Turk Thorac J 2020; 21(1): 14-20 DOI: 10.5152/TurkThoracJ.2019.180136

Original Article

# Therapeutic Treatment with Abdominal Adipose Mesenchymal Cells Does Not Prevent Elastase-Induced Emphysema in Rats

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**Cite this article as:** Yıldız Gülhan P, Ekici MS, Niyaz M, et al. Therapeutic Treatment with Abdominal Adipose Mesenchymal Cells Does Not Prevent Elastase-Induced Emphysema in Rats. Turk Thorac J 2020; 21(1): 14-20.

Abstract

**OBJECTIVES:** Emphysema and chronic bronchitis have different pathophysiologies but both are significant components of chronic obstructive lung disease (COPD). The levels of Matrix metalloproteinase (MMP)-9 in the bronchoalveloar lavage fluid (BALF) and in serum indicate the presence of emphysema. Intratracheal administration of elastase has been used to create a rat model of emphysema. Adipose tissue-derived mesenchymal stem cells (MSC) have been postulated to prevent or reverse emphysema, however, this has not been examined in the rat model of elastase-induced emphysema.

**MATERIALS AND METHODS:** In this study, 31 Wistar albino rats aged 6–8 weeks and weighing 250–300 g were assessed. On day 1, the animals were treated intratracheally with 0.5 mL saline (control group, n=10), i.e., 0.5 mL saline solution containing 0.1 IU porcine pancreatic elastase (PPE) (Elastase group, n=12) or PPE plus MSC (Elastase-MSC group, n=9) was adminstered per animal. MSCs suspended in serum were injected via the caudal vein on day 21. At least $10^6$ cells were injected. All animals were sacrificed on day 42 and the emphysema index (EI) was calculated, along with measuring the BALF and serum MMP-9 concentrations.

**RESULTS:** Porcine pancreatic elastase induced a significant degree of emphysema in the PPE groups as compared to the control group, which was determined by the EI index (p=0.008). This was not reversed by MSC treatment. The EI remained significantly low in comprison with the controls (p=0.001) and measured no different from the Elastase-treated animals. There was no statistically significant difference between the BALF and serum MMP-9 levels between the control and treatment groups.

**CONCLUSION:** Our findings suggest that therapeutic treatment with adipose tissue-derived MSC in rats has no effect on emphysema or on MMP9 expression, which is a known marker of emphysema.

**KEYWORDS:** Chronic obstructive pulmonary disease, emphysema, mesenchymal cell, matrix metalloprotein

***Received:*** *15.08.2018* ***Accepted:*** *06.02.2019*

## INTRODUCTION

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2017 Guidelines, Chronic Obstructive Lung Disease (COPD) is a common, preventable, and treatable disease caused by exposure to damaging particles or gases and is characterized by permanent airway obstruction and respiratory symptoms [1]. Although both emphysema and chronic bronchitis are associated with distinct pathophysiological mechanisms, they are important components of COPD. These pathophysiological mechanisms include a protease-antiprotease imbalance that causes matrix damage and emphysema, oxidative stress that drives inflammatory cell migration and protein oxidation, alveolar matrix destruction and disturbed regenerative capacity in small airways, excessive matrix accumulation in the arteries that leads to pulmonary hypertension, and endothelial and epithelial apoptosis [2,3].

Chronic obstructive lung disease treatment is mainly targeted toward controlling symptoms, therefore, these medicines are not curative [4]. Mesenchymal stem cells (MSCs) have been proposed as a possible treatment for a variety of diseases due to their characteristics, including ease of isolation, ability to replicate in large numbers in different cultures, capacity to differentiate and possess immunosuppressive characteristics, and the ability to migrate to areas of cellular damage [5]. Previous therapeutic studies in rat emphysema models have provided evidence that MSCs are protective against the development of emphysema [6,7].

Autologous MSCs can be readily isolated from the bone marrow and other tissues and have been shown to reduce inflammation and contribute to the repair process in several disease models [5]. Therefore, the use of MSCs, either alone or in

*This study was presented at the Turkish Thoracic Society 17. Annual Congresses, SS-005, 2-6 April 2014, Antalya, Turkey and ERS International Congress 2014, P-1848, 6-10 September 2014, Munich, Germany.*

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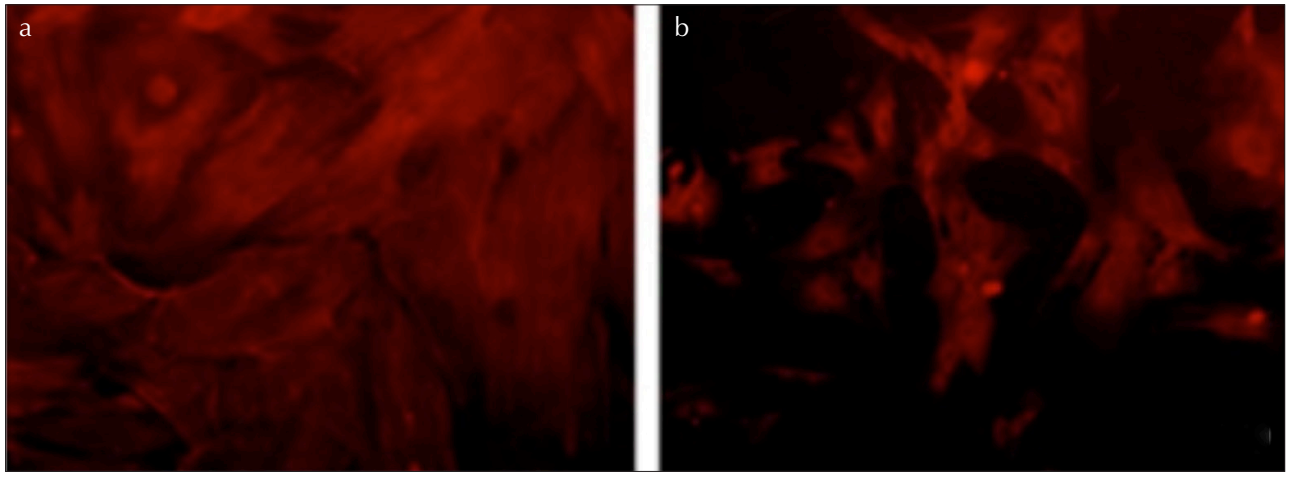


**Figure 1. a, b.** Mesenchymal stem cells after immunofluorescence staining (a) CD13 (b) CD29 positive cells (X20)

combination with novel bioengineering approaches, may have therapeutic potential for pulmonary repair and remodeling [8]. Indeed, a recent phase II clinical study was conducted with MSCs in patients with mild and moderate COPD [9]. MSCs has been evaluated in several therapeutic models of severe pulmonary diseases, including acute pulmonary injury [10], COPD [11], pulmonary hypertension [12], asthma [13], and lung fibrosis [14]. In experimental models, MSCs have been applied to the lungs via both intravenous and intratracheal routes. Improvements in pulmonary damage have been demonstrated by flooding endogenous pulmonary stem cells with MSC phenotype in rat lungs that have been treated by elastase [11].

This study demonstrated a reduction in the inflammatory response of the MSCs such that the MSC treatment was safe in COPD patients, however, no beneficial effects were observed in pulmonary functions.

We hypothesized that MSCs can attenuate emphysema and decrease the levels of bronchoalveolar lavage fluid (BALF) and serum Matrix metalloproteinase (MMP)-9 in a rat model of elastase-induced emphysema. The specific aim was to assess the therapeutic potential of adipose tissue-derived MSCs and the effect on MMP-9 expression, as this would be more akin to the approach required to treat COPD patients.

## MATERIALS AND METHODS

The current study was approved by the Institutional Review Board of Kırıkkale University (Ethics Committee No: 2011/121) and performed in the Animal Laboratories of University between April-May 2010. All procedures were carried out in compliance with the Declaration of Helsinki (1986).

### Isolation of the Adipose Tissue MSC

Mesenchymal stem cells were isolated from subcutaneous adipose tissue in the flanks of the rats (Wistar albino, 300 g, 6-8 weeks, male rats). MSCs were characterized using immunofluorescence staining and flow cytometric analysis as described below.

### Characterization of MSCs by Immunofluorescence Staining

The expression of two MSC-selective surface antigens CD13 and CD29 was analyzed using immunofluorescence staining (Figure 1).

Flow cytometric analyzes of CD29, CD45, CD54, CD90, CD106, MHC Class I, and MHC Class II cell surface markers were performed in the Center for Stem Cell and Gene Therapies Research and Application Center of Kocaeli University (SCGTR, Kocaeli, Turkey) as previously described (Figure 2).

### Study Design

A total of 34 Wistar albino rats weighing 250-300 g were divided into three groups. Animals were anaethetized with ketamine (100 mg/kg; intraperitoneally) before the vehicle (5% Gummi Arabicum adhesive [Arabic Gum]) (n=10) or porcine pancreatic elastase (PPE, CALBIOCHEM, EMD Biosciences Inc., CA) was delivered via the intratracheal route as previously described [15]. The vehicle solution was administered 1 hour prior to the treatment with PPE (0.1 IU/g of body weight in 0.5 ml saline) (n=13). PPE solutions in saline were freshly prepared under sterile conditions just before use. Rats were placed in the Trendelenburg position to enable even distribution of elastase in both lungs. The number of the rats with post-elastase lethality in the elastase and elastase+MSC groups were n=1 and n=2, respectively.

On the 21$^{st}$ day of the study, 1x10$^{6}$ MSCs suspended in physiological saline solution were administered to the elastase+MSC group (n=11) via the tail veins. On day 42 of the study, rats in all three groups were sacrificed under high-dose ketamine (100 mg/kg) anesthesia.

Bronchoalveloar lavage fluid was performed immediately after the rats were sacrificed and the trachea was ligated from

**MAIN POINTS**

- There is no curative treatment of COPD
- Stem cell therapy may be the key for the future curative treatment of COPD
- Mesenchymal cells derived from adipose tissue can be use for COPD treatment
- Different methods are used for the application of stem cells.
- Mesenchymal cells applied through tail vein did not prevent to emphysema

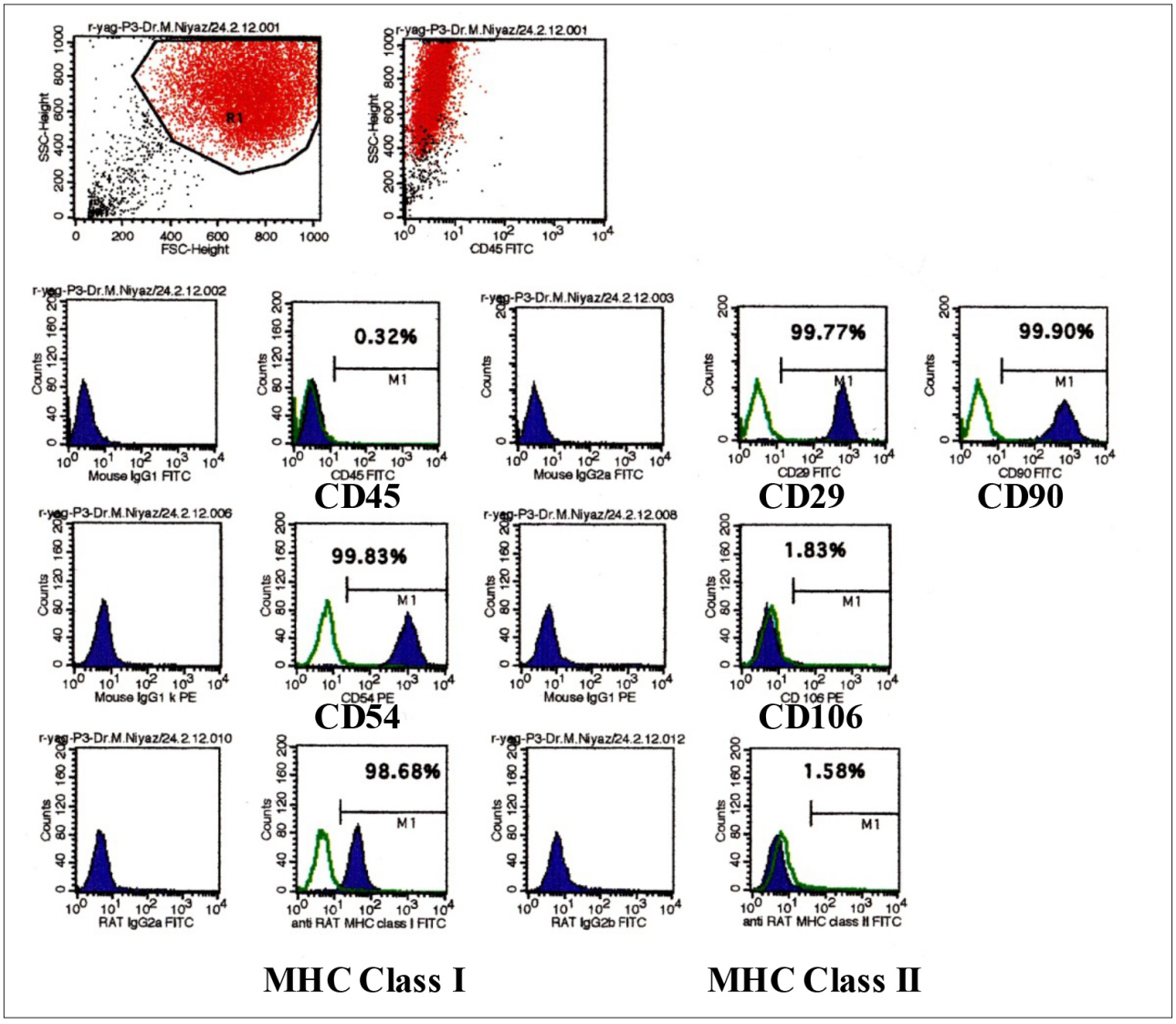


**Figure 2.** The results flow cytometry analysis of the mesenchymal stem cells. CD29, CD90, CD54 and MHC Class I positive; CD45, CD106, and MHC Class I negative MSCs. These results represent 98.68 of the cells and the positive and negative results for mentioned antigens shows MSCs with very high accuracy and homogeneity

the upper end as described previously [16]. BALF was collected using 3x 3 mL saline washes delivered via tracheal cannulae. The pooled BALF was cooled and centrifuged at 1500 rpm for 5 minutes, and the supernatant was kept aside for MMP-9 analysis.

**BALF and Serum MMP-9 Analysis**

Bronchoalveloar lavage fluid and serum MMP-9 levels were measured by commercially available ELISA kits according to the recommendations of the manufacturer (Med-Systems Diagnostics Gmbh, Vienna, Austria).

**Histological Analysis of The Lung**

The right and left lungs of the rats were taken out after sacrifice and fixed in 10% formaldehyde. Lung tissues were divided into 2 mm-thick blocks and embedded in paraffin, following which 5 μm tissue slices were cut and stained with hematoxylin-eosin before being examined under the light microscope [17]. Five random photographs of each slide were obtained and an emphysema index (EI) was calculated using the following formula.

Emphysema Index = (Emphysema area + Normal area) / (Emphysema area + Normal area + Stromal field).

**Statistical Analysis**

All data were analyzed using the Statistical Packages for the Social Sciences software, version 11.5 (SPSS Inc.; Chicago, IL, USA) . After performing the descriptive statistical analyses (frequency, percentage distribution, mean±standard deviation, median [minimum-maximum]), the Kruskal-Wallis Analysis of Variance Test was applied. When the results showed significant differences among the groups, the Mann-Whitney U test was used. The association between continuous variables was evaluated by the Spearman's Correlation Test. A value of $p \leq 0.005$ was considered to be statistically significant.

**RESULTS**

A statistically significant difference was found in the median EI values among the three rat groups (p=0.005) (Figure 3a-c) (Table 1). The median EI of both the elastase (p=0.008) and

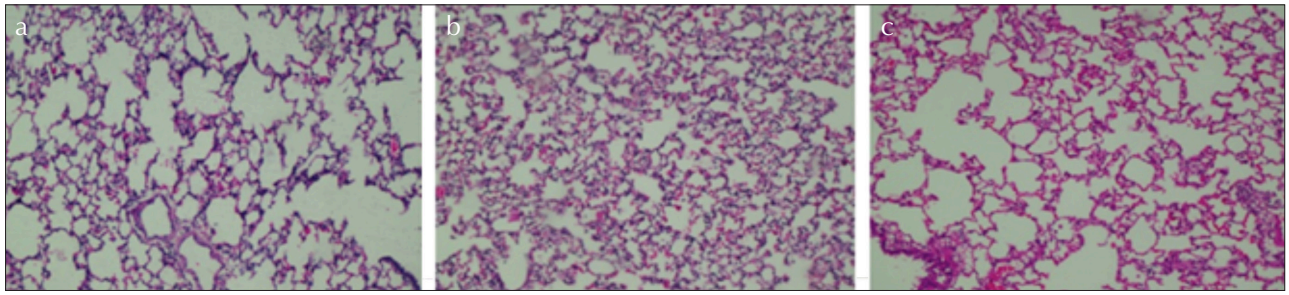


**Figure 3. a-c.** Histological appearance of rat lungs in the (a) Control group (n=10), (b) Elastase group (n=12), and (c) Elastase + MSC group (n=9). Images are representative of 5 images per lung from each animal [Hematoxylin-eosin x 200]

**Table 1.** Comparison of the EI, serum MMP-9, and BAL MMP-9 levels of the three groups

| | | EI (%) | Serum MMP-9 | BAL MMP-9 |
|---|---|---|---|---|
| Elastase (n=12) | Mean | 76.75 | 5.60 | 5.11 |
| | SD | 5.57 | 1.07 | 0.08 |
| | Median | 76.45 | 5.19 | 5.09 |
| | Minimum | 69.50 | 5.08 | 5.02 |
| | Maximum | 87.30 | 8.85 | 5.31 |
| Elastase-MSC (n=9) | Mean | 76.22 | 5.29 | 5.09 |
| | SD | 6.13 | 0.26 | 0.03 |
| | Median | 77.60 | 5.23 | 5.08 |
| | Minimum | 61.70 | 5.06 | 5.04 |
| | Maximum | 84.30 | 5.95 | 5.13 |
| Control (n=10) | Mean | 82.94 | 5.52 | 5.11 |
| | SD | 2.83 | 0.19 | 0.04 |
| | Median | 84.00 | 5.42 | 5.10 |
| | Minimum | 78.70 | 5.36 | 5.06 |
| | Maximum | 85.60 | 5.84 | 5.16 |
| P-value | | 0.005* | 0.018+ | 0.742 |

MMP-9: matrix metalloproteinase; EI: emphysema index; BAL: bronchoalveolar lavage; MSC: mesenchymal stem cells

**Table 2.** The association of the EI, serum MMP-9 and BAL MMP-9 levels in all rats

| | | EI | Serum MMP-9 |
|---|---|---|---|
| Serum MMP-9 | r | 0.205 | |
| | p | 0.269 | |
| BAL MMP-9 | r | -0.069 | 0.184 |
| | p | 0.712 | 0.323 |

MMP-9: matrix metalloproteinase; EI: emphysema index; BAL: bronchoalveolar lavage

elastase+MSC (p=0.001) groups were significantly lower than that of the control group. Therapeutic administration of MSCs had no effect on EI.

There were no statistically significant differences in BALF or serum MMP-9 levels between the three groups (Table 1).

**Table 3.** Association of the EI, serum MMP-9 and BAL MMP-9 values in the Elastase Group

| Elastase | | EI | Serum MMP-9 |
|---|---|---|---|
| Serum MMP-9 | r | 0.214 | |
| | p | 0.505 | |
| BAL MMP-9 | r | -0.260 | 0.030 |
| | p | 0.414 | 0.926 |

MMP-9: matrix metalloproteinase; EI: emphysema index; BAL: bronchoalveolar lavage

**Table 4.** Association of the EI, serum MMP-9 and BAL MMP-9 values in the Elastase-MSC Group

| Elastase-MSCs | | EI | Serum MMP-9 |
|---|---|---|---|
| Serum MMP-9 | r | -0.628 | |
| | p | 0.070 | |
| BAL MMP-9 | r | -0.380 | 0.470 |
| | p | 0.313 | 0.201 |

MMP-9: matrix metalloproteinase; EI: emphysema index; BAL: bronchoalveolar lavage; MSC: mesenchymal stem cells

**Table 5.** Association of the EI, serum MMP-9 and BAL MMP-9 values in the Control Group

| Control | | EI | Serum MMP-9 |
|---|---|---|---|
| Serum MMP-9 | r | -0.600 | |
| | p | 0.067 | |
| BAL MMP-9 | r | 0.200 | -0.600 |
| | p | 0.580 | 0.067 |

MMP-9: matrix metalloproteinase; EI: emphysema index; BAL: bronchoalveolar lavage

When all rats included in the study were evaluated together, no significant associations were observed between the EI values and the serum levels of MMP-9 (r=0.205, p=0.269), between the EI values and the BAL MMP-9 levels (r=–0.069, p=0.712), or between serum MMP-9 levels (r=0.184, p=0.323) and the BAL MMP-9 levels (Table 2).

When rats in the elastase group alone were evaluated, no statistically significant associations were detected between the EI and serum MMP-9 (r=0.214, p=0.505) levels, between

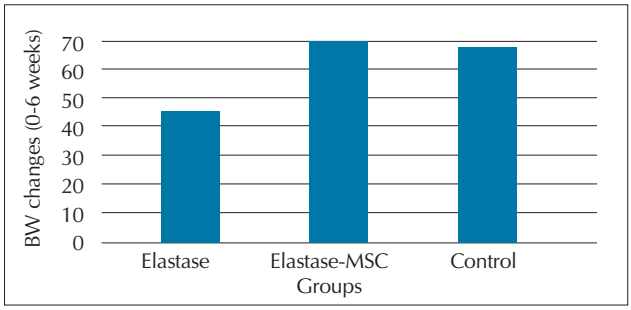


**Figure 4.** Bar graph showing mean values of BW changes (0–6 weeks) in the three groups

the EI and BAL MMP-9 (r=–0.260, p=0.414) levels, and between the serum MMP-9 (r 0.030, p=0.926) and BAL MMP-9 levels (Table 3).

When the rats in the elastase+MSE group were evaluated, no significant associations were observed between the EI levels and serum MMP-9 (r=–0.628, p=0.070) levels, between the EI and BAL MMP-9 (r=–0.380, p=0.313) levels, or between the serum MMP-9 levels (r=0.470, p=0.201) and BAL MMP-9 levels (Table 4).

Finally, there was no correlation between EI and serum or BAL MMP-9 in the control group alone (Table 5).

There were no statistically significant difference between the baseline body weight (BW) (p=0.119) values and the BW change values (p=0.153) in the three rat groups (Figure 4).

## DISCUSSION

In this study, we confirmed the ability of PPE to induce significant levels of emphysema in rat lungs. Contrary to expectations, we did not show any effect of therapeutic administration of adipose tissue-derived MSCs on PPE-induced emphysema, as was determined histologically. In addition, we were unable to demonstrate any correlation of the degree of emphysema with blood or BALF levels of MMP-9. Previous studies have shown that a single-dose of intratracheal PPE results in a degree of airspace enlargement that is similar to emphysema-like lesions [18,19] and that the degree of emphysema is enhanced with repeated doses of PPE [20].

MSCs have been proposed as potential candidates for the treatment of many diseases [5]. Previous studies in rat models of emphysema have provided evidence that prophylactic therapy with MSCs provides protection against the development of emphysema [6,7]. In addition, MSCs suppress inflammation in animal models of acute pulmonary injury [21-23] and partially improve pulmonary emphysema in papain- or elastin-induced animal (mice and rat) emphysema models [24-26]. Intrapulmonary therapeutic administration of MSCs to rats chronically exposed to cigaret smoke is also protective against emphysema [27]. However, although these reports indicate success using prophylactic interventions or adipose tissue-derived MSCs, which are promising options in cell therapy [28], they reported no regression in the emphysema areas induced by PPE. One limitation of our study was that we did not perform immunohistochemical evaluation of the presence of adipose tissue-derived MSCs in the lung tissue. Failure of these cells to migrate to the lung could account for the lack of effect seen, although other studies have reported efficient targeting of the lung by MSCs injected into rat tail veins [29].

The proposed use of MSCs in the treatment of pulmonary diseases, such as acute lung injury, pulmonary fibrosis, and COPD is based on the capacity of these cells to modulate local inflammatory and immunological responses [30]. A phase II, multi-center, randomized, and placebo-controlled study using allogeneic MSCs in patients with moderate to severe COPD demonstrated that this therapy was safe and that the MSC infusion resulted in a significant reduction in the CRP levels [31]. Monthly systemic MSC infusion in these COPD patients had no effect on adverse events, but also did not reduce the exacerbation frequency or alter the course of disease [32]. In contrast, bone marrow-derived stem cells that were infused systemically into 4 patients with COPD/pulmonary emphysema and grade IV dyspnea resulted In only a slight improvement in spirometry over a 12-month period [33].

Autologous lung-derived MSCs are also considered to have potential beneficial effects in the treatment of pulmonary emphysema [34]. These were shown to be safe in a small (n=10) Phase 1 clinical trial involving GOLD stage 3-4 COPD patients, when they were used as an add-on treatment to one-way endobronchial valves (EBV). Allogeneic bone marrow-derived MSCs ($10^8$ cells) were given just before the insertion of one-way EBVs and no adverse effects were seen after 90 days [35].

MMP-9 levels are increased in the lungs [36] and in alveolar macrophages [37,38] of patients with COPD. MMP-9 production is further increased in circulating monocytes of individuals with advanced emphysema [39]. However, in a study of 101 patients with emphysema, although BALF MMP levels were higher in emphysema patients as compared to nonsmoking controls, MMP-9 did not predict the severity or progression of emphysema due to elevated levels, which were also seen in the healthy smoking control group [40]. In addition, the release of MMP-9 from serum platelets or leukocytes was enhanced upon sampling and the measured serum MMP-9 levels may not have accurately reflected the circulating MMP-9 concentrations [41,42]. Accumulation of MMP-9-expressing pulmonary alveolar macrophages has been reported to accompany the development of emphysema in the intraperitoneral mouse model of PPE-induced emphysema [43]. In contrast, in our study, we did not show any link between BALF or serum levels of MMP-9 and emphysema scores in our rat model after 6 weeks. A limitation of our study was that we did not examine the time-course of BALF or serum MMP-9 expression in our model and we may have missed the optimal time point for this analysis.

Loss of body and muscle mass in COPD (pulmonary cachexia) causes skeletal muscle weakness and impaired exercise capacity [44,45]. The association of the pulmonary cachexia to pulmonary inflammation [46] and the increased levels of circulating inflammatory cytokines [47-49] suggests that sys-

temic inflammations may trigger or contribute to muscle atrophy [50]. These systemic abnormalities can also be mimicked by PPE administration in animal models of emphysema [20,51-53]. Although weight gain was the lowest in rats in the elastase group, which was consistent with the literature, this did not reach statistical significance.

The results of our study demonstrated that the emphysematous areas of the elastase and elastase + MSC groups were larger compared to the control group and that the serum and BALF MMP-9 levels were similar in all the three groups of animals. Although evidence in previous animal models of emphysema indicated that prophylactic MSCs prevent the onset or development of emphysema, our results indicate that therapeutic administration of adipose tissue-derived MSCs are ineffective at reversing emphysema. Our results fit with the overall safety and efficacy profile of therapeutic MSC infusion in COPD patients, but future studies should examine the presence of these MSCs in lung tissue to assess whether sufficient MSCs reached the lung tissue to have an effect on emphysema. Our data on BALF and serum MMP-9 levels were partly in agreement with the literature in general. However, our findings regarding the effect of MSCs on MMP-9 levels paralleled our findings on emphysema with no observed effect. Overall, our data does not support the therapeutic benefit of adipose tissue-derived MSCs for the treatment of emphysema in COPD patients.

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**Ethics Committee Approval:** Ethics committee approval was received for this study from the ethics committee of Kırıkkale University (Ethics Committee No: 2011/121).

**Informed Consent:** Written informed consent was obtained from patients who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - P.Y.G., M.S.E., M.N., A.E.; Design - P.Y.G., M.S.E., M.N., A.E.; Supervision - P.Y.G., M.S.E., M.N., M.G.; Resources - P.Y.G., M.S.E., M.N., A.E.; Materials - P.Y.G., M.G., N.A., M.N., M.E.E.; Data Collection and/or Processing - P.Y.G., M.N., M.E.E., N.A.; Analysis and/or Interpretation - P.Y.G., M.S.E., M.H., A.E.; Literature Search - P.Y.G., M.G., M.E.E., N.A.; Writing Manuscript - P.Y.G., M.G.; Critical Review - P.Y.G., M.S.E., M.N., M.G., M.E.E., N.A., A.E.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

**Financial Disclosure:** This study was supported by Kırıkkale University BAP unit (2010/21).

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