

A plant proteinase inhibitor from *Crataeva tapia* (CrataBL) attenuates elastase-induced pulmonary inflammatory, remodeling, and mechanical alterations in mice



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ABSTRACT

Chronic obstructive pulmonary disease (COPD) can lead to chronic and obstructive bronchitis and emphysema resulting in decreased bronchial lumen size.

This study evaluated the effect of CrataBL, a protein isolated from the bark of *Crataeva tapia*, on lung mechanics, inflammation, and remodeling after elastase-induced pulmonary alterations in mice.

The use of CrataBL led to decreased mechanical alterations, alveolar septum disruption (Lm), number of macrophage and neutrophil cells in the BALF, and TNF- α , MMP-9, MMP-12, TIMP-1, eNOS, and iNOS positive cells in the airways and alveolar walls compared to the animals in the ELA group. Moreover, a reduction in MUC-5-positive cells in the airway walls was observed. In conclusion, CrataBL attenuates changes in lung mechanics, inflammation, extracellular lung remodeling, and oxidative stress responses induced by the administration of elastase and decreased the volume fraction of isoprostane, collagen, and elastic fibers in the airways and alveolar walls compared to the animals in the ELA groups. Therefore, CrataBL is a potential therapeutic tool in the treatment of COPD.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory disease that can induce chronic changes in the bronchi (chronic and obstructive bronchitis) and lung parenchyma (emphysema) leading to structural changes and decreased bronchial lumen size. The destruction of lung parenchyma causes the loss of alveolar engagement in the small airways and decreases lung elastic recoil [1,2].

Inflammation affects the large and small airways resulting in goblet cell hyperplasia, increased mucous glands, and infiltration

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of neutrophils, macrophages, and CD8-positive lymphocytes in the upper airways, especially in the later stages of COPD. Inflammatory infiltrates are also found in small airways, with a predominance of macrophages in the early stages of the disease [3]. Activated macrophages release IL-8, LTB₄, tumor necrosis factor α (TNF- α), monocyte chemotactic peptide (MCP1), and enzymes such as elastase, cathepsins (B, K, L and S), and matrix metalloproteinases (MMP-2, -9 and -12) that act as mediators and effectors of lung injury [4]. MMPs selectively degrade components of the extracellular matrix and play a crucial role in the trafficking of inflammatory cells. Imbalance in these enzymes and their inhibitors leads to diseases resulting from the destruction of connective tissue [2,5,6] and stimulate the search for alternative compounds, particularly those from natural sources, which block uncontrolled proteolytic activity and may suppress the cellular signaling pathway activation [7–11]. The increasing number of deaths [2] caused by pulmonary diseases and the economic burden from treatments and loss of productivity in affected patients vindicate the search for qualified therapeutic drugs or biomarkers for early diagnosis [12]. CrataBL is

a bifunctional glycoprotein isolated from the bark of *Crataeva tapia*, which presents lectin activity and inhibits trypsin (K_{iapp} 43 μ M) and the factor Xa (K_{iapp} 8.2 μ M). CrataBL does not interfere in the activity of human plasma kallikrein, tissue kallikrein, human neutrophil elastase, plasmin, or chymotrypsin [11]. However, previous studies have shown that CrataBL binds to glycosaminoglycans (negatively charged compounds bound to cell surfaces) [13,14], particularly to heparin [15], and is effective in neutralizing heparin's anticoagulation activity [11,16].

This study evaluated the effect of CrataBL in elastase-induced pulmonary inflammatory, remodeling, and mechanical alterations in mice with emphysema. We evaluated respiratory mechanics and inflammatory, remodeling, and oxidative stress markers in airways and alveolar walls.

2. Materials and methods

2.1. Plant protein

CrataBL was purified following the methodology described by Ferreira et al. [11]. The concentration of the lectin/inhibitor used was determined by the Lowry assay; the dose used (2 mg/kg) was the same as that reported for BbCl, another proteinase inhibitor [17].

2.2. Animals

Thirty-two C57Black/6 young adult male mice (20–25 g) obtained from the University of São Paulo were used in the study. The 6–8 weeks old animals were held in the laboratory vivarium for 2 weeks prior to the study start. The study was approved by the Ethics Committee for Analysis of Research Projects (CAPPesq) of the Clinical Hospital and Faculty of Medicine at the University of São Paulo. The animals were handled in accordance with the guidelines for the care of laboratory animals [18].

2.3. Animal model

The animals were randomly divided into four groups. 1. Elastase group (ELA) – The animals were anesthetized with isoflurane and the neck region was shaved and disinfected with povidone where an incision of approximately 0.5 cm was made in the mid-region to expose the trachea. Using a 30-unit syringe, a single 50 μ l dose of 0.025 mg of porcine pancreatic elastase (Sigma, CA, USA) was injected directly into the trachea between the cartilaginous rings using a 30-unit syringe and a single 50 μ l dose of 0.025 mg of porcine pancreatic elastase (Sigma, CA, USA). The wound was sutured, and the neck area was disinfected with povidone. 2. Elastase group treated with CrataBL (ELA-CrataBL) – CrataBL (2 mg/kg) was injected intraperitoneally (i.p.) one hour after the intratracheal administration of elastase (0.025 mg in 50 μ l). After 14 days, the animals received two doses of CrataBL (2 mg/kg) every seven days ($n=8$). Control group (SAL) – Saline was injected intraperitoneally (i.p.) one hour after the intratracheal administration of saline (50 μ l). After 14 days, the animals received two doses of saline every seven days ($n=8$). Control group treated with CrataBL (SAL-CrataBL) – CrataBL (2 mg/kg) was injected intraperitoneally one hour after the intratracheal administration of saline (50 μ l). After 14 days, the animals received two doses of CrataBL (2 mg/kg) every seven days ($n=8$).

2.4. Mechanical evaluation

Twenty-eight days after the study start, the animals were anesthetized with thiopental (250 mg/kg i.p.) and tracheotomized. A metal cannula was inserted through the tracheostomy hole and

fixed with cotton thread around the trachea. The animals were connected to a mechanical ventilator for small animals (*FlexiVent*, *Scireq*, Montreal, Canada) and ventilated with a tidal volume of 10 ml/kg at a respiratory rate of 120 cycles/min and inspiratory flow with a sine curve. To induce skeletal muscle relaxation during ventilation, the animals received 0.2 mg/kg pancuronium intraperitoneally. Data recording began when the animal was still. With the expiratory valve in the closed position, signal producing oscillations of different frequencies of airflow material (0.25 to 19.625) for 16 s were applied. The desired pressure values were obtained and impedance in the airway (pressure/flow) was calculated as a function of different frequencies.

Using a pop-up signal of 75% in 16 s, 3 blocks of 8 s were used to calculate the parameters of mechanical oscillation (i) according to the equation:

$$Z(f) = \text{Raw} + i(2\pi f)l_{aw} + \frac{[Gtis - iHtis]}{(2\pi f)^\alpha}$$

In this model, $Z(f)$ is the impedance of air as a function of frequency, i is the imaginary unit ($-1/2$), f is the frequency, l_{aw} is the inertance of airways, and $\alpha = (2/\pi) \times \arctan(Htis/Gtis)$. The obtained parameters were Raw (airway resistance), Gtis (lower airway or tissue resistance), and Htis (lung tissue elastance).

2.5. Determination of exhaled nitric oxide (E_{NO})

During the mechanical ventilation, gas was collected at the expiratory portion of the fan through a balloon impermeable to NO (Mylar bag, Sievers, Instruments Inc., Boulder, CO, USA) for 5 min. After the collection period, the balloons were sealed for later analysis.

Nitric oxide was measured by a chemiluminescence analyzer (280 NOA – Nitric Oxide Analyzer – Sievers Instruments Inc., Boulder, CO, USA). The average concentration of NO was recorded in parts per billion (ppb) as an index of NO concentration in exhaled air.

2.6. Fluid analysis (BALF)

BALF was performed by injecting 1.5 ml of saline (3×0.5 ml) through a tracheal cannula. The total number of cells obtained in the lavage was determined using a *Neubauer* chamber. The cells were differentially counted using a cytospin preparation followed by staining with *Quick-Stain* reagent. Differential cell counts were performed by evaluating more than 300 cells in an optical microscope.

2.7. Morphometric study

The animals were exsanguinated, the chest was opened, and the heart and lungs were removed *en bloc*. The lungs were fixed with 4% formaldehyde at a constant pressure of 20 cm H_2O for 48 h to homogenize the distension of the pulmonary parenchyma and subsequently stored in 10% formalin for up to 7 days. Lungs were cut on the long axis and subjected to histological processing; slides were stained with Hematoxylin–Eosin, Resorcin–Fuchsin (for the analysis of elastic fibers), and Picrosirius (for the analysis of collagen fibers). Additional slides were also prepared for immunohistochemical staining for the analyses on expression of MMP-9, MMP-12, TIMP-1, MUC-5, iNOS, eNOS, and 8-iso-PG2F α .

The morphometric analysis was performed in an optical microscope using a lattice of lines and points (50 lines and 100 points) according to the technique of point counting [19]. We analyzed 20 fields of lung parenchyma. The results were expressed as percentages of positive areas (volume ratio) [20].

Table 1
Bronchoalveolar lavage fluid analysis.

Bronchoalveolar lavage	Groups (n = 8)			
	SAL	ELA	SAL-CrataBL	ELA-CrataBL
Total cells (cells/10 ⁴ μm ²)	0.5 ± 0.2	2.8 [†] ± 0.4	0.7 ± 0.1	1.5 ^{**} ± 0.1
Macrophages (cells/10 ⁴ μm ²)	0.63 ± 0.04	1.47 ± 0.2	0.7 ± 0.1	1.46 ± 0.09
Neutrophils (cells/10 ⁴ μm ²)	0.0007 ± 0.0007	0.50 [†] ± 0.17	0.003 ± 0.001	0.04 ± 0.01
Lymphocytes (cells/10 ⁴ μm ²)	0.0017 ± 0.0007	0.26 [†] ± 0.009	0.003 ± 0.001	0.02 ± 0.004
Eosinophils (cells/10 ⁴ μm ²)	0.0000 ± 0.0000	0.05 [†] ± 0.001	0.005 ± 0.001	0.001 ± 0.005

Mean and standard error values for the BALF analysis in the four experimental groups.

[†] p < 0.05 compared to other groups.

^{**} p < 0.05 compared to the SAL and SAL-CrataBL groups.

The reticle was coupled, adjacent to the wall of the airway from the base of the epithelium, for the evaluation of airways. Three to five airways were randomly selected for evaluation in each animal, and approximately three fields per airway were assessed. This technique was used to count neutrophils, positive cells for TNF-α, MMP-9, MMP-12, TIMP-1, MUC-5, eNOS, and iNOS, and to evaluate the volume fraction of isoprostane, collagen, and elastic fibers.

2.8. Mean alveolar diameter (Lm)

The alveolar diameter analysis was performed using a microscope with an appropriate reticulum to calculate the mean alveolar diameter. The initial area of the lung parenchyma was determined, excluding vessels and airways. A lattice with 50 straight (100 points) lines was placed over the lung parenchyma area, and point intersections were counted in the alveolar wall. The mean alveolar diameter was calculated according to the ratio between the lung parenchyma area and number of intersections between the lines and lung parenchyma.

2.9. Immunohistochemistry

Lung sections were deparaffinized and rehydrated. Step 1 – *Recovery of Antigen*: Sections were treated with Proteinase K for 20 min (37 °C) followed by 20 min at room temperature; slides were washed in PBS. Step 2 – *Blocking and Incubation with Primary Antibody*: Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide (10 v% H₂O₂) (3 × 10 min). This was followed by incubation with primary antibodies, which were applied to sections of experimental and control tissues (positive and negative); slides were incubated overnight. Step 3 – *Incubation with Secondary Antibody and Peroxidase Complex*: The slides were washed in PBS and incubated with secondary antibody using an ABC Kit by Vectastain (Vector Elite-PK-6105 (anti-goat)/PK-6101 (anti-rabbit)). Step 4 – *Visualization*: The slides were washed in PBS and proteins were visualized using 3,3 diaminobenzidine chromogen (DAB) (Sigma

Chemical Co., St. Louis, MI, USA). Step 5 – *Counter-Staining and Mounting of Slides*: The slides were thoroughly washed in tap water and counter-stained with *Harris Hematoxylin* (Merck, Darmstadt, Germany); slides were subsequently washed in water, dehydrated, diaphanized, and mounted using the Entellan microscopy resin (Merck, Darmstadt, Alemanha).

The following primary antibodies were used: anti-mouse macrophage Mac2 (Cedarlane Lab, Ontario, Canada, 1:60,000); anti-mouse neutrophils (AbD Serotec, Kidlington, UK; 1:400); anti-mouse MMP-9 (Santa Cruz Biotechnology, CA; 1:500); antibody anti-mouse MMP-12 (Santa Cruz Biotechnology, CA; 1:100); anti-iNOS (LabVision, NeoMarkers, California; 1:500); anti-eNOS (LabVision, NeoMarkers, CA; 1:100); anti-TIMP-1 (LabVision, NeoMarkers, CA; 1:400); anti-TNF (Santa Cruz Biotechnology, California, U.S.; 1:900); anti-isoprostane-8 (Oxford Biomedical Research, Oxford, United Kingdom; 1:10,000), and anti-MUC-5 (LabVision, NeoMarkers, CA; 1:400).

2.10. Data analysis

Statistical analysis was performed using the *SigmaStat* software (SPSS Inc., Chicago, IL, USA). Multiple comparisons were made by *One-way ANOVA*. The *Holm-Sidak* test was used for comparisons between groups. Data are presented as mean ± standard error and depicted in bar graphs with error bars. Significance was considered at p < 0.05.

3. Results

3.1. Lung mechanics

The values of respiratory system elastance (Ers) in all experimental groups are shown in the Fig. 1A. Ers was significantly higher, as expected, in the ELA group compared to the SAL group (53.6 ± 6.3 cm H₂O ml⁻¹ vs 37.0 ± 3.4 cm H₂O ml⁻¹, respectively; (p < 0.05); Ers was 60.8% attenuated by the CrataBL treatment.

Table 2
Effect of CrataBL treatment on positive cells in inflammation and lung remodeling in alveolar walls.

	Groups (n = 8)			
	SAL	ELA	SAL-CrataBL	ELA-CrataBL
Inflammatory response				
Macrophages (cells/10 ⁴ μm ²)	11.75 ± 0.75	22.88* ± 2.24	7.86 ± 0.38	9.58** ± 1.36
Neutrophils (cells/10 ⁴ μm ²)	0.43 ± 0.05	1.18* ± 0.15	0.57 ± 0.06	0.75** ± 0.1
TNF-α positive cells (cells/10 ⁴ μm ²)	3.11 ± 0.13	12.52* ± 0.42	4.83 ± 0.16	10.41** ± 0.49
Extracellular remodeling markers				
Collagen fibers (%)	9.4 ± 0.1	11.5* ± 0.11	8.8 ± 0.8	10.8** ± 0.13
Elastic fibers (%)	0.3 ± 0.03	0.5* ± 0.03	0.3 ± 0.3	0.3** ± 0.02
MMP-9 positive cells (cells/10 ⁴ μm ²)	6.65 ± 0.70	18.59* ± 1.8	7.12 ± 0.71	10.35** ± 0.65
MMP-12 positive cells (cells/10 ⁴ μm ²)	10.12 ± 0.83	20.17* ± 1.92	7.49 ± 0.63	14.15** ± 0.59
TIMP-1 positive cells (cells/10 ⁴ μm ²)	3.63 ± 0.59	14.42* ± 2.05	5.73 ± 1.95	9.89** ± 2.79

Data are presented as the mean ± SEM.

*p < 0.05 or **p < 0.05 represent significant differences compared to controls.

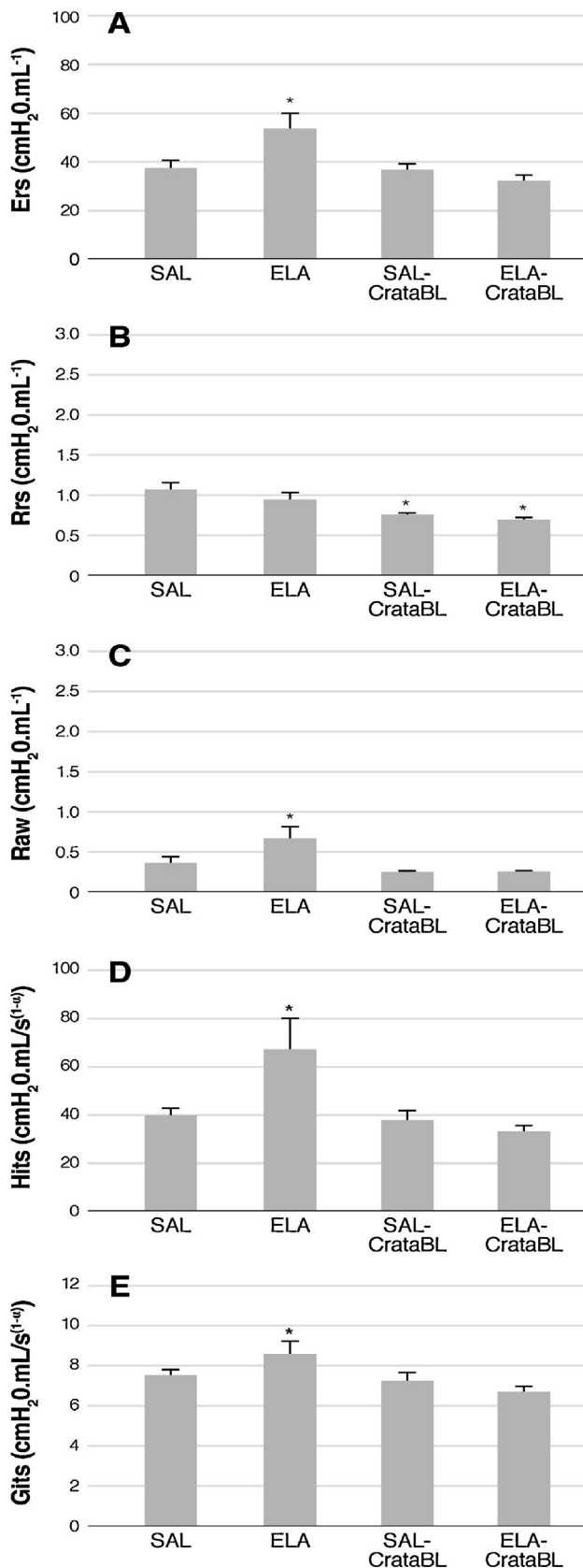


Fig. 1. Mechanical evaluation. The animals were anesthetized with thiopental (250 mg/kg i.p.) and tracheotomized twenty-eight days after the study start; the procedure was performed as described in methods. (A) Respiratory system elastance (Ers), (B) Respiratory system resistance (Rrs), (C) airway resistance (Raw), (D) lung tissue elastance (Htis) and (E) Lung tissue resistance (Gtis). A significant increase of the values in ELA group and a significant decrease ($*p < 0.05$) in all ELA-CrataBL groups was observed.

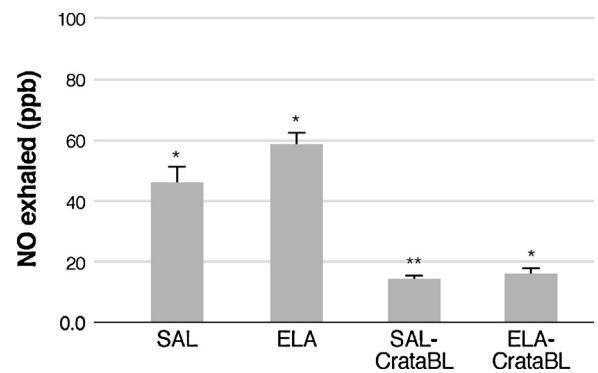


Fig. 2. Determination of exhaled nitric oxide (E_{NO}). Exhaled NO was collected in the four experimental groups 28 days after treatment. The animals treated with CrataBL (SAL-CrataBL and ELA-CrataBL) showed a significant reduction ($**p < 0.05$ and $*p < 0.05$) in exhaled NO compared to the groups SAL and ELA treated groups.

Besides, no significant difference between SAL and SAL-CrataBL were noticed. The values of mechanically ventilated resistance (Rrs) (Fig. 1B) did not differ between the CrataBL groups, but were decreased either among the SAL or ELA groups. Considering the effect of Raw on airway resistance (Raw) shown in Fig. 1C, the increase of Raw provoked by elastase was reduced by the inhibitor to the control level. The same profile was observed in Fig. 1D for lung tissue elastance (Htis) and in the Fig. 1E in the lung tissue damping (Gtis) parameters, which significantly decreased in the ELA-CrataBL group compared to the ELA group ($p < 0.05$).

The Exhaled nitric oxide (E_{NO}) levels are presented in Fig. 2. We observed that E_{NO} levels were lower in the CrataBL groups (around 31% and 73.0%) than in the untreated SAL and ELA groups, respectively.

Table 1 shows the results from the bronchoalveolar lavage fluid analyses. A significant increase in the total bronchoalveolar lavage cell count was observed in the ELA group compared to the control groups (SAL and SAL-CrataBL, $p < 0.05$). ELA-CrataBL group attenuated the number of total bronchoalveolar lavage cells compared to the ELA group ($p < 0.01$).

There was no difference between the number of macrophages in the ELA and ELA-CrataBL groups. However, the increased neutrophils, lymphocytes, and eosinophils accumulation in the ELA group related to the control groups was attenuated by the inhibitor treatment ($p < 0.05$).

Fig. 3 shows the mean alveolar diameter values (Lm) in all experimental groups. A significant increase in Lm was observed in the ELA group ($69.0 \pm 2.7 \mu\text{m}$) when compared to the control groups; moreover, Lm is significantly lower in the ELA-CrataBL group ($49.9 \pm 3.3 \mu\text{m}$; $p < 0.05$).

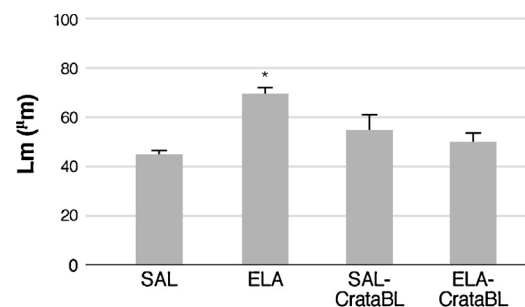


Fig. 3. Alveolar diameter analysis (Lm). Increases in Lm were observed on the 28th after treatment. Mean linear intercept values were measured in the experimental groups. Elastase-induced group shows a significant increase in the values compared to other groups ($*p < 0.05$) decreasing in the ELA-CrataBL group.

Table 3
Effect of CrataBL on inflammatory and extracellular remodeling alteration in airway walls.

	Groups (n = 8)			
	SAL	ELA	SAL-CrataBL	ELA-CrataBL
Inflammatory response				
Neutrophils (cells/10 ⁴ μm ²)	0.23 ± 0.05	0.59* ± 0.10	0.26 ± 0.04	0.46** ± 0.06
TNF-α positive cells (cells/10 ⁴ μm ²)	5.27 ± 0.34	15.82* ± 1.03	6.67 ± 1.49	14.30** ± 1.28
Extracellular remodeling markers				
Collagen fibers (%)	5.72 ± 0.78	8.73* ± 2.59	6.19 ± 1.87	7.80** ± 1.37
Elastic fibers (%)	0.69 ± 0.02	2.56* ± 0.18	1.14 ± 0.11	1.4** ± 0.13
MMP-9 positive cells (cells/10 ⁴ μm ²)	7.39 ± 1.3	14.86* ± 1.77	5.64 ± 0.66	9.93** ± 1.39
MMP-12 positive cells (cells/10 ⁴ μm ²)	12.98 ± 0.59	18.56* ± 1.79	6.99 ± 1.37	12.06* ± 1.15
TIMP-1 positive cells (cells/10 ⁴ μm ²)	0.44 ± 0.09	1.31* ± 0.12	0.55 ± 0.07	0.73** ± 0.05
MUC-5 positive cells (cells/10 ⁴ μm ²)	1.31 ± 0.95	7.09* ± 1.71	1.01 ± 0.42	3.56** ± 0.54

Data are presented as the mean ± SEM * or ** represent statistically significant differences at $p < 0.05$, respectively compared to controls (saline and elastase).

3.2. Inflammatory and remodeling responses in the alveolar and airway walls

The inflammatory and remodeling indicators of alterations in alveolar walls presented in Table 2 showed that the numbers of macrophages, neutrophils, TNF-α, MMP-9, MMP-12, and TIMP-1 positive cells, and the volume fraction of collagen and elastic fibers in the pulmonary parenchyma, significantly increased in the ELA groups compared to controls. The ELA-CrataBL group showed significantly reduced values in all parameters compared to the ELA group ($p < 0.05$).

Table 3 shows inflammatory and remodeling indicators of alterations in the airways. The numbers of neutrophils, TNF-α, MMP-9, MMP-12, TIMP-1, and MUC-5 positive cells, and volume fraction of collagen and elastic fibers significantly increased in the airways in the ELA group compared to the control (SAL, SAL-CrataBL groups; $p < 0.001$). The ELA-CrataBL group showed significantly decreased values in all parameters compared to the ELA group ($p < 0.05$).

3.3. Evaluation of the oxidative stress in the alveolar and airway walls

Fig. 4 shows the graphic and numbers of eNOS- (A); iNOS-positive cells (B), and volume fraction of isoprostane (C) in alveolar walls. The significant increases in eNOS- and iNOS-positive cells, and in the volume fraction of isoprostane observed in the ELA group compared to controls were suppressed by the CrataBL treatment ($p < 0.001$) in all comparisons. Similarly, the increased values of those parameters in the airways (Fig. 5) in the ELA group showed an important effect attenuation in the ELA-CrataBL groups in relation to the ELA groups ($p < 0.001$).

4. Discussion

The involvement of proteinases in the pathophysiology of emphysema is established [5]. However, there is still controversy about the type of proteinases and their specific functions that contribute to the development and progression of emphysema. Recently, new therapeutic strategies for the treatment of COPD have been investigated aiming at the control of events related to the activation of proteinases. The importance of a proteinase-anti-proteinase balance in pulmonary emphysema is confirmed in patients with deficiency in α1-antitrypsin (an inhibitor of neutrophil proteinase) who show destruction in the lung parenchyma [1]. In this study, the natural compound CrataBL was applied in mice with emphysema resulting in a reduction in the respiratory system resistance (Rrs) in animals in the ELA-CrataBL group, regardless of the intratracheal elastase administration. This indicates a bronchodilator effect of the CrataBL proteinase inhibitor, which may be tested in experimental models of asthma. In addition, the Raw

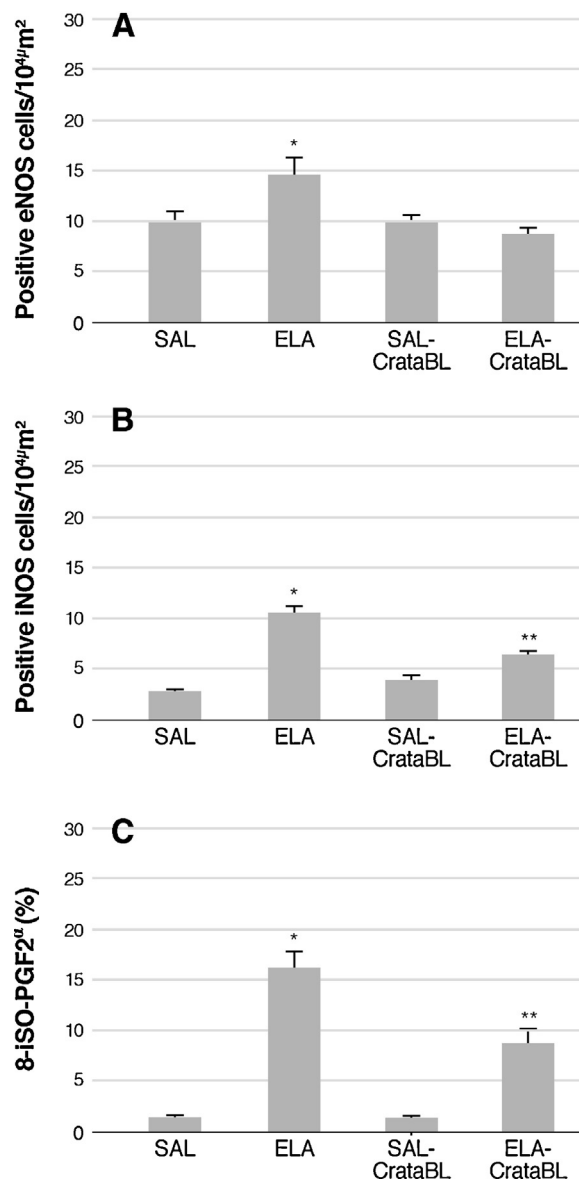


Fig. 4. Oxidative stress: Lung tissue from mice in the elastase-inflamed groups treated with saline and crataBL (SAL-CrataBL and ELA-CrataBL) for 28 days. Mean and standard error of (A) eNOS- and (B) iNOS-positive cells in the experimental groups. * $p < 0.001$ compared to the other groups. (C) Isoprostane volume fraction mean values and standard error, in alveolar walls, in all experimental groups. * $p < 0.05$ compared to other groups.

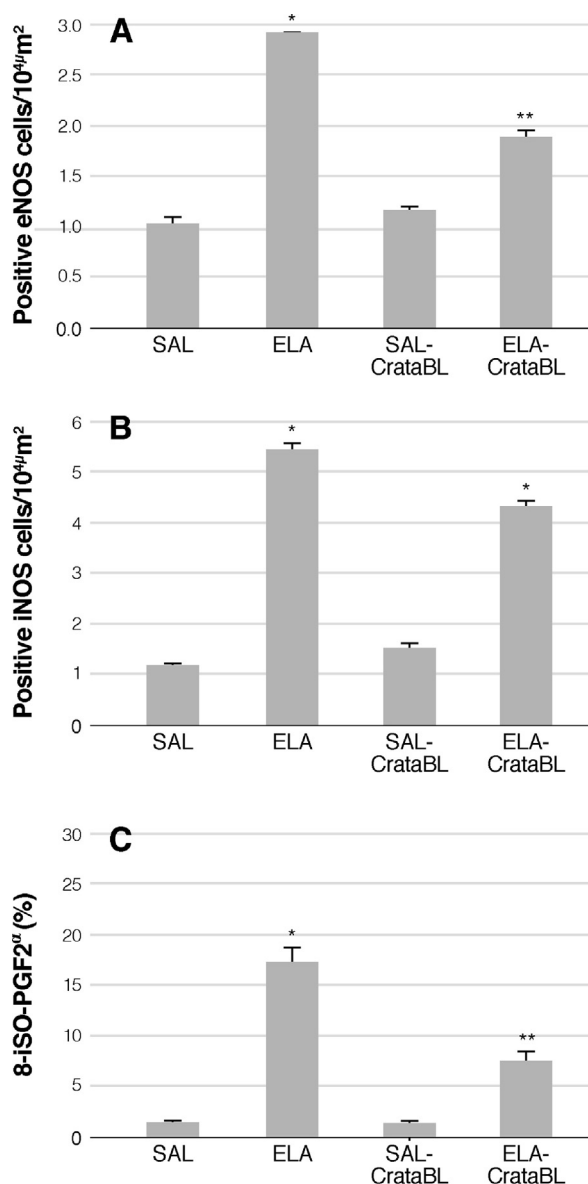


Fig. 5. Oxidative stress in the airways of mice in the elastase-inflamed groups treated with saline and CrataBL (SAL-CrataBL and ELA-CrataBL) for twenty-eight days. Mean and standard error of (A) eNOS-, (B) iNOS-positive cells and (C) isoprostane volume fraction in the experimental groups. The ELA groups showed significantly increased values ($*p < 0.001$) compared to the other groups and attenuation ($*p < 0.05$) of these values were observed in the ELA-CrataBL groups compared to the ELA groups.

values that were enlarged in the ELA group, diminished by 57.1% in the ELA-CrataBL group, which suggests that the airway constriction, caused by intratracheally administered elastase, was controlled by the CrataBL proteinase inhibitor.

The respiratory system elastance (Ers), which assesses changes in distal airways and lung parenchyma, was increased in the ELA group demonstrating the effectiveness of the experimental model by showing enlargement and destruction of alveoli and changes in distal airways. These results are similar to those observed in humans with COPD, particularly under conditions of severe disease. Thus, the evaluation of inflammation, remodeling, and oxidative stress changes in the airways contributes to the understanding of mechanisms involved in the effects of CrataBL.

Tibboel and Post [21] evaluated pulmonary function in mice, that received the same dose of elastase (0.016 mg) used in our study, and showed increased respiratory rate and tidal volume, decreased

tissue elastance, and enlargement of alveolar spaces, similar to what was observed in our experimental model; furthermore, CrataBL attenuated the destruction of lung parenchyma in mice in the ELA group.

Scuri et al. [22] showed that elastase leads to increased production of bradykinin by activating tissue kallikreins, which, in turn, increases lung resistance and elastance, leading to bronchoconstriction. These authors showed that this response is not reversed by the treatment with a histamine antagonist although it was efficiently blocked by antagonists of the bradykinin B2 receptor, therefore confirming the involvement of the kallikrein-kinin system in the process. In the present study, we showed that CrataBL ameliorate lung function corroborating the results obtained by Scuri showing that the kallikrein-kinin system may be involved in the lung function alterations observed in this model of emphysema.

The inflammatory response assessed by bronchoalveolar lavage fluid analyses showed a significant decrease in the number of neutrophils, lymphocytes, and eosinophils in the ELA-CrataBL group compared to the ELA group indicating an important role of CrataBL as an anti-inflammatory protein. This result is similar to that reported by Neuhof et al. [23] in a model of lung edema caused by neutrophil activation, where the BbCI elastase inhibitor (from the Kunitz family of plant inhibitors) decreased the formation of edema. Moreover, Oliveira et al. [17] using models of inflammation in animals pretreated with BbCI (an inhibitor of HLE and cathepsin G) confirmed the main role of these enzymes in the capturing, rolling, transmigration, and cell adhesion of inflammatory cells. While not directly inhibiting the activity of HLE and cathepsin G, CrataBL may significantly interfere in these processes after binding to heparin [24] and glycosaminoglycans on the cell surface. Heparin potentiates the activity of proteases, mainly cathepsins [25] and plasma kallikrein [26]; therefore, CrataBL may interfere in the generation of pro-inflammatory peptides such as kinins (e.g., bradykinin). The indirect interference in proteolytic activity also relates to the finding that the levels of metalloproteinases are diminished by the treatment with an inhibitor in the present study.

Carl et al. [27] showed that bradykinin increased the release of PMN elastase which, in turn, increased microvascular permeability. The authors concluded that blocking the release of bradykinin could be useful in treating inflammatory diseases such as COPD. Additionally, Bhoola et al. [28] showed that neutrophils have all the components to generate kinins, which are involved in local inflammatory response and activation and migration of inflammatory cells. Hence, there are significant implications concerning the pathological conditions, such as in COPD, in which the activation of neutrophils and kallikrein-kinin occurs, reinforcing the importance of the data obtained in the present study.

Tumor necrosis factor alpha (TNF- α) is one of the inflammatory mediators involved in the pathophysiology of COPD [29]. Because macrophages and/or respiratory epithelial cells produce TNF- α , the anti-inflammatory effect of CrataBL was demonstrated through the detection of a decreased number of macrophages in the ELA-CrataBL group. This effect resulted in a decreased number of TNF- α -positive cells and improved pulmonary function status.

The remodeling response showed increased volume fraction of elastic and collagen fibers in the ELA group. These changes were reversed in the ELA-CrataBL group, most likely associated with the CrataBL neutralizing action over heparin. Therefore, CrataBL is effective in blocking the activation of proteases, including neutrophil elastase, and the breakdown of elastic fibers located within the lung parenchyma.

The evaluation of MMP-9 and MMP-12 showed a significant increase in these enzymes in the lung parenchyma and airways of animals in the ELA group, and a significant decrease in the ELA-CrataBL group corroborating the observed effects of CrataBL in blocking protease activation as mentioned above.

In addition to having high affinity for heparin, cathepsins can activate pro-urokinases, which activate plasminogen to form plasmin. In turn, plasmin mainly activates MMP-2 and MMP-9. Plasmin also activates elastase and further increases metalloproteinases and cathepsin activities. Thus, heparin plays an important role in amplifying the network of proteinase activation in pathophysiological processes [30].

We observed an increase in MMP-9 associated with an increase in its inhibitor TIMP-1, which may result from an attempt to repair damages [31]. The imbalance between MMP-9 and TIMP-1 may interfere with cell migration and tissue repair.

The observed increased number of MUC-5-positive cells in the ELA group may suggest a change in the airway wall that occurs later the development of the disease. However, it is important to consider that mice, unlike humans, do not have true goblet cells [32] and that washing the slides during their preparation may also have contributed to clear airways mucus. Thus, we observed a slight change in relation to the number of MUC-5-positive cells, which is perhaps associated with this stage of the disease or due to the limitations of the techniques used.

We demonstrated that the lung injury induced by intratracheal elastase administration caused an increase in exhaled nitric oxide that was reduced by the CrataBL treatment. CrataBL attenuated the oxidative stress response with a reduced number of eNOS- and iNOS-positive cells in the airway and alveolar walls in mice in the ELA group. Our research group previously demonstrated the important role of nitric oxide in experimental asthma [20,33] with increased cellular expression of iNOS and eosinophils in the distal airways of sensitized animals. Furthermore, our group also showed that nNOS-positive cells, although constitutive, were increased among inflammatory cells (eosinophils and lymphomononuclear) [33].

Regarding the response in the airway and alveolar walls from animals with chronic allergic inflammation, the iNOS inhibitors (1400W or L-NAME) attenuated the response to the antigen challenge. This functional impairment was associated with the control of inflammation, remodeling, and oxidative stress [20,34,35]. The attenuation caused by blocking the response of iNOS production appears to also depend on the modulation of the Rho-kinase pathway [36].

Thus, in the inflammation process, the activation of proteinases leads to increased production of bradykinin and subsequent activation of pro-inflammatory cytokines, which thereby increase the numbers of cNOS- and iNOS-positive cells. NO produced by iNOS and cNOS (eNOS and nNOS) leads to increased exhaled NO, which may have triggered the events observed in this model [37].

As previously mentioned, CrataBL counteracted the effects of heparin. Lakshmi et al. [38] showed that low molecular weight heparin inhibits iNOS in activated conditions of injury. These results support the observed reduction in exhaled nitric oxide, in the ELA group after treatment with the CrataBL inhibitor in our study. Moreover, Prado et al. [20] showed that iNOS inhibition reduces MMP-9, TIMP-1, and TGF-beta in vascular walls of animals sensitized with ovalbumin. All these mediators affect the production of collagen and elastic fibers contributing to extracellular matrix remodeling in airways and vessels. The observed reduction of metalloproteinases by CrataBL in this study and TIMP-1 in a previously reported study [33] could result from the effect of iNOS inhibition.

Surprisingly, a significant reduction in exhaled nitric oxide was also associated with the SAL-CrataBL group. Thus, it is likely that the binding of CrataBL to heparin might affect the activity of all nitric oxide synthases, not only constitutive synthases, but also iNOS that produce large amounts of NO. Moreover, it is interesting to note that there was a significant reduction in respiratory system resistance (Rrs), which indicates the response in the proximal airways in animals in the ELA-CrataBL and SAL-CrataBL groups

regardless of the administration of saline or elastase (Sal-CrataBL and ELA-CrataBL groups). The reduction in Rrs and its consequent bronchodilator effect may have resulted from the effect of CrataBL on iNOS- and eNOS-positive cells. The expression of both enzymes in the airway and alveolar walls was significantly reduced by the CrataBL treatment.

Interestingly, the reduction in isoprostane PGF-2 α by CrataBL, which is due to a decrease in peroxynitrite formation [36,37], indicated that the natural protein protects the cell membrane from lipid peroxidation.

The establishment of a relationship between the different parameters evaluated in this study is difficult in *in vivo* studies. CrataBL may have affected these parameters in a direct or indirect way. It is well known that elastase destroys elastic fibers, and that lung repair depends on the recruitment of inflammatory cells to restore lung function. Because CrataBL reduces lung inflammation, it can consequently modulate its remodeling process. Once neutrophils were activated, they induce the release of oxidative stress and nitric oxide. The reduction in oxidative stress could be due to a reduction in iNOS and/or inflammation. The control of the MMP/TIMP balance, induced by CrataBL, controls the remodeling process observed in the lung of emphysematous mice. However, the reduction in lung inflammation and oxidative stress can also control the remodeling process. In fact, the relevant finding in the present study is that, through a direct or indirect effect, CrataBL reduced lung destruction and inflammation as well as oxidative stress. These outcomes probably contributed to the improved lung function observed in CrataBL treated-animals, supporting the substantial relevance of the present study.

5. Conclusion

In this experimental model of lung damage, induced by intratracheal administration of elastase, and treatment with CrataBL, a proteinase inhibitor, decreased lung mechanics alterations associated with a significant reduction in exhaled nitric oxide reduced the inflammatory responses in the airways and alveolar walls and consequently attenuated the destruction of alveolar walls. Furthermore, the CrataBL treatment led to decreased levels of extracellular remodeling markers in the alveolar and airway walls and induced an attenuation of the oxidative stress response.

Our results show that this inhibitor may be a potential therapeutic tool for the treatment of COPD. Moreover, further research possibilities using this experimental model could contribute to the development of therapeutics applied to asthma.

Conflict of interest statement

All authors declare no conflict of interest.

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