediately injected into the chamber. Animals were maintained in this smoke-air condition (± 3%) for 6 minutes. The cover was then removed from the inhalation chamber, and the smoke was evacuated within 1 minute by turning on the exhaust fan of the chapel.

2.3. Bronchoalveolar lavage and lung homogenates

Twenty-for hours after day 120, the animals were euthanized by cervical displacement. Bronchoalveolar lavage (BAL) was performed in the right lung of all animals in each group. Briefly, a cannula was inserted into the trachea, the airspaces were washed three times with buffered saline solution (4^{o}C - 500 µl), and the flow-through (final volume 1.2-1.5 ml) was maintained on ice. The BAL fluid was then centrifuged, and the supernatant was collected and stored at -20°C. The total number of cells in the BAL fluid was determined using a Neubauer chamber. Differential cell counts were performed in cytospin preparations stained with LB Laborclin (cod. 620529). At least 100 cells per BAL fluid sample were counted using standard morphologic criteria [42]. Reactive oxygen species (ROS) detection was performed in BAL cells following the adapted nitroblue tetrazolium chloride (NBT) protocol [44]. Briefly, ROS produced by cells reacted with NBT to generate blue formazan. The optical density was read in a microplate reader at 630 nm. The BAL was also assessed by enzyme-linked immunosorbent assay (ELISA). The right lungs of all animals were removed and homogenized in 1.0 ml of lysis buffer and centrifuged at 210 g for 10 min, and the supernatants were collected for western blot analysis. The lysis buffer consisted of 1 tablet of protease inhibitor (SigmaFAST™), 0.1% triton x-100 in 100 ml of phosphate buffer. The total protein in the samples (tissues and BAL) was determined by the Bradford method [45].

2.4. Tissue processing and morphometry

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The left lungs of all animals were inflated with 10% (pH 7.2) phosphate-buffered formalin at 25 cm H_2O pressure for 2 min and then ligated, removed, and weighed. Inflated lungs were fixed for 48 h before embedding them in paraffin (Sigma-Aldrich, St Louis, USA). Sagittal, 4-µm serial sections of the lungs were stained with hematoxylin and eosin (H&E) and with orcein for histological analyses. Air-space enlargement was quantified based on the mean linear intercept length of the distal air spaces (L_m) in 100 randomly chosen fields of tissue sections per group [46]. The volume densities of the alveolar septa ($Vv_{\text{alveolar septa}}$) and elastic fibers ($Vv_{\text{elastic fibers}}$) were estimated by counting the number of structures that were intersected by the test system (partial points, $P_{p)}$ in 100 randomly chosen fields per group [47]. Hematoxylin and eosin images were captured at 40x magnification. Orcein images were captured at 100x magnification.

2.5. Immunohistochemistry

For immunohistochemistry analysis, lung sections were incubated with primary antibody against Arginase 1 and Nrf2. After the incubation and subsequent washing steps, the attached primary antibody was then linked to dextran polymer by following the manufacturer's protocol (Envision kit, Dako, Carpinteria, CA, USA), and the final reaction was performed by immersing the sections in a solution of 3,3'-diaminobenzidine (DAB) in the case of the Arginase 1 marker. The sections were then counterstained with hematoxylin, and images were captured at 100x magnification. The percentage of arginase-positive macrophages was obtained by counting macrophages stained for arginase (in yellow) in 100 macrophages per group. To detect Nrf2, after incubation with the primary antibody, signal amplification was performed using the Ventana DABMap kit (Ventana Medical Systems, Tucson, AZ, USA) before incubation with secondary antibody. Immunostaining was performed using the automated stainer Discovery XT (Ventana Medical Systems, Tucson, AZ, USA). Thereafter, the stained sections were scanned with NanoZoomer 2.0 RS (Hamamatsu, Tokyo, Japan), and images were captured at 40x magnification. The percentage of Nrf2 localization in the cells was obtained by identifying the marking site in 100 cells per group.

2.6. Western blotting

Proteins (30 µg) were resolved by SDS-PAGE and electrotransferred to PVDF membranes. Primary antibodies against MMP-2 (rabbit, sc-10736), MMP-12 (rabbit, sc-

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30072), Keap1 (rabbit, sc-33569), HO-1 (goat, sc-1796), NQO1 (rabbit, sc-25591), SIRT1 (rabbit, sc-15404) and TGFβ (rabbit, sc-7892) were purchased from Santa Cruz, USA. Primary antibodies against GCLC (rabbit, AV54576), IGF1 (goat, SAB2501424) and β-actin (mouse, A5441) were purchased from Sigma Aldrich, St Louis, USA. Protein detection was performed with the appropriate horseradish peroxidase-conjugated secondary antibody/ECL detection systems (to detect MMP-2, MMP-12, SIRT1, TGFβ and their respective β-actin). Protein detection was also performed with the appropriate fluorescent secondary antibody, and the immunoreactive bands were visualized using a LI-COR-Odyssey infra-red scanner (Keap1, HO-1, GCLC, NQO1 and their respective β-actin). The densitometry of western blottings were quantified at ImageJ program. The protein band of interest was selected and measured according to the program settings. The β-actin was measured in the same way, and all proteins of interest were corrected by β-actin.

2.7. Chemical analysis of the ethanolic extract of propolis

Mass spectrometry with electrospray ionization (ESI-MS) analysis was performed using a MicrOTOF II mass spectrometer (Bruker Daltonics, Inc., Boston, MA, USA). The samples were infused directly into the source at a flow rate of 0.12 ml/h. The source temperature was set at 180°C, the drying gas (nitrogen) flow rate was 4.0 L/min and the nebulizer gas (nitrogen) pressure was 0.4 bar. In negative mode, the capillary voltage was 3.8 kV, the capillary exit voltage was -150 V, the skimmer 1 and 2 voltages were 50 V and 15 V, respectively, the hexapole 1 voltage was -23 V, the hexapole RF voltage was 300 Vpp, the lens 1 transfer was 88 µs and the lens 1 pre plus stage was 15 µs. Data were acquired in negative mode ranging from 50-2000 *m/z*. Mass calibration was achieved by infusing ammonium formate in an isopropanol-water mixture (1:1 v/v) as an external standard. All data were analyzed using Bruker Daltonics ESI Compass Data Analysis Version 4.0 SP 1 (Bruker Daltonics Inc., MA, USA). The mass error (the difference between the measured and the theoretical mass) and sigma (a parameter calculated by software accounting for the difference between theoretical and measured isotopic patterns, in which smaller values of sigma indicate better matching; data not shown) were calculated for each datum.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Interleukin-10 (IL-10) was quantified in BAL using a specific enzyme-linked immunosorbent assay with a rat anti-mouse monoclonal antibody and a detection limit of

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2000 pg/ml, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Mouse recombinant IL-10 standard was used in this assay.

2.9. Statistical analyses

The values for all measurements are expressed as the means ± SEM. All statistical analyses were performed using an unpaired test t ($p < 0.05$ was considered significant). GraphPad Prism software was used to perform the statistical analyses (GraphPad Prism version 5.0, San Diego, CA, USA).

3. Results

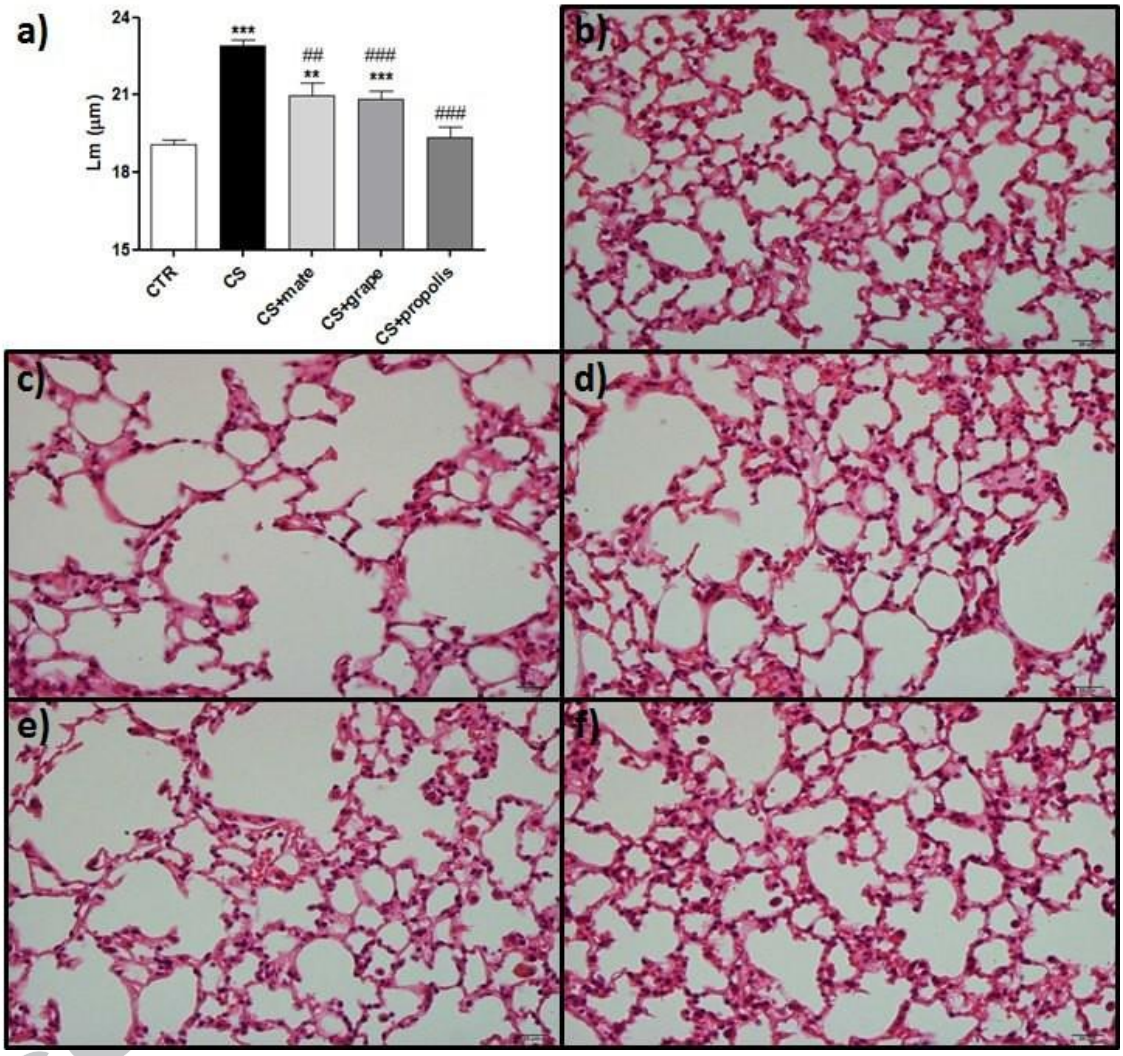
3.1. Propolis repairs lung damage and increases MMP-2 expression

Some natural products are known to protect lungs against acute inflammation and emphysema caused by CS exposure, but it is not clear if these natural resources can repair the lung structure after damage is already established. To investigate whether mate tea (produced from the roasted *Ilex paraguarienesis* herb), grape (from *Vitis labrusca*) and propolis are capable of restoring lung morphology, mice were exposed to 60 days of CS and then treated orally for another 60 days with these products. After 120 days, alveolus enlargement, measured by Lm (Fig. 1a) in lung sections stained with H&E, revealed, as expected, maintenance of septa and intact alveoli in the CTR group (Fig. 1b). However, the Lm in the CS group (Fig. 1c) was significantly increased when compared with the CTR group, demonstrating destruction of the alveolar septa in this lung and consequently an increase in alveoli, thus characterizing emphysema. When mice were treated with mate tea and grape, the treatments were capable of partially reversing the lung histoarchitecture because both were significantly different from the CS group, but they were still high when compared with the CTR group (Fig. 1d and e). However, propolis treatment could effectively completely reverse the lung damage (Fig. 1f), showing a significant decrease in Lm when compared with the CS group. Propolis was the only natural product capable of repairing the lung morphology, reversing the septum and alveolar destruction. Thus, we choose to only investigate propolis treatment.

Analysis of the volume density of alveolar septa ($Vv_{alveolar\ septa}$) showed that treatment with propolis was able to partially reverse the decrease in this parameter caused by CS exposure (Fig. 1g), suggesting that propolis could restore the morphology of the lung but that

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the septa were not completely restructured. The expression of MMP-2 (matrix metalloprotease-2) was analyzed by western blotting. The expression of MMP-2 was augmented in the CS+propolis group, suggesting the participation of this MMP in the lung repair that occurs when the animals were treated with propolis.

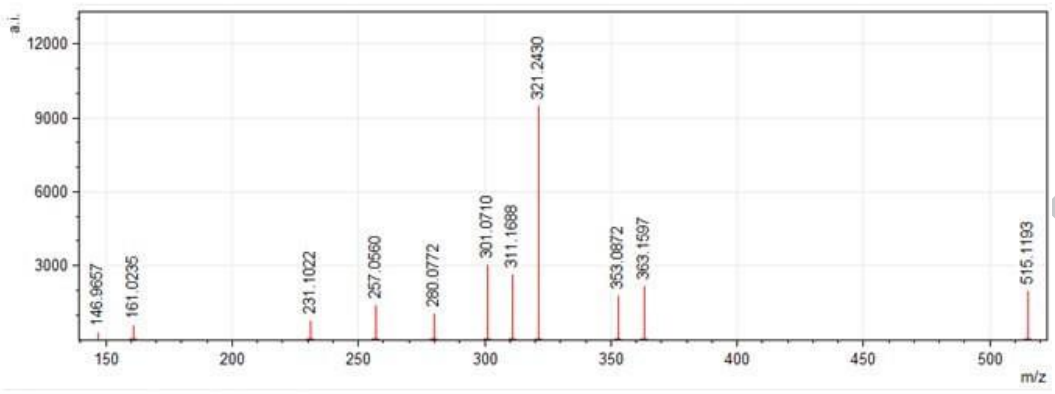


3.2. Full-scan ESI-MS analysis of propolis

Data acquired by ESI-MS analysis enabled us to recognize the major constituents found in Brazilian propolis used in this study (Fig. 2). The most intense ion observed in the samples corresponded to artepillin (Fig. 2a), as previously reported in other studies [23, 26]. Some other compounds found in propolis extract were hesperetin, sideroxylin, dicaffeoylquinic acid isomer and baccharin (Fig. 2b).

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a)



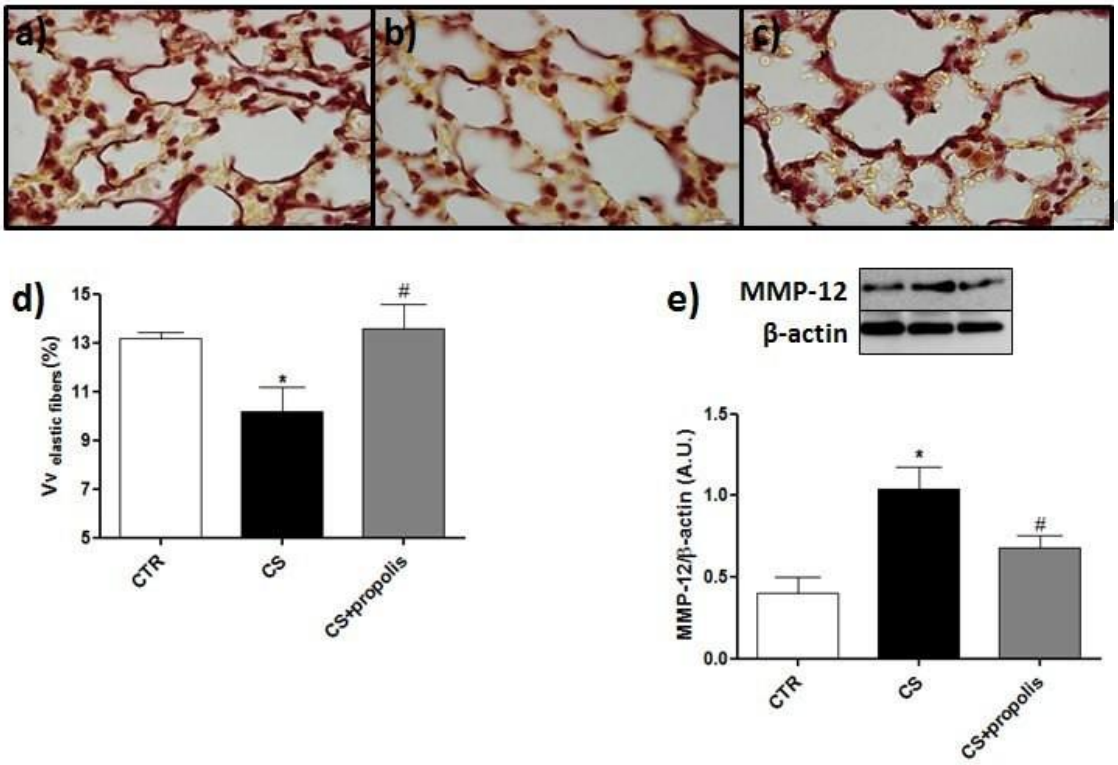
b)

[M-H]⁻	MS/MS fragments	Compound name
231,1022	146,9657	Coumaric acid prenyl ester
257,0560	n.i.	Palmitic acid
280,0772	n.i.	Linolenic acid
301,0710	n.i.	Hesperetin
311,1688	n.i.	Sideroxylin
321,2430	n.i.	Artepillin derivative
353,0872	161,0235	Caffeoylquinic acid isomer
363,1597	n.i.	Baccharin
515,1193	353,0872 / 161,0235	Dicaffeoylquinic acid isomer

3.3 Propolis restores lung elastic fibers and decreases MMP-12 expression

Lung sections were stained with orcein to identify elastic fibers (Fig. 3a-c). The CTR group (Fig. 3a) showed the largest amount of elastic fibers compared with the CS group (Fig. 3b), as expected. However, the lungs of the animals treated with propolis (Fig. 3c) exhibited a recovery of the quantity of elastic fibers, suggesting that propolis was capable of repairing the elastic fiber destruction caused by CS exposure. The volume density of elastic fibers ($Vv_{elastic\ fibers}$) was reduced in the CS group compared with the CTR group (Fig. 3d), but this diminution was reversed in the CS+propolis group. Elastin is the main constituent of elastic fibers. MMP-12 is present in elastase derived from macrophages, and it is capable of degrading elastin [48]. MMP-12 expression was increased in the CS group, and propolis treatment decreased MMP-12 expression (Fig. 3e).

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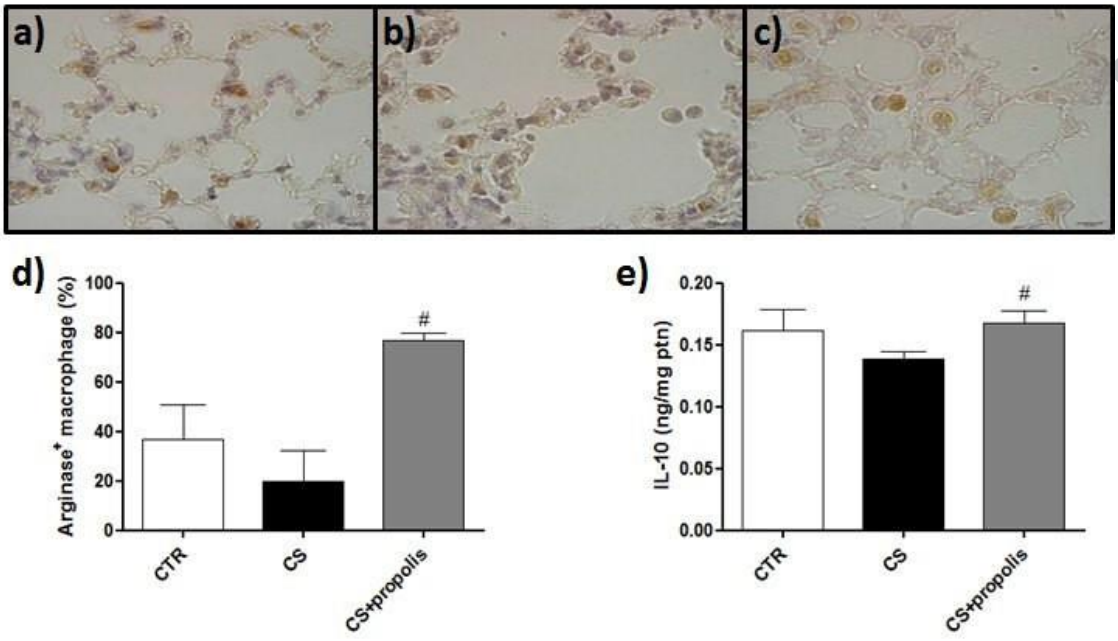
3.4. Propolis induces macrophage alternative activation and an anti-inflammatory microenvironment

To investigate whether propolis could modify the cell profile to restore the lung parenchyma, total leukocytes, macrophages, neutrophils and lymphocytes were quantified in BAL and are represented in Figures 4a-c. Propolis treatment increased the number of total leukocytes, which consisted mostly of macrophages and lymphocytes (Fig. 4d). The number of total leukocytes was not altered in the CS group, nor were the numbers of macrophages and lymphocytes individually when compared with the CTR group. The number of neutrophils remained unchanged in all groups. Surprisingly, the ROS levels were unaltered in the CS+propolis group even with an increase in leukocytes (Fig. 4e).

Therefore, we further investigated whether the increase in macrophages could be related to a profile that would facilitate lung tissue repair since ROS levels were not altered in any of the three groups. Images of immunohistochemistry analysis against arginase (Fig. 5a-c) indicated that propolis was able to induce macrophage alternative activation, increasing the number of arginase-positive macrophages (Fig. 5d). Arginase is a marker for M2 macrophages, a type of macrophage that is related with cytokines and microenvironments with anti-inflammatory profiles. In support of this result, IL-10 levels were increased in the

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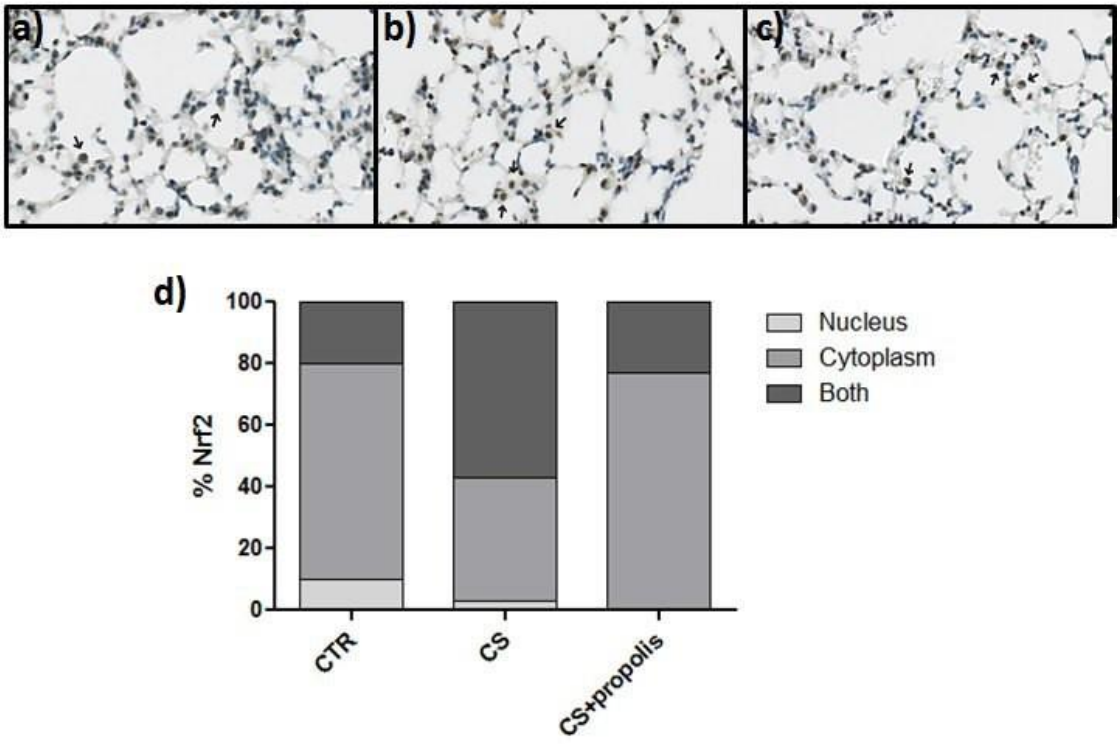
CS+propolis group in comparison to the CS group (Fig. 5e), suggesting that propolis provides an anti-inflammatory microenvironment that could support lung tissue repair.



3.5. Propolis acts independently of the Nrf2 pathway

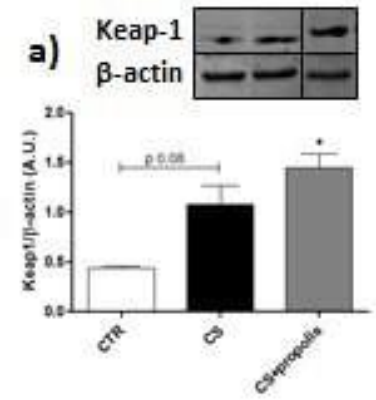
Nrf2 is one of the most important nuclear factors in the regulation of oxidative stress. As described in the literature, emphysema is a disease that is characterized by redox imbalance, and propolis possesses antioxidant properties. Therefore, we decided to examine whether propolis affected the Nrf2 pathway and whether this factor and its related enzymes participated in lung repair. Immunohistochemistry analysis revealed Nrf2 in different compartments of alveolar macrophages (Fig. 6a-c). The percentage of cells with Nrf2 in the cytoplasm and nucleus increased in the CS group (Fig. 6b and d), suggesting that Nrf2 translocation was necessary to regulate the redox imbalance caused by CS exposure. Corroborating these results, the number of cells with Nrf2 only in the cytoplasm decreased in the CS group. However, the most surprising result was that none of the cells in the CS+propolis group displayed Nrf2 only in the nucleus (Fig. 6c and d). This result suggests that propolis functions to restore the pulmonary histoarchitecture in a Nrf2-independent manner.

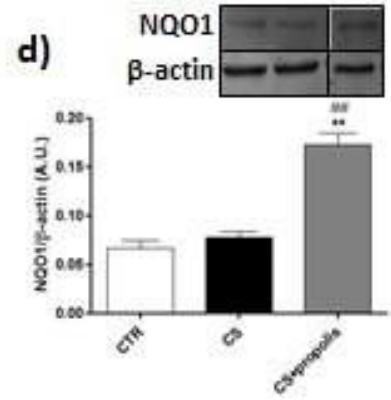
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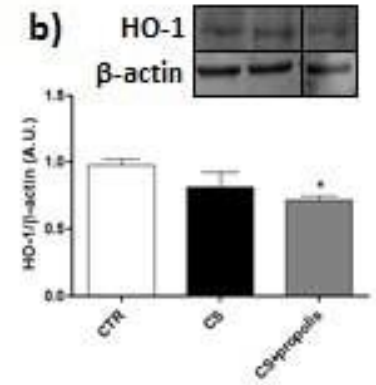


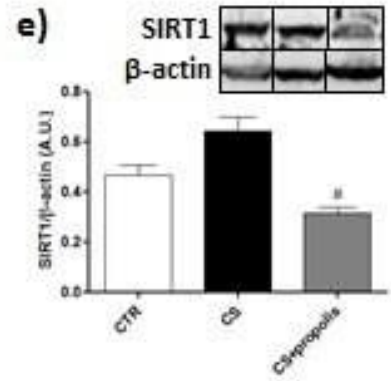
Keap1 protein maintains Nrf2 in the cell cytoplasm under physiological circumstances. Propolis treatment resulted in increased Keap1 expression (Fig. 7a), confirming the retention of Nrf2 in the cytoplasm, as shown in Figure 6d. Furthermore, HO-1 expression was reduced in the CS+propolis group (Fig. 7b). HO-1 is a phase II antioxidant enzyme that requires Nrf2 translocation for its production. GCLC is a subunit of the first rate-limiting enzyme in glutathione synthesis. GCLC expression is reduced only in the CS group (Fig. 7c). Another phase II antioxidant enzyme is NQO1. One of the pathways responsible for the production of NQO1 was the Nrf2 pathway, following the translocation of Nrf2 to the nucleus. However, NQO1 expression was increased in the CS+propolis group (Fig. 7d), contradicting the absence of Nrf2 translocation to the nucleus in this group. Another pathway related to NQO1 expression is sirtuin 1 (SIRT1) [49], but the CS+propolis group showed a reduction in SIRT1 expression (Fig. 7e) and thus still did not justify the increase in NQO1.

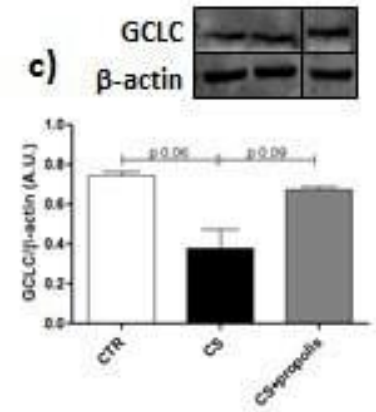
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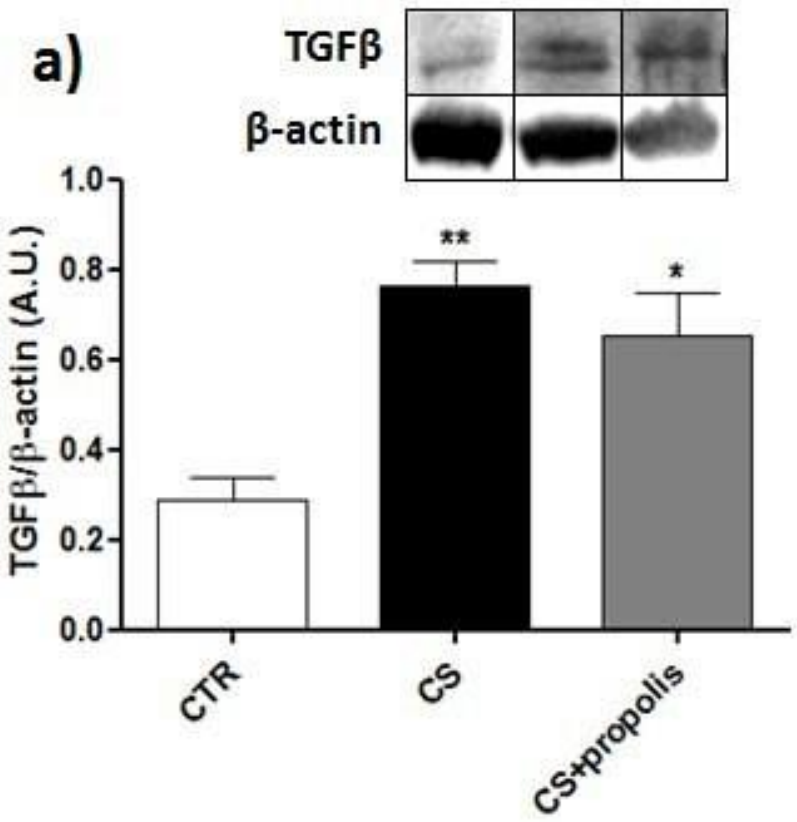
3.6. Lung repair by propolis does not requires TGB-β but necessitates IGF1 reduction

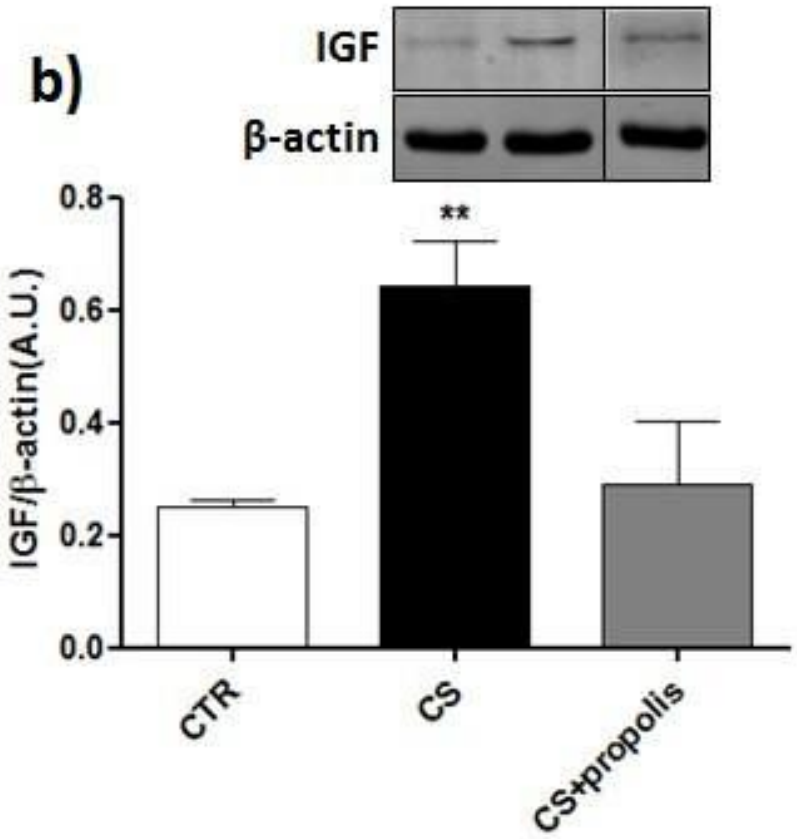
Growth factors play important roles in directing the processes of wound healing and tissue repair. To evaluate the effect of propolis during growth factor expression, TGF-β and IGF1 levels were analyzed. TGF-β expression was increased in the CS group and remained high in the CS+propolis group when compared with the CTR group (Fig. 8a), suggesting that TGF-β did not participate in lung repair in our model. However, IGF1 expression was only

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enhanced in the CS group, whereas this increase was reversed by propolis (Fig. 8b), suggesting an influence of the propolis-induced downregulation of the IGF1 pathway.

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a)
TGFβ
β-actin
TGFβ/β-actin (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
**
*
CTR
CS
CS+propolis


b)
IGF
β-actin
IGF/β-actin(A.U.)
0.8
0.6
0.4
0.2
0.0
**
CTR
CS
CS+propolis

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4. Discussion

Pulmonary emphysema is characterized by enlargement of alveolar spaces with septum destruction and migration of inflammatory cells. The main cause of emphysema worldwide is CS. The present study is pioneer in evaluating a treatment with propolis for emphysema after the disease has already been established. In the literature, there are many studies, including our group, that suggest concomitant treatments with the induction of emphysema, which prevents or attenuates the development of the disease [37-40, 50]. Our model of post treatment with propolis is a novelty in the scientific literature since there are still no studies that reveal the mechanisms involved in lung repair and no effective treatment for patients. Our previous studies have shown that propolis [26] and grape extract [39] were able to reduce mouse acute lung inflammation (ALI), while mate tea was able to reduce ALI and prevent mouse emphysema [37, 38]. However, neither the grape nor the mate tea were able to reverse fully the pulmonary histoarchitecture like the propolis in our study. This result corroborates with literature that shows these activities of propolis in other diseases [27-31]. In the present study, we evaluated whether mate tea, grape and propolis would be able to reverse lung damage caused by CS exposure. As expected, we showed that the mean linear intercept length of the distal alveolar spaces (*L*m) in the CS group was augmented in comparison to the CTR group (Fig. 1a), characterizing the enlargement of alveoli in emphysema. Mate tea and grape treatments were able to partially reverse the Lm because enlargement of the alveoli in those animals was not as severe as in the CS group; however, the lung parenchyma was not completely recovered. In contrast, propolis treatment was able to repair fully the lung parenchyma similarly to the CTR group. For this reason, we decided to evaluate only the propolis extract in subsequent analyses. Metalloproteases (MMPs) are a family of structurally related extracellular matrix (ECM)-degrading enzymes that collectively are capable of degrading essentially all ECM components, and they are subdivided in specific groups [51, 52]. MMP-2 is a gelatinase that is synthesized by a wide variety of cells, including alveolar epithelial cells, endothelial cells, fibroblasts, which play an important role in tissue repair, and to a lesser extent, by macrophages [53, 54]. Propolis was capable of increasing MMP-2 expression, suggesting that this MMP could participate in lung tissue repair promoted by propolis.

Previous studies highlight that oxidative stress and inflammatory cell influx may impair the function of antiproteases, accelerating the breakdown of elastin in the lung

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parenchyma and leading to emphysema [55, 56]. In the present study, we demonstrated that propolis treatment could halt and reverse the breakdown of elastin caused by CS exposure, as evidenced by quantifying the volume density of elastic fibers (Vv[elastic fibers])) (Fig. 2d). Mechanisms by which propolis acts to repair elastic fibers require additional studies; however, we suggest that alternative macrophage activation, which has a resolving effect, should be involved in this elastic fiber recovery. Another metalloprotease that is also involved in emphysema and other pulmonary diseases is MMP-12, which is an elastase (also known as metalloelastase) that mainly functions in the degradation of elastin. MMP-12 is produced by macrophages. Some studies suggest that MMP-12 gene polymorphisms are one of the causative factors of smoking-related injury [57]. Accordingly, Figure 3e suggests that propolis treatment is capable of reversing the increase in MMP-12 induced by CS, justifying the augmented volume density of elastic fibers (Fig. 3d) in comparison to the CS group. When propolis diminishes MMP-12 expression, it is expected that lung elastic fibers could be restored.

To investigate whether propolis treatment could affect inflammatory cell influxes, total and differential BAL counts were performed. CS exposure is known to increase the cell infiltrate, but the cell profiles at 60 days after the end of CS exposure revealed no differences between the CTR group and the CS group. Surprisingly, propolis increased the number of total leukocytes, including macrophages and lymphocytes. Most studies have reported that natural products are capable of reducing inflammatory cells [58-60], but most of these studies were conducted with concomitant treatments. Leukocytes are capable of releasing ROS. Our results showed an increased number of leukocyte in BAL without augmentation of ROS levels, suggesting a novel mechanism by which propolis changes the cell profile and recruits them to repair the site of lung injury without ROS release.

These last results showing increased numbers of leukocytes and macrophages led us to investigate the profile of these cells. Macrophages are key components of the innate immune system that orchestrate both the initiation and resolution of inflammation and can mediate either pro-inflammatory or anti-inflammatory immune responses [61]. Macrophages are also involved in lung formation since embryogenesis [62]. Traditionally associated with host defense, inflammation and scavenging functions, macrophages have a variety of functions, including vital roles in tissue repair [63-65] and organ development [66, 67]. Macrophage “M2 polarization” largely reflects the Th2 response, resolving inflammation

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while promoting cell proliferation and wound healing [61]. In the present study, we showed that propolis treatment was capable of increasing the number of macrophages in BAL, reflecting the enhanced recruitment of these cells. Furthermore, the recruited macrophages presented a M2 profile, which indicates that these cells were probably recruited to mediate lung repair stimulated by propolis. Other studies have also shown the participation of M2 macrophages in lung repair [68-70], corroborating our results. Moreover, it has been reported in literature that resveratrol, the main antioxidant present in grape, skews the macrophage phenotype toward M2 and promotes M1 macrophage apoptosis [71]. Also, a study showed that Brazilian propolis could improve diabetes in obese mice by macrophage polarization [72]. The previously mentioned study also showed that M1 apoptosis was mediated by the release of IL-10 from M2 macrophages. IL-10 is a cytokine with multiple effects on immunoregulation and inflammation by downregulating, for example, the expression of Th1 cytokines and MHC class II antigens. It has been reported that IL-10 influences macrophage M2 polarization and collaborates to repair damage in various tissues [73-75]. Our results demonstrated augmented IL-10 levels in the CS+propolis group, corroborating the increase in macrophage M2 polarization marked by arginase 1, which suggested that propolis might be able to regulate macrophage alternative activation to promote lung repair.

As described in the literature, Nrf2 together with its repressor Keap1 are cellular sensors of oxidative and electrophilic stress that coordinate the induction of a battery of genes that encode detoxifying enzymes, drug transporters, anti-apoptotic proteins, and proteasomes [76]. Under physiological conditions, Nrf2 remains in the cytoplasm attached to Keap1 and then is degraded by the proteasome, and its basal translocation guarantees an antioxidant status. When a redox imbalance occurs, Keap1 releases Nrf2, which translocates to the nucleus, binds to antioxidant response element (ARE) and promotes the generation of many antioxidant enzymes such as HO-1, NQO1 and GCL. Many studies have shown a beneficial effect of Nrf2 activation in various models of tissue repair, including mainly wound healing [77, 78]. Additionally, Nrf2 activation in wound healing, for example, occurs due to antioxidant treatment [79, 80]. Concerning the pathology of emphysema, Nrf2 also plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema [81]. Nrf2 activation is also capable of reversing sinonasal epithelial cell barrier dysfunction induced by CS extract [82], participates in protection against human bronchial epithelial cell injury caused by CS exposure [83], attenuates and protects against lung injury caused by diverse stimuli *in vivo* [84, 85], and participates in the regeneration of other organs such as

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liver [86]. Nrf2 has presented many other functions related to tissue repair and the control of oxidative stress and inflammation. However, all of these models were performed *in vitro* or with concomitant treatments, and thus they do not portray the clinical reality. In the present study, we used a mouse model in which the treatment was administered after the disease was already established and thus more accurately represented patients who seek help for emphysema that is already advanced. We showed that when the mice were exposed to CS without treatment, more cells showed Nrf2 localization in both the cytoplasm and the nucleus as compared with the CTR group, suggesting that CS exposure could induce Nrf2 translocation, potentially to maintain the redox imbalance (Fig. 6). Surprisingly, when the mice were treated with propolis, the cells did not present Nrf2 localization only in the nucleus, and the percentage of cells with Nrf2 in both cellular compartments was very similar to the CTR group. These results suggest that propolis does not function in the Nrf2 pathway, activating it to promote lung repair and macrophage alternative activation, as revealed by the previous results in this study. Corroborating the localization of Nrf2 in the cytoplasm in mice that were treated with propolis, Keap1 expression in this group was augmented even with a concomitant decrease in HO-1 expression since Nrf2 requires Keap1 to remain in the cytoplasm and the absence of Nrf2 translocation precludes HO-1 production. NQO1 is also a phase II enzyme that is generated by the Nrf2 pathway, and thus we did expect a decrease in NQO1 like HO-1. However, NQO1 expression can be affected by other pathways, including the SIRT1 pathway [87]. Consequently, we investigated whether propolis was capable of participating in the SIRT1 pathway leading to increased NQO1 expression, but SIRT1 expression was diminished in the group treated with propolis. One explanation for this increase in NQO1 expression is the activation of aryl hydrocarbon receptor (AhR) by propolis. AhR is a ligand-activated transcription factor in mammalian cells or tissues, and one of its functions is to bind to environmental pollutants; however, the essential functions of this receptor remain largely unknown [88, 89]. Propolis, some teas and processed foods exhibit the ability to activate AhR [90], and activation of the AhR pathway can lead to NQO1 induction [91].

All tissue repair processes require growth factor participation. TGF-β is a multifunctional cytokine that is secreted by many cell types including macrophages. The TGF-β pathway is known to participate in many models of wound healing, tissue repair and fibrosis [92-95]. In the present study, we aimed to investigate whether TGF-β was related to the lung repair promoted by propolis treatment. We showed that propolis treatment did not

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alter TGF-β expression, which also remained high in the animals exposed only to CS. Based on this data we suggest that the TGF-β pathway is not involved in propolis-mediated lung repair. However, IGF1 is a growth factor related to insulin that stimulates cell growth, proliferation and differentiation, and it is essential for normal organism growth and development [96, 97]. Among other functions, IGF1 is important for alveolar formation [98], and it plays important roles in subepithelial fibrosis, airway inflammation, airway hyperresponsiveness and airway smooth hyperplasia [99]. For all these functions of IGF1 related to lung disease, we investigated whether IGF1 could participate in propolis-mediated lung repair. We observed that IGF1 expression was augmented in the CS group, but propolis treatment was able to reverse this increase, demonstrating a downregulation of IGF1 that was accompanied by lung repair. A study performed with fetal lung fibroblasts showed that IGF1 induces smooth muscle cell hyperplasia and increases the expression of type I collagen and alpha smooth muscle actin (α-sma). All these phenomena were mediated by an elevation of NFκB transcription activity induced by the PI-3 kinase and AKT pathways. Although we have not shown this phenomenon, if an increase in type I collagen occurs for remodeling of the emphysematous lung parenchyma, then it will contribute to the fibrosis focal points and worsen the mechanical ventilation. However, the mechanisms by which propolis downregulates IGF1 require further investigation.

5. Conclusion

In summary, all these results support a therapeutic effect of propolis against CS exposure-caused emphysema mediated, at least partially, by a shift in alternative macrophage activation, promoting an anti-inflammatory microenvironment in the lung with the participation of IL-10 and the downregulation of IGF1, resulting in the repair of alveolar septa and elastic fibers. However, the Nrf2 pathway does not participate in the lung repair in this model because propolis was not capable of promoting Nrf2 translocation into the cell nucleus. Therefore, we suggest that propolis post-treatment is efficient for the reversal of lung damage in emphysema caused by CS exposure in mice.

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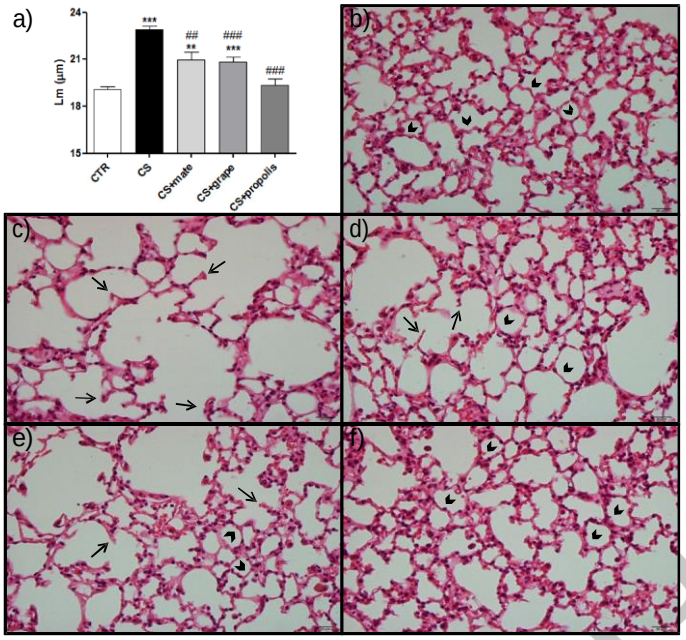
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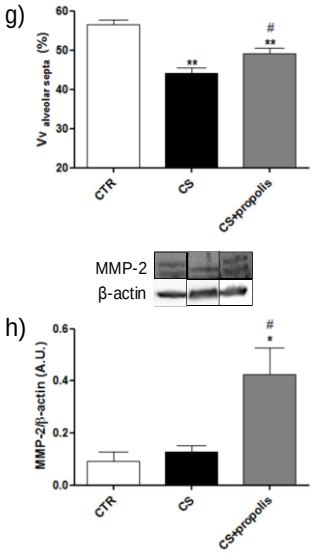
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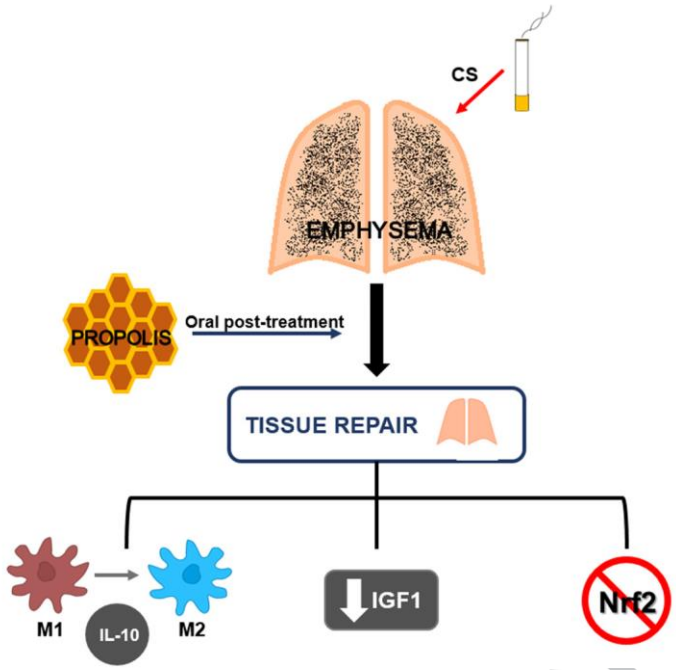
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Figure 1





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Graphical abstract

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