An Acad Bras Cienc (2022) 94(4): e20201914 DOI 10.1590/0001-3765202220201914
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Histomorphometric analysis of the lung of Swiss mice treated with a fibrinolytic protease

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Abstract: Fibrinolytic enzymes are considered promising alternative in the treatment of cardiovascular diseases by preventing fibrin clots. A protease from *Mucor subtilissimus* UCP 1262 was obtained by solid state fermentation and purified by ion exchange chromatography. The purified extract was administered at an acute dose of 2000 mg/mL to evaluate its toxic effects to the lungs of mice. After 14 days of treatment, a histomorphometric study was performed by the type 1 and 2 pneumocyte count and the evaluation of the lung area. As result, the experimental group showed a significant decrease of type 2 pneumocyte and although a decrease in the alveolar area was observed in relation to the control group, no significant pulmonary toxicity, emphysema, and fibrosis characteristics were detected. The *in vitro* tests suggest possible clinical applications for the enzyme.

Key words: Fibrinolytic protease, thrombosis, *Mucor*, pneumocyte, thrombolytic agent.

INTRODUCTION

Fibrinolytic enzymes have been used in the treatment of cardiovascular diseases because their capacity to prevent blood clots. On the other hand, other fibrinolytic agents such as urokinase, streptokinase, plasminogen activators (t-PA) and anticoagulants have been reported for risk of bleeding (Choi et al. 2013, Zapata-Wainberg et al. 2016).

Filamentous fungi are excellent sources of fibrinolytic enzymes (Germano et al. 2003, Liu et al. 2017, Deng et al. 2018) and the fungus *Mucor subtilissimus* UCP 1262 has been extensile researched to produce proteases have reported a promising non-toxic protease (Nascimento et al. 2015, 2016, 2017, 2020, Da Silva et al. 2019) for clinical application with potential to cleave fibrin and fibrinogen, but not through plasminogen activators. Some drugs induce lung diseases such as changes in lung immune cells (Kullberg et al. 2020) causing inflammatory reactions and apoptosis pathways (Zhang et al. 2020), inducing pulmonary fibrosis (Budas et al. 2018). Lung diseases are caused by several routes of administration: intravenous, oral and even inhalation (Bevan et al. 2018).

This study aims to evaluate the acute *in vivo* toxicity of the fibrinolytic protease from *Mucor subtilissimus* UCP 1262 in the lungs of Swiss mice based on histomorphometric study of lung tissue.

MATERIALS AND METHODS

Obtaining fibrinolytic protease

The filamentous fungus *Mucor subtilissimus* UCP 1262 was cultivated in Czapek medium and maintained at 30ºC. The inoculum preparation, fibrinolytic enzyme production by solid state fermentation, and enzymatic extraction, precipitation and purification were performed according to Nascimento et al. (2015).

Animals

Swiss mice (*Mus musculus*) weighting between 38 and 50 g were used, they were obtained from the Keiso-Asami Immunopathology Laboratory (LIKA-UFPE, Recife, Brazil) bioterium and kept in the bioterium of the Federal University of Pernambuco (Academic Center of Vitoria, Brazil) in appropriate polypropylene boxes at 21 ± 1ºC under a photoperiod of 12L:12D with access to food (labine), water and relative humidity of 50 ± 5%. The Animal Ethics Committee of the Universidade Federal de Pernambuco has approved all experimental procedures (process 0058/2018).

Lung evaluation of acute exposure to fibrinolytic enzyme

The fibrinolytic enzyme of *M. subtilissimus* was evaluated following the OECD 423 guideline known as "Acute Toxic Class Method" (OECD 423). Three animals per group were used in duplicate. One group was subjected to the oral application of 2000 mg/kg of water-diluted fibrinolytic protease, and the other group received only water (control). The animals fasted for 3-4 hours before administration of the enzyme. The food was suspended for another 1-2 hours and they were observed for 14 consecutive days. After this period the lung of each animal was removed by means of a thoracotomy and submitted to histological procedures

Histomorphometric analysis

Each lung was cleaved and dipped in formalin solution (10% v/v) for 48h. The fragments were dehydrated into ethyl alcohol in increasing concentrations, diaphanized by xylol, impregnated and embedded in paraffin. The blocks were cut into a microtome adjusted to 4 µm. Thus, the sections were maintained at 60ºC for 24 hours and submitted to the Hematoxylin-Eosin (H.E.) staining technique. The histological images of these slides were captured by a digital camera (Moticam 3000) coupled to the optical microscope (Nikon E-200), under fixed focus and field clarity, obtaining 20 fields per slide with 400X magnification to analyze the amount of type 1 and 2 pneumocytes, and 20 fields per slide in the 100X objective to analyze the area and perimeter of the alveoli. The photomicrographs were evaluated using ImageJ software version 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

A normality test using the Smirnov Kolmogorov program was developed, and then it was verified a non-normal distribution. From this, a non-parametric test (U of Mann-Whitney) was the applied. For this the GraphPad Prism 5.0 program was used and the data were expressed as mean ± SD, p <0.05. compared to the control.

RESULTS AND DISCUSSION

Lung diseases induced by drugs consist of an increasing cause of morbidity (Costabel 2000, Damas et al. 2006). Pacients treated with Amiodarone, a class III antiarrhythmic, showed multiple nodular lesions in the pathological examination (González Gordaliza et al. 2006). At the same time, many medications cause severe acute lung toxicity by presenting pulmonary infiltrates, eosinophilia, and rash (Fernández

Álvarez et al. 1994). Free radicals from oxygen and various cytokines, such as methotrexate (MTX), produce lung toxicity and develop various forms of arthritis and other rheumatic conditions (Kurt et al. 2015). Thalidomide induces interstitial and alveolar alterations and is indicative of partial respiratory failure (Carrión Valero & Bertomeu González 2002). The obtention of non-toxic drugs is a prerequisit for clinical applications and justify the potential of the purified fibrinolytic enzyme from *Mucor subtilissimus*. The present study aims to demonstrate the action of that protease on the lung tissue structure based on its absence of cytotoxicity for tumor cells, kidney, spleen, and liver (Da Silva et al. 2019).

The macroscopic examination of the lung revealed no changes in the color or integrity of the tissue in any of the groups studied (Figure 1a, b, c, d). The lung parenchyma showed no changes in alveolar, septal or bronchiolar architecture. The two pneumocytes types [alveolar epithelial cells I (CEA1) and II (CEA 2)] have been targeted for infections caused by SARS-CoV-2, when a single tape RNA virus has as receptor the angiotensin 2 converting enzyme (ACE2), on the cell surface of the host. The ACE2 is a type I membrane protein expressed in cells in kidneys, heart, TGI, blood vessels and CEA 2 cells which are particularly prone to viral infections (Pascoal et al. 2020, Zhao et al. 2020, Andersen et al. 2020). Figure 1 demonstrates the absence of morphological changes in alveolar epithelial cells.

In the histomorphometric analysis, the lungs of mice exposed to an acute dose of fibrinolytic protease were measured according to the amount of type 1 and 2 pneumocytes. Table I shows that there was a significant decrease in the numner of CEA 2 cells, what is unusual (Castelo Branco et al. 2004). In addition, the alveolar area also showed a significant decrease (Table I). Absence of pulmonary emphysema could be evidenced by the fact that no alveolar changes [dilatation of the air spaces or destruction of the alveolar wall (Monteiro et al. 2004)] were observed. Our results suggest application of the fibrinolytic enzyme in clinical tests against pulmonary diseases.

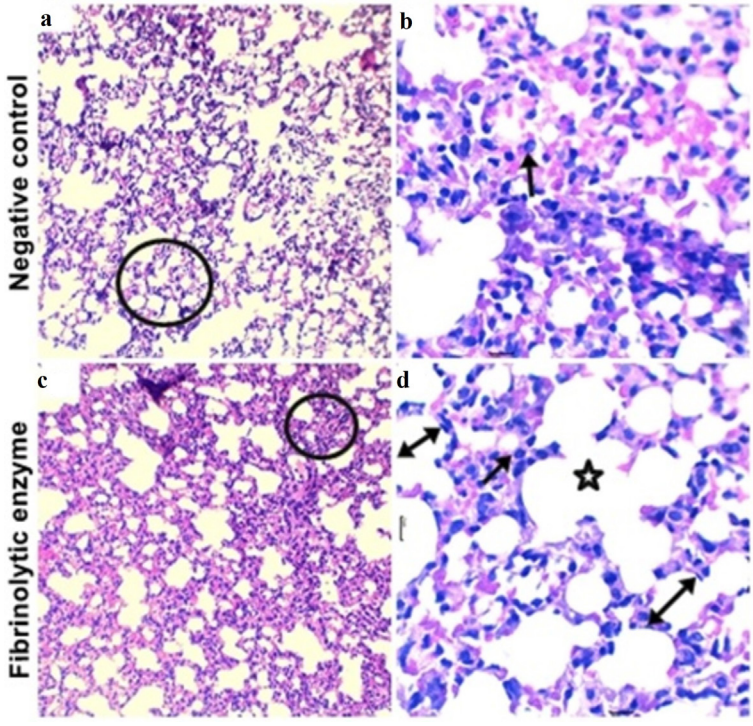

Figure 1. Photomicrographs of lung tissue sections of mice in the different groups studied (H&E). a and c= Preserved lung parenchyma (circle); b and d= Preserved alveolar sac (star), Pneumocyte type 1 (double arrow), Pneumocyte type 2 (arrow). HE staining with magnification of 10X (a and c) and 40X (b and d). Scale bar = 1 µm.

Table I. Number of type and type 2 pneumocytes in the lungs of mice treated with the fibrinolytic enzyme produced by *Mucor subtilissimus* UCP 1262 (2000mg/mL). The results are expressed as mean ± SD. Statistical differences were determined by the Mann-Whitney U-test. CN: negative control; ENZ: fibrinolytic enzyme produced by Mucor subtilissimus. *P<0.05 vs Control.

	Pneumocyte type 1 (n of cells/ μm^2)	Pneumocyte type 2 (n of cells/ μm^2)	Alveolar Area (n of cells/μm^2)
CN	20,38 ± 6,79	29,04 ± 9,58	580,30 ± 230,41
ENZ	17,74 ± 8,44	23,25 ± 11,55*	492,40 ± 242,72 *

CONCLUSIONS

The fibrinolytic protease produced by *Mucor susbtilissimus* UCP 1262 showed potential to reduce pulmonary toxicity, not developing cell characteristics of emphysema or fibrosis, what is indicative of its applicability in clinical tests.

Acknowledgments

We are grateful to the following bodies for the grants awarded: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior): Senior Internship (edital nº16/2016, case number: 88881.119817/2016-01 and the doctoral scholarship; FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco): Researcher's scholarship grant: BFP-0079-5.05/20 CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) process: 427153/2016-6 and we also thank the reviewers for their valuable comments and suggestions as these helped us to improve the manuscript.

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How to cite

DA SILVA MM ET AL. 2022. Histomorphometric analysis of the lung of Swiss mice treated with a fibrinolytic protease. An Acad Bras Cienc 94: e20201914. DOI 10.1590/0001-3765202220201914.

Manuscript received on February 19, 2020
accepted for publication on September 09, 2020

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Author contributions

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