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Title: THERAPEUTIC TREATMENT WITH MESENCHYMAL CELLS ISOLATED FROM ABDOMINAL ADIPOSE TISSUE DOES NOT PREVENT ELASTASE-INDUCED EMPHYSEMA IN THE RAT

Short Title: The effect of Mesenchymal cells on COPD

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ABSTRACT

Background: Emphysema and chronic bronchitis have different pathophysiology but are both significant components of chronic obstructive lung disease (COPD). The levels of matrix metalloproteinase (MMP-9) in bronchoalveloar lavage fluid (BALF) and in serum are associated with emphysema.Intratracheal admnistration 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 but this has not been examined in the rat moel of elastase-induced emphysema.

Methods: In this study, 6/8- week-old 31 Wistar albino rats weighing between 250-300 g were assessed. On day 1 animals were treated intratracheally with 0.5 mls saline (Controls, n=10); 0.5 ml saline solution containing 0.1 IU porcine pancreatic elastase (PPE)

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per animal (Elastase group, n=12) or PPE plus MSC (Elastase-MSC group, n=9). MSCs suspended in serum were injected via the caudal vein at day 21. At least 10^6 cell were injected. All animals were sacrificed on day 42 and the emphysema index (EI) was calculated and BALF and serum MMP-9 concentrations measured.

Results: PPE induced a significant degree of emphysema compared to controls as determined by the EI index (p=0.008). This was not reversed by MSC treatment and the EI remained significantly reduced compared to controls (p=0.001) and no different from Elastase-treated animals. There was no statistically significant difference between serum and BALF MMP-9 levels between the control and treatment groups.

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

Key Words: Chronic obstructive pulmonary disease, emphysema, mesenchymal cell, matrix metalloprotein.

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INTRODUCTION

According to the GOLD 2017 Guidelines, COPD is a common, preventable, and treatable disease caused by exposure to damaging particles or gasses and is characterized by permanent airway obstruction and respiratory symptoms (1). Both emphysema and chronic bronchitis are the important components of COPD although they are associated with distinct pathophysiological mechanisms. These pathophysiological mechanisms include a protease-antiprotease imbalance which causes matrix damage and emphysema; oxidative stress which drives inflammatory cell migration and protein oxidation; alveolar matrix destruction and disturbed regenerative capacity in the small airways, as well as excessive matrix accumulation in the arteries, leading to pulmonary hypertension; and endothelial and epithelial cell apoptosis (2, 3).

COPD treatment mainly targets symptoms but these medications are not curative (4). Mesenchymal stem cells (MSCs) have been proposed as candidate treatment in a variety of diseases due to their characteristics, including easy of isolation; their ability to replicate in large numbers under culture environments; their capacity to differentiate and possess immunosuppressive characteristics and their ability to migrate 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). The use of MSCs, alone or in combination with novel bioengineering approaches, may therefore have therapeutic potential for pulmonary repair and remodelling

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(5). Indeed, a recent phase II clinical study was conducted with MSCs in patient with mild and modarate 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 the pulmonary damage have been demonstrated by administering the endogenous pulmonary stem cells with MSC phenotype to the rat lungs treated by elastase (11).

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

We hypothesized that MSCs will attenuate emphysema and decrease the levels of bronchoalvelar lavage fluid (BALF) and serum 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 Ethics Committee of University (Ethics Committee No: 2011/121) and performe in the Animal Laboratories of University between April-May 2010. All procedures were carried out in compliance with the Declaration of Helsinki (1986).

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Isolation of the Adipose Tissue Mesenchymal Stem Cells

MSCs were isolated from subcutaneous adipose tissue in the flanks of the rats (Wistar albino, 6-8 week old male rats, 300 g). MSCs were characterised 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 analysed using immunofluorescence staining.

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

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Study design

34 Wistar albino rats weighing 250-300g were divided into three groups. Animals were anaethetised with ketamine (100 mg/kg; intraperitoneally) before vehicle (5% Gummi Arabicum adhesive [Arabic Gum]) (n=10) or porcine pancreatic elastase (PPE, CALBIOCHEM,

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EMD Biosciences Inc., CA) were delivered via the intratracheal route as previously described (15). The vehicle solution was administered one hour prior to the treatment with PPE (0.1 IU/g of body weight in 0.5ml 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 the even distribution of elastase into 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 21st day of the study, $1x10^6$ MSCs suspended in physiological saline solution was administered to the Elastase-MSC Group (n=11) via the tail veins. On day 42 of the study, all rats in the three groups were sacrificed under high-dose ketamine (100 mg/kg) anaesthesia.

Bronchoalveolar lavage (BAL) was performed immediately after the rats were sacrificed and the trachea was ligated from the upper end as described previously (16). BALF was collected using 3x 3ml saline washes delivered via a tracheal cannulae. The pooled BALF was cooled and centrifuged at 1500 rpm for 5 min and the supernatant kept for MMP-9 analysis.

BALF and Serum MMP-9 Analysis

BALF and serum MMP-9 levels were measured by commercially available ELISA kits as recommended by the manufacturer (Med-Systems Diagnostics Gmbh, Vienna, Austria).

Histological Analysis of The Lung

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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 embedding in paraffin and 5 µm tissue slices cut and stained with hematoxylin-eosin before being examined under the light microscope (17). 5 random photographs of each slide were obtained and an emphysema index (EI) calculated using the following formula (Prof. Dr Önder Bozdoğan et al., Kırıkkale Pathology AD.): Emphysema Index = (Emphysema area + Normal area) / (Emphysema area + Normal area + Stromal field).

Statistical Analyses

All data were analyzed using "Statistical Packages for the Social Science" (SPSS) 11.5. After performing the descriptive statistical analyzes (frequency, percentage distribution, mean ± standard deviation, median [minimum-maximum]), *the Kruskal Wallis Analysis of Variance Test* was applied. When the results show 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≤0.005 was considered to be statistically significant.

RESULTS:

A statistically significant difference was found in the median Emphysema Index (EI) values among the three rat groups in the study (p = 0.005)(**Figure 3a-c**)(**Table 1**). The median EI of both the Elastase (p=0.008) and Elastase-MSC (p = 0.001) groups were significantly lower than that of the control group. Therapeutic administration of MSCs had no effect on EI.

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

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		Eİ (%)	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
	Min.	69.50	5.08	5.02
	Max	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
	Min.	61.70	5.06	5.04
	Max	84.30	5.95	5.13

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Control (n=10)	**Mean**	82.94	5.52	5.11
	SD	2.83	0.19	0.04
	Median	84.00	5.42	5.10
	Min.	78.70	5.36	5.06
	Max	85.60	5.84	5.16
P value		**0.005***	0.018[+]	0.742

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) and between serum MMP-9 levels (r=0.184, p=0.323) and the BAL MMP-9 levels (**Table 2**).

		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

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 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**).

		EI	**Serum MMP-9**
Serum MMP-9	**r**	0.214	
	p	0.505	
BAL MMP-9	**r**	-0.260	0.030

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	p	0.414	0.926

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; between the serum MMP-9 levels (r=0.470, p=0.201) and BAL MMP-9 levels (**Table 4**).

		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

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

		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

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

DISCUSSION

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In this study we confirmed the ability of PPE to induce significant levels of emphysema in the rat. Contrary to expectations, we did not show any effect of therapeutic administration of adipose tissue-derived MSCs on PPE-induced emhysema as 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 the airspace enlargement similar to emphysema-like lesions (18,19,) and that degree of emphysema is enhanced with repeated PPE doses (20).

Mesenchymal stem cells (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 rat MSCs to rats chronically exposed to cigarette smoke is also protective against emphysema (27). However, although these reports indicate success using prophylactic interventions or study using adipose tissue-derived MSCs, a promising option in cell therapy (28), provided 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. A 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 the rat tail vein (29).

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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, multicenter, randomized, placebo-controlled study using allogeneic MSCs in patients with moderate to severe COPD demonstrated that this therapy was safe and that the MSC infusion gave a significant reduction in the CRP levels (31). Monthly systemic MSC infusion in these COPD patients had no effect on adverse events but did not reduce exacerbation frequency or the course of disease (32). In contrast, bone marrow-derived stem cells were infused systemically into 4 patients with COPD/pulmonary emphysema and grade IV dyspnea and gave 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 (10 patients) phase 1 clinical trial in GOLD stage 3-4 COPD patients when 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 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 compared to non-smoking controls, MMP-9 did not predict severity or progression of emphysema due to elevated levels also being seen in the healthy smoking control group (40). In addition, the

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release of MMP-9 from serum platelets or leukocytes is enhanced upon sampling and measured serum MMP-9 levels may not accurately reflect 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 mouse intraperitoneral PPE-induced emphysema model (43). In contrast, 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 systemic 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, consistent with the literature, this did not reach statistical significance.

6. CONCLUSIONS AND RECOMMENDATIONS

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 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

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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 in part 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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FIGURE LEGENDS

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

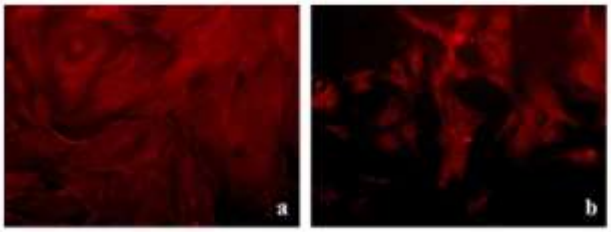

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Figure 2: The results of flow cytometry analysis of the mesenchymal stem cells. CD29, CD90, CD54 and MHC Class I pozitive; CD45, CD 106 and MHC Class I negative MSCs. These results represent 98.68 of the cells and the positive and negative results for mentioned antigens shows MSCs with very high accuracy and homogeneity.

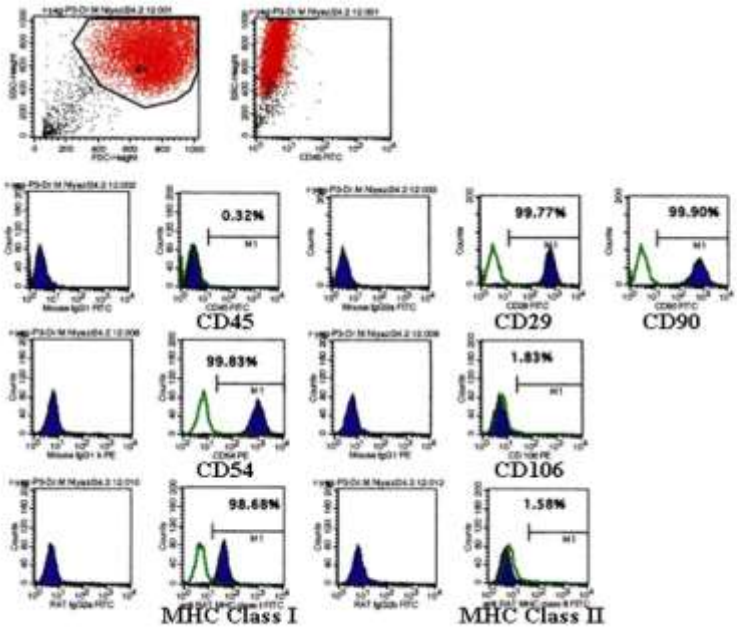

Figure 3: The histological appearance of the lungs of the rats in the (a) Control group (n=10), (b) Elastase group (n=12), (c) The elastase + MSC group (n=9). Images are representative of 5 images per lung from each animal. Hematoxylin-eosin x 200.

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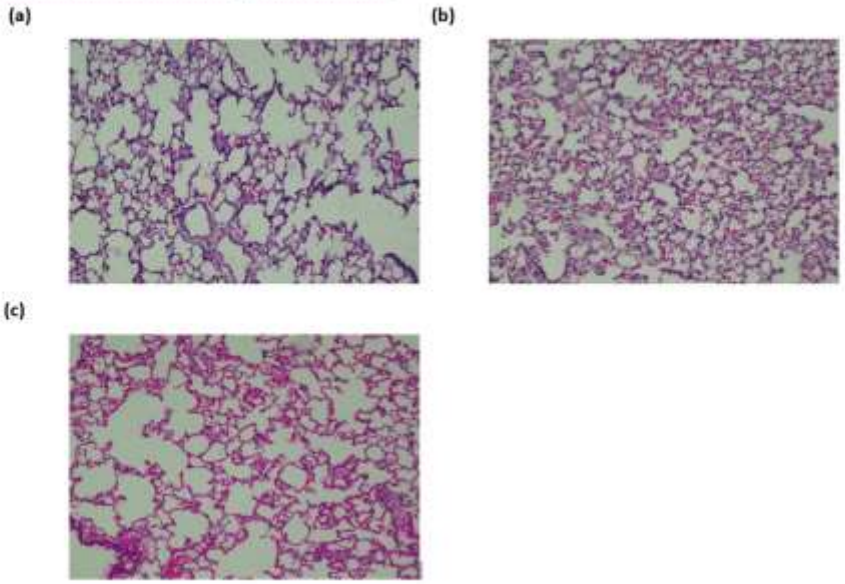
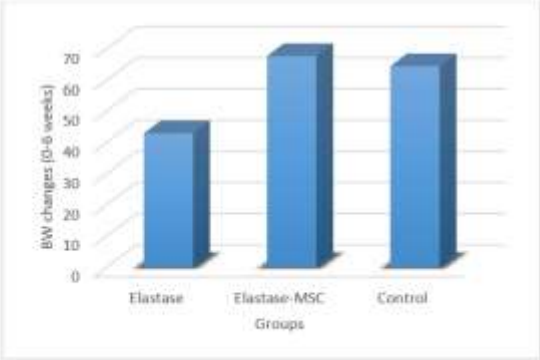


Figure 4: The bar graph of the mean values of the BW changes (0-6 weeks) in the three groups.

Figure 2: The bar graph of the mean values of the BW changes (0-6 weeks) in the three groups



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