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Effect of piperlongumine during exposure to cigarette smoke reduces inflammation and lung injury

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is related to smoking and anti-inflammatory therapy is indicated. Among the mediators with anti-inflammatory properties, we highlight piperlongumine (PL), an alkaloid/amide of *Piper longum*. Here we evaluated the PL administration on an experimental model of respiratory inflammation resulting from exposure to cigarette smoke. Male Balb/c mice were exposed to burning of 10 commercial cigarettes, 2x/day, for five weeks on specific equipment. PL efficacy was evaluated in control, exposed to smoke without treatment and PL treated (2.0 mg/kg, 3x/week) groups. Animals were weighed and plethysmographic analyses performed at the end of the exposure protocol. Inflammatory cells were evaluated in the bronchoalveolar lavage (BAL) and hemoglobin and glucose in the blood. Lung fragments were processed for histopathological studies and AnxA1, COX-2, NF-kB and neutrophil elastase expressions. Plethysmography revealed that PL maintained pulmonary frequency, volume and ventilation parameters similar to controls, with respiratory volume reduction compared to untreated animals. Final weight was reduced in both exposed groups. PL decreased hemoglobin concentration, attenuated the reduction of glucose levels and reduced influx of lymphocytes, neutrophils and macrophages in BAL. Histopathologically occured infiltration of inflammatory cells, increase of the interalveolar septa and intra-alveolar spaces in untreated animals. But, PL administration recovered lung tissues and, immunohistochemically, promoted increased expression of AnxA1 and reduction of COX-2, NF-kB and neutrophil elastase. Together the results indicate that PL attenuates systemic and pulmonary inflammatory changes, partially by modulating the expression the endogenous AnxA1, and may represent a promising therapy in preventing the inflammation induced by cigarette smoke.

1. Introduction

Smoking habit is responsible for 7 million deaths per year. This important global health problem also affects non-smokers who coexist with the smoke produced by smokers and suffer the damage caused by this exposure [56]. Among other conditions, the smoking habit predisposes to chronic obstructive pulmonary disease (COPD), a serious health condition characterized by progressive limitation of airflow and estimated to be the third leading cause of death in 2020 [6,8,36,53]. Patients with COPD also have systemic symptoms and comorbidities, including muscle weakness, weight loss, cardiovascular disease, osteoporosis, hypertension, depression, and cognitive decline [6,18,45,53].

Researches show that the inflammatory process induced by the inhalation of harmful particles and gases leads to pathological alterations such as mucosal hypersecretion and structural changes in the airways and alveoli, [6,8]. The amount of inflammatory cells in bronchial biopsies and induced sputum can be associated with disease severity and lung function decline [8,47]. In addition, other studies have demonstrated that serum biomarkers of inflammation, including tumor necrosis factor (TNF)-α and matrix metalloproteinase (MMP)-9, are

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Abbreviations

µL	microliter
µm	micrometer
mg	microgram
Kg	kilogram
ANOVA	variance analysis
AnxA1	annexin A1
BAL	bronchoalveolar lavage
C	control
CS	cigarette smoking
CS + PL	cigarette smoking + PL
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase
DAB	diaminobenzidine
DMSO	dimethylsulfoxide
H2O2	hydrogen peroxide
Hb	hemoglobin
HE	hematoxylin and eosin
IFN-g	interferon
IkB/IKK	kinases
IL-1β	interleukin 1β
ip	intraperitoneal
JNK	c-jun amino-terminal kinase
LPS	lipopolysaccharide
MMP-9	matrix metalloproteinase
MUC5AC	mucin
NF-kB	nuclear factor kappa B
NO	nitric oxide
OA	ocadaic acid
p38 MAPK	mitogen-activated protein kinases
PBS	Phosphate buffered solution
PI3K	phosphatidylinositol 3-kinase inhibitor
PL	piperlongumine
PMA	phorbol-12-myristate-13-acetate
RPM	rotation per minute
ROS	reactive oxygen species
STAT3	signal transducers and activators of transcription
TGF- β1	tranforming growth fator
TNF-α	tumor necrosis factor
WHO	Word Health Organization

increased in COPD patients and can be correlated with the degree of airflow obstruction and mortality [1,5].

In the development of the disease, activated alveolar macrophages and respiratory tract epithelial cells release chemical mediators, such as TNF-α and interleukins (IL)-1β, IL-6, IL-17, IL, which attract different inflammatory cells into the airways [3,6]. Neutrophils recruited to the lung release elastolytic enzymes such as proteinase-3, cathepsin G and neutrophil elastase, which are closely linked to the destruction of the lung parenchyma, contributing to alveolar destruction [19,44]. Furthermore, proteases are also produced, which in turn stimulate mucus secretion associated with chronic bronchitis, one of the main features of COPD. In these conditions, T-lymphocytes are capable of causing alveolar epithelial cell cytolysis and apoptosis, contributing to disease-related [6,8]. Emphysema can also be caused by alveolar macrophages that produce elastolytic enzymes, such as MMP-2 and MMP-9, which destroy the fibers of the pulmonary connective tissue [6].

Despite advances in the treatment of COPD, there is still a need for new therapies targeting the prevention and progression of the disease, the reduction of exacerbations and mortality [8]. There are several models that contribute to the understanding of the pathophysiological processes involved in COPD and in the development of new treatment strategies. Among them, the exposure of rats to cigarette smoke is a simple and useful model, used by several investigators, including our research group [16,25,40].

In this scenario, a promising therapeutic alternative is piperlongumine (PL), an alkaloid found in roots and stems of several species of the genus *Piper* such as *Piper longum L.*, *Piper retrofractum*, *Piper tuberculatum L.* and *Piper nigrum* [2,38]. Several studies have demonstrated that PL has antitumor, antifungal, anxiolytic, antidepressant, cytotoxic, antiplatelet and anti-inflammatory properties, among others [4,32,41,53]. The anti-tumoral effect of PL has been shown on 14 tumor cell lines without causing any adverse effect on normal cell lines, thus revealing a potent selective activity of this substance in cancer [41]. Other studies have demonstrated antifungal activity on *Cladosporium sphaerospermum* and *Cladosporium cladosporioides* [34,46]. PL has also been shown to be a potent inhibitor of platelet aggregation induced by collagen, arachidonic acid, platelet-activating factor and thromboxane A2 receptor agonist, but not by thrombin, suggesting an antagonistic action on thromboxane A2 receptors [22,37,51].

PL inhibits nuclear factor kappa B (NF-kB) activation induced by carcinogens and inflammatory stimuli such as endotoxin lipopolysaccharide (LPS), ocadaic acid (OA), Phorbol-12-myristate-13-acetate (PMA), hydrogen peroxide (H2O2), and cigarette in different cell lines [20]. The anti-inflammatory activity of PL has also been evaluated in rheumatoid arthritis, showing effective activity in inducing proliferation, migration and invasion, as well as reducing the intracellular production of reactive oxygen species (ROS) induced by TNF-α-and also inhibiting the activation of mitogen-activated protein kinases (p38 MAPK), c-jun amino-terminal kinase (JNK), signal transducers and activators of transcription (STAT3) and NF-kB in fibroblast-like synoviocytes [54].

In this study, we sought to investigate PL as a systemic treatment of respiratory inflammation induced by exposure to cigarette smoke, in search of a new therapeutic strategy in COPD.

2. Materials and methods

2.1. Animals

Male Balb/c mice (20–25 g body weight) were divided into three groups (n = 6/group) and kept in individual cages in a controlled environment (24–25 °C, 12 h light/dark cycle) with water and food ad libitum.

All experimental procedures were conducted according to the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committees on Animal Use at São Paulo Federal University (Certificate nº 8531240217) and University Center Padre Albino (Certificate nº 04/16), Brazil.

The experiments were designed to minimize the number of animals used and their suffering during the execution of the protocols. All animals were daily evaluated by the institution's veterinarian.

2.2. Experimental model of pulmonary inflammation by exposure to cigarette smoke

Two groups of animals were exposed for five weeks to the consecutive burning of 10 commercial cigarettes (containing 0.8 mg of nicotine, 10 mg of tar and 10 mg of carbon monoxide), resulting in approximately 1 h of exposure, twice a day (total of 20 cigarettes/day). The first exposure was performed in the morning (7 a.m.) and the second in the early evening (6 p.m.). The control group was kept in the same conditions but exposed to compressed air only [40].

The exposure apparatus consists of an animal containment system

and a cigarette smoke release system with an external cigarette holder connected to a dynamic suction pump. The pump can be programmed so that cigarette suction periods alternate with periods of clean air suction to prevent asphyxiation [25,40].

2.3. Pharmacological treatment with piperlongumine

The therapeutic efficacy of PL (Sigma - SML 0221) was evaluated by intraperitoneal (ip) administration of PL at the dosage of 2,0 mg/Kg, diluted in 100 µl of 10% DMSO [41] once a day and immediately before the first exposure to cigarette smoke. The treatment protocols were performed three times a week for five weeks. Twenty hours after the last cigarette exposure, the animals were euthanized by excessive dose of anesthetic.

2.4. Physiological evaluations of plethysmography and weight

All animals were weekly weighed and at 24 h after the last exposure to cigarette smoke all mice were evaluated by plethysmography for the measuring of lung ventilation, breathability, frequency and inspired air volume (PowerLab, AD Instruments-Gas Analyzer, Sydney, Australia).

2.5. Quantitative analyses of bronchoalveolar lavage (BAL)

BAL was obtained at the end of the experiment. The trachea was cannulated and the right lung clamped. The left lung was washed 3 times with 500 µl of PBS, and the liquid collected was centrifuged for 10 min at 1,500 rpm. The pellet was resuspended in 50 µl of PBS and aliquots of 10 µl were stained in Turk (1:10) for quantification of inflammatory cells in a Neubauer camera (values as number of cells x 10^4/ml).

2.6. Biochemical blood assays

Blood was collected by cardiac puncture in heparinized syringes and separated in aliquots for the analysis of hemoglobin by a commercial Kit (LAB test, Cat No: 43, Minas Gerais, Brazil). Other aliquots were centrifuged for 15 min at 3,000 rpm and the plasma frozen at −70 °C for further glucose measurement that was performed by commercial kit (LAB test, Cat No: 133-1/500).

2.7. Histopathological and morphometric studies

Fragments of the right lung were fixed in 4% formaldehyde and processed for paraffin inclusion. Sections of 5 µm were used for histopathological, morphometric and immunohistochemical analyses in a Leica microscope (DM500). For histopathological evaluations the tissue sections were stained with Hematoxylin and Eosin (HE). Morphometric studies were performed by means of pulmonary alveolar area measurements using an image analyzer (Software Leica Image Analyses).

2.8. Immunohistochemical analysis

Immunohistochemical studies were used to evaluate the expressions of AnxA1, COX-2, NF-kB and elastase. Lung sections were prepared on silanized slides and processed for antigenic recovery with citrate buffer pH 6.0, blockade of the endogenous peroxidase activity and incubation with the rabbit polyclonal primary antibodies: anti-AnxA1 (1:2000) (Zymed Laboratories, Cambridge, UK), anti-COX-2 (1:350) (Invitrogen), phospho anti- NF-kB p65-S636 (1:1000) (Abcam-ab86299) for the activated, p65 (1:1000) (cat number 14-6731-81- *Invitrogen*) for the total NF-kB, overnight at 4 °C and mouse anti-neutrophil elastase (1: 200) (Abcam-ab21595) for 2 h at 4 °C. Then they were incubated with the biotinylated secondary antibodies (Histostain Kit, Invitrogen for AnxA1 and COX-2 or Sigma-Aldrich, SAB4600006 for neutrophil elastase and NF-kB) and immersed in conjugated streptavidin peroxidase complex. The substrate diaminobenzidine (DAB Kit Invitrogen) was used for the development and, thereafter, the sections were stained with Hematoxylin.

The COX-2 and AnxA1 were quantified by densitometry as arbitrary units from 0 to 255, while the neutrophil elastase and NF-kB (total/activated) were quantified by percentage of positive expression area using the Axiovision Software. For these analyses three slides from each animal were used and 20 points on the alveoli were analyzed in five random fields for an average related to the intensity of immunoreactivity [40].

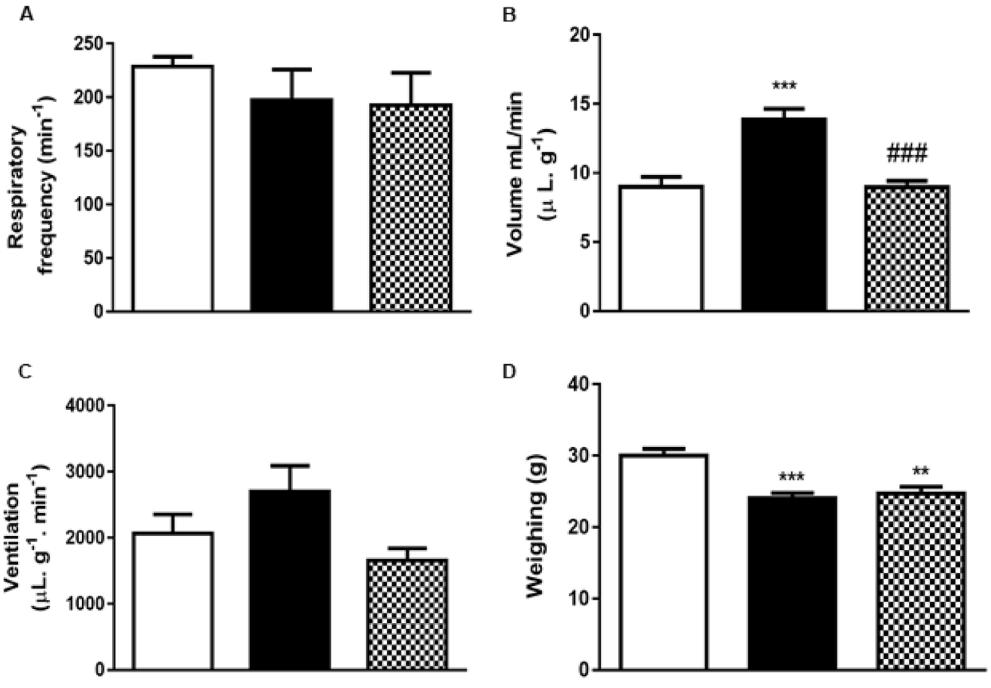

Fig. 1. Physiological analysis of plethysmography and weight - [A] Evaluation of lung frequency per minute, **[B]** inspiratory air volume capacity per ml/minute, **[C]** lung ventilation capacity per minute and **[D]** final weighing. Groups: (C) control, (CS) untreated exposed to smoke and (CS + PL) treated exposed to smoke. The results were presented as mean ± S.E.M. (n = 6/group) and determined by *one-way anova* followed by *Bonferroni.*

2.9. Statistical analysis

Statistical analysis was performed by one-way Anova followed by Bonferroni post-test or Tukey's post hoc for samples with normal distribution and the Kruskal-Wallis test, followed by the Dunn test for data with non-parametric distribution. *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Treatment with PL maintains respiratory volume similar to controls

No significant differences among groups were found regarding the respiratory rate (Fig. 1A) however, the inspired air volume (Fig. 1B) was significantly increased in the untreated group (CS) in relation to control ($p < 0.01$) and also a significant reduction in the inspired air volume was observed in PL treated mice compared to untreated ones ($p < 0.001$). There was no significant difference in the final pulmonary ventilation among groups (Fig. 1C).

Final weighing assessments indicated weight loss in treated animals ($p < 0.01$) or not ($p < 0.001$) relative to controls (Fig. 1D).

3.2. PL reduces hemoglobin levels and attenuates the reduction in blood glucose concentration

Dosages of hemoglobin showed an increase in the blood of animals exposed to cigarette smoke without treatment (Fig. 2A) and decreased concentrations in PL-treated animals compared to untreated ($p < 0.5$). Exposure to cigarette smoke reduced glucose levels, especially in the untreated group versus control ($p < 0001$; Fig. 2B).

3.3. PL reduces the influx of inflammatory cells and the activity of the neutrophil elastase enzyme in the lungs

The alterations of the histoarchitecture of the lung were analyzed histopathologically, with emphasis on alveolar modifications, pulmonary septa thickness and infiltration of inflammatory cells (Fig. 3A–E). Control lungs indicated normal appearance with no significant alterations of the tissue architecture (Fig. 3A). In contrast, after exposure to cigarette smoke we observed significant changes in the tissue, such as influx of inflammatory cells, increased thickness of the pulmonary septa and intra-alveolar spaces, (Fig. 3B and C). Treatment with PL prevented alveolar changes, reduced influx of inflammatory cells, and maintained histological characteristics similar to controls (Fig. 3D).

We also investigated the effect of cigarette smoke on the release of the enzyme neutrophil elastase that is released primarily by neutrophils in the lung tissue. In animals without treatment (Fig. 3G) there was a significant increase ($p < 0.05$) in the expression of the enzyme compared to controls (Fig. 3F). The anti-inflammatory effect of PL (Fig. 3H) was observed by a significant reduction ($p < 0.05$) in elastase expression in relation to untreated mice, which was statistically shown by densitometric analysis (Fig. 3J). The specificity of the reaction was confirmed by sections not incubated with the primary anti-elastase antibody (Fig. 3I). Our results indicated the preservation of the pulmonary structure promoted by the administration of PL in animals exposed to cigarette smoke.

3.4. Reduction of inflammatory cells in BAL after treatment with PL

The quantification of the inflammatory cells in the BAL showed increased influx of lymphocytes ($p < 0.001$, Fig. 4A), neutrophils ($p < 0.01$, Fig. 4B) and macrophages ($p < 0.001$; Fig. 4C) in untreated animals compared to controls.

The anti-inflammatory action of PL was observed by the reduction of the number of lymphocytes ($p < 0.05$) and neutrophils ($p < 0.001$) in relation to the untreated mice. Although not significant, administration of PL also reduced the amount of extravasated macrophages.

3.5. PL reduces the expression of NF-kB and COX-2 and modulates the endogenous AnxA1 protein

NF-kB was evaluated by immunohistochemistry in control (C), exposed to cigarette smoke untreated (CS) and treated with PL (CS + PL). The expression of NF-kB in the CS group was increased (Fig. 5B) compared to the controls (Fig. 5A). The treatment with PL was effective in significantly reducing the immunoreactivity of NF-kB in relation to CS group ($p < 0.05$; Fig. 5C), data that were confirmed by positive expression area (Fig. 5M).

As expected, there was an increase in COX-2 expression ($p < 0.001$) in untreated animals (Fig. 5F) compared to controls (Fig. 5E). The anti-inflammatory effect of PL administration was observed by a significant reduction ($p < 0.001$) in COX-2 expression in lung tissue (Fig. 5G) compared to the untreated group.

Because AnxA1 has an important role in the resolution of the inflammatory process, we analyzed whether PL treatment could affect the expression of this protein. In the treated animals ($p < 0.001$, Fig. 5K) or not ($p < 0.001$, Fig. 5J) there was an increase in protein expression compared to the controls (Fig. 5L). Interestingly, treatment with PL increased the expression of AnxA1 ($p < 0.01$) relative to the untreated group. The specificities of the immunomarcations were verified by the respective reaction controls (Fig. 5D, H and L).

The densitometric studies (Fig 5M, N and O) confirmed the observations described above, statistically evidencing the reduction of NF-kB and COX-2 in lung inflammation as well the increase of endogenous AnxA1 after treatment with PL.

4. Discussion

Initially, physiological data related to weight and lung parameters were analyzed and, as expected, weight loss occurred especially in

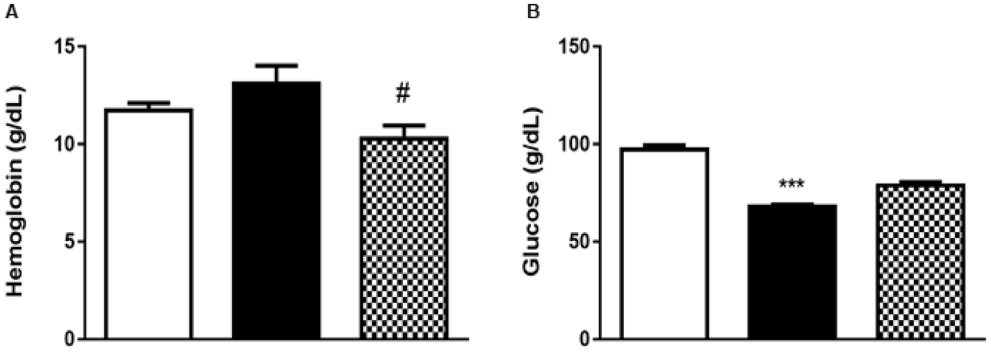

Fig. 2. Biochemical blood tests - **[A]** Level of hemoglobin in whole blood per g/dl and **[B]** glucose measurement in blood plasma per mg/dL. Groups: (C) control, (CS) untreated exposed to smoke and (CS + PL) treated exposed to smoke. The results were presented as mean ± S.E.M. (n = 6/group) and determined by *Kruskal-Wallis* followed by *Dunn*.

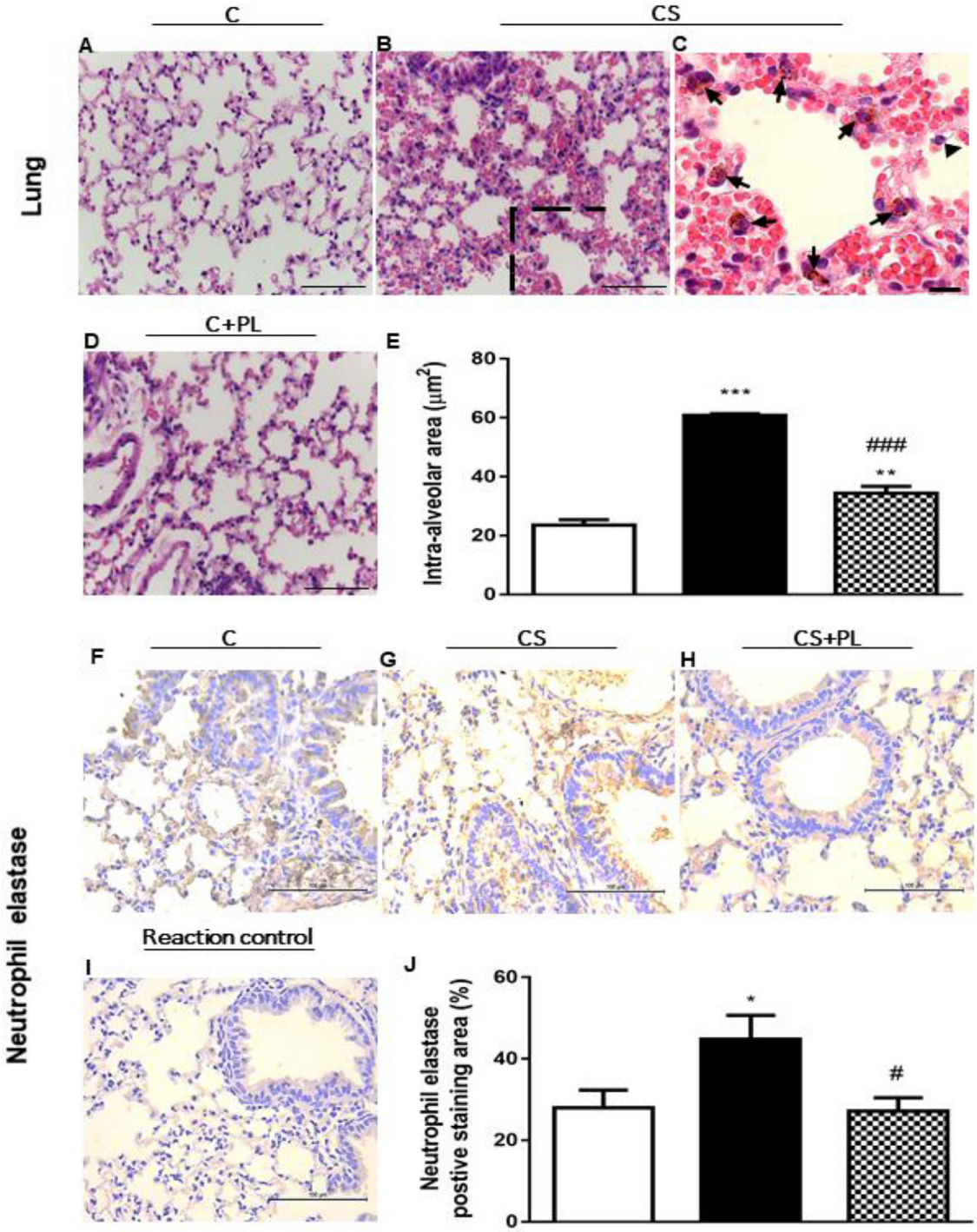

Fig. 3. Structural analyzes of the lung. Histopathological [A] Control with normal appearance. **[B]** untreated exposed to smoke showing increase in inter-alveolar septum thickness and influx of inflammatory cells. **[C]** Details of figure B show alveolar macrophages (arrows) and neutrophil (arrowhead). **[D]** PL preserves lung structures and reduces inflammatory cells. Color: Hematoxylin-Eosin. **Immunohistochemistry**: **[F]** elastase expression in the control. **[G]** increase in the untreated group. **[H]** reduction after PL treatment. **[I]** absence of immunostaining in the reaction control. Countercoloration: Hematoxylin-Eosin. Groups: (C) control, (CS) untreated exposed to smoke and (CS + PL) treated exposed to smoke. Bars: 100 μm (F–I), 50 μm (A, B and D) and 20 μm (C). **[E] Morphometric of the intra-alveolar area**. [**J] Percentage of neutrophil elastase expression**. The results were presented as mean ± S.E.M. (n = 6/group) and determined by *one-way anova* followed by *Bonferroni* or *Tukey's post hoc.*

untreated animals. Data from the literature show that malnutrition often occurs in obstructive pulmonary disease, with difficulty in chewing and swallowing due to dyspnoea, coughing and fatigue, factors that may lead to inadequate food intake and, consequently, weight loss [33]. Another mechanism related to malnutrition is the increased energy expenditure, which leads to a decrease in respiratory performance due to depletion of muscle proteins [14,21,55]. In addition, studies have suggested that increased inflammatory mediators may alter the metabolism of leptin in patients with COPD, contributing to weight loss [9,50]. Leptin, a protein synthesized by adipose tissue, plays an important role in energy metabolism. This hormone represents a signal to the brain and peripheral tissues regulating food intake, basal energy

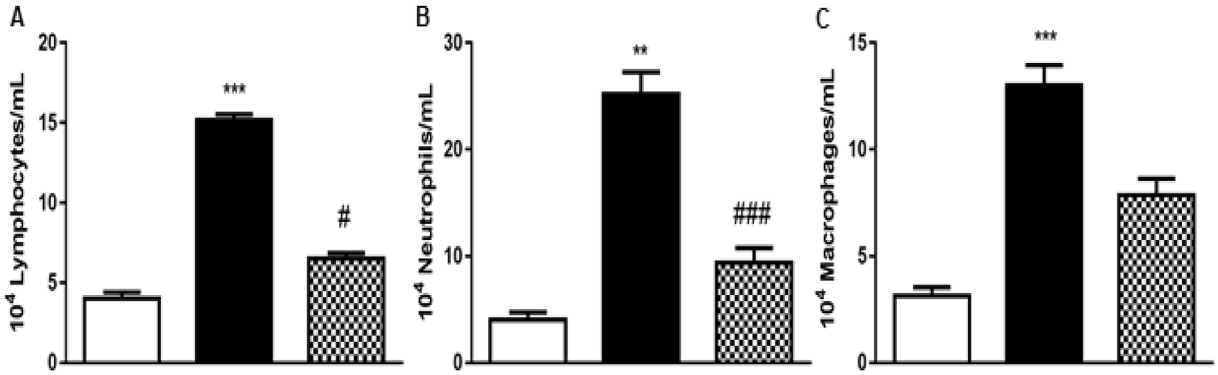

Fig. 4. Quantitative analysis of LBA - **[A]** quantification of lymphocytes, **[B]** neutrophils and **[C]** macrophages. Significant increase of these cells in animals without treatment and significant reduction of lymphocytes and neutrophils after PL treatment. Groups: (C) control, (CS) untreated exposed to smoke and (CS + PL) treated exposed to smoke. The results were presented as mean ± S.E.M. (n = 6/group) and determined by *Kruskal-Wallis* followed by *Dunn* or o*ne-way anova* followed by *Bonferroni.*

expenditure and body weight.

In untreated animals the respiratory volume was increased during the period of exposure to cigarette smoke, reducing significantly after administration of PL. Our physiological analyzes reinforce the harmful effects and comorbidities that accompany the smoking habit [6,18,45,53]. With these considerations, our data show that after cigarette exposure and PL administration, lung weight and ventilation were similar to the control group, demonstrating the efficacy of treatment against the systemic actions of smoking exposure.

Smoking is associated with elevated levels of Hb, which occurs due to the high affinity of carbon monoxide, resulting from the burning of tobacco, that binds to Hb and produces carboxyhemoglobin. This association makes the binding site for the oxygen molecule unavailable by decreasing its concentrations in the blood. Thus, to compensate for this reduction, smokers present increased levels of hemoglobin [29]. In our study, biochemical measurements of blood plasma showed that in untreated animals exposed to smoke, hemoglobin (Hb) levels were increased confirming observation made in smokers, Incontrast, treatment with PL reduced significantly the levels of Hb suggesting an ameliorative effect of PL in carbon monoxide levels. In glucose levels, analyzed biochemically, reduction occurred mainly in the group without treatment, compared to the controls. These results corroborate investigations by Montaño and colleagues [31] who observed a reduction of glucose in rats exposed to smoking.

Histopathological analysis of the lung evidenced an influx of inflammatory cells (mainly macrophages and leukocytes), increased thickness of interalveolar septa and intra-alveolar spaces in animals exposed to untreated cigarette smoke compared to controls. Under these conditions occurs modification of normal cellular physiology such as airway remodeling, excessive oxidative insults and inflammatory mediators [39]. Remodeling promotes thickening of the basement membrane due to deposition of extracellular matrix proteins, mucosal hypersecretion of mucus including MUC5AC mucin by goblet cells and hyperplasia of these cells, resulting in airflow limitation and decline in lung function, in addition to increased recruitment of neutrophils [23,49]. The degeneration that occurs may be related to the imbalance between proteases that destroy the pulmonary parenchyma and antiproteases that inhibit the action of these proteolytic enzymes and may cause pulmonary emphysema [25].

Our findings also reinforce the beneficial effects of PL on the lung tissues analyzed in relation to the inhibitory activity of the neutrophil elastase enzyme. This enzyme is a serine protease capable of cleaving elastin, a collagen-like connective tissue protein that helps in structural formation around the alveoli and airways, allowing the lung to expand in all directions without developing excess of retroceded tissue. Our data show the ability of PL in reducing lesions promoted by elastase, confirming its action in the preservation of the pulmonary parenchyma. These data are important and corroborate with investigations that showed a high increase of this enzyme in the lung tissue of mice exposed to cigarette smoke [28]. Moreover, in patients with cystic fibrosis, the increased activity of this enzyme is correlated with airflow limitation, hyperinflation and severity of lung disease [13].

Our results also revealed that exposure to cigarette smoke and absence of PL treatment leads to an intense inflammatory response. In contrast PL treatment suppressed the cell migration capacity observed by the reduction of inflammatory cells in the bronchoalveolar lavage (BAL).

Neutrophils, macrophages and epithelial cells are activated in the airways of patients with COPD producing large amounts of reactive oxygen species (ROS) during oxidative stress, which leads to the oxidation of arachidonic acid, a lipid present in cell membranes, which can be transformed into prostaglandin by the action of COX-2 [24]. Our results confirmed that PL significantly inhibited the expression of COX-2, confirming its action as a substance able to regulate the synthesis or activity of this enzyme. Studies have shown that increased expression of COX-2 is associated with the high degree of inflammation in COPD [7,30]. Han et al. [20] also showed that PL regulated the expression of COX-2 by inhibiting nuclear factor kappa B (NF-kB) and reducing IL-6 production in a dose-dependent manner in tumor cells. In addition, the investigations showed that the anti-inflammatory activity of *Camellia sinensis* (green tea) was mediated by the reduction in expression of COX-2, prostaglandin 2 and overexpression of AnxA1 [27].

To better understand the role of PL in pulmonary inflammation, we evaluated the expression of NF-kB, a key regulator in the inflammatory response. Our data suggest that systemic administration of PL acts on important members of the inflammatory cascade, demonstrated by the reduction of NF-kB expression in lung tissue. When triggered, many intracellular signaling pathways, such as NF-kB, lead to the release of cytokines and inflammatory mediators. In the cytoplasm the transcription factor is inactive because it is bound to its inhibitor (IkB). After a suitable stimulus, the IkB is phosphorylated by the action of specific kinase proteins (IKK), thereby releasing NF-kB, which translocates to the nucleus promoting gene transcription [43]. The expression and activation of NF-kB are increased in bronchial biopsies of smokers and COPD patients, which is correlated with airflow limitation [42]. In an animal model, exposure to cigarette smoke increased expression of this factor in lung tissue, which was reduced after treatment with Asian acid, a terpenoid extracted from *Centella asiatica* [26]. In fact, previous studies of our group have also shown increased expression of NF-kB in rats exposed to cigarette smoke and reduced immunostaining after treatment with herbal mixture [40]. Again, our data suggest that systemic administration of PL acts on important members of the inflammatory cascade, demonstrated by the reduction of NF-kB expression in lung tissue. Don Ju Son and colleagues [48] also showed that PL

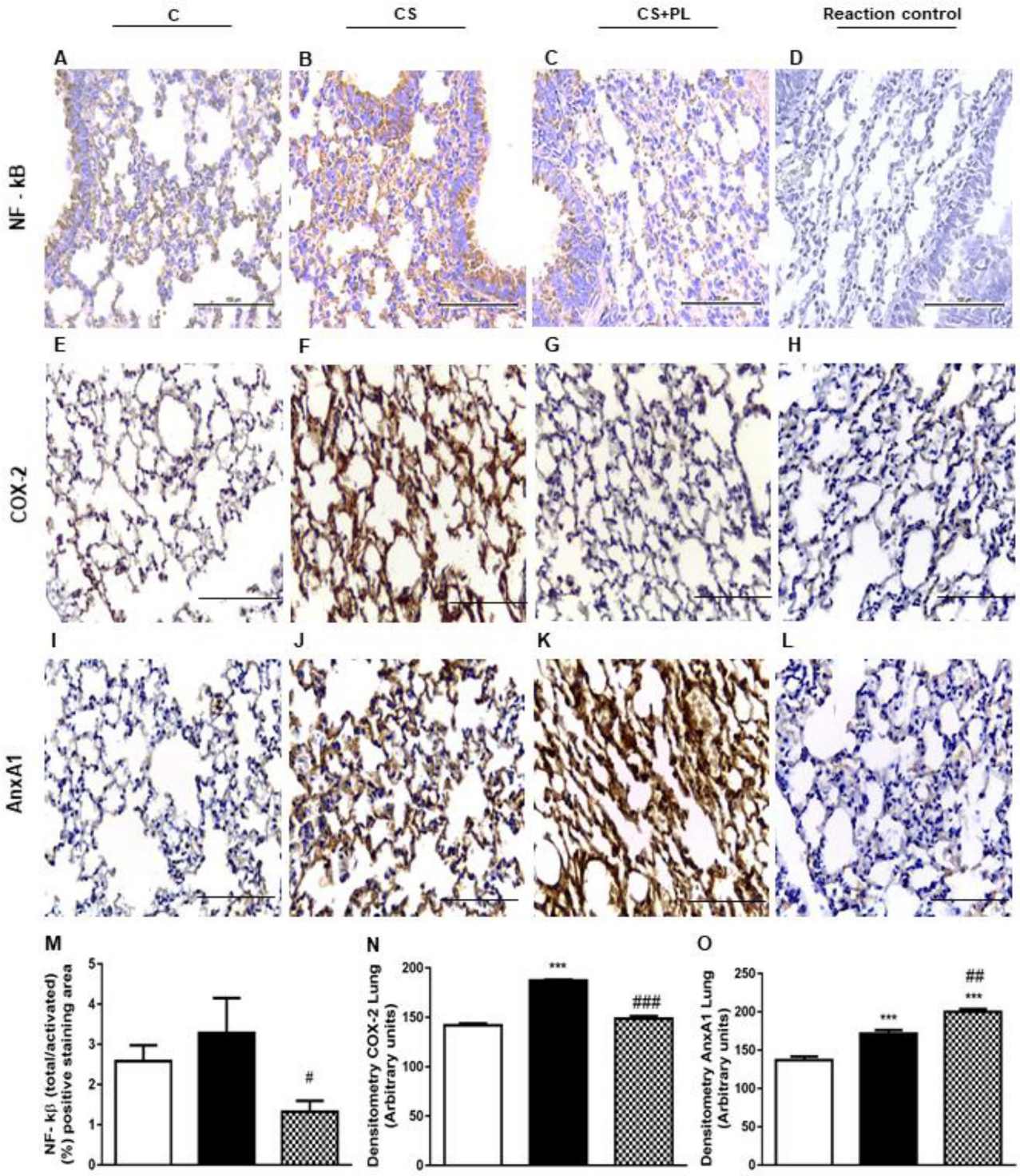

Fig. 5. Expressions of the mediators NF-kB, COX-2 and AnxA1 in lung tissue: [A, E and **I]** expression of NF-kB, COX-2 and AnxA1 in the control groups. **[B, F** and **J]** increase in untreated groups. **[C, G]** reduction of NF-kB and COX-2 expression. **[K]** increase of anxA1 after treatment with PL. **[D, H** and **L]** absence of immunolabeling in the controls of the reactions. Groups: (C) control, (CS) untreated exposed to smoke and (CS + PL) treated exposed to smoke. Contra-staining: Hematoxylin. Bars: 50 μm **[M] Percentage of neutrophil elastase expression**. **[N** and **O] Densitometry COX-2 and AnxA1.** The results were presented as mean ± S.E.M. (n = 6/group) and determined by o*ne-way anova* followed by T*ukey's post hoc* or *Bonferroni.*

inhibited the activation of NF-kB in atherosclerotic lesions, and Han et al. [20] evaluated their action on several cell lines induced by different stimuli, including cigarettes, and showed that PL inhibits the phosphorylation of Ikβ, preventing the activation of NF-kB.

Finally, immunohistochemical studies were conducted to investigate whether PL modulates expression of AnxA1 and, consequently, affects inflammatory responses. The results confirmed that PL significantly increases the expression of the endogenous protein AnxA1, indicating its participation in tissue preservation and in the control of inflammation. Using Balb/c mice induced to pleurisy, Vago et al. [52] demonstrated that treatment with wortmannin, an anti-inflammatory phosphatidylinositol 3-kinase inhibitor (PI3K), increased

expression of the AnxA1 protein, reduced neutrophil number in the cavity pleural and induced cellular apoptosis.

The pharmacological effects of AnxA1 and its mimetic peptides have been explored in several investigations in inflammatory processes including our laboratory [12,15,17]. However, there are no reports of the expression of AnxA1 after treatment with PL in a respiratory inflammatory model favoring or attenuating its effects.

The importance of AnxA1 was inferred in a model of pulmonary fibrosis using AnxA1 $^{-/-}$ animals, which developed more intense inflammation, with increased dosages of IFN-g, TNF-α and TGF-β1 and formation of fibrotic tissue in the lung compared to wildtype animals [11]. This study highlights the importance of endogenous AnxA1 for the control of the fibrotic process. Other investigations have also shown an increase in the expression of AnxA1 in acute-phase inflammatory processes in several experimental models experimentais [10,35,40]. Similarly, we observed in our experimental model an increase of AnxA1 concomitant with the reduction of COX2, suggesting the anti-inflammatory role of PL through the modulation of AnxA1.

5. Conclusions

Together, our results show the efficiency of the experimental model, which promoted important systemic and inflammatory pulmonary alterations that were minimized by the administration of PL and modulation of the endogenous protein AnxA1. In addition, our data point to anti-inflammatory actions of PL in the preservation of the physiological, biochemical and structural characteristics of the lungs as a possible therapeutic strategy for respiratory inflammations.

Author contributions

M.S. performed all experiments and wrote the manuscript. H.R.S. and L.P. contributed with histological studies. Y.V. contributed with immunohistochemical analysis. M.L.C contributed with statistical analyzes. A.P.G. designed, provided mice, coordinated the project, and contributed to write the manuscript. S.M.O. designed, coordinated the project, and contributed to write the manuscript.

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