A murine model of elastase- and cigarette smoke-induced emphysema

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INTRODUCTION

Animal models of emphysema have been extensively used in order to provide a better understanding of the pathogenesis of the disease. This is due to the fact that studies involving human participants focus exclusively on morphological and molecular analysis of lung tissue fragments from patients undergoing surgical procedures or are in vitro studies conducted at a single time point.

The cigarette smoke (CS) and elastase models of emphysema are the most commonly used murine models of the disease, and both can produce pathological changes resembling human emphysema. However, given that neither can closely mimic the disease in humans, it is important to understand the advantages and disadvantages of each.¹ Although CS-induced emphysema models appear to best represent the pathogenesis of human emphysema, one major limitation of such models is that, regardless of how long animals are exposed to CS, the resulting alveolar enlargement is mild in comparison with that resulting from animal models of elastase-induced emphysema.¹⁻³ Depending on the dose, intratracheal or intranasal instillation of elastase can induce severe emphysema in a short time,¹⁻⁴⁻⁷ as well as a significant increase in alveolar enlargement, collagen fibers, and elastic fibers, suggesting a process of lung parenchymal remodeling.⁵⁻⁶ However, the main disadvantage of elastase models of emphysema is that they do not trigger all of the physiological events that CS models do, their relevance for therapeutic approaches therefore being limited.¹

Animal models of CS- and elastase-induced emphysema have been used not only to elucidate the structural changes in lung tissue but also to clarify the mechanistic insights involved in emphysema development. Although the protease-antiprotease imbalance hypothesis remains the most widely accepted hypothesis to explain the parenchymal destruction of emphysema,⁸⁻¹¹ oxidative stress should also be taken into account, given that the oxidant burden is increased in smokers as a response to CS compounds.¹²⁻¹³ In an attempt to reduce the smoke exposure time required to induce emphysema and mimic as closely as possible the pathological features of human emphysema,¹⁻³
A murine model of elastase- and cigarette smoke-induced emphysema, we developed an experimental model of emphysema induced by a combination of instillation of porcine pancreatic elastase (PPE) and exposure to CS for 2 months only.

**METHODS**

The present study was approved by the Human and Animal Research Ethics Committee of the University of São Paulo School of Medicine, located in the city of São Paulo, Brazil. Six- to eight-week-old male C57BL/6 mice (weighing 20-25 g) were used in the study. All animals received humane care in compliance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised in 1996).

For emphysema induction, the animals were anesthetized with a combination of xylazine and ketamine (i.m., 5 mg/kg and 40 mg/kg, respectively) and then challenged with an intranasal instillation of 50 µL of type I PPE (E1250; Sigma-Aldrich, St. Louis, MO, USA) at a lower dose (i.e., 0.33 IU) than that used in other models of PPE-induced emphysema, given that our goal was to induce emphysema by combining instillation of PPE and exposure to CS. The animals received a total of two doses of PPE (one dose at day 0 and one dose at day 30). Control animals received 50 µL of 0.9% saline solution (vehicle).

For animals undergoing CS exposure, the protocol began on day 1. The animals were exposed to CS in a 28-L inhalation chamber with two inlets (one for air and one for smoke), one outlet, and a fan for better mixing of air and smoke inside the chamber. One of the inlets was set to deliver synthetic air flow at 2 L/min, and the other was set to deliver synthetic air flow coming from a Venturi system connected to a lit cigarette, succioning the CS into the chamber. It was possible to change that flow rate to increase or decrease the amount of smoke in the chamber. After several measurements of the concentration of CO in the chamber, the flow rate was set to 1.5 L/min, which produced CO levels ranging from 250 ppm to 350 ppm. Carboxyhemoglobin levels were maintained in the chamber, the flow rate was set to 1.5 L/min, and fixed in 4% formaldehyde at a constant pressure of 20 cmH2O for 24 h, conventional histology being subsequently performed. In brief, lower- and upper-lobe specimens were embedded in paraffin and cut into 5-µm sections that were stained with hematoxylin and eosin in order to measure the mean linear intercept (Lm), which is an indicator of mean alveolar diameter.

After calculation of the aforementioned parameters, a 2-cm incision was made in the abdomen and the animals were euthanized by exsanguination from the abdominal aorta. Subsequently, the anterior chest wall was opened and the lungs were removed en bloc and fixed in 4% formaldehyde at a constant pressure of 20 cmH2O for 24 h, conventional histology being subsequently performed. In brief, lower- and upper-lobe specimens were embedded in paraffin and cut into 5-µm sections that were stained with hematoxylin and eosin in order to measure the mean linear intercept (Lm), which is an indicator of mean alveolar diameter.

The lung tissue was immunostained with the following antibodies: rat anti-mouse macrophage (MAC)-2 monoclonal antibody (1:50,000; CEDARLANE®, Burlington, ON, Canada); polyclonal goat anti-mouse matrix metalloproteinase (MMP)-12 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and polyclonal goat anti-mouse glycosylated 91-kDa glycoprotein (gp91phox; 1:300; Santa Cruz Biotechnology, Inc.).

**Figure 1.** Timeline of the experimental protocol. PPE: porcine pancreatic elastase; and SS: saline solution.
A biotin-streptavidin-peroxidase method was used. Secondary antibodies included anti-rabbit VECTASTAIN® ABC kit, anti-goat VECTASTAIN® ABC kit, and anti-rat VECTASTAIN® ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA), which were also used without a primary antibody, serving as a negative control.\(^{19}\)

For histomorphometry, a 100-point ocular grid of known area was placed in the microscope eyepiece.\(^{20}\) For each animal, 20 randomly selected nonoverlapping fields of lung parenchyma were examined under light microscopy (magnification, \(\times200\)). The Lm was measured by counting how many times the grid lines intercepted the alveolar walls, being calculated by the following equation:

\[
Lm = \frac{L_{total}}{NI}
\]

where \(L_{total}\) is the sum of all grid segments, calculated by measuring each segment with a ruler (Carl Zeiss Microscopy GmbH, Jena, Germany) under the microscope, and \(NI\) is the average number of times that the lines intersected the alveolar walls. All Lm values were expressed in micrometers (\(\mu m\)).

Histomorphometry was also used in order to determine the numbers of cells that were immunoreactive to MAC-2, MMP-12, and gp91\(\text{phox}\) in the distal lung parenchyma and peribronchial region by a point-counting technique with the aforementioned grid placed in the microscope eyepiece (magnification, \(\times400\)). For each animal, 15 fields of lung parenchyma and 5 airways were randomly selected. The results were expressed in cells/\(\mu m^2\).\(^{21-24}\)

Statistical analysis was performed with the program SigmaStat, version 11 (Systat Software, Inc., San Jose, CA, USA). The four groups of mice were compared by one-way ANOVA. Differences were considered significant at \(p < 0.05\).

**RESULTS**

On day 60 of the experimental protocol, no significant differences were found among the four groups of mice regarding the respiratory mechanics parameters assessed (i.e., airway resistance, tissue damping, and tissue elastance; Figure 2). The Lm was found to be higher in the CS + PPE group than in the other groups (\(p < 0.05\); Figure 3), an increased Lm being a hallmark of pulmonary emphysema.

Figure 4 shows the numbers of cells that were positive for MAC-2 in the peribronchial region and distal lung parenchyma. An increased number of macrophages in the peribronchial region (\(p < 0.05\)) and distal lung parenchyma (\(p < 0.005\)) were found in the CS + PPE group.

There were no significant differences among the four groups regarding the number of cells that were positive for MMP-12 in the peribronchial region (Figure 5A). However, in the distal lung parenchyma, the number of cells that were positive for MMP-12 was higher in the CS + PPE group than in the control group (\(p = 0.007\); Figure 5B).

The number of cells that were positive for gp91\(\text{phox}\) in the peribronchial region was higher in the CS group than in the control and PPE groups (\(p = 0.001\); Figure 6A). In the distal lung parenchyma, the number of cells that were positive for gp91\(\text{phox}\) was higher in the CS group than in the control group (\(p = 0.03\)), as well as being higher in the CS + PPE group than in the control and PPE groups (\(p < 0.003\); Figure 6B).

**DISCUSSION**

In the present study, we tested an experimental model of emphysema induced by a combination of short-term exposure to CS and instillation of PPE. After 2 months, there was an increase in the Lm, as well as macrophage infiltration in the peribronchial region and distal lung parenchyma, together with an increase in the numbers of cells that were positive for MMP-12 and gp91\(\text{phox}\) in the distal lung parenchyma.

The fact that no functional changes were found in the present study is probably due to the fact that there

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Respiratory mechanics parameters in the four experimental groups, expressed as mean ± SE. In A, airway resistance (Raw); in B, tissue damping (Gtis); and in C, tissue elastance (Hits). There were no significant differences in any of the parameters assessed among the experimental groups. PPE: porcine pancreatic elastase; and CS: cigarette smoke.
A murine model of elastase- and cigarette smoke-induced emphysema was less alveolar enlargement in our study than in studies involving models of PPE-induced emphysema and higher doses of elastase or in studies involving models of CS-induced emphysema and longer exposure times.

In addition, some studies have shown that assessment of respiratory mechanics does not reflect the presence of emphysema as well as does morphometric analysis. Foronjy et al. found no changes in lung compliance despite the presence of significant emphysema, with no correlation between emphysema as measured by morphometric analysis and lung compliance. They concluded that this lack of correlation occurs because the mechanisms involved in anatomic emphysema might be distinct from those that cause the loss of elastic recoil.

An imbalance between protease and antiprotease activity in the lung remains the most widely accepted mechanism for parenchymal destruction in emphysema. In addition, studies have shown that MMPs, particularly MMP-12, play an important role in attacking the protein components of the lung parenchymal extracellular matrix. MMP-12 is mainly produced by alveolar macrophages and is recognized to play an important role in emphysema. One group of authors exposed MMP-12 knockout mice to CS 6 days a week for 6 months and observed no increase in macrophage number or parenchymal destruction. In addition, there have been reports of increased MMP-12 expression in macrophages in smokers and greater MMP-12 activity in the sputum of patients with COPD than in that of smokers without airflow limitation.

In the present study, there was an increase in macrophages in the peribronchial region and distal lung parenchyma, as well as an increase in the number of cells that were positive for MMP-12 in comparison
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8. Our model of emphysema induced by a combination of CS exposure and PPE instillation results in a significant degree of parenchymal destruction in a shorter time frame than that employed in previous studies, reinforcing the importance of protease-antiprotease imbalance and oxidant-antioxidant imbalance in the pathogenesis of emphysema. Given the diversity of experimental models in the literature, it is important to choose carefully the best model for each purpose. A murine model of emphysema induced by a combination of CS exposure and PPE instillation might be useful for evaluating structural changes occurring during the processes of parenchymal destruction and remodeling in emphysema.
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