Tissue and Cell 89 (2024) 102452

Contents lists available at ScienceDirect

Tissue and Cell

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

Stem cell treatment reduces T cell apoptosis in COPD patients with chronic bronchitis but not with emphysema

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ARTICLE INFO

Keywords:
Stem cell
Apoptosis
COPD
Chronic bronchitis
Emphysema

ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a prevalent and preventable condition. Mesenchymal stem cell (MSC) therapy is being explored to aid in the regeneration of lung cells and airway structure, aiming to restore lung function.
Aim: To examine varied responses of MSCs when cultured with peripheral blood mononuclear cells (PBMCs) from different COPD phenotypes, patients were grouped into ACOS, emphysema, and chronic bronchitis categories.
Methods: PBMCs from these groups and controls were co-cultured with MSCs derived from dental follicles, revealing differing rates of apoptosis among COPD phenotypes compared to controls.
Results: While the chronic bronchitis group exhibited the least lymphocyte viability ($p<0.01$), introducing MSCs notably enhanced viability across all phenotypes except emphysema, with the chronic bronchitis group showing the most improvement ($p<0.05$).
Conclusion: Stem cell therapy might reduce peripheral lymphocyte apoptosis in COPD, with varying responses based on phenotype, necessitating further research to understand mechanisms and optimize tailored therapies for each COPD subtype.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a widespread and persistent respiratory disorder marked by prolonged respiratory symptoms and airflow limitation. This limitation mainly stems from structural changes in the airways and alveoli, typically due to prolonged exposure to harmful particles or gases. Such exposure, often combined with factors impacting aberrant lung development, exacerbates the condition (Global Initiative for Chronic Obstructive Lung Disease GOLD, 2023). COPD poses a substantial public health challenge, exerting a considerable impact on healthcare systems due to frequent hospitalizations prompted by acute exacerbations and the enduring nature of the condition (Buist et al., 2007).

Presently, effective therapeutic strategies targeting the permanent loss of lung function and the partially reversible expiratory airflow limitation associated with COPD remain elusive. While current medications play a crucial role in managing symptoms, they do not target the fundamental pathophysiological alterations (Riley and Sciurba, 2019). In response to this therapeutic void, the pursuit of new treatment avenues has amplified. Mesenchymal stem cell (MSC) therapy has emerged as a potential beacon.

Mesenchymal stromal cells (MSCs), derived from adult sources, are lauded for their multipotency, with capabilities to self-renew and morph into a range of cell types. While they can be extracted from an assortment of tissues such as bone marrow, umbilical cord, and adipose tissue (Ding, D.C., Shyu, W.C., Lin, S.Z., 2011. Mesenchymal stromal cells. Cell Transplant 20, 5–14.), their promise extends beyond origin. Recent advances in both lab and clinical settings highlight the therapeutic potential of MSCs, especially as immunotherapeutic agents for treating diverse autoimmune, atopic, and inflammatory conditions (Akkoc, T., Genc, D., 2020. Asthma immunotherapy and treatment approaches with mesenchymal stromal cells. Immunotherapy 12, 665–674.; Girdlestone,

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https://doi.org/10.1016/j.tice.2024.102452
Received 15 November 2023; Received in revised form 3 June 2024; Accepted 17 June 2024
Available online 26 June 2024

J., 2016. Mesenchymal stromal cells with enhanced therapeutic properties. Immunotherapy 8, 1405–1416.).

MSCs, particularly those derived from bone marrow (BM-MSCs), have exhibited remarkable attributes, both in vitro and in vivo (Ma et al., 2011). Furthermore, preclinical investigations using animal models of COPD have shown that MSCs possess the potential to attenuate inflammation, curb apoptosis, and enhance overall lung function and tissue repair (Zhen et al., 2008; Gu et al., 2015; Furuya et al., 2012). These encouraging findings have paved the way for clinical studies, wherein MSC treatment has been administered safely and tolerated well. These studies have also demonstrated a significant reduction in circulating C-reactive protein levels, though they have not yet established substantial differences in disease severity or exacerbation rates when compared to placebo groups (Weiss et al., 2013). For example, Abbaszadeh et al. (2022) highlight the potential of MSCs and MSC-derived extracellular vesicles to modulate inflammation and promote tissue regeneration in COPD and asthma (Abbaszadeh et al., 2022). Additionally, Cruz (2019) highlights the promising potential of MSC therapy in chronic lung diseases and emphasizes that further research is needed to optimize treatment parameters (Cruz and Rocco, 2020).

The path ahead for MSC therapy in COPD presents several challenges, including the optimization of treatment parameters such as dosage, administration route, and long-term safety and efficacy. Moreover, questions regarding the most appropriate criteria for selecting both MSC recipients and donors persist (Glassberg et al., 2021). Clinical trials in this field encounter complexities stemming from the notable diversity in physiological characteristics, pathological manifestations, and inflammatory states across individuals with COPD. Although current research involving COPD patients has not amassed sufficient data to conclusively confirm therapeutic efficacy, results from rigorously replicable animal models consistently affirm the safety and promise of MSC-based interventions.

Recognizing the likelihood of individualized responses to treatment, future clinical investigations will necessitate comprehensive patient characterization to identify potential biomarkers or patient phenotypes that may demonstrate favorable responses to MSC therapy. Collectively, it is now widely acknowledged that COPD encompasses a spectrum of phenotypes and endotypes, each presenting distinct prognostic implications and varying therapeutic responses (Han et al., 2010).

In light of these considerations, our study aims to probe the potential differential effects of MSC co-culture on PBMCs from patients with varied COPD phenotypes. Specifically, we strive to uncover discrepancies in the MSC co-culture response among those with emphysema, chronic bronchitis, and the Asthma-COPD (ACOS) phenotype. Such findings could shed light on tailored applications of this emerging therapeutic modality.

2. Materials and methods

2.1. Patient demographic distribution

This study received approval from the Ethics Committee of the University of Marmara's Medical Faculty in compliance with the principles outlined in the Helsinki Declaration (Approval No: 09.2019.929).

The study's experimental group comprised newly diagnosed patients who had not undergone any prior treatment for their ailment. Patients were classified into three specific phenotypic categories: Asthma-COPD (ACOS), emphysema, and chronic bronchitis. Patients satisfying the American Thoracic Society (ATS) guidelines were categorized as follows: those with chronic bronchitis, evidenced by sputum symptoms lasting a minimum of 3 months over two successive years; those with emphysema visible on thoracic computed tomography (CT); and those displaying bronchial hyperresponsiveness and airflow obstruction during respiratory tests, which indicated the asthma-COPD overlap phenotype. A control group, age- and gender-matched, was integrated into the study. Pulmonary function test results were used to validate the diagnosis of COPD (Soriano et al., 2003).

The study included all COPD patients who had previously smoked. The patients had no history of infection or acute exacerbation within the last three months. Emphysema observations varied in intensity and character across the emphysema phenotypic. None of the patients utilized oral or intravenous steroids.

When radiological findings of patients with emphysema phenotypic were evaluated, four patients had a paraseptal pattern, two had a centriacinar pattern, two had a panacinar pattern, and two had both a paraseptal and a panacinar emphysema.

2.2. Extraction and cultivation of dental follicle-derived mesenchymal stromal cells (DF-MSC)

Dental follicle tissues, a source of DF-MSCs, were extracted from three impacted teeth from healthy donors aged 18–25 years. Ensuring sterile conditions within a Class II laminar flow cabinet, the follicle tissues were carefully separated from the tooth structures. The procured tissues were subsequently fragmented into approximately 1 mm^3 pieces. Concurrently, a collagenase solution (1 mg/mL, Gibco) was formulated using Hank's Balanced Salt Solution (HBSS, Multicell). This enzymatic solution was introduced to the minced tissue samples, initiating their enzymatic breakdown. The mixture was subsequently placed in a 37°C incubator with a 5 % CO2 environment for a duration ranging between 45 and 60 minutes, ensuring the tissue reached a homogenous consistency. After the digestion process, the enzyme-tissue solution was thoroughly washed using phosphate-buffered saline (PBS) followed by centrifugation at 1500 RPM for 5 minutes. The resulting sediment was strained using a 22-μm sterile mesh into a pristine 15 mL Falcon tube. The filtrate, containing the isolated cells, was then inoculated into a T25 flask filled with a nourishing medium. This medium was routinely replaced every 72 hours. Cells were allowed to proliferate and were subcultured upon reaching 80–90 % confluence, up to the third passage.

Our choice of DF-MSCs was informed by their distinctive attributes, which offer specific advantages in treating COPD. These cells are known for their high proliferation rate, substantial multipotentiality allowing differentiation into various lineages, and a robust immunomodulatory capacity that could be especially beneficial for mitigating the chronic inflammation characteristic of COPD. The accessibility of dental follicle tissues, typically sourced from routine wisdom teeth extractions, presents a practical and ethically favorable option for MSC acquisition, further motivating our selection. This ease of access, combined with the potential of DF-MSCs to contribute to lung tissue repair and regeneration through their potent immunomodulatory effects and ability to secrete growth factors, underscores their promising role in exploring new treatments for COPD.

2.3. Phenotypic characterization and functional differentiation assays of DF-MSC

Phenotypic profiling of DF-MSCs was conducted at the third passage. Cells were enzymatically dissociated using 0.25 % Trypsin-EDTA and subsequently centrifuged. The resultant cell pellet, containing approximately 1x10^6 cells, was resuspended in PBS. For characterization, cells were exposed to a series of surface markers. Positive indicators comprised CD90 (FITC-conjugated), CD73 (APC-conjugated), and CD105 (PerCP.Cy5.5-conjugated). Conversely, negative markers included CD45, CD11b, CD19, and HLA-DR (all PE-conjugated). The staining procedure involved adding 10 μl of the appropriate antibody to tubes with around 5x10^5 DF-MSCs, combined with a staining buffer. This mixture was then incubated at room temperature for 15 minutes in a light-protected environment. Following this, a centrifugation at 1500 RPM for 5 minutes was performed. The supernatant was carefully discarded, and the cell pellet was reconstituted in staining buffer. The resultant phenotypic profile of DF-MSCs was analyzed using flow cytometry.

2.4. The differentiation capacity of DF-MSCs was assessed through the following procedures

2.4.1. Adipogenic differentiation

At the third passage, DF-MSCs were dispersed at a concentration of 50,000 cells per well across six wells, with each well furnished with collagen type I coverslips. For instigating adipogenic differentiation, the regular culture medium was supplanted daily with an adipogenesis-specific differentiation medium procured from the Stempro Adipogenesis Differentiation Kit (Thermo Fisher). This regime persisted for a week. Subsequent to a cumulative four-week incubation span, differentiated adipocytes were assessed under an inverted microscopy setup and were subsequently accentuated with a 0.5 % Oil Red stain (Sigma), in line with the directives provided by the manufacturer.

2.4.2. Chondrogenic differentiation

Once the DF-MSC culture progressed to passage 3, cells were allocated at a rate of 50,000 cells per well across a set of six wells, wherein each was lined with a collagen type I coverslip. The induction of chondrogenic differentiation was initiated by systematically switching the standard culture medium with a chondrogenesis-tailored differentiation medium sourced from the Stempro Chondrogenesis Differentiation Kit (Thermo Fisher). This replacement continued daily for a week. Post a month-long incubation, differentiated chondrocytes were distinguished using Alcian Blue (Sigma) staining, which was then inspected under inverted microscopy.

2.4.3. Osteogenic differentiation

By the time the DF-MSC culture matured to its third passage, cells were arranged at a density of 50,000 cells for every well, distributed over six wells, each pre-coated with collagen type I coverslips. The onset of osteogenic differentiation was marked by the daily substitution of the base culture medium with an osteogenesis-focused differentiation medium, as part of the Stempro Osteogenesis Differentiation Kit (Thermo Fisher), over the span of a week. After incubating for four weeks, osteocytes were brought into prominence using Alizarin Red (Sigma) and were subsequently appraised under an inverted microscope.

2.4.4. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples were aseptically drawn into heparinized tubes from patients for the purpose of isolating Peripheral Blood Mononuclear Cells (PBMCs). These blood samples were then diluted at a 1:1 (volume/volume) ratio with sterile PBS and carefully layered over a ficoll solution within 15 mL tubes, facilitating density gradient separation. Subsequent to centrifugation at 2000 rpm for 20 minutes, the resulting buffy coat, located at the interface, was meticulously collected. It underwent two successive washing steps utilizing complete RPMI 1640 (cRPMI) medium, composed of RPMI supplemented with 10 % FBS and 1 % penicillin/streptomycin. Each wash was performed by centrifuging the cells at 1500 rpm for 5 minutes, followed by discarding the supernatant. The remaining cell pellet was subsequently resuspended in cRPMI medium for subsequent culturing. A cell density of 1x10^6 cells/mL was enumerated in the culture medium.

2.4.5. Co-culture of PBMCs with DFSCs

DFSCs were initially seeded at a density of 5 × 10^4 cells per well within a 48-well plate, 48 hours prior to the addition of lymphocytes in the culture medium. Co-cultures of DFSCs and PBMCs were established at a ratio of 1:10 (DFSCs:PBMCs) and incubated for a duration of 72 hours. The cultures were subjected to simulation with 0.5 μg/mL of CDmix (consisting of anti-CD3 and anti-CD28).

2.4.6. Assessment of cell viability via flow cytometry

Following a 4-day incubation period, supernatants were harvested, and lymphocytes were isolated through centrifugation at 1500 RPM for 5 minutes. Post-centrifugation, the supernatant was decanted, leaving the lymphocytes as pellets, which were subsequently resuspended in a solution comprising 10 % FBS and DPBS (used as a washing solution). This washing step was repeated for thorough purification. After the second centrifugation, the supernatant was once again removed, and the lymphocytes were resuspended in 500 μL of staining solution (BD).

Subsequently, a staining solution consisting of 10 μL of Annexin V (FITC, Biolegend) and 10 μL of PI (PE, Biolegend) was introduced to the cells. Following this addition, the cells were incubated for 30 minutes at room temperature in a light-protected environment. After incubation, the cells were subjected to centrifugation, and the supernatant was discarded. The cell pellets were reconstituted in 100 μL of staining solution. Assessment of lymphocyte viability was carried out using the FACS Calibur Flow Cytometry system.

Table 1
Demographic data of COPD phenotypes.

COPD PHENOTYPES			
	ACOS	Emphysema Dominant	Chronic Bronchitis Dominant
Gender			
Male, no(%)	4 [50]	8 [80]	6 (85.7)
Female, no(%)	4 [50]	2 (20)	1 (14.3)
Age, years (mean)	56.6	60	58.8
BMI (kg/m2) (mean)	26,6	25,7	26,45
FEV1, % predicted (mean)	47.5	49.4	60.8
FEV1/FVC ratio, % (mean)	56.8	55.7	60.5
FVC % predicted (mean)	65,5	77,7	87
FEV1 predicted liters (mean)	1,65	1,52	2,01
Post-bronchodilator FEV1/FVC ratio, % (mean)	65	62,8	64
Post-bronchodilator FEV1 liters (mean)	1,98	1,88	2,15
Post-bronchodilator FVC liters (mean)	2,7	2,61	2,95

2.4.7. Statistical analysis

The statistical analyses were conducted using R version 4.2.2. Compatibility with a normal distribution was assessed through the Shapiro-Wilk test and QQ plots. The impact of Chronic Obstructive Pulmonary Disease (COPD) and its interaction with MKH on cell viability were evaluated for each stage of Cell Viability, Early Apoptosis, Late Apoptosis, and Necrosis using a generalized linear model. Statistically significant effects were further scrutinized through post hoc tests, and p-values were adjusted for false discovery rate (fdr).

The level of significance was predetermined at $p < 0.05$.

3. Results

3.1. Clinical characteristics of COPD patients by phenotype

Table 1 shows subject characteristics of the COPD patients enrolled in the study, categorized by phenotypes. While the male gender was dominant in emphysema and chronic bronchitis phenotypes, male and female genders were equally frequent in the ACOS type.

3.2. Isolation, characterization, and differentiation of DFSCs

Fig. 2. shows isolation, characterization, and differentiation of DFSCs.Mesenchymal stromal cells (MSCs) were successfully isolated from dental follicle tissues, and their proliferation resulted in the gradual formation of small colonies within three days. By the seventh day after the initial plating for the first passage, these MSCs reached a confluence rate of approximately 70 % in the primary culture. In the third passage (P3), the majority of the MSCs exhibited a fibroblast-like morphology (Fig. 2**a**).

Flow cytometry analysis of the MSCs demonstrated positive staining

Table 2
Evaluation of cell viability rates between 2 different groups.

Live Cell Rate				
	MSC (-) Mean (SD)	MSC (+) Mean (SD)	Total Mean (SD)	p[1]
Healthy Control	76.4 (7.1)[a]	86.8 (5.8)[a]	81.6 (8.2)[a]	0.066
Emphysema	73.8 (9.4)[ab]	76.4 (10.9)[ac]	75.0 (9.8)[ab]	0.660
ACOS	61.0 (10.7)[bc]	75.6 (11.9)[ac]	68.3 (13.2)[bc]	0.066
Chronic Bronchitis	53.8 (11.3)[c]	74.9 (4.8)[bc]	64.4 (13.7)[c]	0.007
Total	65.6 (13.3)	77.9 (9.5)		

a-b-c: There is no difference between the phenotype groups with the same letter.
[1]: p-value of comparison between MSC groups within each phenotype group

for CD29, CD90, CD146, CD73, and CD106, indicating their mesenchymal stem cell identity. Conversely, these cells displayed negative staining for CD34, CD45, CD14, CD28, and CD25 (Fig. 2**b).**

The multipotent differentiation potential of these MSCs was confirmed in vitro. Osteogenic differentiation was observed during a 28-day culture period in osteogenic induction medium, as evidenced by the formation of calcified bone nodule structures, visualized through alizarin red staining. Adipogenic differentiation, induced by culturing the cells in adipogenic induction medium and staining with oil red O, revealed the presence of intracellular lipid droplets. Furthermore, in vitro chondrogenic differentiation was successfully achieved following a 14-day culture period in chondrogenic induction medium, with alcian blue staining confirming the differentiation of these cells into chondrocytes. Intracellular proteoglycans were evident in the stained chondrocytes **(**Fig. 2**c).**

3.3. Comparison of cell viability ratios among phenotypes

The cell viability ratio was compared between each phenotype, and the results are presented in Table 2**.** and Fig. 1. In the Emphysema phenotype group, there was no statistically significant difference between the group with MSCs and the group without MSCs (Cohen's d = −0.26). The mean cell viability ratio for the MSC group (M = 76, SD = 12) was slightly higher than that for the non-MSC group (M = 61, SD = 11). In the ACOS phenotype group, these results were statistically significant (p = 0.049) with a large effect size (d = 1.3), where the mean cell viability ratio for the MSC group (M = 75, SD = 5) was slightly higher than that for the non-MSC group (M = 54, SD = 11). Similarly, in the chronic bronchitis phenotype group, the results were statistically significant (p = 0.002) with a large effect size (d = 2.4), and the mean cell viability ratio for the MSC group (M = 75, SD = 5) was slightly higher than that for the non-MSC group (M = 54, SD = 11). In summary, based on the effect sizes, stem cells were most effective in increasing viability in the chronic bronchitis phenotype. Fig. 2

3.4. Evaluation of early apoptotic cell and late apoptotic cell ratios

The early apoptotic cell ratio was compared between each phenotype, and the results are presented in **(**Table3**.and** Table 4**) and (**Fig. 1**.) S**tem cells were most effective in decreasing early apoptotic and late apoptotic cell ratios in the chronic bronchitis phenotype. Fig. 3, Fig. 4, Fig. 5 and Fig. 6

3.5. Evaluation of necrotic cell ratios

There is no difference in any of the groups in terms of necrotic cell ratios **(**Fig. 1**.) and** Table5**.**

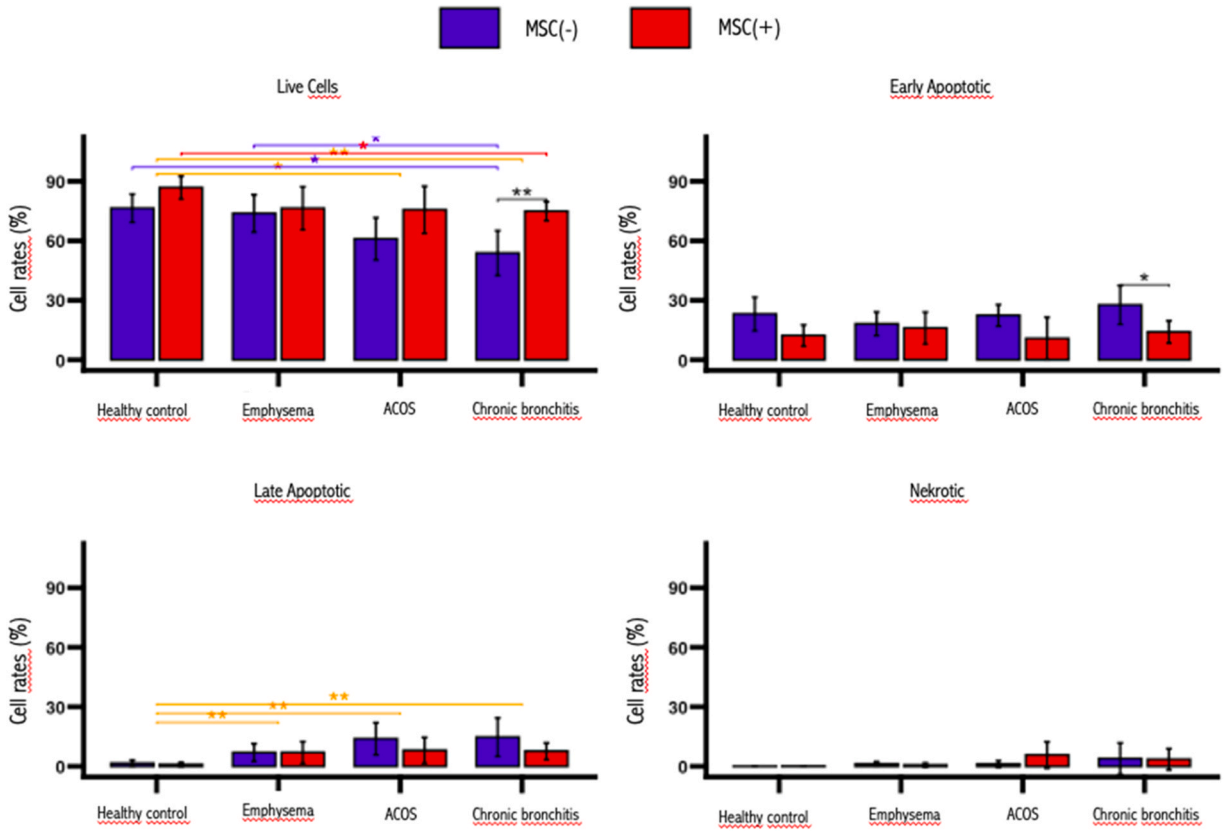

Fig. 1. Comparison of disease sub-phenotypes according to the presence of stem cells and comparison of disease sub-phenotypes regardless of the presence of MSCs.

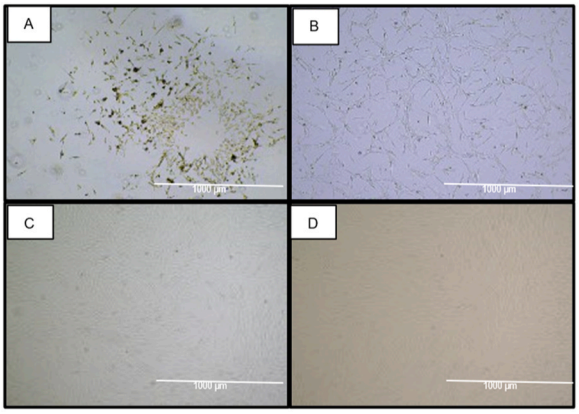

Fig. 2. Morphological appearance of DF-MSC (10X). A.) DF-MSC fibroblast-like morphology in passage 0 (P0) B.) DF-MSC fibroblast-like morphology in passage 1 (P1) C.) DF-MSC fibroblast-like morphology in passage 2 (P2) D.) DF-MSC fibroblast-like morphology in passage 3 (P3).

Table 3
Evaluation of early apoptotic cell rates between 2 different groups.

Early Apoptotic Cell Rate				
	MSC (-) Mean (SD)	MSC (+) Mean (SD)	Total Mean (SD)	p[1]
Healthy Control	23.1 (8.5)	12.3 (5.3)	17.7 (8.8)	0.063
Emphysema	18.1 (6.0)	16.0 (8.0)	17.2 (6.8)	0.605
ACOS	22.4 (5.4)	10.8 (10.6)	16.6 (10.1)	0.063
Chronic Bronchitis	27.7 (9.8)	14.0 (5.6)	20.8 (10.5)	0.040
Total	22.8 (8)	13.3 (7.5)		

MSC (-): PBMCs of the experimental groups were cultured in non-MSC medium.
MSC (+): PBMCs of the experimental groups were cultured in medium containing MSC.
[1]: p-value of comparison between MSC groups within each phenotype group

Table 4
Evaluation of late apoptotic cell rates between 2 different groups.

Late Apoptotic Cell Rate				
	MSC (-) Mean (SD)	MSC (+) Mean (SD)	Total Mean (SD)	p[1]
Healthy Control	1.5 (1.6)[a]	0.9 (1.1)	1.2 (1.3)[a]	0.637
Emphysema	6.9 (4.5)[ab]	7.0 (5.5)	7.0 (4.7)[b]	0.99
ACOS	13.9 (8.1)[b]	8.1 (6.5)	11.0 (7.6)[b]	0.406
Chronic Bronchitis	14.7 (9.7)[b]	7.6 (4.2)	11.1 (8.1)[b]	0.406
Total	9.7 (8.4)	6.2 (5.3)		

a-b: There is no difference between the groups with the same letter.
*significant
MSC(-): PBMCs of the experimental groups were cultured in non-MSC medium.
MSC(+): PBMCs of the experimental groups were cultured in medium containing MSC.
[1]: p-value of comparison between MSC groups within each phenotype group

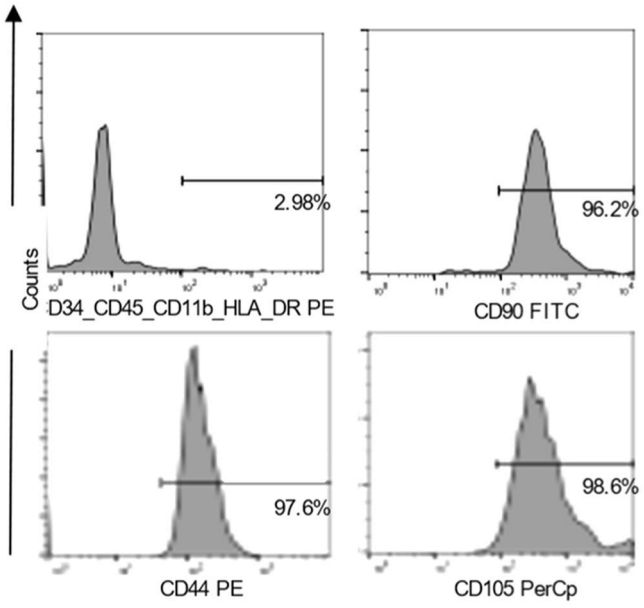

Fig. 3. Determination of the immunophenotypic characteristics of DF-MSC by flow cytometry. CD90, CD105, and CD44 represent positive markers of DF-MSCs. which are expressed higher than 90 %; HLA_DR, CD34, CD45, and CD11b represent negative markers of DF-MSCs described lower than 3 %.

4. Discussion

In our study, we aimed to investigate differences in cell viability, early apoptosis and late apoptosis rates in peripheral blood lymphocytes among different COPD phenotypes and the control group. We observed significant variations in early apoptosis rates and apoptotic cell counts between the COPD phenotypes and the control group. Specifically, the chronic bronchitis group exhibited the lowest lymphocyte viability rate, both with and without the presence of stem cells. Notably, the introduction of MSCs to the culture significantly enhanced lymphocyte viability rates across all groups, except for the emphysema subgroup. The most pronounced effect was observed in the chronic bronchitis phenotype, suggesting that MSC therapy may possess immunomodulatory effects on lymphocyte function.

COPD is a progressive lung disease characterized by chronic inflammation, oxidative stress, and apoptosis of lung cells (Global Initiative for Chronic Obstructive Lung Disease GOLD, 2023). Apoptosis is a programmed cell death process critical for maintaining tissue homeostasis. However, excessive apoptosis can lead to tissue damage and dysfunction, believed to contribute to COPD pathogenesis.

Several studies have explored the role of apoptosis, particularly peripheral lymphocyte apoptosis, in COPD. These studies consistently demonstrate that COPD patients exhibit higher levels of T-cell apoptosis compared to healthy individuals. For instance, Hodge et al (Hodge et al., 2003). showed an increase in peripheral blood lymphocyte apoptosis in COPD patients, which was associated with upregulated apoptotic pathways, TGF-β, TNF-α, and Fas in COPD peripheral blood. Additionally, In their experimental study, Gao et al. reported that the transplantation of CXCR4-overexpressing BMSCs during COPD model generation significantly inhibited apoptosis via the extrinsic apoptosis pathway (Gao et al., 2023).

Lymphocytes can migrate between the bloodstream and the bronchoalveolar space, potentially explaining the increased apoptosis observed in peripheral T-cells of COPD patients (Lehmann et al., 2001). The distinctions between Th1, Th2, Th17, and regulatory T cells (Tregs) and the role of T cells in the pathogenesis of COPD and asthma have been extensively studied. For example, Xiaoyan Li et al. by showing how MSCs can inhibit the proliferation of T cells exposed to nicotine, it may

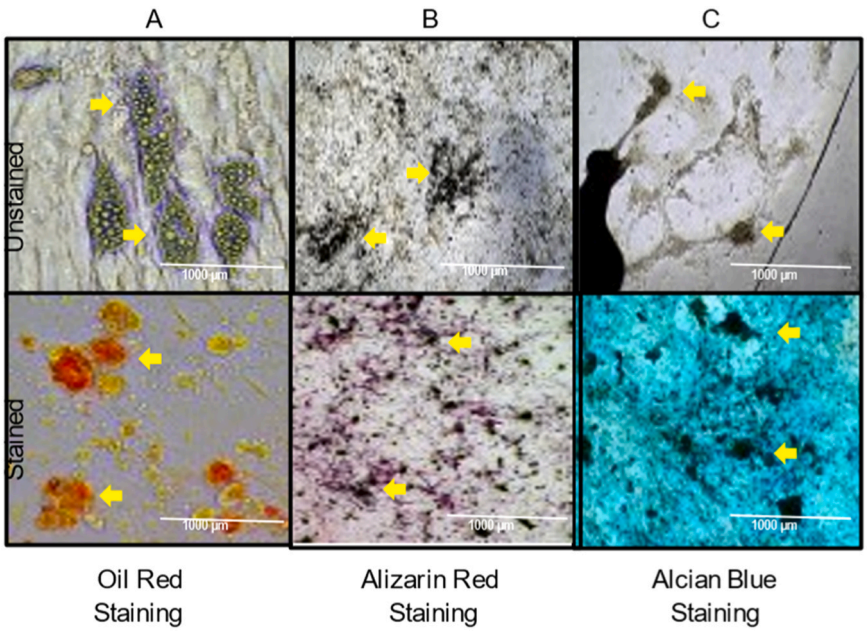

Fig. 4. Differentiation of MSCs. Adipogenic differentiation of DF-MSCs. stained with oil red O, Osteogenic differentiation of DF-MSCs. stained with Alizarin red, Chondrogenic differentiation of DF-MSCs. stained with alcian blue, 1000 µm.

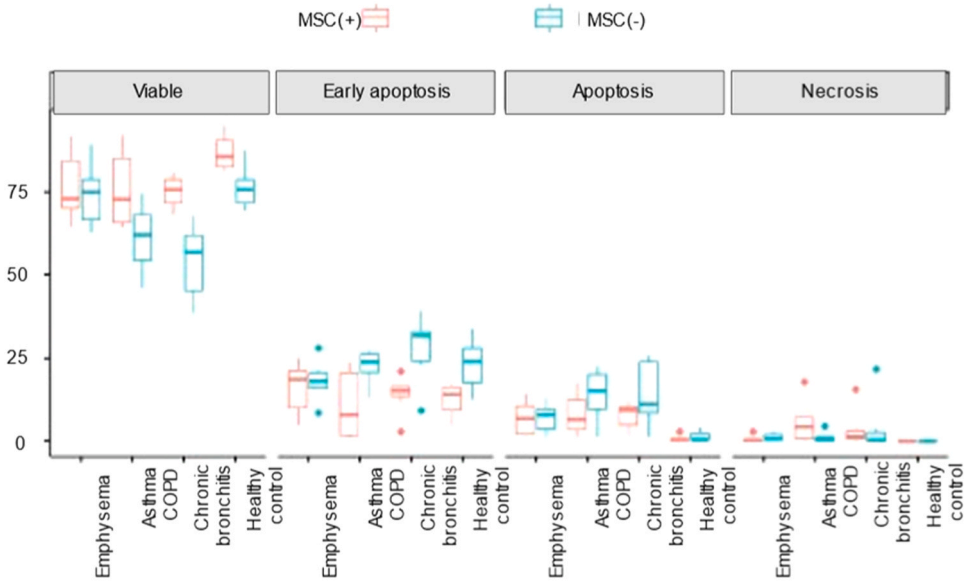

Fig. 5. A chart showing the statistical analysis of PBMC with and without DF-MSC generated by the viability flow analysis.

provide insight into potential therapeutic mechanisms (Li et al., 2020). Additionally, airway-derived T-cells in COPD patients exhibit elevated apoptosis rates. Our study also observed increased late apoptosis in all COPD subgroups compared to the control group. COPD is now recognized as a systemic disease, with recent research investigating its effects on peripheral blood. Studies have shown increased T-cell apoptosis in both airways and peripheral blood in COPD patients, alongside airway epithelial inflammation indicated by intraepithelial T-cell increase (Hodge et al., 2005; Van Stijn et al., 2003). Furthermore, studies have shown that T-cells can travel between the peripheral circulation and the lungs, with lymphocytes from the bronchoalveolar space able to re-enter lung tissue, migrate to regional lymph nodes, and rejoin the systemic immune system (Lehmann et al., 2001). Therefore, increased peripheral blood T-cell apoptosis in COPD may result from apoptotic stimuli originating either in peripheral blood or from local apoptotic triggers in the airways.

COPD is a heterogeneous disease with various phenotypes, each potentially contributing to disease development and progression through distinct mechanisms. Our study revealed that apoptosis increased in all phenotypes compared to the control group, without significant differences among the phenotypes. However, we found that the addition of MSCs mitigated apoptosis in phenotypes other than emphysema. Considering the potential future clinical use of our findings, preclinical studies play a crucial role in providing essential evidence concerning the safety, toxicity, therapeutic efficacy, and mechanism of MSC action. Studies conducted on rodent animal models of COPD have consistently shown that intravenous injection or intratracheal instillation of rodent bone marrow MSCs (BM-MSCs) or adipose-derived MSCs (AD-MSCs) were both safe and effective in mitigating airway injury by reducing airway inflammation and apoptosis

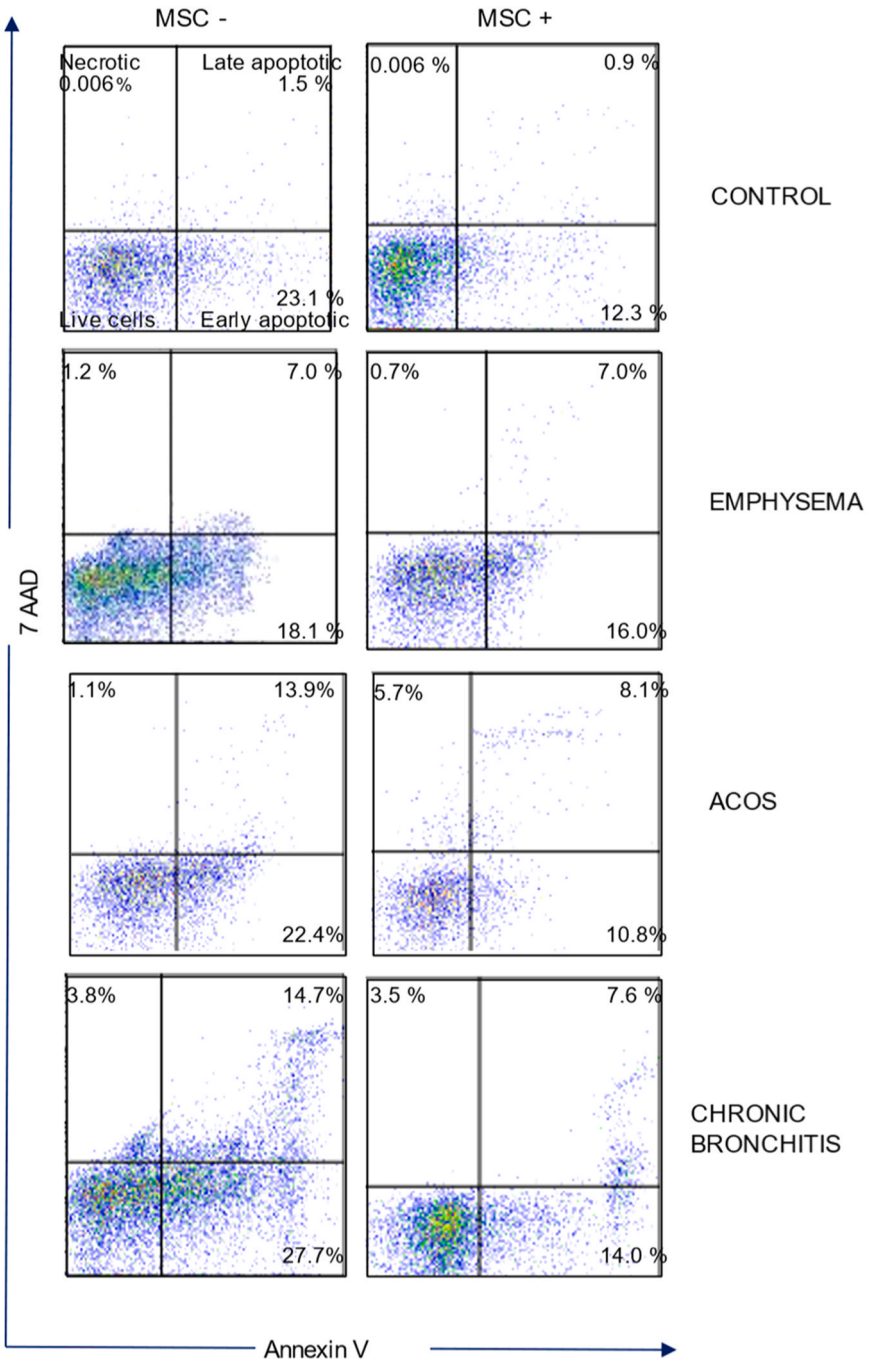

Fig. 6. The viability of PBMC with or without DF-MSC was assessed by flow analysis.

(Antunes et al., 2014; Guan et al., 2013).

There may be various explanations for the difference in the decrease in apoptosis rates in the emphysema and chronic bronchitis groups with the presence of stem cells. Notably, there may be differences in apoptotic pathways between emphysema and chronic bronchitis, two subtypes of COPD. In emphysema, apoptosis of alveolar cells, particularly type I epithelial cells and endothelial cells, is a prominent feature. This process is believed to be mediated by increased oxidative stress and inflammation, both of which are elevated in emphysema. Conversely, in chronic bronchitis, apoptosis of airway epithelial cells takes precedence, thought to be mediated by increased oxidative stress, proinflammatory cytokines, and viral infections (Segura-Valdez et al., 2000; Demedts et al., 2006). Notably, there may be differences in apoptotic pathways between emphysema and chronic bronchitis, two subtypes of COPD.

Collectively, these studies suggest that stem cell therapy could potentially benefit peripheral T-cell apoptosis in both emphysema-dominant and chronic bronchitis-dominant COPD patients, although the therapeutic response may differ between the two phenotypes. Further research is warranted to comprehensively grasp the underlying mechanisms and determine the optimal stem cell type, dosage, and administration method for each COPD phenotype.

This statement underscores an important limitation of our study and acknowledges the necessity for additional research to fully comprehend the potential therapeutic implications of stem cell therapy in COPD patients. It underscores that COPD is a multifaceted and complex disease, and assessing the efficacy of stem cell therapy through a single mechanism may not be sufficient to grasp its potential benefits. Additionally, it acknowledges that various COPD phenotypes may encompass

Table 5
Evaluation of necrotic cell rates between 2 different groups.

Necrotic Cell Rate				
	MSC (-) Mean (SD)	MSC (+) Mean (SD)	Total Mean (SD)	p[1]
Healthy Control	0.0 (0.0)	0.1 (0.1)	0.0 (0.1)	0.316
Emphysema	1.2 (1.2)	0.6 (1.0)	0.9 (1.1)	0.531
ACOS	1.1 (1.7)	5.7 (6.7)	3.4 (5.2)	0.316
Chronic Bronchitis	3.8 (8.0)	3.5 (5.4)	3.7 (6.5)	0.933
Total	1.7 (4.3)	2.6 (4.7)		

multiple pathogenetic mechanisms, highlighting that evaluating stem cell activity through a single mechanism may not adequately determine efficacy. Nevertheless, the statement also emphasizes the significance of recognizing that stem cell therapy may exhibit varying levels of efficacy in different phenotypes, which could guide future treatment planning and research endeavors.

In conclusion, this statement emphasizes the need for ongoing research and investigation into the potential therapeutic advantages of stem cell therapy in COPD patients, recognizing the intricate and multifactorial nature of the disease.

After identifying statistically significant effects, pairwise comparisons were conducted. False discovery rate (FDR) adjusted p-values were calculated to account for multiple comparisons. Results are reported using letters instead of p-values. Groups sharing the same letter are considered not statistically different according to the pairwise comparisons.

MSC (-): PBMCs of the experimental groups were cultured in non-MSC medium.

MSC (+): PBMCs of the experimental groups were cultured in medium containing MSC.

p-value: Comparison of whether MSC was present for each experimental group

MSC(-): PBMCs of the experimental groups were cultured in non-MSC medium.

MSC(+): PBMCs of the experimental groups were cultured in medium containing MSC.

p-value: Comparison of whether MSC was present for each experimental group

Ethics approval and consent to participate

This study received approval from the Ethics Committee of the University of Marmara's Medical Faculty in compliance with the principles outlined in the Helsinki Declaration (Approval No: 09.2019.929) and the patients signed an informed consent form.

Funding

This study was supported by the Turkish Thoracic Society.

CRediT authorship contribution statement

Tunç Akkoç: Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Esin Çetin:** Validation, Supervision, Methodology. **Zeynep Mercancı:** Data curation. **Zeynep Tunca:** Formal analysis, Data curation. **Emel Eryuksel:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nur Ecem Öztop:** Data curation. **Esra Akdeniz:** Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Derya Kocakaya:** Data curation. **Sabriye Senem Kılıç:** Data curation.

Declaration of Competing Interest

We declare that we have no conflict of interest.

Data Availability

Data will be made available on request.

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