Development of different degrees of elastase-induced emphysema in mice: a randomized controlled experimental study

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ABSTRACT

Introduction: Mouse models of emphysema are important tools for testing different therapeutic strategies. The aim of this study was to develop a mouse model of emphysema induced by different doses of elastase in order to produce different degrees of severity.

Methods: Thirty female mice (C57BL/6) were used in this study. Different doses of porcine pancreatic elastase were administered intratracheally once a week for four weeks, as follows: 0.1 U (n=8), 0.15 U (n=7), and 0.2 U (n=7). Control mice (n=8) received 50 microL of sterile saline solution intratracheally. Lung mechanics were analyzed by plethysmography. Mean linear intercept and volume fraction occupied by collagen and elastic fibers were determined.

Results: An increase in lung resistance was observed with 0.2 U of elastase [median (P-25-P75): 2.02 (1.67; 2.34) cmH2O.s/mL], as well as a decrease in tidal volume and minute ventilation. Peak expiratory flow increased significantly in the groups treated with 0.15 U and 0.2 U of elastase. Mean linear intercept was higher with 0.15 U and 0.2 U of elastase, with destruction of alveolar walls [median (P-25-P75): 30.31 (26.65-43.13) microm and 49.49 (31.67-57.71) microm respectively]. The volume fraction occupied by collagen and elastic fibers was lower in the group receiving 0.2 U of elastase.

Conclusion: Four intratracheal instillations of 0.2 U of elastase once a week induced changes in lung function and histology, producing an experimental model of severe pulmonary emphysema, whereas 0.15 U resulted in only histological changes.

Keywords: *Pulmonary emphysema; lung mechanics; pancreatic elastase*

Pulmonary emphysema is a chronic obstructive pulmonary disease marked by persistent airflow limitation and histologically characterized by permanent enlargement of airspaces distal to the terminal bronchioles, accompanied by destruction of their walls, and without obvious fibrosis[1,2]. Decreased alveolar radial support affects the elastic recoil properties of the lung, leading to expiratory collapse of small airways and subsequent air trapping. A striking feature of the emphysematous lung is the exacerbation of communicant airflow between the surrounding alveolar sacs, a mechanism known as collateral ventilation[3]. It is estimated that 210 million people currently have chronic obstructive pulmonary disease worldwide. The World Health Organization predicts that by 2030 this disease will become the

Clin Biomed Res. 2014;34(3):297-306

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third leading cause of death in the world, with an increase in health care costs in industrialized and developing countries[1,2,4].

So far, there has been no effective therapy able to mitigate morphological damage, improving lung function and symptoms. Smoking cessation and pulmonary rehabilitation programs can be of some benefit; however, most patients in advanced stages of the disease have dyspnea with minimal effort and poor quality of life[2,5-8]. Experimental models have been developed in an attempt to enhance our understanding of the pathophysiology of emphysema and to test new therapies, including genetic models and experimental emphysema induced by smoke exposure or enzymes such as papain and elastase[9]. The use of multiple doses of elastase has the ability to rapidly induce panacinar emphysema[10], but high doses of elastase have been associated with increased mortality in mice[10,11]. Moreover, response to different treatments depends on the degree of emphysema[2].

The aim of this study was to develop a mouse model of emphysema induced by different doses of elastase in order to produce different degrees of disease severity.

METHODS

This study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre under protocol number 13-0086. All animals received humane care in compliance with Brazilian Law No. 11.794/2008, the Brazilian Guidelines for the Care and Use of Laboratory Animals, the Guidelines for the Euthanasia of Animals, and the Guide for the Care and Use of Laboratory Animals prepared by the U.S. National Academy of Sciences. The experiments were carried out in the Experimental Animal Unit of the Experimental Research Center of Hospital de Clínicas de Porto Alegre.

Thirty-one two-month old female C57BL/6 mice weighing between 17 and 20 g were used in this study. Animals were anesthetized with isoflurane (5% for induction and 2% for maintenance) and received 10 mg/kg of tramadol intraperitoneally. With the mouse in the supine position, the trachea was accessed through a longitudinal incision in the midline of the neck, and the anterior aspect was exposed. The middle third of the cervical trachea was punctured with an insulin syringe-needle for infusion of saline or diluted elastase. The skin incision was closed with 5-0 nylon suture. While the mouse was still under sedation, positioning maneuvers were performed for better distribution of elastase in the lungs.

The mice were randomly divided into the following groups: control (C) and elastase (E10, E15, E20). In the C group (n=8), sterile saline solution (0.9% NaCl; 50 µL) was instilled intratracheally (i.t.). In E groups, three different doses of porcine pancreatic elastase (Sigma-Aldrich, St. Louis, MO, USA) were diluted with 50 µL of 0.9% NaCl and instilled i.t., as follows: E10 (n=8): 0.1 U of elastase; E15 (n=7): 0.15 U of elastase; and E20 (n=8): 0.2 U of elastase. Each mouse received one infusion per week for four weeks.

One week after the fourth i.t. instillation, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), intraperitoneally. Lung mechanics were analyzed by plethysmography using the FinePointe™ Series Resistance and Compliance system (Buxco, Wilmington, NC, USA). The system was calibrated for a maximum volume of 0.25 mL and air pressure of 40 cmH2O. Tracheotomy was performed and a cannula inserted into the trachea. Mice were then mechanically ventilated in volume-controlled mode with a respiratory rate of 140 breaths per minute (bpm) and a positive end-expiratory pressure (PEEP) of 2 cmH2O. Airway measurements were recorded over a 3-minute period after a 5-minute acclimation period followed by saline nebulization.

At the end of the experiment, mice were euthanized with an overdose of the same anesthetic mixture. The lungs were inflated and the trachea was clamped at end-expiration. The right lung was removed and fixed in 10% formalin for histological analysis. The left lung was immediately cryopreserved in liquid nitrogen and stored at -80°C for further analysis.

Three-µm-thick slices were cut from the peripheral part of the right lung and analyzed for (a) alveolar diameter, (b) volume fraction occupied by collagen fibers, and (c) volume fraction occupied by elastic fibers.

To determine alveolar diameter, sections were stained with hematoxylin and eosin. An optical microscope with a high-resolution camera (Axioplan, Zeiss, Thornwood, NY, USA) was used for morphometric analysis, and digital images were obtained. Overlay images with the reticulum were obtained using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The reticulum contained a reference system with 100 points and 50 line segments arranged in parallel.

Subsequent quantitative analysis was performed by the point-counting technique[12].

For mean linear intercept (Lm) determination, the number of alveolar intercepts was counted across 20 random fields per slide at 400x magnification, where Lm = Σ length of the line segments (1250 µm) / number of intercepts. Collagen (Sirius Red) and elastic fibers (Orcein dye) were quantified in the alveolar septa across 10 random fields per slide at 400x magnification using the ImageJ software. The results were expressed as a percentage of the amount of collagen and elastic fibers per area (%).

Because the data were nonparametric, they were analyzed using the Kruskal-Wallis test followed by Dunn's test. Nonparametric data were and expressed as median and interquartile range (25th-75th percentile). All tests were performed using the Statistical Package for the Social Sciences (SPSS) version 20.0. The significance level was set at $p<0.05$.

RESULTS

No mice died during anesthesia or surgical procedures. One mouse in the E20 group was excluded due to the development of pneumonia. Thus, a total of 30 mice were available for analysis, eight in the C group, eight in E10, seven in E15, and seven in E20.

Lm increased with higher doses of elastase (figure 1). Lm was higher in E15 and E20 groups when compared to control values ($p=0.028$ and $p=0.001$, respectively). There was no significant difference between controls and E10 mice. Lm was higher in the E20 group than in E10 group ($p=0.017$) (figure 2).

The volume fraction of elastic fibers reduced significantly in the E20 group compared to all other groups ($p=0.020$). There was a significant difference between E20 and E10 groups ($p=0.026$) (figure 3). The volume fraction of collagen fibers also decreased in E15 and E20 groups compared to the C group ($p<0.0001$ and $p<0.0001$, respectively) (figure 4).

Lung resistance (R) was higher in the E20 group than in the E10 group ($p=0.008$) (figure 5). No differences were observed in dynamic lung compliance (Cdyn) (figure 6). Tidal volume (TV; $p=0.021$), minute ventilation (MV; $p=0.013$) and peak expiratory flow (PEF; $p=0.000$) showed a greater decrease in the E20 group compared to all other groups (figures 7 and 8).

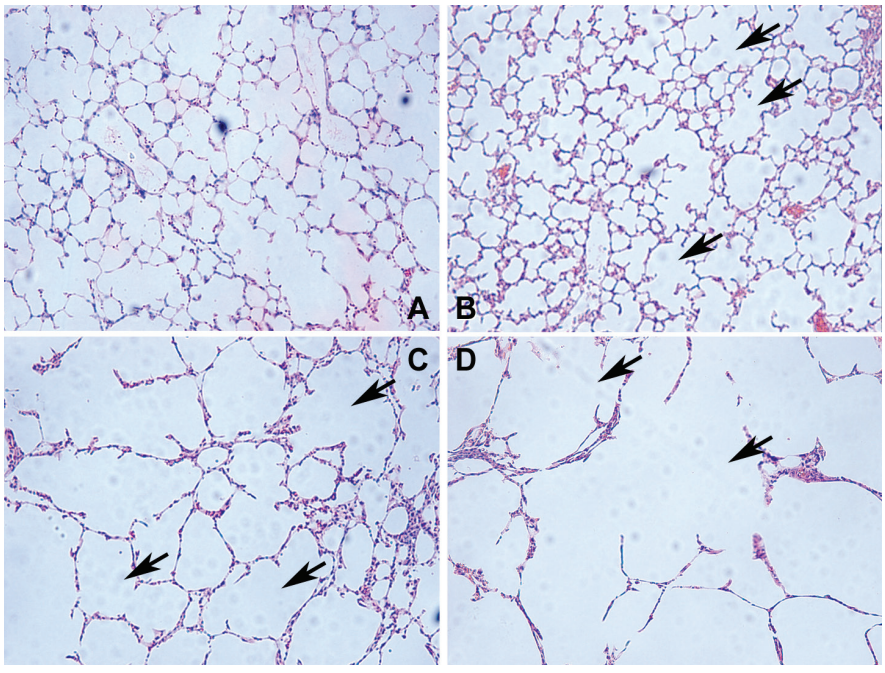

Figure 1: Lung parenchyma in an elastase-induced mouse model of emphysema
Micrographs showing samples stained with hematoxylin and eosin one week after the last instillation of elastase or saline, which were administrated once a week for four weeks; original magnification x200.

A – normal lung architecture in the control group; B – discrete alveolar enlargement in animals treated with 0.1 U of elastase; C – moderate bronchiolar changes in animals treated with 0.15 U of elastase; D – severe alveolar destruction in animals treated with 0.2 U of elastase. Arrows show areas of emphysema.

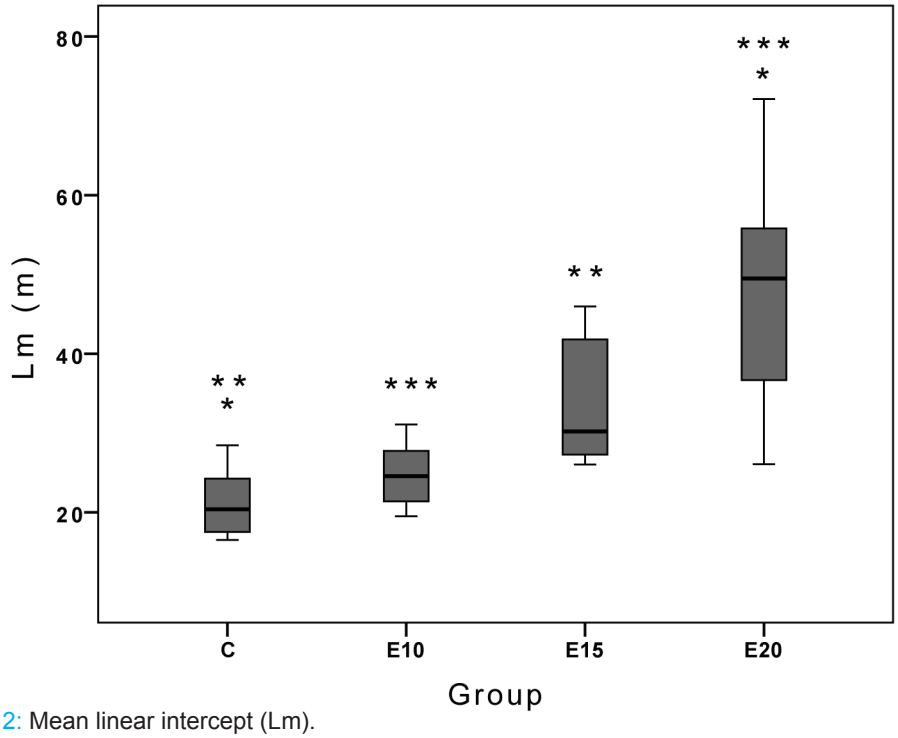

Figure 2: Mean linear intercept (Lm).

Values are the median of 7-8 animals (25th-75th percentile). Statistically significant difference between: *C versus E20 (p=0.001); **C versus E15 (p=0.028); ***E10 versus E20 (p=0.017). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks.

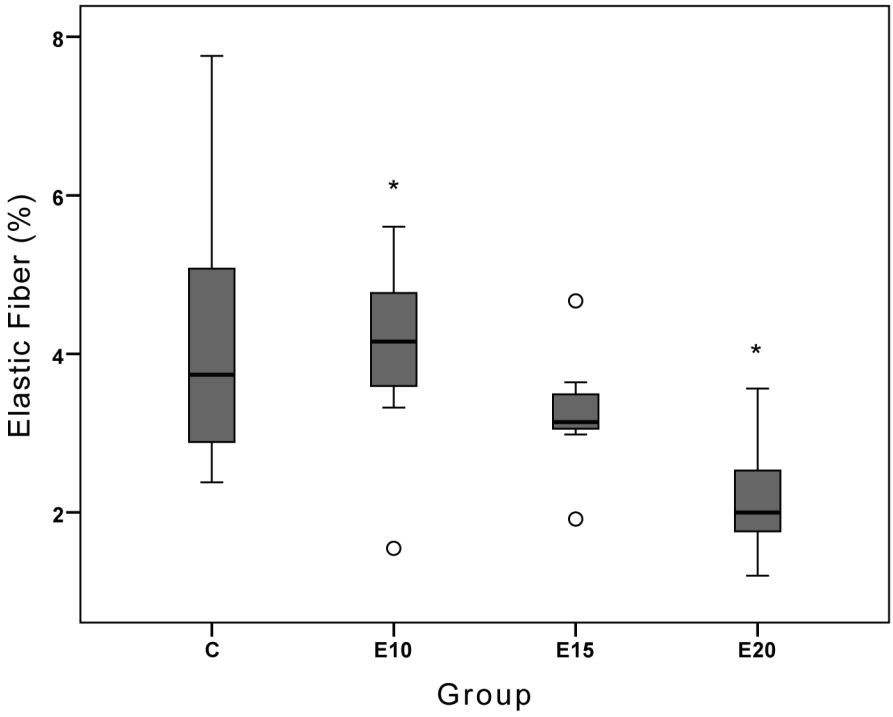

Figure 3: Volume fraction of the lung occupied by elastic fibers.

Values are the median of 7-8 animals (25th-75th percentile). Statistically significant difference between: *E10 versus E20 (p=0.026). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks. o = outlier values.

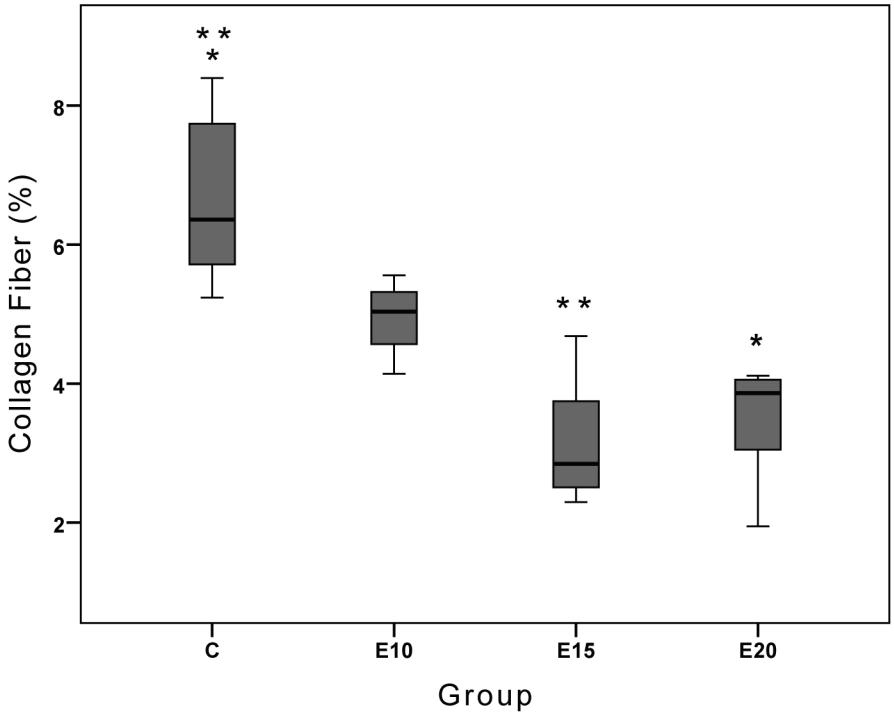

Figure 4: Volume fraction of the lung occupied by collagen fibers.

Values are the median of 7-8 animals (25th-75th percentile). Statistically significant difference between: *C versus E20 ($p<0.0001$); **C versus E15 ($p<0.0001$). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks.

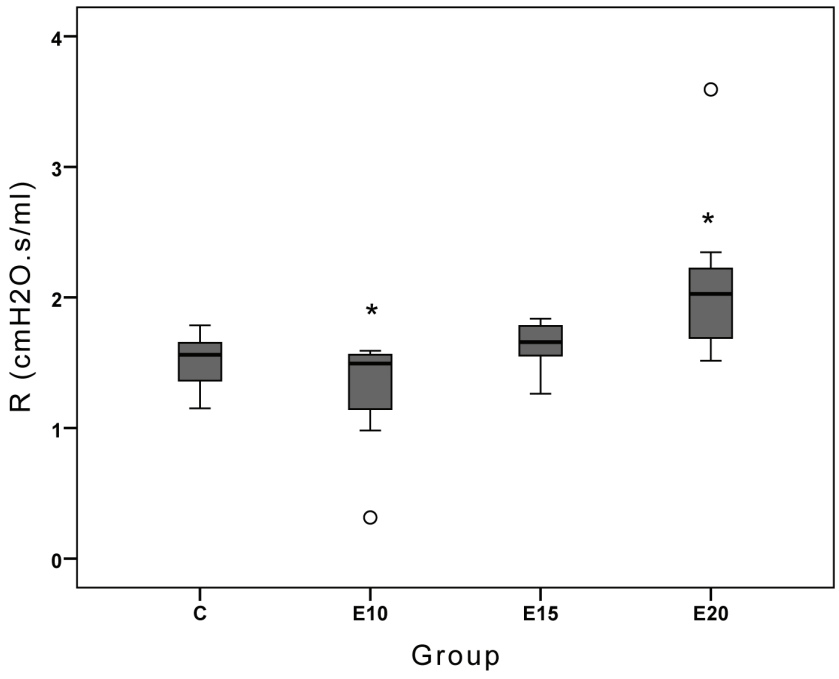

Figure 5: Lung resistance (R).

Values are the median of 7-8 animals (25th-75th percentile). Statistically significant difference between: *E10 versus E20 ($p=0.006$). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks. o = outlier values..

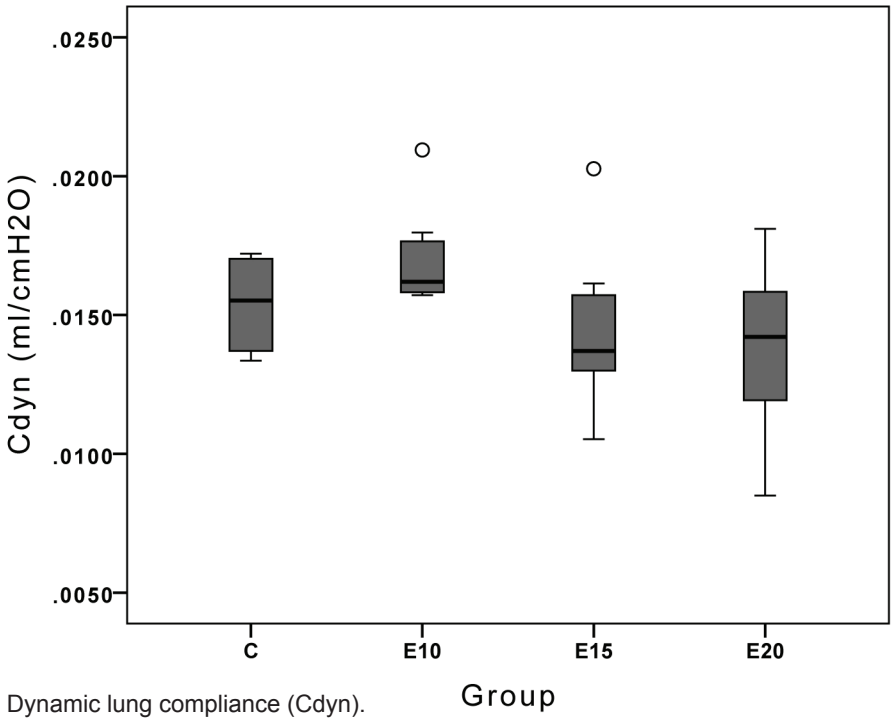

Figure 6: Dynamic lung compliance (Cdyn).

Values are the median of 7-8 animals (25th-75th percentile). No statistically significant difference between groups was detected. C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks. o = outlier values.

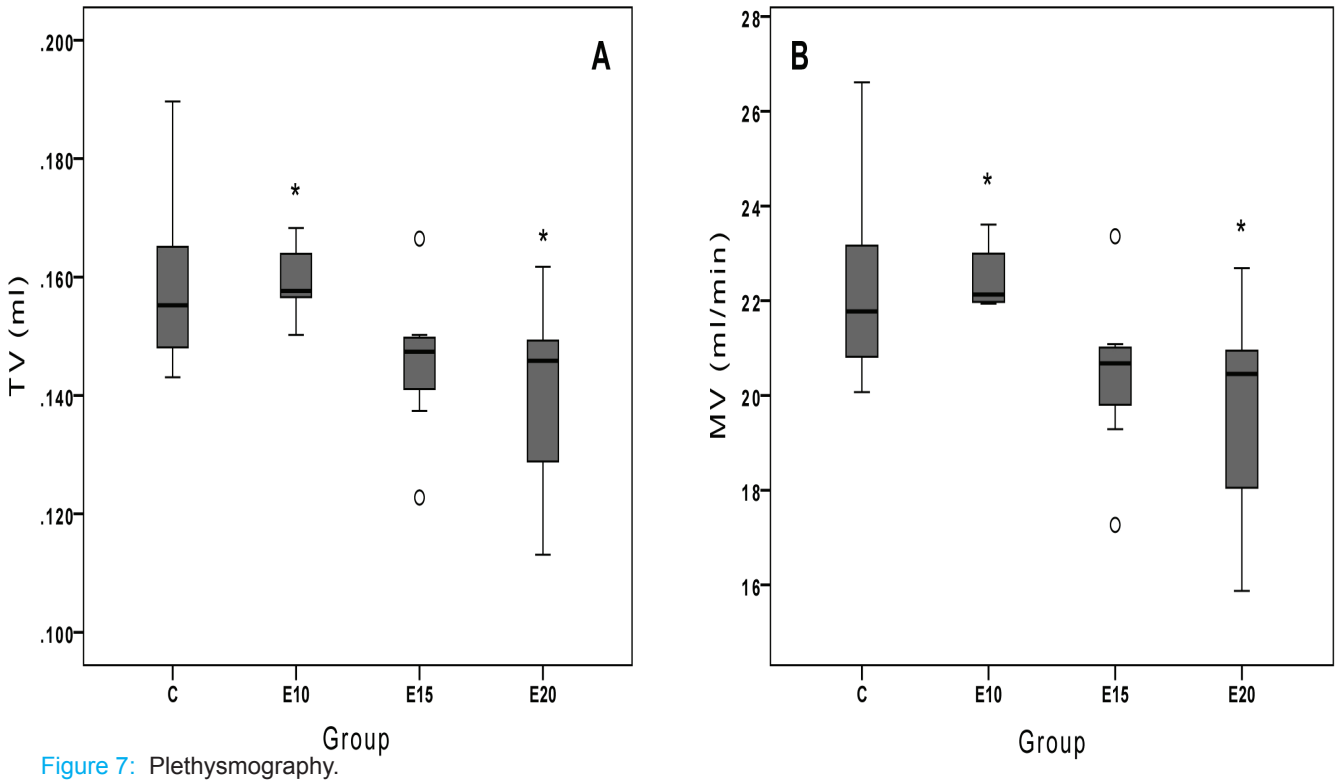

Figure 7: Plethysmography.

Values are the median of 7-8 animals (25th-75th percentile). A – tidal volume (TV). Statistically significant difference between: *E10 versus E20 (p=0.041). B – minute ventilation (MV). Statistically significant difference between: *E10 versus E20 (p=0.024). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks. o = outlier values.

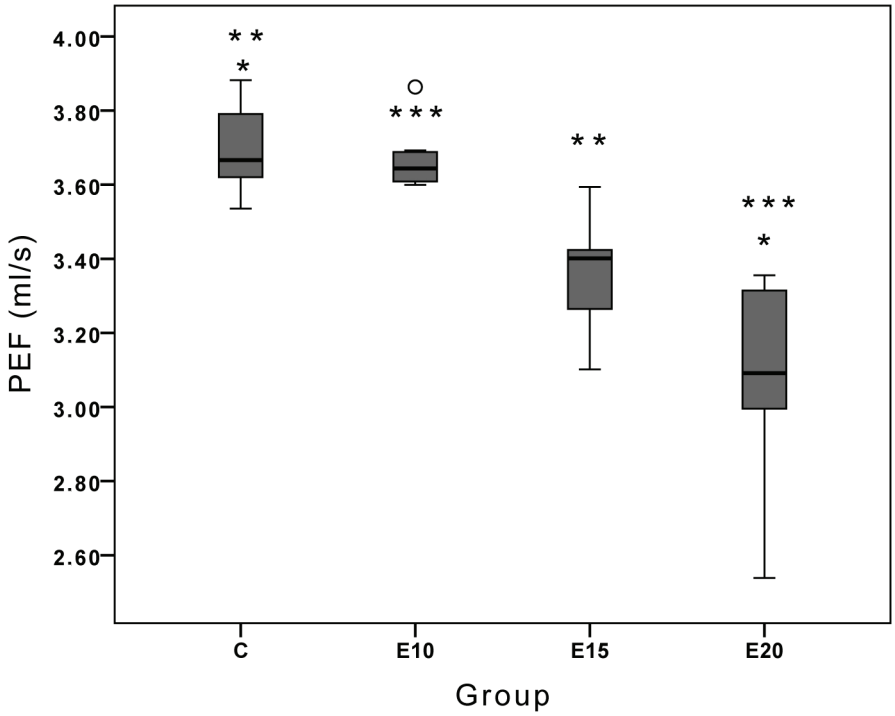

Figure 8: Peak expiratory flow (PEF).

Values are the median of 7-8 animals (25th-75th percentile). Statistically significant difference between: *C versus E20 (p<0.0001); **C versus E15 (p=0.032); ***E10 versus E20 (p=0.001). C – control group; E10 – 0.1 U of elastase once a week for four weeks; E15 – 0.15 U of elastase once a week for four weeks; E20 – 0.2 U of elastase once a week for four weeks. o = outlier values.

DISCUSSION

In the present study, we developed models of different degrees of emphysema induced by different doses of elastase in mice. For this purpose, lung mechanics and histology were analyzed. The highest dose (0.2 U) resulted in changes in lung mechanics associated with elastolysis and alveolar hyperinflation. An elastase dose of 0.15 U resulted in histological changes, but with no significant changes in lung mechanics. The lowest dose (0.1 U) led to no significant morphofunctional changes.

Induction of emphysema with multiple doses of elastase has some advantages over a single-dose model, such as increased Lm, a pattern of damage similar to that of progressive emphysema in humans, and systemic changes[10,11,13]. The doses of elastase used in our study were selected based on a previous study by Cruz et al., who reported morphological changes in the lung parenchyma, with increased Lm and collagen fiber content and a decreased amount of elastic fibers in the alveolar septa, using 0.1 U of elastase[14]. In our study, however, no statistical difference was observed in these parameters when an elastase dose of 0.1 U was used. Luthje et al. used an elastase dose of 3.3 U/100g (0.66 U per mouse) administered once a week for five weeks. The mice developed emphysema with a higher Lm (260.7 µm) than controls (24.7 µm), but also with a higher mortality rate due to pneumothorax and pulmonary hemorrhage (30%)[11]. In our study, mean Lm was 47.5 ± 15.8 µm in the E20 group and no deaths were observed, demonstrating the safety of our model.

We observed that an increased dose of elastase was able to produce emphysema with a reduction in the volume fraction occupied by collagen fibers in the alveolar septa. The remodeling phase of healing involves collagen deposit and begins approximately eight days after injury. Initially, type III collagen fibers are synthesized, followed by type I collagen fibers (more mature and resistant collagen)[15]. Hamakawa et al., in an elastase-induced mouse model of emphysema, reported that the ratio of type III to type I collagen decreased in the early phase (two days after treatment with elastase) and then gradually returned to control values (21 days after elastase administration)[16]. Thus, the reduction

in collagen fiber content seven days after the last instillation in our study is in accordance with that described by Hamakawa et al.[16]. However, other studies have reported an increased amount of collagen content in experimental emphysema due to the inflammatory process[17-19]. These differences among studies may be attributed to the time points used for analysis, as most studies have evaluated collagen fiber content at least 14 days after disease induction[17-19].

Elastic fiber content also reduced one week after the last i.t. instillation of elastase in our study, which may be attributed to the release of metalloprotease[16,20]. Since there was no sufficient time for lung remodeling, we believe that loss of elastin led to enlargement of alveolar spaces, supporting the hypothesis that elastolysis promotes the development of emphysema. Furthermore, Kononov et al. showed that the elastin-collagen network exhibited deformation and distortion during stretching[21]. Therefore, in addition to a reduction in collagen and elastic fibers, the efficiency of these fibers may be compromised, resulting in changes in lung mechanics[22].

In this study, R increased in mice receiving the highest dose of elastase (0.2 U). This mechanical change may be attributed to the decrease in collagen and elastic content, leading to loss of airway tethering, which may limit airway expansion. Furthermore, we observed a reduction in TV, MV, and PEF. R is inversely proportional to airflow[23]; therefore, since R was higher, TV, VM and PEF were consequently lower in the group with greater enlargement of alveolar spaces. In humans, R values may remain unaltered due to compensation resulting from hyperinflation, but this did not occur in the E20 group. However, similarly to what occurs in human emphysema, there was great involvement of the peripheral component[23]. Ingenito et al. reported the same pattern of R in sheep treated with papain, suggesting an underlying airway obstruction[24]. No other studies using mice and whole-body plethysmography have reported increased R. Some studies have employed other evaluation methods that use muscle paralysis as an adjunct to anesthesia, mainly influencing the assessment of airway resistance[25-27]. Ito et al. showed decreased R values with increasing PEEP (higher than 6 cmH2O), but this was not significant[19]. In our study, we used a PEEP level only sufficient to maintain functional residual capacity, thus contributing to evaluate solely the effects of emphysema.

In the present study, no differences were observed in Cdyn values when compared to those of controls. It is known that Cdyn is strongly related to elastic tissue (especially collagen fibers)[19,28]. There was no increase in the volume fraction occupied by collagen fibers in this study. Thus, the stable behavior of Cdyn may be associated with the absence of an increasing amount of collagen fibers as well as with a balance between areas of hyperinflation and collapse.

Perez-Padilla et al., using data from large population studies, tested whether pre-bronchodilator PEF could identify spirometrically confirmed post-bronchodilator airflow obstruction. Since patients with higher risk for chronic obstructive pulmonary disease had lower PEF, the authors pointed out the usefulness of PEF measurement as an indicator of mortality risk in hospitalized patients, in the emergency room screening of exacerbations, and for home assessment of patients[29]. In our study, PEF had a significant decrease proportional to the increase in the elastase dose, closely resembling the behavior of pulmonary emphysema in humans.

The present study has some limitations: (1) all analyses were performed one week after the last instillation, and therefore there was insufficient time for increase in collagen content and elastogenesis, and (2) inflammation was not evaluated as to whether it correlated with lung function and remodeling.

In conclusion, four i.t. instillations of 0.2 U of elastase once a week produced an effective and safe experimental model of severe pulmonary emphysema characterized by changes in lung mechanics and histology and elastolysis. Additionally, an elastase dose of 0.15 U induced a model of emphysema characterized by only histological changes. We believe that this study can serve as a platform for future research in the field of therapy targeting different degrees of pulmonary emphysema.

Acknowledgment

This study was supported by the Research Incentive Fund, Hospital de Clínicas de Porto Alegre (FIPE/HCPA).

Conflicts of interest

All authors declare that they have no competing interests.

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Received: 14/07/2014
Accepted: 12/09/2014