ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2017; 16(3):205-218.

Effects of Intraperitoneal Injection of Allogeneic Bone Marrow-derived Mesenchymal Stem Cells on Bronchiolitis Obliterans in Mice Model

Sakine Işık[1], Nevin Uzuner[1], Meral Karaman[2], Özkan Karaman[1], Müge Kıray[3], İlknur Kozanoğlu[4], Hüsnü Alper Bağrıyanık[3], Zeynep Arıkan-Ayyıldız[1], Melis Kartal Yandım[5], and Yusuf Baran[5]

[1] Department of Pediatric Allergy and Immunology, Dokuz Eylul University, İzmir, Turkey
[2] Department of Microbiology, Dokuz Eylul University, İzmir, Turkey
[3] Department of Histology, Dokuz Eylul University, İzmir, Turkey
[4] Department of Hematology, Başkent University, Adana, Turkey
[5] Department of Molecular Biology and Genetics, İzmir Institute of Technology, İzmir, Turkey

Received: 22 July 2016; Received in revised form: 27 January 2017; Accepted: 15 March 2017

ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSCs) can ameliorate a variety of lung diseases such as asthma, lung fibrosis, and acute lung injury by its anti-inflammatory and immunmodulatory effects. In this study, we developed a mouse model of bronchiolitis obliterans (BO) and evaluated the effects of the intraperitoneal administration of BMSCs on lung histopathology and cytokine levels.

25 BALB/c mice were divided into four groups; control group (Group I), BO developed and $1x10^6$ BMSCs-injected group (Group II), non-BO, $1x10^6$ BMSCs-injected group (Group III), and BO developed and saline-injected group (Group IV). Histological and immunohistochemical findings of the lung tissue and the migration of BMSCs to the lung were evaluated using light and confocal microscopy techniques.

Confocal microscopy evaluations showed that there was no noteworthy amount of BMSCs in the lung tissue of group III while significant amount of BMSCs was detected in group II. Wall thicknesses of terminal bronchiole and periterminal bronchiolar collagen deposition were significantly lower in group II compared to the group IV ($p<0.05$). Furthermore, according to the immunohistochemical staining results, CD3, CD4, CD8, CD20, CD68 and neutrophil elastase positive immune cells of group II were stained more positive than group IV cells ($p<0.05$). IFN-γ IL-2 and TNF-α levels in bronchoalveolar lavage fluid (BALF) were significantly lower in group II compared to group IV ($p<0.05$).

The findings of this study indicate that intraperitoneally administered BMSCs have potent effects on histopatological changes of the lung tissue and cytokine levels in the murine model of BO.

Keywords: Bronchiolitis obliterans; Intraperitoneal administration; Mesenchymal stem cells; Murine model

Corresponding Author: Sakine Işık, MD;
Department of Pediatric Allergy and Immunology, Dokuz Eylul University, Balçova, 35330, İzmır, Turkey.

Tel: (+90 232) 4126059, Fax:(+90 232) 412 6005, E-mail: drsakinekar83@hotmail.com

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Bronchiolitis obliterans (BO) is a rare disorder characterized histologically by a fibrosing/inflammatory process that constricts and eventually obliterates the small airways.[1] The functional loss of these airways results in long-term wheezing, chronic cough, and often severe respiratory distress. Etiological causes of BO include especially *Mycoplasma* and *Adenovirus* infections (the most common cause in children); toxic fume inhalation; drugs; and complications in allograft recipients undergoing heart-lung, lung and bone marrow transplantation (the most common causes in adults).[1,2]

Patients with BO have poor prognosis and significant long-term morbidity is reported in 78% to 92% of patients.[3,4] A variety of medications including immunosuppressive, anti-inflammatory, and anti-fibrotic agents such as corticosteroids, calcineurin inhibitors, azathioprine, methotrexate, pirfenidone, monoclonal antibodies, macrolides, and chloroquine have been used.[5-9] However, these therapeutic approaches applied to patients with severe BO generally fail and the outcome is usually disappointing. These treatment approaches are based mainly on supportive care. This failure can be the result of delayed diagnosis and there can be irreversible fibrotic changes and airway obliteration.

Mesenchymal stem cells (MSCs), multipotent progenitor cells, are readily isolated from bone marrow and it is proved that MSCs are useful in the regeneration and maintenance of various tissues.[10,11] MSCs produce large quantities of bioactive factors, which cause immunsupression in T cells as well as anti-scarring, angiogenic, anti-apoptotic, and regenerative effects. These factors can also suppress the production of some cytokines such as TNF-α and IFN-γ[11,12] Stem cell plasticity refers to the ability of some stem cells to give rise to cell types, formerly considered outside their normal repertoire of differentiation for the location, where they are found. Previous studies demonstrated that MSCs can show better plasticity than it is known. For instance; it is possible to differentiate them into alveolar and bronchial epithelium, vascular endothelium, and interstitial cell types.[13] Furthermore, they have positive effects on various pulmonary diseases such as asthma, acute lung injury/acute respiratory distress syndrome, and chronic obstructive pulmonary disease.[14-17]

The purpose of this study was to evaluate the migration of allogeneic bone marrow-derived MSCs (BMSCs) to the lung tissue upon intraperitoneal injection and effects of BMSCs on lung histopathology along with TH1 immune responses in a murine model of BO.

MATERIALS AND METHODS

Animals and Experimental Procedures

25, male, BALB/c mice of 6-8 weeks of age were equally randomized into four groups as following: Control group (Group I) consisted of healthy animals (n=5); bronchiolitis obliterans (BO)-developed and $1x10^6$ allogeneic BMSCs-injected group (Group II) (n=7); non-BO and $1x10^6$ allogeneic BMSCs-injected group (Group III) (n=7); and BO-developed and saline-injected group (Group IV) (n=7). In BO-developed groups, animals received 20 µL nitric acid at a concentration of 2% intranasally through a volumetric pipette. Furthermore, in non-BO groups, animals underwent the same experimental procedure but received an intranasal instillation of saline solution (0,09 % NaCl).[18] BO model was established eight weeks after the administration of the nitric acid. At that time, $1x10^6$ allogeneic BMSCs in physiological saline solution were respectively administered to group II and III mice by an intraperitoneal injection. Saline was also administered intraperitoneally to group IV mice. One week after the model establishment, all mice were sacrificed and tissue harvesting was done (Figure 1). All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the Dokuz Eylul University.

Isolation and Culture of BMSCs

Mesenchymal stem cells were isolated from femurs and tibias of sacrificed 6 to 8-week-old BALB/c mice. Briefly, femurs and tibias were stored in phosphate buffered saline (PBS) with 1% penicillin/streptomycin (Invitrogen, USA) and the ends of bones were carefully removed. Then, 5 mL of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) including 15% fetal bovine serum and 1% penicillin/streptomycin and 2 mM L-glutamine, was used to flush cells from femurs by using a 18-gauge needle. Bone marrow cells were collected in a falcon tube and then centrifuged at 1000 rpm for 10 min. The

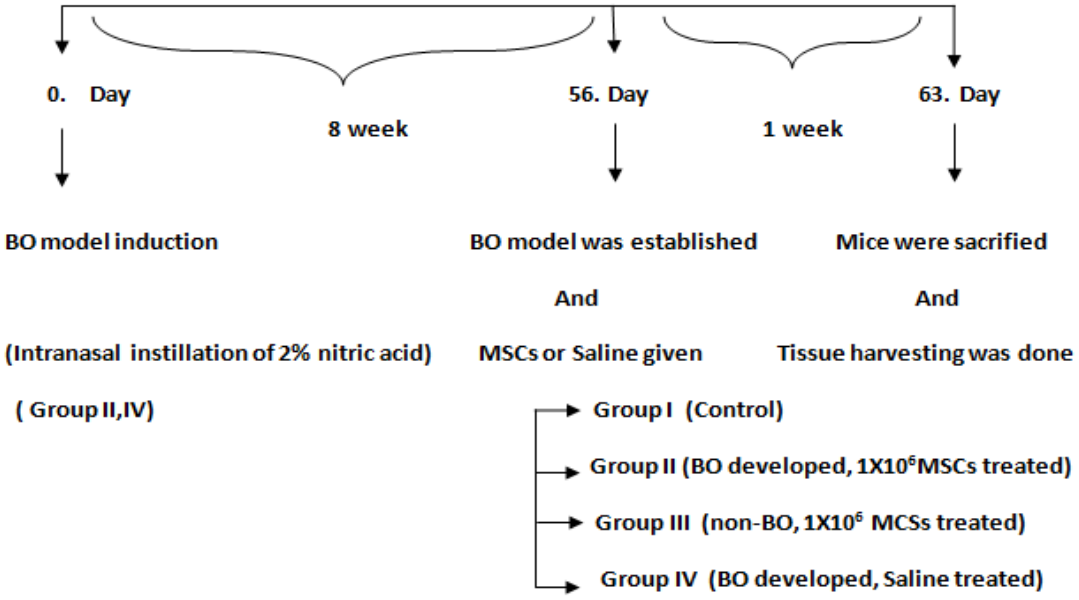

Figure 1. Time table for the bronchiolitis obliterans (BO) murine model and treatments with bone marrow-derived mesenchymal stem cells (MSCs) and saline

cell pellet was homogenized in DMEM including 15% fetal bovine serum and 1% penicillin/streptomycin and 2 mM L-glutamine. Then, cells were transferred into 75 cm^2 culture flasks and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.[19]

Upon 72 h of incubation, the non-adherent cells were removed by changing the medium. The medium was completely replaced every 3 days and non-adherent cells were discarded. When cells became 70-80% confluent, adherent cells were detached from the flasks by 0,25% trypsin-EDTA, collected in 50-mL-falcon tubes, and centrifuged at 1000 rpm for 10 min. The cell pellet was homogenized in full DMEM medium, transferred into 75-cm^2-culture flasks, and incubated at 37°C in a humidified atmosphere of 5% carbon dioxide.[20]

Transfection of BMSCs by Green Fluorescent Protein

In order to track the stem cells in vivo, BMSCs were transfected with pEGFP-N2 vector including green fluorescent protein (GFP) encoding gene (ClonTech, USA) by using Metafectene Pro transfection reagent (Biontex Laboratories GmbH, Germany). 5×10^5 MSCs were transferred into 75-cm^2-flasks and incubated at 37°C and 5% CO_2 till they became 70-80% confluent. On the day of transfection, the medium was discarded from the flasks, and 12 mL of blank DMEM medium was transferred onto the cells. 6 µg plasmid DNA and 20 µL of transfection reagent were mixed in order to form transfection complexes, and these complexes were transferred dropwise onto the cells. The flasks were gently swirled to ensure the uniform distribution of the complexes. The transfected cells were incubated at 37°C in 5% CO_2 incubator for 4 h, and then medium containing transfection complexes were replaced with fresh full DMEM medium. 72 h after the transfection, transfected cells were visualized under the fluorescent microscope.[21]

Characterization of BMSCs

Cells were incubated with antibodies recognizing NG2 PE (Beckman Coulter), CD45 PC5 (Beckman Coulter, Marsillia, France), CD73 PE (Becton-Dickinson, Bioscience Pharmingen, San Diego, CA, USA), and CD105 FITC (Serotec, Oxford, UK). Fluorescence histograms were obtained by recording $2x10^4$ cells/sample at a flow rate of approximately 200 cell events/s. Experiments were performed with the

FACS Canto II device and flow cytometric data were examined using the FACS DIVA software (BD Bioscience, San Jose, USA).[24] Flow cytometry analysis revealed that expression levels of MSC-specific CD105, CD73, and NG2 antigens were significantly increased and none of the cells were stained with CD45 hematopoietic marker antigen.[19] 4',6-diamidino-2-phenylindole (DAPI) staining was used to determine the number of nuclei and to assess gross cell morphology (Figure 2).

Histopathological and Immunohistochemical Analysis of Lung Tissue

Lungs of each mouse were removed and examined for histomorphological changes. Mice were anesthetized with the overdose of ketamine after 1 week of the last MSCs and saline administration. Two investigators, who were blinded to the treatment groups, interpreted the histopathological changes. Tissue specimens were obtained from the middle zone of the left lung of mice. Samples were fixed in 10% formalin for light microscopic evaluations. After fixation, samples were embedded in paraffin and serial sections of 5-µm thickness were prepared. Sections were stained with hematoxylin and eosin (H&E), and masson's trichrome stain. In histological analyses, diameter and wall thickness of terminal bronchiol and terminal artery, and peribronchovascular collagen depositions were evaluated. For immune cell infiltration, paraffin sections were stained by CD3 (1:100, Santa Cruz, USA), CD4 (1:100, Santa Cruz, USA), CD8 (1:100, Santa Cruz, USA), CD20 (1:100, Santa Cruz,USA), CD 68 (1:100, Novus, USA), and Neutrophil Elastase (1:100 Novus, USA) antibodies for immunhistochemical analyses. Seconder marking sections were finally stained with diaminobenzidine (DAB) (Invitrogen, USA). Immune cell densities were assessed in 10 different areas per section and expressed as percentage. Photomicrographs were taken with JVC TK-890-E camera (JVC, Japan) that was adapted on Olympus BH-2 RFCA model microscope (Olympus Optical, Japan). The histological analysis was carried out with UTTHSCSA Image Tool for Windows Version 3.00 Software (UTHSCSA Dental Diagnostic Science, USA).

Measurements of Cytokines and Differential Cell Count in Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was immediately collected from euthanized mice by instillation and recovery of 1 mL 0,09% NaCl through the tracheal cannula. BALF was centrifuged at 3000 × g for 10 min and supernatant was removed and stored at −80◦C. Levels of Interleukin-2, TNF- α, and IFN-γ were quantified in BALF by using standard ELISA protocols with the help of commercial mouse Interleukin-2, TNF-α, IFN- γ kits (eBioscience Mouse ELISA kit, USA).

Detection levels were 2.0 pg/mL for IL-2, 8.0 pg/mL for TNF-α and 15 pg/mL for INF- γ Histological smear samples were also prepared. A differential cell count was performed by using May-Grünwald Giemsa staining and cells were classified as eosinophils, neutrophils, and lymphocytes on the basis of morphologic criteria and staining characteristics. Differential cell counts were performed in a blinded fashion by counting at least 200 cells per slide under light microscopy.

Statistical Analysis

SPSS 15 package program was used for statistical analyses (SPSS Inc, Chicago, Illinois, USA). The data were presented as mean±standard error of mean (SEM). All data were analyzed by using one-way analysis of variance (ANOVA) posthoc Bonferroni test. $p<0,05$ was considered statistically significant.

RESULTS

Characterization and Migration of BMSCs

Flow cytometry analysis revealed that expression levels of MSC-specific CD105, CD73, and NG2 antigens were significantly increased and none of the cells were stained with CD45 hematopoietic marker antigen (CD105; %99,7, CD73; %99,9, NG2; %98,9, CD 45; %0,1). According to the results of the confocal microscopy, group III mice did not have significant BMSCs in their lung tissue, while it was observed that all mice in group II had green dots and this finding indicates that labeled BMSCs migrated to the lung tissues of group II mice upon their intraperitoneal administration (Figure 2).

Histopathological and Immunohistochemical Analysis of Lung Tissue

Morphometric measurements of terminal bronchiole (TB) and terminal arteriole (TA) diameters and wall thicknesses of the groups are presented in Table 1 and Figure 3, 4. The TB wall thicknesses and diameters were significantly lower (p<0.05) in group II when compared to group IV, whereas TA wall thicknesses and TA diameters were similar to group IV. Peribronchovascular collagen deposition significantly was decreased in group II compared to group IV (Table 1) (p<0.05). Measurements of the total amount of immune cell airway infiltration (lymphocytes, macrophages and neutrophils) and their immunophenotyping are presented in Table 2 and Figure 5, 6. The percentage of all immune cells significantly was increased in group IV compared to group I (control group) (p<0.05). Additionally, the number of immune cells significantly decreased in group II mice compared to group IV mice (p<0.05).

Cytokine Levels and Differential Cell Count in BALF

All of the cytokine levels (IL-2, IFN-γ, TNF- α) of group II were significantly lower compared to group IV (Table 3, Figure 7) (p<0.05). The number of eosinophils, lymphocytes, and neutrophils in BALF significantly was reduced in group II (BO developed and BMSCs treated group) compared to group IV (BO-developed and saline treated group) (Table 4, Figure 8) (p<0.05).

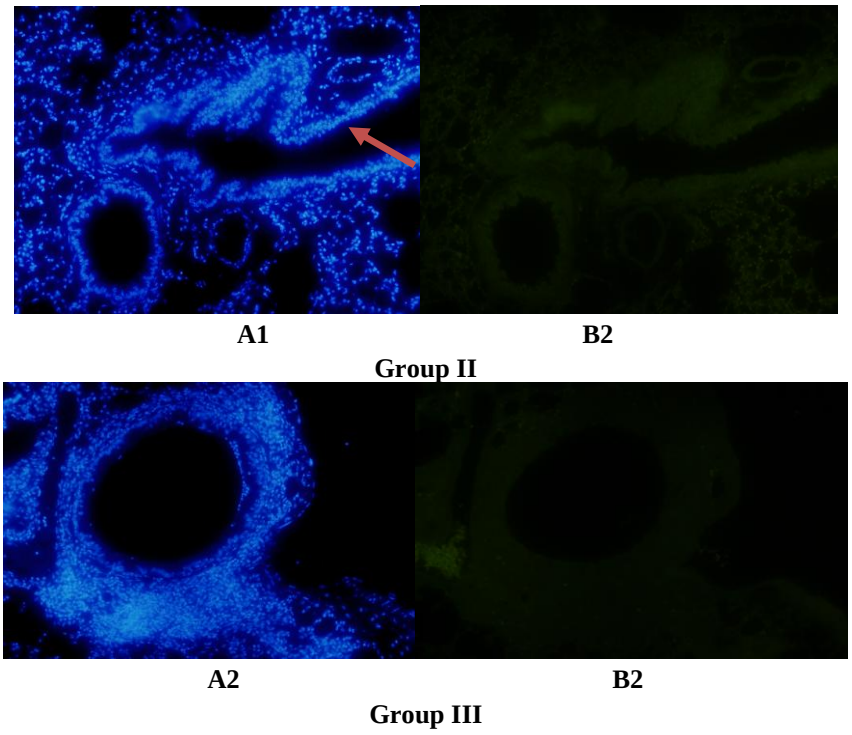

Figure 2. Confocal microscopic images after intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells (MSCs) in bronchiolitis obliterans mice model; non-bronchiolitis obliterans developed and $1x10^6$ allogeneic bone marrow-derived MSCs- injected group (Group III) did not have significant bone marrow-derived MSCs in their lung tissue while it was observed that all mice in bronchiolitis obliterans-developed and $1x10^6$ bone marrow-derived --MSCsinjected group (Group II) had green dots, which indicates that labeled bone marrow-derived MSCs migrated to the lung tissues of group II mice.

Table 1. Histopathological findings after intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in mice model of bronchiolitis obliterans

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group (mean±SD)

	Group I (Control) (Mean ±SD)	Group II (Bronchiolitis Obliterans+, Mesenchymal stem cell) (Mean ±SD)	Group III (Bronchiolitis Obliterans -, Mesenchymal stem cell) (Mean ±SD)	Group IV (Bronchiolitis Obliterans +, Saline) (Mean ±SD)
Diameter of terminal bronchiole (µm)	118.9±38.43	125.78±14.37	116.31±9.82	124.38±18.27
Wall thickness of terminal bronchiole (µm²)	10.52±1.28*	8.95±0.77*	9.39±0.95*	32.94±5.15
Diameter of terminalarteriol (µm)	59.19±3.77	66.91±2.6	57.61±5.99	70.97±8.78
Wall thickness of terminalarteriol (µm²)	6.59±0.80	8.24±1.87	7.88±0.41	8.61±1.45
Peribronchiolar collagen deposition (µm²/µ m)	3.49±0.20*	3.71±0.36*	3.44±0.22*	9.33±2.14
Periarteriolar collagen deposition (µm²/µm)	3.57±0.63*	5.1±0.69*	4.93±0.90*	16.67±1.86

*p<0.05 compared to group BO +, Saline (Group IV)

Table 2. Immunohistochemical findings of the intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group (mean±SD)

	Group I (Control) (Mean ±SD)	Group II (Bronchiolitis Obliterans+, Mesenchymal stem cell) (Mean ±SD)	Group III (Bronchiolitis Obliterans -, Mesenchymal stem cell) (Mean ±SD)	Group IV (Bronchiolitis Obliterans +, Saline) (Mean ±SD)
CD3 (%)	0.11±0.01*	0.29±0.01*	0.16±0.02*	0.50±0.02
CD4 (%)	0.14±0.01*	0.30±0.01*	0.18±0.02*	0.46±0.03
CD8 (%)	0.11±0.01*	0.32±0.02*	0.16±0.01*	0.44±0.03
CD20 (%)	0.13±0.02*	0.26±0.01*	0.13±0.01*	0.41±0.01
CD68 (%)	0.12±0.01*	0.25±0.02*	0.14±0.02*	0.37±0.02
Neutrophil Elastase (%)	0.14±0.01*	0.24±0.01*	0.16±0.02*	0.42±0.01

*p<0.001 compared to group bronchiolitis obliterans +, Saline (Group IV)

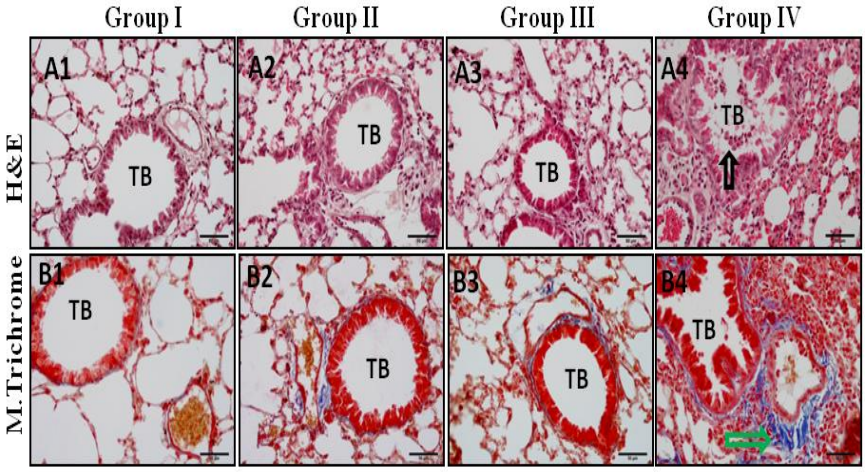

Figure 3. Light microscopic findings after intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model groups (40X)

I; Control, II; bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells-injected group, III; non-bronchiolitis obliterans and bone marrow-derived mesenchymal stem cells -injected group, IV; bronchiolitis obliterans-developed and saline-injected group. In representative histological images lung tissues were stained with hematoxylin & eosin (H&E) (A; 1st row) and Masson's Trichrome (B; 2nd row). In control group; light microscopic findings revealed a regular, normal bronchiole wall thickness, bronchiole lumen, collagen deposition (A1,B1). In group IV; H&E staining revealed thickened bronchiole wall, reduction of diameter of bronchiole lumen and inflammatory cells around the bronchiole wall (black arrow, A4). In group IV; Masson's trichrome staining revealed increased periarteriolar collagen deposition (blue color- green arrow, B4). In group II, bronchiole wall thickness and peribronchiolar and periarteriolar collagen deposition (A2,B2) were significantly less than group IV (A4,B4) and no statistical difference was found in terms of the diameter-wall thickness of arteriol and diameter of bronchiol between both groups.

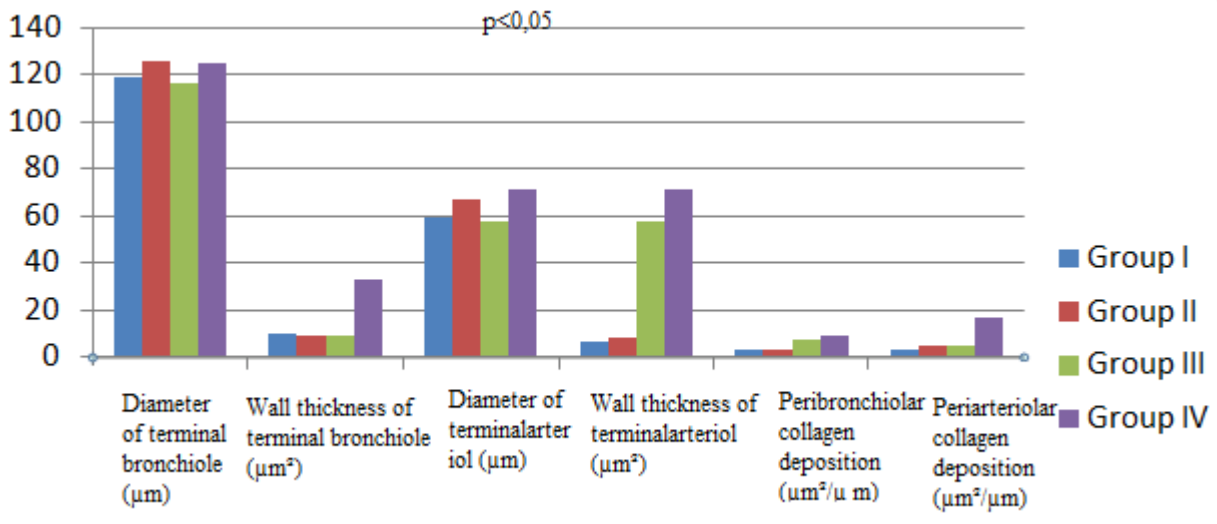

Figure 4. Correlation graphics of histopathological findings in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group.

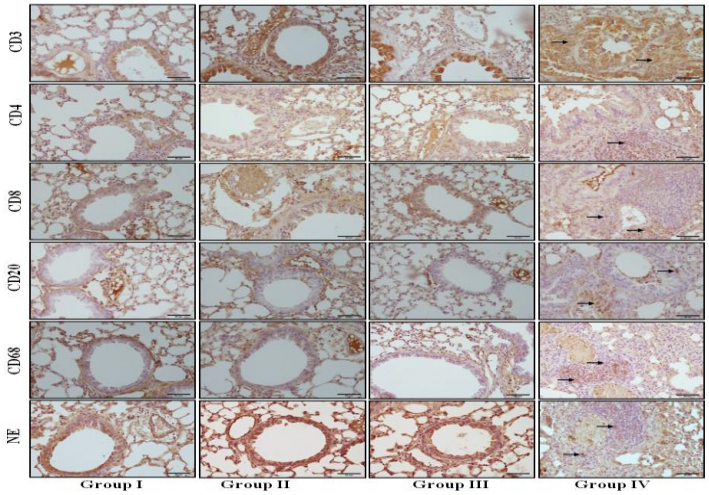

Figure 5. The immunohistochemical staining after the intraperitoneal injection of allogeneic bone marrow- derived mesenchymal stem cells in bronchiolitis obliterans mice model groups (40X)

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group. Representative pictures show the immunohistochemical staining of CD3, CD4, CD8, CD20, CD68, neutrophil elastase. Black arrows indicate the staining of group IV in comparison with group II and group III. The percentage of all immune cells significantly was increased in group IV compared to group I (control group). Additionally, the number of immune cells significantly decreased in group II mice compared to group IV mice.

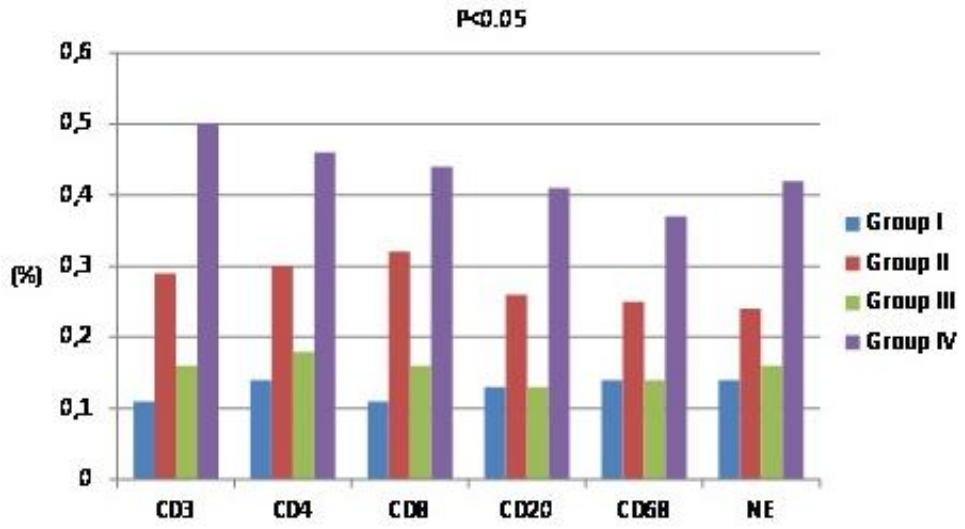

Figure 6. Correlation graphics of immunohistochemical findings in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells inbronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group.

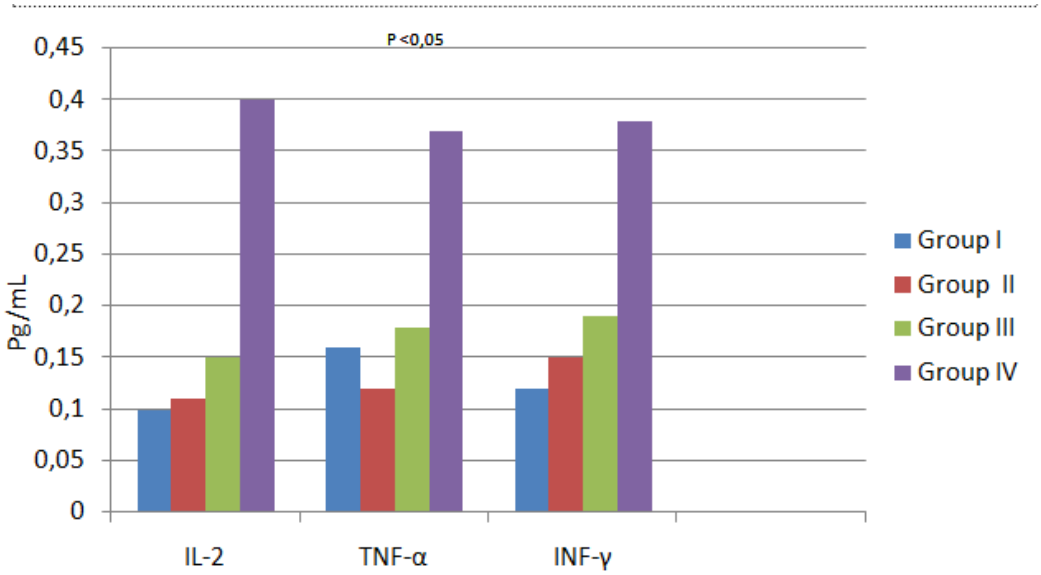

Figure 7. Correlation graphics of cytokine levels in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group.

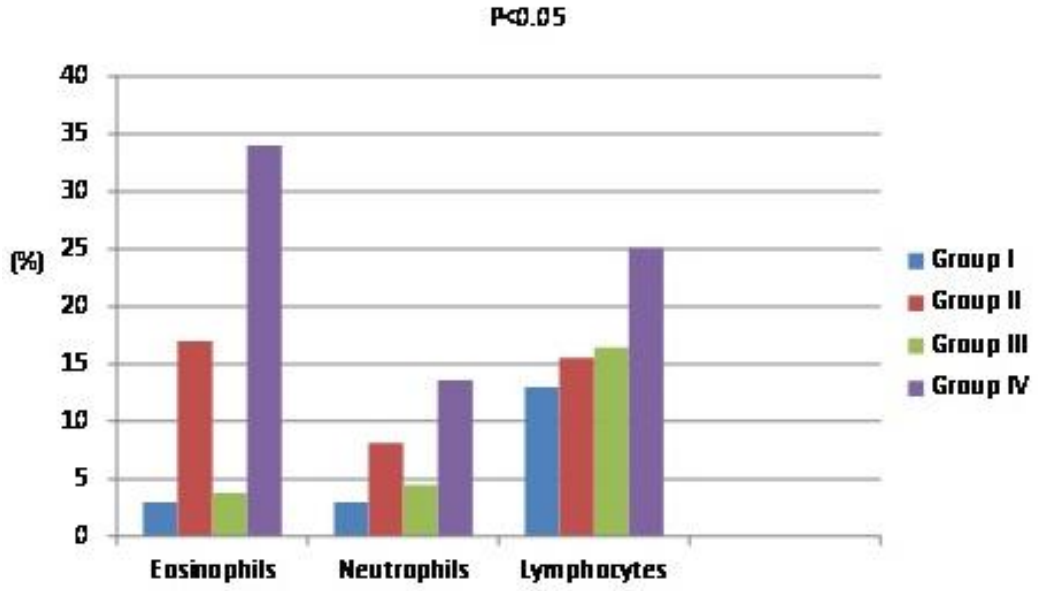

Figure 8. Correlation graphics of differential cell counts of bronchoalveolar lavage fluid in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group.

Table 3. Cytokine levels in bronchoalveolar lavage fluid (BALF) (pg/ mL) in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group (mean±SD)

	Group I (Control) (Mean ±SD)	Group II (Bronchiolitis Obliterans+, Mesenchymal stem cell) (Mean ±SD)	Group III (Bronchiolitis Obliterans -, Mesenchymal stem cell) (Mean ±SD)	Group IV (Bronchiolitis Obliterans +, Saline) (Mean ±SD)
IL-2 (BALF)	0.10±0.04	0.11±0.04*	0.15±0.05*	0.40±0.04
TNF-α (BALF)	0.16±0.03	0.12±0.02*	0.18±0.05*	0.37±0.09
IFN-γ (BALF)	0.12±0.02	0.15±0.04*	0.19±0.02*	0.38±0.06

*p<0.05 compared to group bronchiolitis obliterans +, Saline (Group IV)

Table 4. Differential cell counts of bronchoalveolar lavage fluid (BALF) in the study of intraperitoneal injection of allogeneic bone marrow-derived mesenchymal stem cells in bronchiolitis obliterans mice model

I; Control; II, bronchiolitis obliterans-developed and bone marrow-derived mesenchymal stem cells -treated group, III; non-bronchiolitis obliterans developed and bone marrow-derived mesenchymal stem cells-given group, IV; bronchiolitis obliterans-developed and saline-treated group (mean±SD)

% Cells	Group I (Control) (Mean ±SD)	Group II (Bronchiolitis Obliterans+, Mesenchymal stem cell) (Mean ±SD)	Group III (Bronchiolitis Obliterans -, Mesenchymal stem cell) (Mean ±SD)	Group IV (Bronchiolitis Obliterans +, Saline) (Mean ±SD)
Eosinophils	3.0±0.44*	17±1.97*#	3.8±0.67*	34±3.02#
Neutrophils	3.0±0.45*	8.1±1.31*#	4.5±0.71*	13.6±1.05#
Lymphocytes	13.6±1.12*	15.5±1.93*	16.4±1.15*	25.1±3.37#

*p<0.05 compared to group bronchiolitis obliterans +, Saline (Group IV)

#p<0.05 compared to group control (Group I)

DISCUSSION

The pathogenesis of BO has been extensively studied in both populations which were either organ transplanted or not transplanted. In the pathogenesis of BO, bronchiolar epithelial cells are damaged, and T lymphocytes and neutrophils are recruited to the site of the injury. Various cytokines and chemokines such as TNF- α, IL-2, IL-8, and regulated on activation, normal t cell expressed and secreted (RANTES) are activated and create an inflammatory response. The repeated and persistent inflammation can lead to an excessive fibroblastic response which can further cause the peribronchiolar fibrosis and obliteration of airways.[23-25]

Stem cells are undifferentiated cell groups that show varying degrees of self-renewal and differentiation capacity. MSCs are capable of adopting the morphology and phenotype of parenchymal cells of many non-hematopoietic tissues, including the lung tissue,[26,27] where they can differentiate into bronchial epithelial cells[28] and alveolar type I and II pneumocytes.[26,27,29] Furthermore, MSCs exhibit immunosuppressive properties through a mechanism that involves the paracrine inhibition of T- and B-cell proliferation.[30] Recently, most studies have investigated the role of MSCs on tissue repair, regeneration, and remodeling in respiratory diseases.[31,32] Stem cell therapy appears to be a promising strategy for the

treatment of many respiratory diseases which have no effective treatment, such as acute lung injury (ALI)[16] and its severe forms such as acute respiratory distress syndrome (ARDS),[17] and chronic obstructive pulmonary disease (COPD).[15] Some clinical trials test the safety and feasibility of cell-based therapy in respiratory diseases. Recently, a Brazilian research group has tested the safety of bone marrow-derived mesenchymal stem cell administration in four patients with severe COPD. However, they did not show the efficacy of the stem cell therapy.[33] On the other hand, another clinical study suggested that the intratracheal transplantation of allogeneic human umbilical cord blood-derived MSCs is safe and feasible for bronchopulmonary dysplasia in preterm infants.[34]

BO is a devastating disease with high mortality rate and no effective-curative therapy can reverse or delay the natural course of this disease. MSC therapy could be a possible therapeutic tool for BO, because MSCs can yield anti-inflammatory and immunmodulatory effects, and they can also inhibit the fibrogenesis. A recent study has shown that the treatment with placenta-derived human mesenchymal stem cells (PMSCs) can reduce the development of BO in a heterotopic tracheal transplantation murine model.[35] Grove et al.[36] also evaluated the effects of bone marrow-derived MSCs on the BO murine model. They carried out a retro-orbital injection of $5x10^5$ cells in allogeneic mice. They detected a 60% decrease in the intraluminal obstruction in the experimental group of animals, which were sacrificed at the 7 and 14 days of the MSCs administration, compared to controls. There are many controversial studies in the literature, which have evaluated effects of stem cell therapy in experimental murine model of BO. Espinel at al. showed that the regular injection of epididymal adipose tissue-derived stem cells did not significantly reduce the severity of allograft inflammation in seven days. However, it increased the allograft inflammatory process in heterotopic tracheal transplant bronchiolitis obliterans model in 21 days.[37] In their study, they obtained MSCs from epididymal adipose tissue and it was suggested that different sources of MSCs may change the immunomodulating potency due to the standard production of growth factors and interleukins. In our study, we observed that BMSCs administered by intraperitoneal route significantly decreased TH1 immune cytokine (such as IL-2, TNF- α and IFN- γ levels in group II (BO-developed and BMSCs-treated group) compared to group IV (BO-developed and saline-treated group). MSCs prevent fibrosis at an early stage of lung injury by reducing the neutrophil influx, inflammation, and collagen deposition. In a late stage of lung injury, MSCs can prevent the fibrosis by downregulating the cytokines such as IL-1β, TGF- β, VEGF, IL-6, TNF-α, and nitric oxide synthase (NOS).[38] The bleomycin-induced lung fibrosis model is a well-characterized and commonly used model for understanding the mechanism of MSCs. It was shown that MSCs inhibited the inflammation and the fibrosis in the lung tissue in animals with bleomycine-induced lung injury by inhibiting the production of two fundamental pro-inflammatory cytokines such as IL-1 and TNF-α and reducing the neutrophil and lymphocyte trafficking in the lung.[39] Furthermore, the treatment with MSCs decreased the bleomycin-mediated collagen deposition and decreased the levels of TGF-β, MIP, and IFN-γ cytokines.[40] Even though some researchers claim the antifibrotic effect of MSC, some others support the idea that MSC therapy have profibrotic effects.[41,42] In our study, we showed that periarteriolar and peribronchiolar collagen deposition and wall thickness of terminal bronchiole were significantly decreased in Group II (BO-developed and BMSCs-treated group) compared to the Group IV (BO-developed and saline-treated group) in murine model of BO.

The optimal route for stem cell transplantation in a lung injury has not yet been determined. In contrast to a normal lung tissue, an injured lung produces soluble factors, which can cause MSCs to proliferate and migrate towards an injured lung.[43] It has recently been shown that human MSCs administered by intraperitoneal injection could be implanted in the lungs of newborn rats and cells could effectively prevent the hyperoxia-derived lung injury in rats.[44] Several studies have demonstrated that the pulmonary engraftment of MSCs in the lung tissue is very low and thus the effects of these cells on organ systems are currently attributed to a paracrine effect. In other words, secretion of soluble factors in different diseases can modulate various immune responses such as inhibition or activation of dendritic cells and T lymphocytes, and inflammatory cytokine secretion (TNF- α, IFN-γ), and increase in the release of anti-inflammatory cytokines (IL-10, IL-4).[39,45,46] This mechanism was first identified when MSCs were systemically administered and they could inhibit the expression of several pro-inflammatory and pro-

fibrogenic cytokines in acute lung injury and pulmonary fibrosis models.[29,39] TNF- α, a mesenchymal growth factor that is known to participate in matrix remodeling, has been shown to be elevated in the early stage of BO in both human and animal studies.[29,47] Upregulation of TH11 cytokines and chemokines, such as IFN- γ and IL-2, were also observed in post-transplant airway obliteration.[48] In the present study, BMSCs were intraperitoneally administered to mice in order to evaluate the migration. In our study, we showed that the intraperitoneally injected BMSCs migrated to the injured lung tissue in group II (BO-developed and BMSCs-treated group). Similar to the literature, cells showed beneficial effects on lung histopathology and TH1 immune response in murine model of BO. In our opinion, beneficial effects of intraperitoneally injected BMSCs on the lung tissue may be related the autocrine or paracrine effects of these stem cells on inflammation and fibrosis.

In the present study, immunohistochemical and histopathological examinations demonstrated that intraperitoneally administered BMSCs migrated to the lung tissue, and they have beneficial effects on inflammation and fibrogenesis in murine model of BO. However, further experimental studies are necessary in order to support the effective transfer of cellular therapy for BO. Additionally, many new trials will be necessary to assess the favorable effects of the use of BMSC therapy in human BO. However, route of administration, as well as the cell type, the best dose to be administered, the nature of the signals involved in MSC recruitment to the lung, the extent of MSC engraftment, and the role of the injury or disease in these processes are all issues to be clarified.

ACKNOWLEDGEMENTS

This work (project no.112S148) was supported by a research grant that was received from the Scientific and Technological Council of Turkey. We are grateful to the Scientific and Technological Council of Turkey for their financial support and Bioengineering and Biotechnology Application and Research Center staff of Izmir Institute of Technology for their help and technical support.

REFERENCES

1. Ryu JH, Myers JL, Swensen SJ. Bronchiolar disorders. Am J Respir Crit Care Med 2003; 168(11):1277-92.
2. Chan PWK, Muridan R, Debruyne JA. Bronchiolitis obliterans in children: clinical profile and diagnosis. Respirology 2000; 5(4):369-75.
3. Kotloff RM, Ahya VN, Crawford SW. Pulmonary complications of solid organ and hematopietic stme cell transplantation. Am J Respir Crit Care Med 2004; 170(1):22-48.
4. Yalçın E, Doğru D, Haliloğlu M, Özçelik U, Kiper N, Göçmen A. Postinfectious bronchiolitis obliterans in children: clinical and radiological profile and prognostic factors. Respiration 2003; 70(4):371-75.
5. Sarahrudi K, Carretta A, Wisser W, Senbaklavaci O, Ploner M, Neuhauser P, et al. The value of switching from cyclosporine to tacrolimus in the treatment of refractory acute rejection and obliterative bronchiolitis after lung transplantation. Transplant Int 2002; 15(1):24-8.
6. Ding IB, Baumgartner RA, Schwaiblamir M, Behr J. Admistration of anti-interleukine 2Rα monoclonal antibody in bronchiolitis obliterans syndrome after lung transplantation. Transplantation 2003; 75(10):1767-9.
7. Dosanh A, Ikonen T, Wan B, Morris RE. Pirfenidone: a novel anti-fibrotic agent and progressive chronic allograft rejection. Pulm Pharmacol Ther 2001; 15(5):433-7.
8. Gerhardt SG, McDyer JF, Girgis RE, Conte JV, Yang SC, Orens JB. Maintenance azithromycin therapy for bronchiolitis obliterans syndrome. Am J Respir Crit Care Med 2003; 168(1):121-5.
9. Hardy KA, Schidlow DV, Zacri N. Obliterative bronchiolitis in children. Chest 1988; 93(3):460-6.
10. Delorme B, Charbord P. Culture and characterization of human bone marrow mesenchymal stem cells. Methods Mol Med 2007; 140:67-81.
11. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411):143-7.
12. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006; 98(5):1076-84.
13. Loebinger MR, Aguilar S, Janes SM. Therapeutic potential of stem cells in lung disease: progress and pitfalls. Clin Sci (Lond) 2008; 114(2):99-108.
14. Mohammadian M, Boskabady MH, Kashani IR, Jahromi GP, Omidi A, Mejad AK, et al. Effect of bone marrow derived mesenchymal stem cells on lung pathology and inflammation in ovaalbumin-induced asthma in mouse. Iran J Basic Med Sci 2016; 19(1):55-63.
15. Ghorbani A, Feizpour A, Hashemzahi M, Gholami L, Hosseini M, Soukhtanloo M, et al. The effect of adipose derived stromal cells on oxidative stress level, lung

emphysema and white blood cells of guinea pigs model of chronic obstructive pulmonary disease. Daru 2014; 22(1):26.

16. Matthay MA, Thompson BT, Read EJ, McKenna DH Jr, Liu KD, Calfee CS, et al. Therapeutic potential of mesenchymal stem cells for severe acute lung injury. Chest 2010; 138(4):965-72.
17. Horie S, Laffey JG. Recent insights: mesencyhmal stromal/stem cell tharapy for acute respiratory distress syndrome. F1000Res 2016; 28:5.
18. Garippo AL, Parra ER, Teodoro WR, Veloza AP, Yoshinari NH, Capelozzi VL. Immune cell infiltration and broncovascular remodeling after nitric acid nasal instillation in a mouse bronchiolitis obliterans model. Lung 2006; 184(4):229-38.
19. Firinci F, Karaman M, Baran Y, Bagriyanik A, Ayyildiz ZA, Kiray M, et al. Mesenchymal stem cells ameliorate the histopathological changes in a murine model of chronic asthma. Int Immunopharmacol 2011; 11(8):1120-6.
20. Sunay O, Can G, Cakir Z, Denek Z, Kozanoğlu İ, Erbil G, et al. Autologous Rabbit Adipose Tissue Derived Mesenchymal Stem Cells For The Treatment Of Bone Injuries With Distraction Osteogenesis. Cytotherapy 2013; 15(6):690-702.
21. Baran Y, Ural AU, Avcu F, Sarper M, Elçi P, Pekel A. Optimisation of Transfection of Green Flourescent Protein in Pursuing Mesenchymal Stem Cells, In Vivo. Turk J Haematol 2008; 25(4):172-5.
22. Loebinger MR, Aguilar S, Janes SM. Therapeutic potential of stem cells in lung disease: progress and pitfalls. Clin Sci (Lond) 2008; 114(2):99-108.
23. Aris RM, Walsh S, Chalermskulrat W, Hathwar V, Neuringer IP. Growth factor upregulation during obliterative bronchiolitis in the mouse model. Am J Respir Crit Care Med 2002; 166 (3):417-22.
24. Hertz MI, Henke CA, Nakhleh RE, Harmon KR, Marinelli WA, Fox JM, et al. Obliterative bronchliolitis after lung transplantation: a fibroobliterative disorder associated with platelet-derived growth factor. Proc Natl Acad Sci USA 1992; 89(21):10385-9.
25. Laohaburanakit P, Chan A, Allen RP. Bronchiolitis obliterans. Clin Rev Allergy Immunol 2003; 25(3):259-74.
26. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, et al. Multiorgan,multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001; 105(3):369–77.
27. Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC, et al. Bone marrow derived cells as progenitors of lung alveolar epithelium. Development 2001; 128(24):5181–8.
28. Theise ND, Henegariu O, Grove J, Jagirdar J, Kao PN, Crawford JM, et al. Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow. Exp Hematol 2002; 30(11):1333–8.
29. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A 2003; 100(14):8407-11.
30. Ishizawa K, Kubo H, Yamada M, Kobayashi S, Suzuki T, Mizuno S, et al. Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells. Biochem Biophys Res Commun 2004; 324(1):276–80.
31. Mei SH, Haitsma J, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, et al. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 2010; 182(8):1047–57.
32. Krasnodembskaya A, Song Y, Fang X, Gupta N, Serikov V, Lee JW, et al. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells 2010; 28(12):2229–38.
33. Ribeiro-Paes JT, Bilaqui A, Greco OT, Ruiz MA, Marcelino MY, Stessuk T, et al. Unicentric study of cell therapy in chronic obstructive pulmonary disease/ pulmonary emphysema. Int J Chron Obstruct Pulmon Dis 2011; 6:63–71.
34. Chang YS, Ahn SY, Yoo HS, Sung SI, Choi SJ, Oh WI, et al. Mesenchymal stem cells for bronchopulmonary dysplasia: phase 1 dose-escalation clinical trial. J Pediatr 2014; 164(5):966-72.
35. Zhao Y, Gillen JR, Harris DA, Kron IL, Murphy MP, Lau CL. Treatment with placenta-derived mesencyhmal stem cells mitigates development of bronchiolitis obliterans in a murine model. J Thorac Cardiovasc Surg 2014;147(5):1668-1677.e5.doi:10.1016/j.jtcvs.2013.09.041.
36. Grove DA, Xu J, Joodi R, Torres-Gonzales E, Neujahr D, Mora AL, et al. Attenuation of early airway obstruction by mesencyhmal stem cells in a murine model of heterotopic tracheal transplantation. J Heart Lung Transplant 2011; 30(3):341-50.
37. Espinel Jde O, Uribe C, Meyer FS, Bringheti R, Kulczynskı J, Saueressig MG. Cell therapy in thetreatment of bronchiolitis obliterans in a murine model. Rev Col Bras Cir 2015; 42(3):181-8.

38. Lee SH, Jang AS, Kim YE, Cha JY, Kim TH, Jung S, et al. Modulation of cytokine and nitric oxide by mesenchymal stem cell transfer in lung injury/fibrosis.Respir Res 2010; 8:11-6.
39. Ortiz LA, Dutreil M, Fattman C, Pandey AC, Torres G, Go K, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. Proc Natl Acad Sci USA 2007; 104(26):11002–07.
40. Moodley Y, Atienza D, Manuelpillai U, Samuel CS, Tchongue J, Ilancheran S, et al. Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury. Am J Pathol 2009; 175(1):303-13.
41. Salama M, Andrukhova O, Jaksch P, Taghavi S, Kelpetko W, Dekan G, et al. Endothelin-1 governs proliferation and migration of bronchoalveolar lavage-derived lung mesenchymal stem cells in bronchiolitis obliterans syndrome. Transplantation 2011; 92(2):155-62.
42. Walker N, Badri L, Wettlaufer S, Flint A, Sajjan U, Krebsbach PH, et al. Resident tissue-specific mesenchymal progenitor cells contribute to fibrogenesis in human lung allografts. Am J Pathol 2011; 178(6):2461-9.
43. Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, et al. Bone marrow-derived mesenchymal stem cells in repair of the injured lung. Am J Respir Cell Mol Biol 2005; 33(2):145–52.
44. Tian ZF, Du J, Fu XM, Wang B, Hong XY, Feng ZC. Influence of human bone marrow-derived mesenchymal stem cells on the lung of newborn rats damaged by hyperoxia. Zhonghua Er Ke Za Zhi 2008; 46(1):4-8.
45. Abreu SC, Antunes MA, Maron- Gutierrez T, Maron-Gutierrez T, Cruz FF, Carmo LG, et al. Effects of bone marrow-derived mononuclear cells on airway and lung parenchyma remodeling in a murine model of chronic allergic inflammation. Respir Physiol Neurobiol 2010; 175(1):153–63.
46. Mistchenko AS, Diez RA, Mariani AL, Robaldo J, Maffey AF, Bayley-Bustamante G, et al. Cytokines in adenoviraldisease in children: association of interleukine-6, interleukine-8 and tumor necrosis factor alpha levels with clinical outcome. J Pediatr 1994; 124(5):714-20.
47. Boehler A, Bai XH, Liu M, Cassivi S, Chamberlain D, Slutsky AS, et al. Upregulation of T-helper 1 cytokines and chemokine expression in post-transplant airway obliteration. Am J Respir Crit Care Med 1999; 159(6):1910-7.
48. Zhao Y, Gillen JR, Harris DA, Kron IL, Murphy MP, Lau CL. Treatment with placenta-derived mesenchymal stem cells mitigates development of bronchiolitis obliterans in a murine model. J Thorac Cardiovasc Surg 2014; 147(5):1668-77.