Pulmonary Pharmacology & Therapeutics 70 (2021) 102075

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Mesenchymal stromal cells-based therapy in a murine model of elastase-induced emphysema: Simvastatin as a potential adjuvant in cellular homing

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ARTICLE INFO

Keywords:
Chronic obstructive pulmonary disease
COPD
Cell therapy
Mesenchymal stromal cells
MSC
Simvastatin
Cellular homing
~~Mouse~~

ABSTRACT

Chronic Obstructive Pulmonary Disease - COPD is characterized by the destruction of alveolar walls associated to a chronic inflammatory response of the airways. There is no clinical therapy for COPD. In this context, cell-based therapies represent a promising therapeutic approach for chronic lung disease. The goal of this work was to evaluate the effect of simvastatin on cell-based therapy in a mice emphysema model. Female FVB mice received intranasal instillation of elastase (three consecutive doses of 50 μL) in order to promote pulmonary emphysema. After 21 days of the first instillation, the animals were treated with Adipose-Derived Mesenchymal Stromal Cells (AD-MSC, 2.6×10^6) via retro-orbital infusion associated or not with simvastatin administrated daily via oral gavage (15 mg/kg/15d). Before and after these treatments, the histological and morphometrical analyses of the lung tissue, as so as lung function (whole body plethysmography) were evaluated. PAI-1 gene expression, an upregulated factor by ischemia that indicate a low survival of transplanted MSC, was also evaluated. The result regarding morphological and functional aspects of both lungs, presented no significant difference among the groups (AD-MSC or AD-MSC + Simvastatin). However, significant anatomical difference was observed in the right lung of the both groups of mice. The results shown a higher deposition of cells in the right lung, with might to be explained by anatomical differences (slightly higher right bronchi). Decreased levels of PAI-1 were observed in the simvastatin treated groups. The pulmonary ventilation was similar between the groups with only a tendency to a lower in the elastase treated animals due to a low respiratory frequency. In conclusion, the results suggest that both AD-MSC and simvastatin treatments could promote an improvement of morphological recovery of pulmonary emphysema, that it was more pronounced in the right lung.

Author contributions

The authors CAF, WASJ, DLZ and JTRP contributed with the experimental design, data analysis, discussion and manuscript writing and review. The authors KBCAC, MB and EC contributed with data analysis, discussion and manuscript review. The authors are grateful to Wagner Nagib de Souza Birbeire for his contribution to the elaboration of the figures.

Funding

The author CAF was financially supported by the National Council for Scientific and Technological Development – CNPq – Grant GM/GD 140348/2012–13.

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https://doi.org/10.1016/j.pupt.2021.102075
Received 17 February 2021; Received in revised form 31 May 2021; Accepted 18 August 2021
Available online 21 August 2021

1. Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response of the airways and lung to noxious particles or gases. The airflow limitation results from the interaction between the small airway disease (bronchiolitis) and parenchymal destruction (emphysema), without apparent fibrosis [1]. From the epidemiological viewpoint, COPD is a leading cause of morbimortality worldwide. World Health Organization (WHO) – Top 10 causes of deaths - places COPD as the third leading cause of death in the world [2], characterizing this pathological condition as a serious global public health problem [1,3].

It is worth noting that, despite significant advances in the therapeutic arsenal and rehabilitation techniques, there is, to date, no proven effective treatment for COPD/emphysema. The main clinical treatments ~~for COPD~~ are ~~only~~ palliative and supportive care, which aim the symptoms improvement and the quality of life of the patients [1]. In this context, cell-based therapies emerge as a potentially promising ~~therapeutic~~ alternative for COPD/emphysema and, since the pioneering studies published at the beginning of this century, a large number of cell-based therapies have been conducted in preclinical experimental models of COPD/emphysema [4–8]. The results in experimental models provided the basis for translational studies and the implementation of cell-based clinical trials in human patients [9–14] and this theme of cell-based therapy in ~~COPD~~ emphysema has been the subject of detailed critical reviews [4,15–25].

Despite great advances and some promising results from cell-based clinical trials [12,13,16], there are doubts and gaps underlying the mechanisms of action of the Mesenchymal Stromal/Stem Cells (MSC) and a series of questions to be better defined, which refer to the best source for obtaining the cells, culture conditions (cell quality), best route and number of cells to be delivered to the patient [4,6,21,26–31]. One of the questions that remain unanswered refers to the mechanisms inherent to cell migration, homing, engraftment and viability and survival of cells in an inhospitable microenvironment after transplantation [27,32–34]. Thus, improving the survival and viability of MSC in the target tissue is a crucial factor for the success of cell-based therapies. In this way, the advancement of knowledge about the molecules and regulatory mechanisms involved in this process should contribute to improving the viability and survival of transplanted cells and increase therapeutic effectiveness [27,32,33,35,36].

As proposed by Copland et al. (2007), one of the factors that determine the low survival of transplanted MSC is the ischemic condition of the target tissue, such as infarcted myocardium. The authors, using microarray techniques and proteomic screens, proposed that Plasminogen Activator Inhibitor 1 – (PAI-1) is an upregulated factor in ischemia-mimicking conditions in vitro. In complementing this study, using techniques of genetic analysis and chemical manipulation, Copland et al. (2009) proposed that PAI-1 acting as a key negative regulatory factor on MSC viability and survival in vivo [35,36]. Moreover, Yang and colleagues showed that the therapeutic efficacy of MSC transplantation in acutely infarcted hearts is improved by simvastatin treatment, with increased MSC survival [37].

A systematic review and meta-analysis showed that statins reduce plasma levels of PAI-1 [38]. Simvastatin is a drug from the group of statins which, besides the effect of reducing cholesterol levels, has several pleiotropic effects, such as decreased circulating pro-inflammatory cytokines (Interleukin 1 (IL-1), Interleukin 6 (IL-6) and Tumor Necrosis Factor-α (TNF-α). In vitro and in vivo studies have shown the anti-inflammatory effects of statins, which were able to reduce the activities of Cyclooxygenase 2 (COX-2) and Matrix Metalloproteinase 9 (MMP-9) ~~activities~~, improve Nitric Oxide (NO) bioavailability and reduce Nuclear Factor-κB (NF-κB) activation. These effects were clinically related to the prevention of stroke and coronary artery disease [32,38,39]. In addition to these effects, simvastatin has been shown to negatively modulate PAI-1, reducing the inflammatory response, stimulating cell migration and increasing the homing of transplanted cells [32]. It should be emphasized that PAI-1 gene expression was negatively regulated while Tissue Plasminogen Activator (PLAT) was positively regulated in MSC treated in vitro with simvastatin [40], which may indicate an inhibition of senescence by simvastatin, since PAI-induces senescence by preventing Insulin-like growth factor-binding protein 3 (IGFBP3) proteolysis by PLAT [41].

Based on these considerations and previous experimental findings, we hypothesized that reduced plasma levels of PAI-1 induced by simvastatin improves migration and homing of ~~Bone Marrow Mononuclear Cells (BMMC) or~~ Mesenchymal Stromal/Stem Cells (MSC) and, as consequence simvastatin, could act as a positive adjuvant effect (additive or synergistic) in cell-based therapies in ~~COPD~~ lung emphysema. In this context, the aim of this study was to evaluate the ~~beneficial~~ effect on homing and therapeutic efficacy of MSC-based therapy as a function of previous administration of simvastatin in an experimental model of emphysema in mice as summarized in Fig. 1.

2. Material and methods

2.1. Simvastatin and PAI-1 in vivo dose assay

For this study, 23 male 8 weeks old C57Bl/6 mice were randomly divided into four groups, one control group with 5 animals and 3 treated groups with 6 animals each. During 15 days, control animals received one daily oral gavage of 100 μL vehicle (water/ethanol 1%) while treated animals received one daily oral gavage, of vehicle containing simvastatin, as shown in Table 1.

All animals were euthanized by cervical dislocation on day 15 after the beginning of the experiments, 1 h after the administration of the last dose of the drug. Immediately after death, blood was collected from heart to evaluate PAI-1 circulating levels. The lungs and liver were removed and kept in liquid nitrogen for further analysis of PAI 1 gene expression and protein analysis. Additionally, tissue samples of the organs were submitted to histological analysis.

2.2. Elastase-induced emphysema and cell MSC therapy

For the elastase-induced emphysema, 16 female C57Bl/6 mice approximately 8 weeks old received intranasal instillation of three consecutive doses of 50 μL of porcine pancreatic elastase (Sigma Aldrich, São Paulo, Brazil, as previously described by Longhini-dos-Santos et al. [42]. After 21 days of the first instillation, the animals were subjected to retro-orbital infusion of Bone Marrow-derived Mesenchymal Stromal Cells (BM-MSC) or vehicle, according to the experimental group. The animals were obtained from the Biotério Central da Faculdade de Medicina de Ribeirão Preto – FMRP USP and randomly divided into four groups of four animals each, as presented in Table 2.

After 21 days of cell therapy (42 days after the beginning of the experiment), the lung function of the animals was evaluated in vivo by the whole body plethismography method. Following, the animals were euthanized by cervical dislocation and had their lungs removed for histological and morphometrical analysis.

2.3. Bone marrow and adipose-derived mesenchymal stromal cells isolation

The adipose derived and bone marrow derived mesenchymal stromal cells were obtained from 7 male FVB/Luc+ (CAG-Luc-EGFP) mice 6 weeks old from the Fundação Hemocentro de Ribeirão Preto. The bone marrow derived mesenchymal stromal cells were obtained and cultivated as previously described by Longhini-dos-Santos et al. [43] and the Adipose-Derived Mesenchymal Stromal Cells – AD-MSC were obtained and cultivated as previously described by Marcelino et al. [7]. At passage 3, the cells were frozen in −80 °C and remained frozen until use.

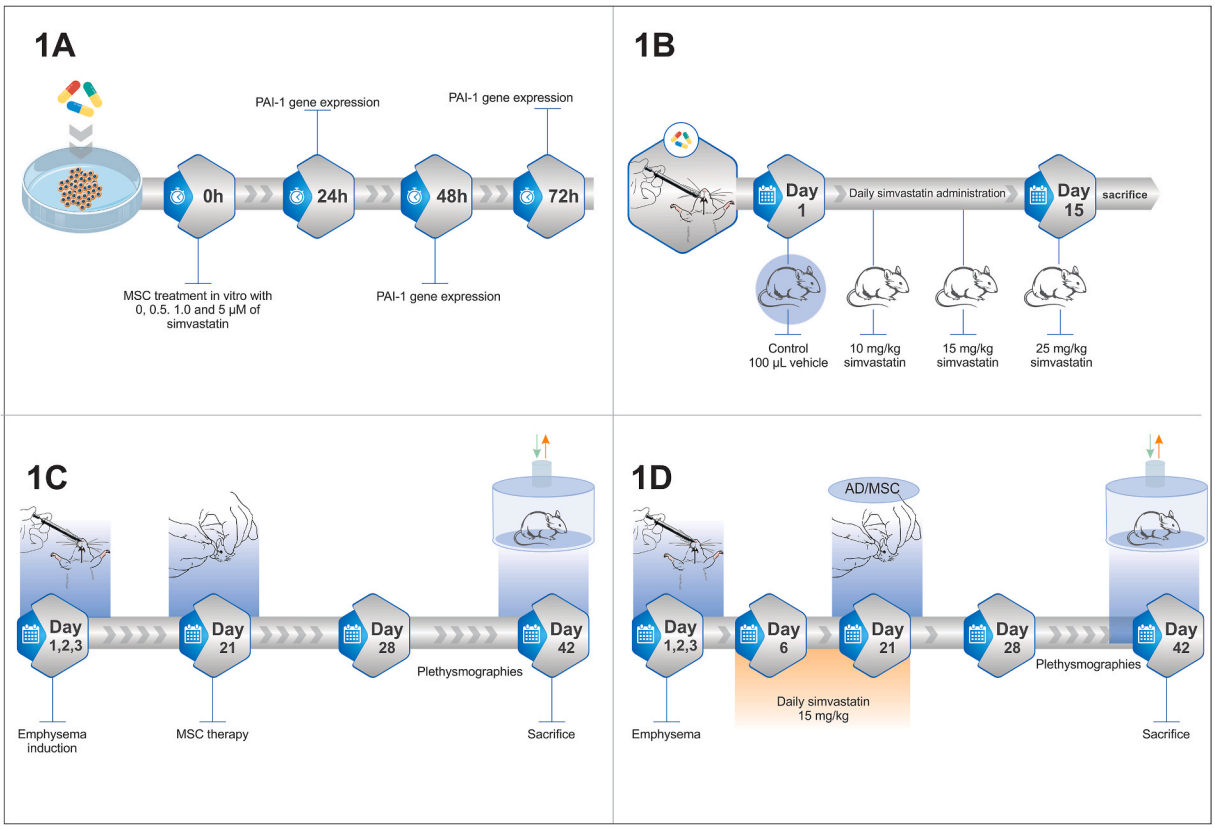

Fig. 1. Experimental design **(A)** Simvastatin dose optimization in vitro. **(B)** Simvastatin dose optimization in vivo. **(C)** MSC therapy for elastase-induced mouse emphysema model. **(D)** Cell therapy and simvastatin.

Table 1
Experimental groups for the simvastatin dose assay in vivo.

Group	Animals (n)	Simvastatin dose (mg/Kg)	Dose per animal (mg/Animal)
Control	5	0	0
SINVA 1	6	10	0,250
SINVA 2	6	15	0,375
SINVA 3	6	25	0,625

Table 2
Experimental groups of induced-emphysema and mesenchymal stromal cell therapy.

Group	Animals (n)	Intranasal instillation 3 doses	Retro-orbital infusion
Vehicle	4	50 μL saline	–
Vehicle/Media	4	350 μL saline	100 μL of RPMI
Elastase	4	50 μL elastase	100 μL of RPMI
Elastase/MSC	4	50 μL elastase	2.6×10^6 MSC 100 μL of RPMI

Three vials of each culture were thawed and underwent cell differentiation test with the StemPro® (Gibco, Life technologies) kit, according to the manufacturer's instructions. One vial of each cell culture was thawed and cultivated for 48 h for luciferase imaging test.

2.4. Effect of simvastatin in an experimental model of MSC cell therapy

The For this assay, 32 female C57Bl/6 mice approximately 8 weeks old were obtained from the Ribeirão Preto Medical School – FMRP USP were randomly divided into four groups of eight animals each, as presented in Table 3. One hour, 24 h and 48 h after cell infusion, the animals

Table 3
Experimental groups for the effect of simvastatin in experimental model Mesenchymal Stromal Cells therapy assay.

Group	Animals (n)	Intranasal instillation	Gavage	Retro-orbital infusion
Normal/Control	8	Saline	Vehicle	4×10^6 AD-MSC
Normal/ Simvastatin	8	Saline	15 mg/kg of simvastatin	4×10^6 AD-MSC
Emphysema/ Control	8	4 IU of elastase	Vehicle	4×10^6 AD-MSC
Emphysema/ Simvastatin	8	4 IU of elastase	15 mg/kg of simvastatin	4×10^6 AD-MSC

underwent in vivo imaging for luciferase and the infused cells were traced. After 21 days of cell therapy (42 days after the beginning of the experiment), the animals were evaluated by hole body pletismography and were euthanized at day 42 by cervical dislocation and had their lungs removed for histological and morphometrical analysis.

2.5. PAI 1 gene expression analysis

Total RNA was isolated from cells or tissues using Trizol Reagent® (Life Technologies, Invitrogen), according to manufacturers' instructions. RNA quality was assessed by microfluidics electrophoresis in a Bioanalyzer® System (Agilent Technologies, Santa Clara, CA, USA), with the Eukaryote Total RNA Nano Chips. The RNA (500 ng) was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Byosistems, Life Technologies, São Paulo, SP, Brasil), according to manufacturers' instructions. Diluted cDNA (1:5) was used in real time PCR reactions with TaqMan® Gene Expression Master Mix

(Applied Byossistems, Life Technologies) and PAI-1-specific FAM TaqMan® probe Mm00435860_m1 (Applied Byossistems, Life Technologies). As endongenous control, Taqman® GAPD-VIC®, NM_008084.2 (Life Technologies) probe was used. All reactions were run in duplicates, in a FAST 7500 System (Life Technologies). Total RNA input was normalized based on the mean of Ct value of GAPDH, used as endogenous control. The fold change was calculated using 2-ΔCt method, as described by Wu and colleagues [44].

2.6. PAI 1 protein analysis

Total protein form cells and tissue samples were obtained using Trizol Reagent® (Life Technologies, Invitrogen), according to manufacturers' instructions. To address PAI 1 circulating levels, blood was collected from animals by heart puncture immediately after euthanasia, and the plasma fraction was isolated by centrifugation. Proteins obtained from cells, tissue or plasma were quantified using BCA Protein Assay (Thermo Scientific, Thermo Fisher Scientific Inc., Rockford, IL, EUA), according to manufacturers' instructions and read in a Versa MaxPLUS ROM equipment (Molecular Devices, Sannyvale, CA, EUA). The primary antibody used was (PAI-1 sc-8979, Santa Cruz Biotechnology Inc., Dallas, TX, EUA) and the control was GADP sc-25778 antibody (Santa Cruz Biotechnology), both diluted 1:1000 in TBS/T. The secondary antibodies were goat anti-rabbit HRP SC-2004, Santa Cruz Biotechnology Inc. diluted 1:2000 and anti-biotine antibodies (cell signaling #7075) diluted 1:10000. Detection was performed with Luminol and the image was captured in a CCD camera using the ImageQuant 4000 (GE Healthcare).

2.7. Whole body plethysmography

In order to assess the lung function of the animals treated with the MSC therapy, the respiratory frequency, tidal volume and pulmonary ventilation were evaluated by the whole body plethysmography method in freely moving mice as previously described by Malan [45]. Briefly, the animals were acclimated to a plethysmography chamber (3 L) for 30 min. After this period, the ports of exit or entrance for gas in the chamber were closed to produce an internal constant volume and the recordings were performed during 1 min. The chamber temperature was around 1 °C above room temperature (approx. 25 °C), with non-significant variation over the period (5 min) that all measurements were made. Rectal temperature was constant, and no significant changes were observed between groups. The signals of breathing frequency (fR) and tidal volume (VT) were measured by changes in the pressure inside the chamber due to both temperature and humidification changes in the inspired/expired gases. A spirometer (model ML141; AD Instruments, Colorado Springs CO, USA) was used for measurements, and the signals were analyzed using Powerlab software (Ad Instruments, Colorado Springs, CO, USA). The system was calibrated with injections of 0.2 mL of room air with the animal inside the plethysmography chamber. Minute ventilation was reported as the product of Fr and VT. Baseline VE measurements were obtained with the animals breathing room air (normoxic conditions).

2.8. In Vivo Imaging System

In order to detect luciferase activity in cultured cells to be used in cell therapy, cell cultures were exposed to 1 mM luciferin in the culture medium. For the detection of transplanted cells expressing luciferase in the recipient's tissues, the luciferase substrate was provided as an intraperitoneal injection of saline containing D-Luciferin 150 mg/kg (Perking Elmer) and the animals were kept under sedation with 4% isofluorane (Cristalia) in oxygen. The images were then captured using In Vivo Imaging System (IVIS 100) (Perkin Elmer, São Paulo, SP, Brazil). When the signal strength reached its maximum levels, the photons emitted by the recipient tissue or culture plate were located, quantified and compared using the manufactures software.

2.9. Histological and morphometrical analysis

The obtained tissues were perfused with fixative solution (paraformaldehyde 4%), and followed histological routine. Histological sections of 7 μm were obtained, stained with Hematoxylin/Eosin (HE) and morphometric analyzes were performed by a single investigator in a blind test. Ten random non-coincident fields of the lung tissue histological slides of each animal were evaluated in 400-fold magnification for the measurement of the mean linear intercept in a light-field optical microscope of the Zeiss model axioskop 2 plus. The morphometric analysis of emphysema was performed using the LM (in micrometers), an indicator of the average alveolar diameter, as proposed by Weibel [46]. For the other tissues, qualitative analyzes were performed.

2.10. Statistical analysis

The data obtained were compared by applying analysis of variance considering a significance level of 5% using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

2.11. Ethical aspects

All experiments were carried out following ethical standards, according to the National Institutes of Health guide for the care and use of Laboratory animals [47]. All animals were kept under controlled light and temperature conditions (12 h light/dark cycle and 24 °C) in the Laboratório de Estudo Experimentais em Animais (LEEA), from Fundação Hemocentro de Ribeirão Preto. Animals were fed with NuvilabCR1R (Nuvital Nutrientes S/A, Colombo, PR, Brasil) and with filtered sterile water ad libitum. The project was approved by the Ethics Committee on the Use of Animals - CEUA, of the University of São Paulo, Ribeirão Preto Campus, under protocol Nº. 14.1.447.53.1.

3. Results

3.1. In vivo experiments for the optimization of simvastatin oral dose

There were no significant morphological or structural changes in the organs of the normal animals treated or not with simvastatin, according to the macroscopic and histological analysis. The RNAs extracted from the lung and liver were of good quality, whereas the pancreatic RNA was of low quality and was not used (Supplementary data). Fig. 2B shows a significant decrease in PAI-1 expression in the lungs of animals treated with simvastatin compared to controls. No difference was observed in the livers. Simvastatin at 15 mg/kg was chosen as the ideal dose used to be used in the next phases of the study. Additionally, we observed that the oral administration of simvastatin significantly decreased the circulating levels of PAI-1 in normal mice, according to western blotting results (Fig. 2C).

MSC therapy for elastase-induced mouse emphysema model.

The optimal elastase concentration was 6.75 U/μL in 150 μL as determined previously (supplementary data). Emphysema induction was confirmed by histological sections of lung tissue, which showed lung parenchyma alterations consistent with emphysema (alveolar destruction, increase in alveolar spaces and inflammatory cell infiltration). The animals subjected to elastase and MSC therapy had histological morphologies similar to control (Fig. 3B). Fig. 3C shows a trend for decreased respiratory frequency, expiratory volume and tidal volume in emphysema-induced animals, but there were no significant differences between the groups.

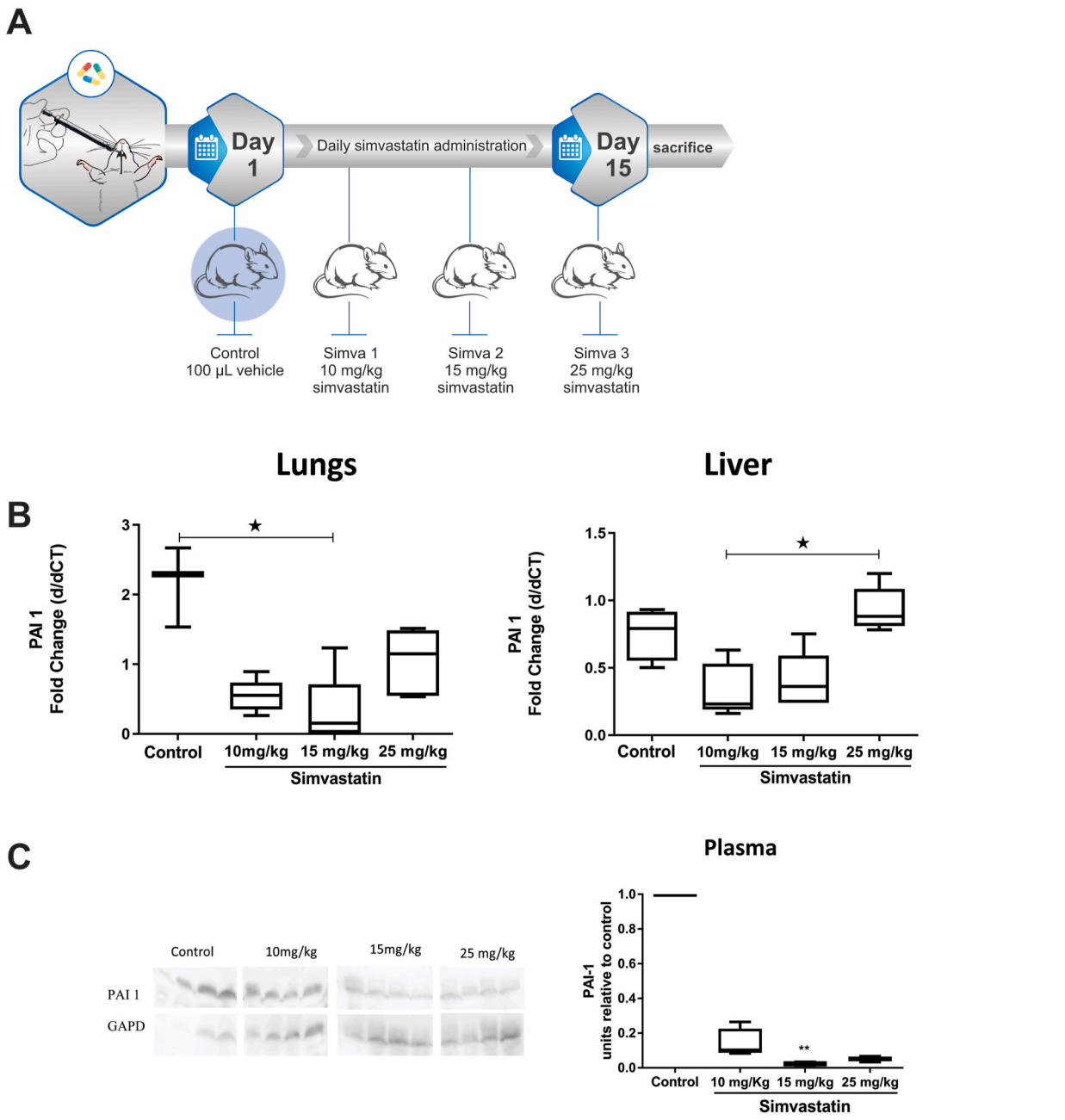

Fig. 2. **(A)** Experimental design of simvastatin dose optimization in vivo. **(B)** ~~Relative~~ PAI-1 gene expression normalized based on the mean of Ct value of GAPDH, used as endogenous control (the fold change was calculated using 2-ΔCt method, as described by Wu and colleagues [44]) in lung and liver tissues from simvastatin-treated mice: vehicle (controls), or simvastatin at 10 mg/kg, 15 mg/kg and 25 mg/kg. **(C)** Photograph of Western blotting membrane showing PAI-1 protein and GAPD (control) protein expression in plasma from mice. The graph shows the western blotting results in arbitrary units related to GAPD.

3.2. Cell therapy and simvastatin

Before cell therapy in vivo experiments, bone marrow (BM-MSC) and adipose derived MSC (AD-MSC) were treated in vitro with simvastatin to confirm the modulation of PAI-1 gene expression by simvastatin, as shown in supplementary data. Right lung after cell therapy with BM-MSC, there was a predominance of the infused cells in the animals' right lung (compared to the left), as assessed by IVIS 100 images, which also show the drop in the presence of signals after 24 and 48 h (Fig. 4B). Fig. 4C shows there was no significant difference between the groups considering the same time of analysis (Oneway ANOVA, Tukey test with a significance level of 5%). The lung function was accessed by unrestrained whole body plethysmography and histomorphological analysis were performed, as shown in Fig. 5.

4. Discussion

One of the main challenges to the success of cell-based therapies is the low survival rates of the infused cells during homing and engraftment, mainly due to the inhospitable microenvironment found in the target tissues, not favorable to cell proliferation [32,36,48]. Two possible ways of addressing this problem include manipulating cells before transplantation, or modulating processes such as cell migration and inflammation of the recipient tissue before infusion, increasing the chances of transplanted cells survival [32,33].

There are few reports in the literature regarding the role of plasmin pathways and drugs that interfere in these pathways, such as simvastatin, in the homing and engraftment processes in cell therapy [32,40]. The results obtained in the present study through the real-time PCR

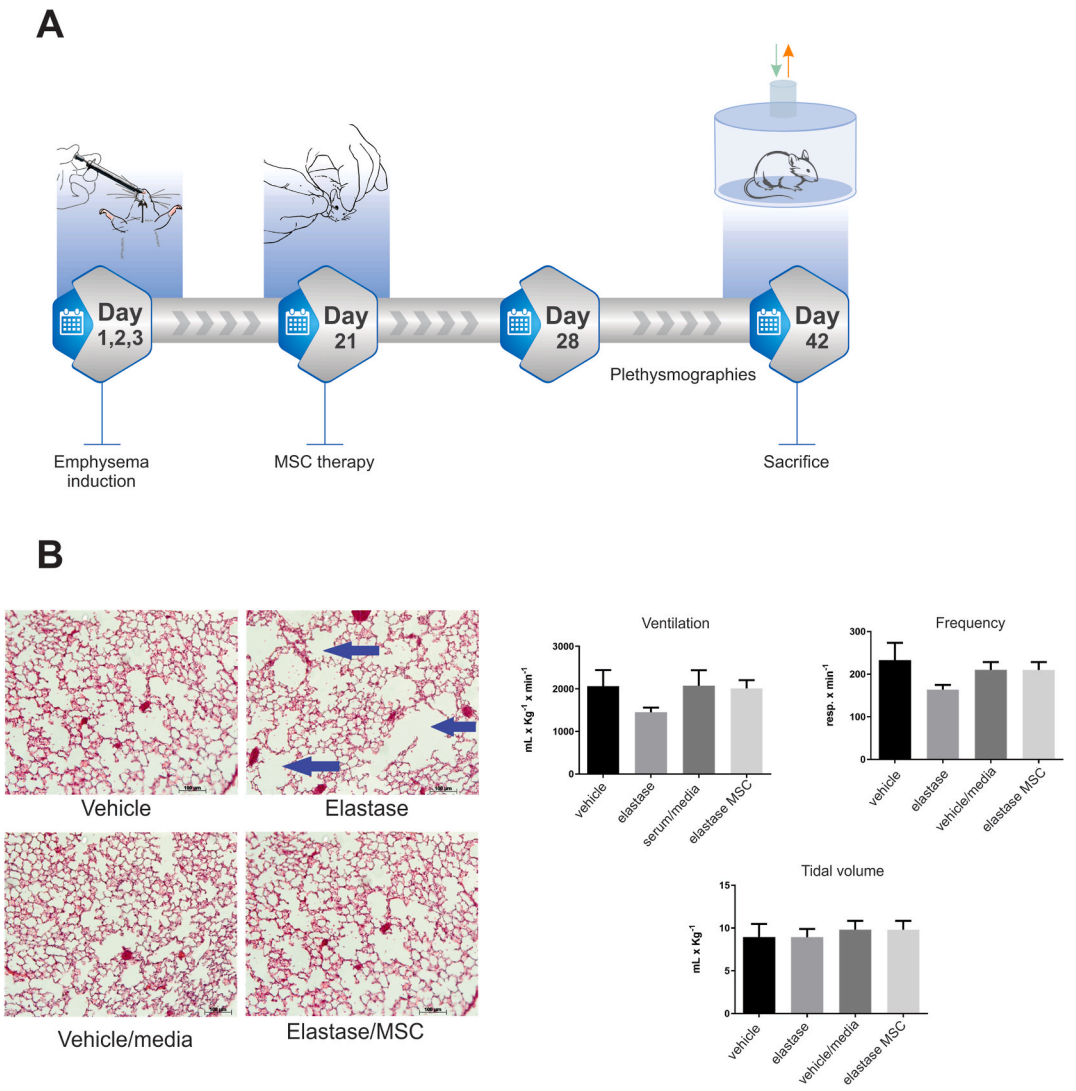

Fig. 3. **(A)** Experimental design of pulmonary emphysema. **(B)** Representative photomicrographs in 40X magnification of histological sections of the lung tissue stained with Hematoxylin-Eosin. Two groups of control animals were used: Vehicle, submitted only to intranasal instillation of saline; and Vehicle/media, subjected to intranasal instillation of saline and RPMI culture medium. Experimental groups were composed of animals subjected to elastase instillation (Elastase) and those submitted to intranasal instillation of elastase and MSC infusion (Elastase/MSC). The blue arrows indicate regions of alveolar septa degradation, characterized by the increase in air spaces and the absence of epithelial cells. **(C)** Plethysmographies results for control and experimental groups of animals 21 days after cell infusion (experimental day 42).

technique showed a significant decrease of PAI-1 gene expression levels in the lungs of the animals subjected to simvastatin administration in doses of 10 mg/kg; 15 mg/kg and 25 mg/kg~~, as presented in~~ (Fig. 2B). It was also possible to observe significantly decreased PAI-1 protein levels in the blood plasma of these animals by means of western blotting, as shown in Fig. 2C. There was a reduction in the levels of PAI-1 48 and 72 h after exposure of the cells to the different doses of simvastatin, with the most pronounced reduction being 48 h after exposure to the 1 μM dose of simvastatin in both cells (Bone marrow and adipose tissue derived, as can be seen in supplementary data). These results are in agreement with previous publications reporting that pretreatment with simvastatin decreased the levels of PAI-1 in culture of HepG2 cells previously increased by insulin induction [49,50]. Also in agreement with those findings, Yu and colleagues [51] observed that simvastatin decreased PAI-1 levels and septic shock in rats induced by Lipopolysaccharide – LPS.

The evaluation of the animal model of cell therapy for pulmonary-induced emphysema shows that the animals developed histomorphological patterns consistent with pulmonary emphysema 21 days after intranasal elastase instillation. Also, the animals exposed to elastase showed regions of enlargement of the alveolar spaces resulting from the proteolysis of the alveolar septa, as can be seen in Fig. 3B. It is also possible to observe that the histomorphological pattern was recovered in the group exposed to elastase and subjected to AD MSC infusion. These data are in agreement with previous reports regarding the morphological pattern of the elastase-induced lesion, as well as the recovery of morphological patterns of the alveolar lesions 21–28 days after cell therapy [5,6,42,52–56].

Despite of histomorphopathological changes promoted due to elastase treatment in mice, the pulmonary ventilation evaluated in vivo animals was preserved. The tidal volume it was similar between the groups and the respiratory frequency tended to be lower after elastase (Fig. 3). We can speculate that under air room condition (normoxic air) the lung commitment demonstrated in the anatomohistological analyses was not reflected on based ventilation. Unfortunately, in this protocol the pulmonary ventilation was not tested under challenge conditions, such as hypoxia and hypercapnia, that represent a forced breathing function which could promote results closer to those of histological

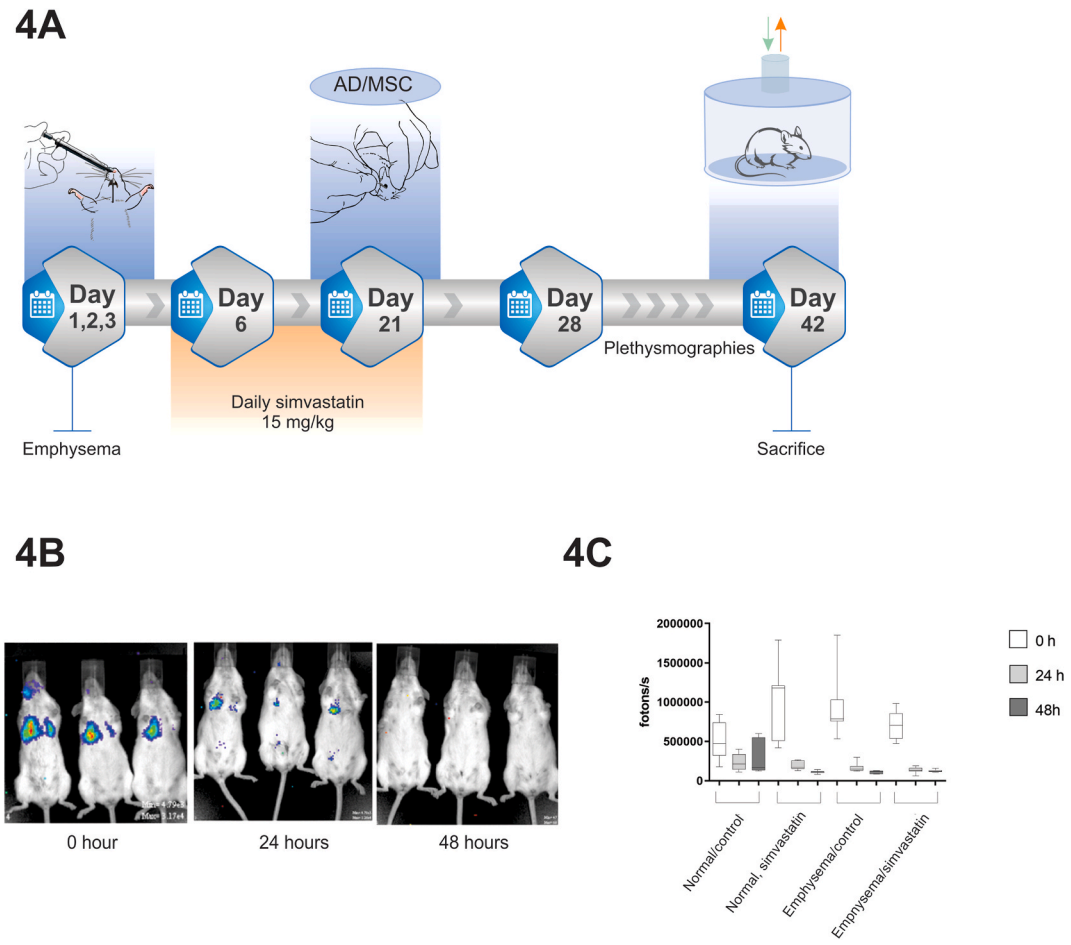

Fig. 4. **(A)** Experimental design of emphysema induction and daily treatment of animals with simvastatin 15 mg/kg for 15 days, followed by cell therapy with AD MSC applied on day 21 **(B)** Luminescence detection of MSC infused in animals. Representative photographs of luminescence detection in recipient animals at 0, 24 and 48 h after cell therapy. The Normal-Control group received intranasal instillation of saline and vehicle gavage, the Normal-Simvastatin group received intranasal instillation of saline and vehicle gavage containing 15 mg/kg of simvastatin. The Emphysema-Control group received intranasal instillation of saline solution containing 4 IU of elastase and vehicle gavage. The Emphysema-Simvastatin group received intranasal instillation of saline solution containing 4 IU of elastase and vehicle gavage containing 15 mg/kg of simvastatin. **(C)** Graph of luminescence in recipient animals: Graph representing the detection of luminescence in the lung tissue of recipient animals 0, 24 and 48 h after cell therapy, in the same groups of animals described in B.

analysis.

Although the mouse is widely used as an animal model of human diseases, there are important differences to be considered. For example, the anatomical differences in the respiratory tract that can have an important impact on the location and deposition of particles and gases in the lung parenchyma. In mice, the main bronchus is wider, shorter and more vertical on the right side than on the left side of the lung, resulting in more pronounced effects on the right side of the lungs after experimental procedures involving the intranasal and intratracheal routes [53, 57,58]. Thus, it is possible to hypothesize that the elastase was deposited in a more concentrated form on the right side during the induction of COPD in mice, which would lead to a more accentuated inflammatory process and injury in the right lung ~~and~~ as well as a greater presence of chemo attractants on the right side, which could further generate better results regarding homing and cell engraftment in the right lung.

The comparative graph of the average alveolar diameter of the animals calculated using the LM allows to confirm that there was a significant difference between the groups submitted to intranasal instillation of elastase and the control groups, indicating the successful induction of emphysema with elastase (Fig. 5). There was no difference between the groups submitted to intranasal instillation of elastase that received or not simvastatin, indicating that simvastatin had any effects on morphological alveolar parameters in the emphysema model. The combined effect of simvastatin and cell therapy~~,~~ was also evaluated by examining the presence of the infused cells in the lung tissue of recipient mice . The results show that the cells remained in the lung tissue of the recipient mice up to 24 h after the infusion and that there was a predominance of the infused cells in the right lung (Figs. 4 and 5).

Anatomical differences, as previously discussed, may lead ~~led~~ to a greater deposition of cells in the right lung, as evidenced by the results obtained in the bioluminescence tests. Thus, it can be proposed that the right lung received a higher proportion of cells and simvastatin. Therefore, it was decided to analyze the average alveolar diameter of the groups considering the right and left lungs separately. As can be seen in the LM comparative graphs, there was no statistically significant difference between the right lungs of control groups and of animals treated with elastase and those that received simvastatin and MSC infusion, indicating no morphological signs of emphysema. There was no difference between the control without emphysema and the control with emphysema that received cells + simvastatin, but there was a difference between control without emphysema and emphysema with cells only, that is, the group with emphysema that received cells + simvastatin regressed until there was no difference to the control group, but only cells did not regress so much. This effect is not detected if we look at both lungs at the same time, can be detected if we look only at the right lung, but it disappears if we look at the left lung only.

In short, by the results obtained from the evaluated animal model, it can be proposed that inflammatory mediators, characteristic of the

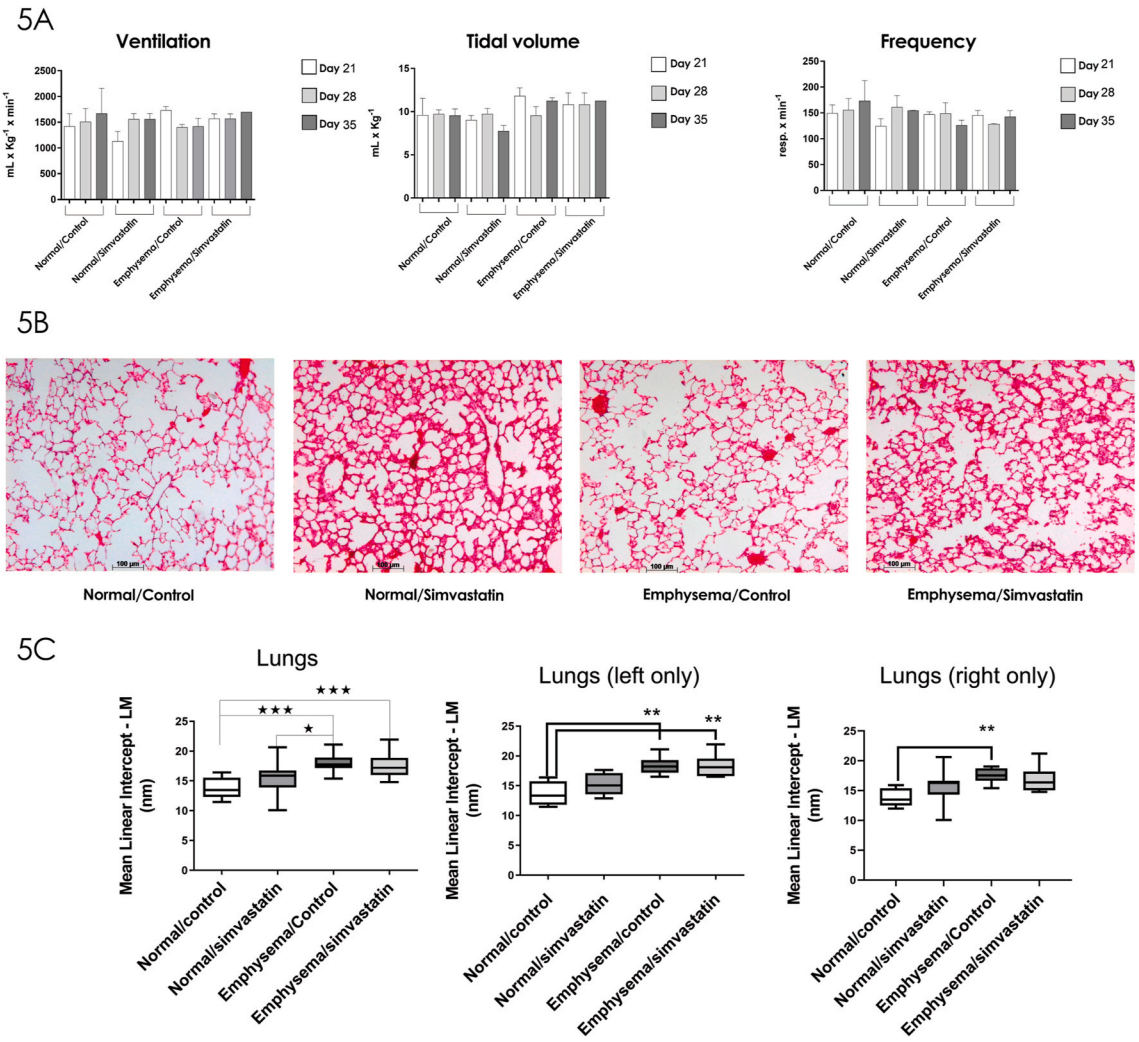

Fig. 5. **(A)** Representative graph of the results of the plethysmographies of the animals one week before and two weeks after cell therapy, with day zero being considered the day of intranasal instillation. The Normal-Control group received intranasal instillation of saline and vehicle gavage, the Normal-Simvastatin group received intranasal instillation of saline and vehicle gavage containing 15 mg/kg of simvastatin. The Emphysema-Control group received intranasal instillation of saline solution containing 4 IU of elastase and vehicle gavage. The Emphysema-Simvastatin group received intranasal instillation of saline solution containing 4 IU of elastase and vehicle gavage containing 15 mg/kg of simvastatin. **(B)** Representative photomicrographs in 400X magnification of histological sections of lung tissue stained with HE - Hematoxylin-Eosin. **(C)** Graphs of the comparative analysis of the mean alveolar diameter considering both sides together – Lungs, and the right and left lung separately – Lungs (left versus right).

COPD/emphysema, would act as chemotactic factors for the migration of the AD-MSC to the injury site. In parallel, the hypothesis that simvastatin would act as a negative modulation factor for PAI-1, thus increasing the viability and survival of MSC in the target organ [36,37] could not be confirmed in this study. Thus, simvastatin did not show an adjuvant role in this model of cell-based therapy, as hypothesized initially. However, it must be emphasized that this study corresponds to an initial approach and new methodological alternatives should be used in future studies, such as different treatment schemes with simvastatin (different doses). Also, to more accurately verify the possible adjuvant effect of simvastatin in cell therapy with AD-MSC, new methods should be incorporated to access lung function in animals from control and treated groups.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pupt.2021.102075.

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