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DOI: 10.14670/HH-18-493
Article type: ORIGINAL ARTICLE
Accepted: 2022-07-11
Epub ahead of print: 2022-07-11

This article has been peer reviewed and published immediately upon acceptance. Articles in “Histology and Histopathology” are listed in Pubmed.
Pre-print author’s version
Therapeutic Effect of Adipose-Derived Mesenchymal Stem Cells (AD-MSCs) compared to Pirfenidone on corticosteroid resistance in a mouse model of Acute Exacerbation of Idiopathic Pulmonary Fibrosis

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Running title: AD-MSCs on corticosteroid resistance in AE-IPF.

Word count: 7832
**Abstract**

**Introduction:** Acute exacerbation-idiopathic pulmonary fibrosis (AE-IPF) is a life-threatening condition. In the treatment of AE-IPF, corticosteroid medication is commonly utilized. However, there is insufficient evidence to justify its usage. Pirfenidone (PFD) has recently been discovered to be effective in the treatment of AE-IPF patients. However, regenerative therapy, such as stem cell therapy or tissue engineering, is necessary due to ineffective and limited therapies. Combining MSC transplantation with pharmacological therapy may also give additional benefits; nevertheless, its use must be proven experimentally. As a result, the goal of this study was to assess the therapeutic effects of adipose-derived mesenchymal stem cells (AD-MSCs) on corticosteroid resistance in an animal model of AE-IPF caused by bleomycin compared to PFD.

**Materials and methods:** Seventy C57BL/6J male mice were randomly divided into seven groups, control, BLM, methylprednisolone (MP), PFD, AD-MSCs, PFD +MP, and AD-MSCs +MP.

**Results:** In terms of survival, collagen deposition, the acute lung injury score (ALI), and the Ashcroft score, AD-MSCs exceeded PFD. AD-MSCs + MP provided protection and preserved the lung’s architecture in BLM-induced AE. In addition, AD-MSCs successfully decreased chemokine (CC motif) ligand-2 (CCL2) positive cells and lower pro-fibrotic and pro-inflammatory cytokines.

**Conclusions:** AD-MSCs enhanced histological structure, Ashcroft and ALI scores, lung collagen deposition, survival, and cytokines in an animal model of AE-IPF. As a result, we believe that AD-MSCs may be more therapeutically helpful for AE-IPF than presently available therapies, either alone or in conjunction with MP.

**Keywords:** Acute exacerbation, Idiopathic Pulmonary Fibrosis, Adipose-derived mesenchymal stem cells, Pirfenidone, Bleomycin, corticosteroids
1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, severe lung disease with a poor prognosis and a median survival period of three to four years following diagnosis. IPF has a wide range of natural histories, ranging from chronic, stable symptoms to progressive respiratory failure or acute exacerbation (AE). IPF-related AE affects 5–10% of people per year (Kishaba, 2019).

Several animal models have been utilized to explore the biology of IPF during the last few decades. These models are used to evaluate and understand biological fibrogenesis mechanisms and the efficacy of treatments (Moore and Hogaboam, 2008). Common methods to induce fibrosis include systemic or intratracheal instillation of Bleomycin (BLM) (Della Latta et al., 2015), radiation (Dadrich et al., 2016), intratracheal administration of silica (Zelko et al., 2016), and transgenic mice (Yoon et al., 2016). BLM can be administered in a variety of ways. As a result, it is frequently employed as an animal model in IPF (Della Latta et al., 2015) and AE-IPF studies (Wei et al., 2016).

Only supportive care and high-dose corticosteroids, such as methylprednisolone (MP) and prednisolone, are currently recommended by international consensus, according to the American Thoracic Society, the European Respiratory Society, the Japanese Respiratory Society, and the Latin American Thoracic Association (ATS/ERS/JRS/ALAT) guidelines. Even though no randomized controlled clinical studies have been conducted to evaluate the efficacy of corticosteroids therapy for AE-IPF, worldwide guidelines have issued a weak recommendation for corticosteroids treatment (Raghu et al., 2011). Furthermore, several studies have linked corticosteroid usage to worse outcomes in AE-IPF patients (Papiris et al., 2012; Atkins et al., 2014). There was no clear definition of AE in retrospective studies of larger cohorts of patients.
treated for AE-IPF with corticosteroids in addition to other therapies, and mortality rates were extremely high. For example, in 65 patients who had MP pulse or prednisolone in high or low dosages as only immunosuppressive treatment, documented in-hospital mortality was 55% (Song et al., 2011). Furthermore, in BLM-induced animal models, corticosteroids were ineffective in reducing fibrosis (Oku et al., 2008; Zhu et al., 2013).

The Food and Drug Administration has authorized pirfenidone (PFD) and nintedanib, two new medicines for IPF. Several trials have demonstrated that the anti-fibrotic medicines nintedanib and PFD substantially minimize the loss in forced vital capacity, reduce AE, and improve death rates in patients with IPF (Richeldi et al., 2014; Noble et al., 2016). Furthermore, PFD possesses anti-fibrotic, antioxidant, and anti-inflammatory properties in chronic IPF (King Jr et al., 2014). PFD was recently shown to be beneficial for a patient with AE-IPF; however, this finding warrants more investigation (Ohkubo et al., 2014; Furuya et al., 2017).

There is an urgent need for creative techniques and new medicines that can give complete remission due to a lack of effective and restricted medications. Because of its immunomodulatory, anti-inflammatory, and anti-fibrotic characteristics, stem cell treatment might be one method (Wakitani et al., 2007). Mesenchymal stem cells (MSCs) have sparked a lot of interest in the treatment of a variety of ailments (Ahmed et al., 2014; Fikry et al., 2016). MSCs might be a viable therapeutic option for IPF (Sroul and Thébaud, 2015). MSCs, such as adipose-derived (AD) (Dong et al., 2015), bone marrow (BM) (Zhang et al., 2019), lung spheroid cells (Henry et al., 2015), and human umbilical MSCs (Moodley et al., 2009), have been studied extensively for the treatment of IPF, but their effects have not been demonstrated in the treatment of AE-IPF.
Previous research has shown the benefits of both stem cell and pharmaceutical therapies, but there is currently a dearth of evidence on combining the two. So, this study aimed to investigate the therapeutic effect of AD-MSCs in combination with corticosteroids on corticosteroid resistance in an animal model of AE-IPF induced by BLM compared to PFD.

2. Materials and methods:

2.1. Ethics statement

The study was ethically reviewed by Medical Research Center (MASRI), faculty of medicine, Ain Shams University, Cairo, Egypt. The experimental protocol was approved by the institutional of Animals Care and Use Committee (ACUC) and Research Ethics Committee (FMASUS REC) with Federal wide assurance No. 000175. 85 (Reference No. FMASU R40/2022).

2.2. Drugs and Chemicals

Bleomycin (BLM) was purchased from Nippon Kayaku Co. (Ltd., Tokyo, Japan). Pirfenidone (Esbriet) (brand name: Pirfenex Cipla ® Capsule 200 mg) was purchased from a local pharmacy, Cairo, Egypt. Methylprednisolone (MP) was purchased from Pfizer Manufacturing Belgium NV. Sterile phosphate-buffered saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Penicillin /streptomycin mixture, and 0.1% collagenase type I were purchased from Lonza Company, Switzerland. CD44 and CD34 monoclonal mouse anti-rat antibodies were purchased from Thermo Fisher Scientific, Lab vision, USA. Paul Karl Horan 26 (PKH-26) and Sigma Aldrich chemical Co. provided the hydroxyproline (Hyp) (St. Louis, MO, USA). Ketamine (Ketalar 50 mg/ml; Pfizer, New York, USA) and xylazine 10 mg/kg body weight (Xylaject 20 %; Adwia, Co., Egypt).) Immunohistochemistry (rabbit polyclonal antibody) was performed on lung tissue sections for detection of chemokine (CC motif) ligand-2 (CCL2) and was
purchased from Abcam, Cambridge, MA. Transforming growth factor-β1 (TGF-β1) and Lung Tumor necrosis factor-alpha content (TNF-α) in mice were analyzed using enzyme-linked immunosorbent assay (ELISA) commercial kits from eBioscience (San Diego, CA, United States).

2.3. Isolation and culture of AD-MSCs:

Isolation of AD-MSCs from mice subcutaneous fat and preparation of their culture media were performed at the Unit of Stem cells at the Department of histology and cell biology, Faculty of Medicine, Ain shams University, Egypt. The AD-MSCs in the present study were isolated from subcutaneous fat and cultured using methods that have been proven to be effective (Park et al., 2012). Wild-type C57BL/6J male mice aged 5–12 months had their subcutaneous fat removed and washed three times with sterile PBS to remove contaminated debris and red blood cells. The cleansed tissues were cut into 1mm³ pieces and digested with 0.1 percent collagenase type I in PBS for 45-60 minutes at 37°C with periodic shaking every 10 minutes. 5 mL complete media including DMEM, 10% fetal FBS, and 1% Penicillin/streptomycin combination was employed to neutralize the enzyme activity. To eliminate undigested tissues, the suspension was filtered through a 100 m nylon mesh filter and subsequently a 40 m filter. At 25°C, the filtered suspension was centrifuged for 10 minutes at 1800 rpm. The supernatant containing mature adipocytes was discarded. The pelleted cells were resuspended in complete media. The cells were then plated at a density of 8000-10000 cells/cm² in 75ml cell culture flasks and incubated at 37°C with 5% CO₂. AD-MSCs developed a characteristic fibroblastic-like shape after many days of incubation. The media was changed every 3-4 days, and the cells were passaged when they reached >70% confluence, and they were designated as first passaged cells (P1). P3 cells were used in this experiment.
2.3.1. **Paul Karl Horan 26 (PKH-26) for Labeling of AD-MSCs:**

Before injection into mice, cells were labeled with PKH-67 (red fluorescence cell linker) fluorescent cytosolic dye to examine the homing ability of systemically given AD-MSCs (Haas et al., 2000). After centrifugation, the cells were washed twice in a serum-free medium. After pelleting, they were distributed in a dye solution. Before using AD-MSCs in this study, we cooled them to 4°C. Lung tissue was examined with a fluorescent microscope (Olympus BX50F4, No. 7M03285, Tokyo, Japan) at the Department of Biochemistry, Faculty of Medicine, Kasr Al-Ainy, Cairo, Egypt, to identify and track the cells labeled with PKH26.

2.3.2. **Characterization of cultured AD-MSCs:**

2.3.2.1. **Characterization of AD-MSCs by Immunohistochemical stain:**

The primary antibodies CD44 and CD34 monoclonal mouse anti-rat antibodies were used to characterize the cultivated AD-MSCs using the streptavidin-biotin immunoperoxidase method. The third passaged cells were fixed for 15 minutes in a pre-cooled mixture of acetone and methanol (1: 1, v: v). After the fixative was removed, the cells were washed twice with PBS. The Petri-dishes were covered in 10% H₂O₂ for 15 minutes to suppress endogenous peroxidase. After that, the primary antibody was added (in a ready-to-use formulation) and incubated at 37 °C for one hour. The cells were washed three times in PBS before being incubated for one hour at 37 °C with a 1:400 dilution of the secondary antibody (biotinylated goat anti-mouse). The cells were washed three times in PBS. The location of immunostaining may be determined by applying Streptavidin horseradish peroxidase conjugate for 15 minutes and then rinsing twice with PBS. With 1-2 ml of newly synthesized diaminobenzidine, color was generated for 10 minutes. The cells were then
thoroughly cleaned with distilled water. The hue of positive cytoplasmic responses was brown. The 2nd antibody was used as a negative control to check for any cross-reactions. (Sakr et al., 2017). In preparation for injection, cells were counted and resuspended in PBS using a modified version of a previously described procedure (Reddy et al., 2016).

2.3.2.2. Characterization of ADMSCs by flow cytometry analysis:

We used monoclonal antibodies specific to CD90, CD105, CD34, and CD45 to analyze the undifferentiated ADMSCs using a flow cytometer after the third passage. Fluorescein isothiocyanate-conjugated monoclonal antibodies (CD90 PE and CD45 PeCy7 IgG1 (Beckman Coulter, USA) were used to label adherent cells at 1x10^5 cells/ml. The cells were then trypsinized, fixed for 30 minutes in ice-cold 2 percent formaldehyde, washed in flow cytometry buffer, and incubated for 30 minutes in flow cytometry buffer containing the antibodies at 4°C in the dark. It was then centrifuged and resuspended in 500 µl PBS that included 2% FBS solution for each tube of monoclonal treated cells. For cell analysis, Beckman Coulter's CYTOMICS FC 500 flow cytometer (FL, USA) and CXP Software version 2.2 were utilized (Tucker and Bunnell, 2011).

2.4. Animal Model of Bleomycin-Induced AE-IPF:

On day 1, an intraperitoneal (IP) injection of ketamine/xylazine was used to anesthetize the mice, followed by a single intratracheal administration (IT) of 4 mg/kg BLM, diluted in 50 µL 0.9% NaCl. We waited for a gasp, which indicates that the liquid has been delivered through the tracheal tube. The same volume of NaCl was injected into control mice instead of the BLM solution. To prevent hypothermia, the animals were kept warm until they recovered from anesthesia using a
heating light or pad. On day 21, an AE-IPF model was established by delivering the second dose of BLM to all experimentally treated groups, whereas the control group received NaCl (Wei et al., 2016).

2.5. Experimental animals and grouping:

In the current study, seventy C57BL/6J male mice weighing 25–30g (52–54 weeks) were utilized. They were kept in plastic cages with mesh wire coverings. The animals were kept in normal circumstances for the tests, with a regulated light-dark cycle and a temperature of 22-25°C and allowed free access to food and water. The ethics committee of Ain Shams University in Egypt allowed the use of laboratory animals, which were used in compliance with the standards for care. Mice was divided into seven equal groups of 10.

**Control group I:** On day 1, the animals were given 50 µL of 0.9 % NaCl by IT. On day 21, they were given a second dose of NaCl by IT, followed by intravenous (IV) 200 µL PBS by tail vein and twice-daily oral gavage with 50 ml of 0.9 % NaCl for four weeks till day 52 (the end of the experiment).

**BLM group II:** Establishment of an AE-IPF model (Wei et al., 2016).

**MP group III:** Mice were given 10 mg/kg MP dissolved in saline twice daily by oral gavage until the end of the experiment on day 24 after the AE-IPF model had been established (Aytemur et al., 2012).

**PFD group IV:** On day 24 after AE-IPF induction, 100 mg/kg PFD was given twice daily for four weeks till the completion of the trial (Reddy et al., 2016).
AD-MSCs group V: On day 24 after AE-IPF induction, mice were given intravenous (IV) injection of $40 \times 10^6$ cells/kg body weight in 200 µl PBS (corresponding to a human dosage of $2 \times 10^6$ cells/kg body weight) (Reddy et al., 2016).

PFD + MP group VI: On day 24, mice were given a combination of PFD and MP at the same dosages and delivery routes as before until the completion of the trial.

AD-MSCs+ MP group VII: On day 24, mice were given a combination of AD-MSCs and MP at the same dosages and routes as before until the completion of the experiment.

2.6. Histological Examination and Mallory's trichrome Staining:

On day 52 of the experiment, a thoracotomy was done by elevating the sternum and cutting both sides of the ribs. IT received a 0.5 ml 10% formalin installation until the lungs had filled the chest cavity. The left lung was removed, and paraffin slices were created. Before staining, the sections were dewaxed with xylene and rehydrated using a series of ethanol solutions. After that, sections with a thickness of 4-5 µm were cut and stained with hematoxylin and eosin (H&E) and Mallory's trichrome stain and examined by optical microscope (Olympus CX22; Olympus Corporation, Tokyo, Japan) (Suvarna et al., 2018). For immunohistochemical staining, other sections were cut on positively charged slides. The additional right lung lobes were maintained frozen at -80 until tissue homogenization was completed.

2.7. Immunohistochemical (IHC) analysis:

To evaluate chemokine (CC motif) ligand-2 (CCL2), a standard avidin-biotin-peroxidase complex system followed by DAB visualization was used. Five µm thickness sections were deparaffinized,
followed by rehydration using descending grades of alcohol and washed in PBS. Endogenous peroxidase was inactivated by immersing sections in 3 percent hydrogen peroxide and then washing them with PBS. After that, the slides were boiled for 20 minutes in 10mM citrate buffer (pH 6.0) and then allowed to cool for another 20 minutes at room temperature. The addition of 5% bovine serum albumin to Tris-buffered saline prevented nonspecific binding. Primary anti-CCL2 antibodies were used at a 1:100 dilution for 60 minutes at room temperature before being washed with PBS. After that, the slides were treated with secondary antibodies. After that, the sections were rinsed and visualized with DAB. The slides were counterstained with hematoxylin. The primary antibodies were not used in the negative control sections (Suvarna et al., 2018).

2.8.Morphometric analysis:

Lung sections were examined using an image Leica Q win V.3 application installed on a computer linked to A Leica DM2500 microscope (Leica Microsystems, Germany) at Ain Shams University's Faculty of Medicine's Histology and Cell Biology Department. A total of ten specimens from six individual mice in each group (n=6) were analyzed. Five separate non-overlapping fields were collected from each section. Five different readings from each taken snapshot were counted for each specimen, and the mean was computed. To provide an unbiased evaluation, measurements at magnification x400 were collected by an impartial observer who was blinded to the specimens' features.
2.8.1. **Pulmonary fibrosis scoring (Ashcroft score)**

In a semiquantitative study of histological fibrotic changes, the Ashcroft score (Ashcroft et al., 1988) was employed to measure the degree of lung fibrosis. Section evaluation necessitates the completion of all 30–35 fields. Pulmonary fibrosis was graded according to the following criteria: Grade=0 normal lung, grade 1=minimal fibrous thickening of alveolar or bronchial walls, grade 2–3=moderate thickening of walls without obvious damage to lung architecture, grade 4–5=increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass, grade 6–7=severe distortion of structure and large fibrous areas, “honeycomb lung” was placed in this category, grade 8=total fibrous obliteration of the field. The fibrosis score for that lung region was calculated using the average of the scores from all fields.

2.8.2. **The acute lung injury (ALI) scoring**

Wei et al. reported ALI scoring criteria. The four categories mentioned below were evaluated on H&E-stained sections: (1) congestion; (2) hemorrhage; (3) inflammatory cells infiltrate; and (4) thickened alveolar wall or formation of the hyaline membrane. The scoring damage ratings ranged from zero to four: zero for no harm, one for moderate damage, two for severe damage, and four for maximum damage. The overall score was then calculated by summing together the individual scores (Wei et al., 2016).

2.9. **Tissue biochemical analysis:**

Right lung tissues were minced and homogenized, separately, in ice-cold sodium and potassium phosphate buffer (pH 8) at a concentration of 15% (w/v). The homogenate was centrifuged at
110000 x g for 20 minutes at 4 °C, and the supernatant was utilized to determine collagen content and cytokine markers.

The manufacturer's instructions were followed to calculate the amount of hydroxyproline (Hyp). First, the absorbance of each sample was read at 550 nm by a microplate reader, and the amount of Hyp per protein content (ng Hyp /mg protein) was determined.

In tissue homogenate, the levels of Tumor Necrosis Factor Alpha (TNF-α) as an indication of inflammation and Transforming Growth Factor beta-1 (TGF-β1) as an indicator of fibrosis were measured according to the manufacturer's procedure.

2.10. Statistical Analysis:

All data were collected, reviewed, and statistically analyzed using one-way analysis of variance (ANOVA)-one-way analysis and post-hoc least significant difference (LSD) using the SPSS.26 software (IBM Inc., Chicago, Illinois, USA). The data were summarized using the mean and standard error of the mean (SEM). Over the course of experiment, the number of mice that died and those that survived were counted each day, the body weight was recorded at day 0, 21, 38, 52, and the weight of the lung was recorded at the end of the experiment. The Kaplan-Meier method was used to create the survival curve, and the log-rank test was used to analyze the differences between them. Pearson's test was used to examine the correlation between the Hyp and area percentage of collagen fibers and the levels of TGF-β1 and TNF-α. Also, we examined the correlation between the body weight and lung weight with the Hyp content at the end of the experiment. Statistically significant differences were defined as those with p values less than 0.05. GraphPad Prism 9 was used to create the graphs (CA, USA).
3. **Results:**

3.1. **Identification of AD-MSCs:**

Patches of aggregated cells were seen during the microscopic inspection for flushing and estimating the centrifugation AD-MSCs yield shortly before incubation. Elongated, fusiform, and spindle-shaped cells, on the other hand, proliferated and stuck to the flask wall after 3 days of incubation. After 7 days of incubation, a reasonably homogeneous cell culture of AD-MSCs with fibroblast-like shape emerged. On the 14th day of incubation, the cell population had attained 70–80% confluence and had a fibroblastic shape (Figures 1A&B). AD-MSCs were consistently positive for CD44 (Figure. 1C) and negative for CD34 (Figure. 1D) when identified and examined by immunohistochemical analysis based on cell surface marker expression. Anti-CD90 and CD105 were detected in ADMSCs and anti-CD45and CD34 antibodies were not detected in ADMSCs by flow cytometry (Figure E).

3.2. **Fluorescence detection:**

To detect and track PKH26-labeled AD-MSCs, researchers used a fluorescence microscope to examine unstained paraffin sections. For groups, I, II, III, and VI, fluorescence microscopy of slides produced from lung sections after deparaffinization with unstained sections and no treatment with AD-MSCs revealed no auto-fluorescence. There were also apparent red fluorescent spots in both the AD-MSCs-only treated mice and the AD-MSCs + MP treated animals (Figures 2A&2B, respectively), indicating the homing of AD-MSCs into the lung tissue.
3.3. Body weight and Lung weight observation

In the present study, after the BLM treatment, there was a significant decrease (P<0.0001) in the BW in all experimental groups (BLM, MP, PFD, AD-MSCs, PFD + MP, and AD-MSCS + MP) on day 21 (15.10± 5.425, 14.90± 0.73, 15.30± 0.94, 15.10±0.99, 15.00±0.94, 14.50±0.84; respectively) as compared to the control (32.10± 1.91). While after the second dose of BLM, the average BW in the AE-IPF model (BLM group) and MP treated group decreased to (11.00±3.916 and 10.30± 3.71; respectively) that in the control group increased to 34.20± 1.47 on day 38; the difference between the two groups was statistically significant (P < 0.0001). More importantly, IV administration of AD-MSCs alone or with MP on day 38 (after 2 weeks from start of the treatment) showed a significant (P < 0.0001) increase in BW (17.10± 0.99 and 17.20± 0.78; respectively) as compared to the BLM and MP groups. Interestingly, there were non-significant differences (P=0.99) between PFD treatment alone or with MP (17.10± 0.87 and 17.00± 0.94; respectively). Interestingly, AD-MSCs treated groups alone or in combination with MP showed non- significant differences (P=0.99) compared to the PFD alone or in combination with MP. BW subsequently increased in all experimental groups, and it reached its highest point on day 52 in (BLM, MP, PFD, AD-MSCs, PFD + MP, and AD-MSCS + MP), with BW values of (8.10± 4.35, 7.20 ± 3.8, 16.90±5.99, 19.10±0.99, 17.00±6.03, 20.10±0.99; respectively) when compared to control group (36.60± 1.57). More importantly, IV administration of AD-MSCs alone or with MP on day 52 (after 4 weeks from start of the treatment) showed significant (P < 0.0001) increase in BW as compared to the BLM and MP groups. Interestingly, AD-MSCs treated groups alone or in combination with MP showed non- significant difference (P=0.86, 0.51; respectively) compared to the PFD alone or in combination with MP (Fig. 3A).
Consistent with these observations, AE-IPF model (BLM group) and MP treated group (1.45±0.09, 2.54±0.15; respectively) showed significant (P < 0.0001) increases in lung weights versus control group (0.10±0.008). A significant (P<0.001) decrease in collagen deposition was seen after intravenous treatment of AD-MSCs in both the AD-MSCs treated alone group (0.14±0.03) and the AD-MSCS +MP group, with the latter showing significantly lower lung weights. The pirfenidone-treated group performed considerably worse than the AD-MSCs group (Figure 3B).

3.4. Survivability and mortality rate:

Seventy mice were included in the current study. There were three deaths in the BLM group (d20-30), four deaths in the MP (d28–32), one in the PFD (d 46), one in the PFD + MP (d 47) and no deaths were recorded in the remaining experimental treated groups by AD-MSCs (d1-52). The overall survival rate was 87.14 % (61/70). Survival rates in the control, BLM, MP, PFD, AD-MSCs, PFD +MP, and AD-MSCS +MP were 100% (10/10), 70 % (7/10), 60% (6/10), 90% (9/10), 100% (10/10), 90% (9/10), and 100% (10/10), respectively. The survival curve showed a significant difference in survival among the experimental groups (χ² = 15.16, p = 0.019) (Figure 3C). From each group, six mice were chosen for statistical analysis.

3.5. AD-MSCs Ameliorates Corticosteroid Resistance in a model of AE-IPF.

3.5.1. Assessment of H&E:

As shown in Figures 4A and B, the architecture of lung tissue in control group I was normal, with thin inter-alveolar septa (IAS) and patent alveoli. The alveoli were lined with type I pneumocytes with flattened nuclei and type II pneumocytes with rounder nuclei protruding into the alveolar
lumen. Bronchioles appeared patent, with simple columnar epithelium lining them and nearby pulmonary blood vessels. With their dome-shaped apices, Clara cells in between the epithelial lining of the bronchioles were seen. Severe alveolar damage and increased alveolar cell cellularity and thickened IAS were seen in BLM group II (Figure 4C and D). Lung parenchyma and alveoli showed substantial local cellular infiltration with collapsed alveoli, with compensatory dilatation of nearby ones, and the hyaline membrane that is formed of eosinophilic fibrinous exudate and necrotic debris of cells. Cellular infiltration around the bronchioles was clearly seen. Polymorph nuclear leucocytes were also seen in the bronchioles harboring vacuolated eosinophilic exudate (pulmonary edema fluid). Furthermore, in MP group III, therapy with MP did not ameliorate the AE-IPF caused by BLM. Most alveoli seemed collapsed with compensatory dilatation of surrounding ones harboring vacuolated eosinophilic exudate entangled with polymorph nuclear leucocytes, resulting in widespread degenerative alterations and distortion of lung architecture in a patchwork manner. In addition, most regions of the lung tissue had enlarged IAS with significant cellular infiltration and broad patches of eosinophilic exudates (hyaline membrane). The bronchial revealed significant disorder and degeneration, necrosis, and desquamation of the epithelial lining (Figures 4E and F). While the lung architecture of PFD group IV improved, it was not as comprehensive as the control group's (Figure 4G and H). Surprisingly, AD-MSCs had a significant ameliorative impact on lung structure in group V. There were few localized regions of thickened inter-alveolar septa, inflammatory cellular infiltration, and an increase in pneumocyte type II numbers. Furthermore, the bronchial lining epithelium was virtually identical to that of the control group. At the same time, few inflammatory cells were seen around the bronchiole (Figures 4I and J). However, compared to groups II and III, lung architecture improved slightly in PFD + MP group VI (Figures 4K and L). In the thickened IAS and around the bronchioles, the lung
sections exhibited few localized aggregations of inflammatory cell infiltration. In the bronchioles, there were regions with normal lining epithelium and a few vacuolated goblet cells, as well as a few eosinophilic exudates visible in the lung parenchyma. In AD-MSCs + MP group VII, microscopic examination of sections from this group showed that the combination of AD-MSCs with MP caused a marked improvement in lung parenchyma that showed nearly normal alveoli and bronchioles lining epithelium. They were almost identical to the control group (Figures 4M and N).

3.5.2. Assessment of pulmonary fibrosis:

Few collagen fibers were seen in the IAS and surrounding the bronchiole and blood arteries in control mice lung sections (Figure. 5A). In the BLM and MP groups, significant collagen fiber deposition was seen in the lung interstitium, along the bronchial tubes (indicating lung fibrosis), and around the alveoli (Figures. 5B & 5C). Compared to the BLM group, sections from the PFD and AD-MSCs groups exhibited an apparent reduction in collagen fibers in the IAS and around the bronchi (Figures. 5D & 5E). Compared to the MP group, some collagen fibers were detected in the IAS and along the bronchial wall in the PFD + MP group (Figure. 5F). Interestingly, the AD-MSCs a+ MP group revealed an apparent decrease in collagen fibers and appeared similar to the control group (Figure. 5G).

3.5.3. Morphometric analysis:

Collagen quantification analysis revealed that there was a significant (P<0.0001) increase in percentage of collagen area in BLM (31.88±0.88) and MP (46.17±0.28) compared to control
(4.40±0.14), but the MP group was significantly higher than in the BLM group. Moreover, PFD (16.78±0.32) and AD-MSCs (5.16±0.04) exhibited a significant decrease compared to the BLM group. Also, MP+ PFD (16.76±0.34) and MP+ AD-MSCs (4.533±0.16) exhibited a significant (P<0.0001) reduction in contrast to the MP. Interestingly, AD-MSCs treated groups alone or in combination with MP showed a significant decrease compared to the PFD alone or in combination with MP (Figure 6A).

The collagen content (Hyp) in the BLM (252.3±2.61) and MP (350.8±11.55) groups was increased significantly (P<0.0001) compared with the control (84.83±1.55), PFD (99.50±2.78), and AD-MSCs group (87.33±2.61). Also, notably AD-MSCs+ MP (86.50±2.78) significantly (P<0.0001) decreased collagen content as compared to PFD+ MP (109.83±3.57). Interestingly, there was no significant difference between AD-MSCs (P=0.72) and AD-MSCs +MP (P=0.61) groups and the control (Figure 6B).

As shown in Figure 6C, the ALI scores were assessed in all treated groups. Comparing the BLM group to the other treated groups, except for the MP group, the ALI score increased significantly (P<0.0001). There was a non-significant (P=0.99) difference between BLM and MP groups. PFD (1.33±0.21) and AD-MSCs (1.16±0.30) groups had significantly decreased (P<0.0001) ALI score in contrast with BLM (3.50±0.22) and MP (3.66±0.21) groups. Moreover, PFD+ MP (1.33±0.21) and AD-MSCs +MP (0.50±0.22) significantly (P<0.0001) decreased the ALI score in contrast with MP group.

Ashcroft fibrosis score was significantly higher in BLM (4.83±0.30) and MP (6.66±0.33) groups than all treated groups, but the MP group was significantly (P<0.0001) higher than those in the BLM group. Moreover, AD-MSCs (3.33±0.21) groups exhibited significant (P<0.01) reduction
versus BLM. PFD (4.50 ± 0.22, P=0.89) showed no significant difference with BLM. Meanwhile, PFD + MP (2.00 ± 1.33) and AD-MSCs + MP (0.83 ± 0.16) groups exhibited significant (P<0.0001) reduction versus MP animals. Interestingly, AD-MSCs alone or in combination with MP showed a significant (P<0.0001, P<0.001; respectively) decrease in contrast with the PFD alone or in combination with MP (Figure 6D).

3.5.4. **Immunohistochemical analysis for chemokine (CC motif) ligand-2 (CCL2)**

On day 52 of each group, immunohistochemical labeling for CCL2 was used to detect the cellular origins of CCL2. There were no positive brownish cells in the control group (Figure 7A). In the BLM and MP groups, alveolar and bronchiolar epithelial cells and macrophages showed increased CCL2 positivity (Figure 7B and 7C). In each section, treatment with PFD or AD-MSCs alone or in combination with MP reduced the positive staining (Figure 7D-G). The lungs of the AE-IPF animal model generated CCL2 from alveolar and bronchiolar epithelial cells as well as macrophages, according to these findings.

3.6. **Cytokines levels outcomes:**

TNF (35.33 ± 0.66, 44.66 ± 0.66) and TGF-1 (6.00 ± 0.57, 8.16 ± 0.30; respectively) in tissue homogenate increased significantly (P< 0.0001) in the BLM and MP groups versus control TNF-α (9.50 ± 0.56) and TGF-1 (0.54 ± 0.18). Interestingly, PFD significantly dramatically decreased the lung TNF-α (20.00 ± 1.15, P<0.0001) and TGF-β1 (3.33 ± 0.21, P<0.0001) versus BLM mice. The administration of AD-MSCs, on the other hand, significantly reduced the lung TNF-α (12.50 ± 0.84, P<0.0001) and TGF-β1 (1.38 ± 0.15, P<0.0001) compared with the BLM and PFD groups.
Interestingly, AD-MSCs +MP exhibited a markedly significant decrease in the lung TNFα (10.66 ±0.33, P<0.0001) and TGF-β1 (0.94 ±0.29, P<0.0001) content as compared with PFD+ MP (2.50 ±0.42). There was no significant difference in TNFα and TGF-β1 (P=0.26, P=0.41; respectively) between AD-MSCs +MP and the control group. We wanted to see a link between TGF-β1, and collagen mean area % and Hyp content in this investigation. TGF-β1 (r= 0.922, P<0.0001) and mean area % of collagen fibers (r= 0.955, P<0.0001) had a strong positive association with Hydroxyproline concentration, as illustrated in Figures 8 C and D. In addition, there was a strong positive association (r= 0.97, P<0.0001) of lung weight and strong negative (r= -0.7087, P<0.0001) correlation of body weight with Hydroxyproline concentration (Figures 8 E).

4. Discussion:

Because of its severity, increased mortality rate, and drastically decreased survival, AE-IPF has sparked widespread concern worldwide. Despite the lack of proof, supportive care with the addition of high-dose corticosteroids, broad-spectrum antibiotics, and occasionally additional immunosuppression are the current foundations of AE-IPF therapy (Collard et al., 2016). On the other hand, Corticosteroids do not appear to reduce overall mortality or prevent AE (Atkins et al., 2014). Furthermore, in BLM-induced IPF animal models, corticosteroids are ineffective in reducing fibrosis (Oku et al., 2008).

Recently, stem cell therapy in experimental IPF has received much attention since it looks to be amenable to treatment. Transplantation of BM-MSCs has been shown to be a potential therapeutic option for IPF patients (Zhao et al., 2013; Baranowska et al., 2017). However, present procedures make obtaining stem cells from BM difficult, which limits the utility of BM-MSCs. AD-MSCs,
on the other hand, come from a larger variety of sources and are more easily available. Furthermore, in practical practice, liposuction is more likely to be accepted by patients than BM puncture. AD-MSCs are also a new therapeutic option, having anti-inflammatory, antioxidant, and anti-fibrotic properties (Ikarashi et al., 2019; Elshemy et al., 2021). As a result, it is predicted to be beneficial in the treatment of IPF patients. We used AD-MSCs in the current study to take advantage of these benefits.

Male aged C57BL/6 mice were chosen as the model system for AE-IPF because they have been shown to be more susceptible to BLM-induced IPF than other strains. According to Redente and colleagues (Redente et al., 2011), aged male C57BL/6 mice develop a more severe form than age-matched female or young mice of either sex. In addition, they reported that more neutrophil recruitment into the air passages was linked to increased mortality in elderly male mice. Furthermore, recent studies have found that estrogen is protective and that androgens aggravate fibrotic responses (Voltz et al., 2008).

In the present study, mice were given single $40\times10^6$ cells/kg IV, equivalent to the human dose of $2\times10^6$ /kg in IPF) via tail vein on day 24 post BLM induction AE-IPF (Reddy et al., 2016). Therapeutic options of BLM-induced IPF with single MSC therapy is now being investigated as a possible treatment option (Min et al., 2015; Gad et al., 2020). In a sheep model of severe ARDS, researchers observed that human MSCs reduced the severity of ALI (Asmussen et al., 2014). hUMSCs substantially increased the therapeutic benefits of linezolid on MRSA-induced pneumonia in a rabbit model, according to Kong et al. (Kong et al., 2019). MSCs may still have a therapeutic impact on ALI in species other than rodents, according to these findings. It's interesting that Zheng et al. published the findings of a single-center randomized, double-blind, placebo-controlled study in which 12 patients with moderate to severe ARDS were given either allogeneic
human AD-MSCs or saline placebo (Zheng et al., 2014). An open-label, dose-escalating phase 1 study was conducted with nine patients suffering from ARDS to assess the safety and efficacy of MSCs. Using three distinct MSC dosages (low dose: $1 \times 10^6$ cells/kg, intermediate dose: $5 \times 10^6$ cells/kg, and high dose: $10 \times 10^6$ cells/kg), patients were separated into three groups, each with three patients. Two of the nine individuals in the trial died within 60 days after receiving the study infusion. In the low-dose group, the first fatality occurred on day 9; on day 31, the second occurred. Each of the three groups had a decrease in lung damage scores, with the biggest decrease occurring in those given a high dosage (Wilson et al., 2015). MSCs are now being tested in clinical studies for the treatment of ALI in patients with severe COVID-19, and some improvement has been made in these trials. Sengupta et al. used exosomes generated from allogenic BMMSCs in a prospective non-randomized open-label cohort research. Efficacy and safety were assessed in 24 confirmed COVID-19 patients who received a single intravenous injection of 15 mL. The overall survival rate was 83%, with a recovery rate of 71% and a stability rate of 13% as well as a death rate of 16% unrelated to therapy (Sengupta et al., 2020).

Previous studies have proved the long-term survival of AD-MSCs engraftment in lung parenchyma (Schweitzer et al., 2011; Sun et al., 2011). This could be explained by the homing of AD-MSCs into the lung tissues and its appearance on day 52 at the end of the experiment as red fluorescent spots in both AD-MSCs and AD-MSCs +MP groups. As compared to the untreated mice, animals with BLM-induced AE-IPF survived longer when AD-MSCs were administered IV. This may have been due to a slow injection and precooling of AD-MSCs before administration (Kotani et al., 2017).

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS) are syndromes of acute respiratory failure, which was defined clinically by alveolar edema.
Alveolar airspace is filled with proteinaceous fluid and blood because the type I pneumocytes and the vascular endothelium are damaged. Hyaline membrane and interstitial edema, as well as alveolar hemorrhage, are other possible findings (Huang et al., 2018). Human illness and the laboratory workbench are linked by animal models. Matute-Bello et al. identified four major features of experimental animal models of ALI in the BLM-induced lung injury model (Matute-Bello et al., 2011) and applied in previous preclinical ALI studies (Matute-Bello et al., 2008). Histological evidence of tissue damage, alterations to the alveolar capillary barrier, an inflammatory response, and evidence of physiological dysfunction are the four "major characteristics" associated with ALI (Matute-Bello et al., 2011).

Wei et al. created an AE-IPF mouse model by administering a second intratracheal perfusion of BLM (4 mg/kg) to mice that had already acquired PF as a result of the first perfusion. This study demonstrated significant levels of PF, diffuse alveolar damage (DAD), and huge volumes of hyaline membrane in mice raised on the AE-IPF model (Wei et al., 2016). In the fibrotic lung, BLM is the best method for imitating ALI (Kondoh et al., 1993; Chen et al., 2017). Furthermore, because it closely reflects human fibrotic lung disease, intratracheal injection of a single BLM dosage in rats is a well-established, well recognized, and frequently utilized model of lung fibrosis (Williamson et al., 2015). A non-infectious model of AE-IPF was thus developed utilizing two doses of BLM.

In the present study, we choose day 52 (after 4 weeks of treatment) to verify outcomes according to Wei et al. (Wei et al., 2016) who reported that AE-IPF was induced by two BLM stimulations and examined the mouse model on day 24, 28, and 35 from the second dose. In addition, Ohkubo et al. (Ohkubo et al., 2014) who reported a case study for start of the use of the pirfenidone on day 23 after AE-IPF, they reported that chest CT showed improvement in the ground-glass opacities.
on day 49 (after 26 days from the start of the treatment) and on day 101 the patient was discharged. The effects of HUMSCs and anti-fibrotic drugs (pirfenidone and nintedanib) were also studied by Chu and colleagues in a model of PF in rats. These researchers compared the effects of pirfenidone/nintedanib administration and the intratracheal transplantation of $2.5 \times 10^7$ HUMSCs between days 2 and 4 after the injection of the BLM on day 21 (Chu et al., 2020).

Furthermore, our findings revealed that by day 52, following two doses of BLM, fibrosis in the lungs had considerably increased (Ashcroft score, ALI score, Hyp, percentage of collagen fibers deposition). Furthermore, we show that higher collagen levels in the lungs of mice given BLM correspond with H&E staining, Mallory trichrome staining, and increased positive CCL2 cells. These findings corroborate Wei and colleagues, who found that collagen levels rise sharply in the BLM-induced AE-IPF mice model. AE-IPF group had an increase in the number of inflammatory cells, congestion, and serious damage to alveoli (Wei et al., 2016). Previous studies using FITC and HV68 in mice generated primary fibrosis, which was followed by ALI clinical signs (McMillan et al., 2008). Our approach of constructing an AE-IPF model with two BLM injections is non-infectious, has fewer experimental settings, is easier to carry out, has higher repeatability, and is safer than McMillan's. An AE-inducing lipopolysaccharide was administered to mice seven days following BLM treatment (the inflammatory stage) (Kimura et al., 2015). To generate the ALI based on PF, a better mimic to AE-IPF in people, we picked day 21, the PF stage, for the second dosage based on Kimura's study. Another study used multiple intratracheal BLM injections. They reported that greater lung fibrosis appeared in multiple repeated injections more than standard (one dose) (Degryse et al., 2010). In addition, our findings revealed that TGF-β1 and TNF-α levels were considerably higher in the BLM-group compared to the control group. Bonifazi et al. findings are consistent with these results. They reported that MSCs were shown to be
significantly more likely to secrete cytokines directly linked to inflammation, such as TNF-α and TGF-β1 in fibrotic (IPF-MSCs) than in control (C-MSCs) which isolated from lung tissue (Bonifazi et al., 2020). Different characteristics of TGF-β1 play a key role in IPF. Pro-inflammatory cytokines, such as TNF-α and IL6, are also stimulated by TGF-β1 in order to maintain the fibrotic response, which is further facilitated by the stimulation of fibroblasts toward the formation of myofibroblasts (Fernandez and Eickelberg, 2012).

Although MP did not reverse the AE-IPF model in the present research, the histological examination, HYP, survival rate, Ashcroft, and ALI scores were all exacerbated as a result of the treatment. These data show that concurrent MP treatment cannot decrease BLM-induced AE-IPF. In bronchiolar epithelial cells, prednisolone has been shown to impair healing processes, which is in line with our own findings (Oku et al., 2008). Aytemur et al. also looked at the effects of IP iloprost treatment on BLM-induced PF and compared the effects of iloprost to MP. They found that iloprost decreased PF and that the fibrosis score in the iloprost group was lower than in the MP group (Aytemur et al., 2012).

Pirfenidone, a broad-spectrum anti-fibrotic drug, is effective in animal models of fibrosis and is now being tested in humans with AE-IPF (Furuya et al., 2017). The impact of PFD alone or in conjunction with MP on the AE-IPF model was assessed in this study, and it was compared to AD-MSCs. According to our findings, PFD significantly reduced BLM-induced increases in hydroxyproline, histological structures, and Ashcroft and ALI scores. These findings showed that PFD might reduce BLM-induced lung inflammation and fibrosis in mice while also improving resistance. These findings matched Oku et al. (Oku et al., 2008), who examined the anti-fibrotic effects of prednisolone and PFD in mice with BLM-induced lung damage. PFD also inhibits inflammatory cytokines such as TGF-β1 and basic fibroblast growth factor (b-FGF), which are
associated with the formation of fibrosis and have anti-inflammatory and anti-fibrotic properties (Inoue et al., 2002). TGF-β1 stimulates collagen expression and cell proliferation in a variety of cells, including fibroblasts (Mitani et al., 2008). Furthermore, Vaidya et al. suggested that PFD in conjunction with prednisolone might be used to treat BLM-induced lung injury (Vaidya et al., 2016). PFD in conjunction with corticosteroids appears to be the available therapeutic choice for patients with AE-IPF since it prevents diffuse alveolar damage and decreased serum level of Krebs von den Lungen-6 (KL-6) which originates from type 2 pneumocytes. In patients with interstitial lung disease, it was shown to be a helpful indication of disease activity (Song et al., 2011).

Early experimental findings only indicate AD-MSC treatment having a possibly beneficial effect on fibrotic lung illnesses during active inflammation when given early in the disease (Lee et al., 2014). Most individuals with respiratory symptoms, on the other hand, have already acquired PF to various degrees. Furthermore, recent research showed that administering AD-MSCs to fibrotic lungs may not be helpful (Uji et al., 2013) because most of the mice died after being infused with AD-MSCs at the fibrotic stage days 14, 17, and 20 after BLM (Reddy et al., 2016). When AD-MSCs were given two to three weeks after BLM instillation, they may have failed to migrate sufficiently into the fibrotic region to block growing interstitial fibrosis (Uji et al., 2013). Our attempt to infuse AD-MSC on day 24 (at the fibrotic stage) revealed anti-fibrotic properties in the AD-MSC and MP groups. Compared to the PFD treated group, AD-MSCs alone or in combination with MP produced a significant improvement in the histological examination with the restoration of alveolar architecture and reduction of excessive collagen deposition in the inter-alveolar septa and intensive downregulation of the Ashcroft and ALI scores. Quantitative imaging analysis of lung fibrosis sections stained with Mallory trichrome stain among the various research groups corroborated it. These findings are consistent with Chu et al. In their study, umbilical cord (UC)-
MSCs were given 21 days after the intratracheal instillation of BLM. The host macrophages were stimulated by UC-MSCs, which produced MMP-9 and degraded existing collagen (Chu et al., 2019). The results of late MSC implantation in a silica-induced fibrosis model were similarly encouraging (Zhang et al., 2018).

In contrast to the current study, Reddy et al. found that AD-MSCs are more effective than PFD in treating lung fibrosis, but only if they are given early in the process of active inflammation rather than after the fibrosis has developed (Reddy et al., 2016). Rathinasabapathy et al. developed IPF mice by airway administration of BLM to 8-week-old mice. Intravenously administering $1 \times 10^6$ AD-MSCs collected from 8–9-week-old mice and AD-MSCs conditioned medium 3 (early phase) or 7 (late phase) days after BLM administration and discovered that pulmonary fibrosis was inhibited. They reported that, while early MSC intervention may be more successful, MSC application at a later stage may be helpful, indicating that such methods to treat PF in AE-IPF are feasible (Rathinasabapathy et al., 2016). Our results demonstrated that, AD-MSCs after engraftment at the site of injury cause significant reduction in pro-inflammatory and pro-fibrotic markers, including TNF-$\alpha$ and hydroxyproline concentration. (this section is unclear and requires revision) Gazdhar and colleagues showed that AD-MSCs block the signaling pathway through hepatocyte growth factor and have the capacity to prevent TGF-$\beta 1$ induced fibroblast differentiation as well as disrupt its pro-fibrotic activity (Gazdhar et al., 2014).

In monocytes, macrophages, epithelial cells, and fibroblasts, the CCR2 receptor mediates the potent mononuclear cell chemoattractant CCL2 (Rose Jr et al., 2003). CCL2 may impact fibrosis directly through various methods, including its capacity to influence fibrocyte recruitment (Schupp et al., 2015). Macrophages, alveolar epithelial cells, and bronchiolar epithelial cells were all positive for CCL2 in the lungs of BLM-caused animals with acute respiratory distress syndrome.
A study by Inomata et al. demonstrated that fibrocytes from bone marrow migrate into BLM-induced fibrotic lesions in the lungs in response to chemokine production. Several chemokines are involved in the recruitment of fibrocytes, and CCL2 is one of them. Fibrosis is caused by the proliferation and differentiation of fibroblasts into myofibroblasts by circulating fibrocytes. They discovered that PFD reduces the fibrocyte pool in BLM-treated mice lungs by reducing CCL2 and CCL12 production (Inomata et al., 2014).

When AD-MSCs were administered to BLM-treated mice at the end of day 24, immunohistochemical analysis indicated a significant reduction in CCL2 in the mice lungs. Murine BLM damage model was used by Garcia et al. in order to induce parenchymal remodeling, increased collagen expression, and increased CCL2 synthesis, all of which are seen in human IPF. Next, they gave the cohorts intravenously injected mouse amniotic fluid stem cells (AFSCs) to test if AFSCs might decrease experimentally induced PF progression. During "acute" or "chronic" fibrotic remodeling events, AFSC therapy decreased changes in histology, collagen deposition, and pulmonary function, all of which are linked with pulmonary fibrosis development. It has been discovered by researchers that AFSC express CCR2, which has a strong affinity for fibrous regions in vivo and decreases elevated CCL2 levels in the BAL following BLM-induced lung damage (Garcia et al., 2013).

5. **Conclusion:**

In conclusion, in an animal model of AE-IPF, a regimen of PFD combined with corticosteroids may enhance survival and histological status. Our findings show that AD-MSCs home into the site of injury after systemic administration, engraft in the lung parenchyma, inhibit the production of
pro-inflammatory and pro-fibrotic mediators, and effectively alleviate the histological findings of PF in BLM-induced AE-IPF and on corticosteroid resistance models. Interestingly, our data show that AD-MSCs, alone or with corticosteroids, had a stronger effect on AE than PFD. Furthermore, our data indicate that AD-MSCs play a unique mechanistic role in regulating pro-inflammatory (TNF-α) and pro-fibrotic mediators (CCL2 and TGF-β1), resulting in the inhibition of both remodeling of the parenchyma and the development of PF. These findings shed light on the feasibility of using a new AD-MSCs-based therapy to target the CCL2 pathway in fibrotic lung diseases, as well as a therapeutic strategy that we believe warrants further investigation.

6. **Recommendation**

In the present study, we did not demonstrate data within the first 30 days. So, further studies are required to evaluate the histological and biochemical changes in the first 30 days to explain the earlier efficacy of AD-MSCs either alone or with MP. Also, future clinical research is needed to evaluate the efficacy of AD-MSCs with PFD in the prevention of AE-IPF in patients either alone or with MP. As such, we leave open the possibility that future studies may refute these recommendations.

7. **Acknowledgements**

The authors would like to thank Enago (www.enago.com) and True Language Edits (TLE) editing services for the English language review.
8. **Contributors**

The authors confirm contribution to the paper as follows: study conception and design: Dr. Heba Fikry, Dr. Sara Abdel Gawad, Dr. Lobna A. Saleh; data collection: Dr. Sara Abdel Gawad, Dr. Lobna A. Saleh; analysis and interpretation of results: Dr. Heba Fikry; manuscript draft preparation: Dr. Heba Fikry. All authors reviewed the results and approved the final version of the manuscript.

9. **Conflict of Interest**

The authors declare that they have no relevant or material financial interests that relate to the research described in this paper.

10. **Role of Funding Source**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

11. **References:**


Della Latta V., Cecchettini A., Del Ry S. and Morales M.A. (2015). Bleomycin in the setting of


Henry E., Cores J., Hensley M.T., Anthony S., Vandergriff A., de Andrade J.B.M., Allen T.,


Ohkubo H., Kunii E., Moriyama S. and Niimi A. (2014). Efficacy of combined corticosteroid and


Rose Jr C.E., SUNG S.J. and Fu S.M. (2003). Significant involvement of CCL2 (MCP-1) in


Cancer Res. Ther. 12, 1198.


Zhao M., Cui J., Cui Y., Li R., Tian Y., Song S., Zhang J. and Gao J. (2013). Therapeutic effect

Figure legends:

**Figure 1:** Phase contrast micrograph of AD-MSCs showing: most cells have granular cytoplasm and vesicular nuclei (A and B) (x200, scale bar 50 µm). (C and D) a positive cytoplasmic brownish immunological reaction for CD44 (C) and negative immunological reaction for CD34 the hematopoietic cell marker (D) streptavidin-biotin peroxidase (x200, scale bar 50 µm). (E) Representative histogram plots for Characterization of ADMSCs by flow cytometry analysis for CD45, CD 34, CD105, and CD90.

**Figure 2:** As varied-sized red fluorescence dots, PKH26 tagged stem cells (yellow arrow) in lung tissue of AD-MSCs group (A) and AD-MSCs +MP group (B) mice suggest homing of the AD-MSCs into the lung tissue.

**Figure 3:** (A) Change in average mouse body weight weekly. (B) changes in lung weight at the end of the experiment. Statistical analysis was carried out using One-Way ANOVA. ****p<0.0001 statistically significant from the corresponding control group. (C) Kaplan-Meyer survival curve after BLM exposure in male mice with log-rank (Mantel-Cox) test in all experimental groups.

**Figure 4:** Images of lung tissues (H&E x 10, 20, 40; scale bar 100, 50, 50 µm) showing:
Control group (A&B): Expanded alveoli (A) and alveolar sacs (s) separated by thin interalveolar septa (IAS) (↑), bronchioles (B), and a neighboring pulmonary blood vessel (V) are shown in the control group. Clara cells, with their dome-shaped apices, are found between the lining cells (dot arrow). The alveoli lumen is bordered by thin type I pneumocytes (yellow ↑) with flat nuclei and cuboidal type I pneumocytes (red ↑) with rounded nuclei.

BLM group (C&D): All the animals developed severe acute lung injury (ALI), thickened IAS (↑), infiltration of inflammatory cells (*), and clear deeply eosinophilic fibrinous exudate (E) and the necrotic debris of cells (Hyaline membrane). The bronchiolar lumen (B) becomes clogged with acidophilic exudate (yellow arrow) entangled with polymorph nuclear leucocytes (white arrow). Some alveoli (A) look to have collapsed, while others appear to be widening. There is deeply acidophilic cytoplasm with darkly stained nuclei (curved arrow) in the epithelial lining of the bronchiole (B), detached epithelial cells with pyknotic nuclei (green arrow), and extensive cellular infiltration (*) in the interstitial tissue around the bronchiole are seen.

MP group (E&F): The bronchiolar passage (B) shows vacuolated cells with eukaryotic nucleus (curved arrow) with extensive Hyaline membrane (E). Congested blood vessels (V), thickened IAS (yellow arrows), and extensive cellular infiltration (yellow *) around the bronchiole are seen. Most alveoli are obliterated, with some collapsing (A), while some alveoli appear to enlarge.

PFD (G&H): most of the bronchiolar (B) epithelial is normal compared to BLM and MP groups. Some areas show congested blood vessels (v) and some with inflammatory cell (*) in thickened IAS (↑). The alveoli are mostly similar to control.

AD-MSCs (I & J): The bronchioles (B) were seen intermixed with the alveoli similar to control. Few inflammatory cells (*). Bronchiole are lined by intact respiratory epithelium (curved arrow) and Clara cells (dot arrow). The alveoli are mostly lined with type I pneumocyte (yellow arrow) and pneumocytes type II (red arrow). Some areas show thickened IAS (↑).

PFD+ MP (L & N): Most of the IAS is thin, with just a few thickened (↑). Note patent alveoli (A). It was possible to detect residual hyaline membrane (E). Clara cells (dot arrow) and a few vacuolated cells with deep pyknotic nuclei (curved arrow) border the bronchioles (B) with few cellular infiltrations (*).

AD-MSCs + MP (M & N): Normal histological structure of the bronchioles (B) that are lined by intact simple columnar epithelium (curved arrows) and Clara cells (dot arrow). The majority of IAS are thin (↑). Notice the normal structure of alveolar sacs and patent alveoli (A).

Figure 5: Lung tissue stained with Mallory's trichrome-stain (x 20; bar= 50 µm): (A) Control: little collagen fiber (↑) deposition around alveoli (A) in the IAS, the bronchiole (B) and??? the walls of the blood vessels (V). (B) BLM: apparent increase in collagen fiber (↑) deposition. (C) MP: apparent extensive collagen fiber (↑) deposition. (D) PFD: apparent decrease of collagen fiber (↑) deposition as compared to BLM group. (E) AD-MSCs: little collagen fiber (↑) deposition as compared to BLM and MP groups. (F) PFD + MP: moderate collagen fiber (↑) deposition as
compared to BLM and MP groups. (G) **AD-MSCs +MP**: marked improvement in collagen fiber (↑) deposition, which appears similar to the control group.

**Figure 6:** (A) Morphometric analysis of collagen fibers. (B) Hydroxyproline content in the tissue homogenate. Scoring of (C) pulmonary fibrosis (Ashcroft score) and (D) acute lung injury (ALI) score. Data are presented as mean ± SEM of the number of animals (n)=6 in each group. Statistical analysis was carried out using One-Way ANOVA. ns: non-significant, **: statistically significant from the corresponding control group at p<0.01   ****: statistically significant from the corresponding control group at p<0.0001.

**Figure 7:** Alveolar cells (arrowhead), bronchiolar epithelial cells (↑), and macrophages (arrows) are seen in lung tissue stained with immunohistochemical analysis for chemokine (CC motif) ligand-2 (CCL2) (x 20; bar 50 µm): CCL2 immunoreactivity is negative in control (A). BLM (B): a positive brownish response that appears to be increasing. **MP(C):** a similar rise in positive brownish immunoreactive cells is seen in the MP group and the BLM group. Compared to the BLM group, there is an apparent reduction in positive brownish cells in the **PFD (D) group.** Compared to the BLM group, there is an evident reduction in positive brownish immunoreactive cells in **AD-MSCs (E).** When comparing the **PFD + MP (F) group** to the MP group, there is an apparent reduction in positive brownish immunoreactive cells. There are fewer positive brownish immunoreactive cells in the **AD-MSCs + MP (G) group** than in the MP group.

**Figure 8:** (A) The levels of Tumor Necrosis Factor Alpha (TNF-α) as an indicator of inflammation and (B) Transforming Growth Factor beta (TGF-β) in all experimental groups. Data are presented as mean ± SEM of the number of animals (n)=6 in each group. Statistical analysis was carried out using One-Way ANOVA. ****: statistically significant from the corresponding control group at p<0.0001. Pearson correlations between Hydroxyproline content (in the homogenate) and (C) the area percentage of collagen fibers, (D) TGF-β in mice, (E) body weight, and (F) lung weight in different groups.
**A**

Body weight (gm)

Weeks

- Control
- BLM group
- BLM + MP group
- BLM + PFD group
- BLM + AD-MSCs
- BLM+MP+ PFD
- BLM+MP+ AD-MSCs

**B**

Lung weight (gm)

- Control
- BLM group
- BLM + PFD group
- BLM + AD-MSCs
- BLM+MP+ PFD
- BLM+MP+ AD-MSCs

**C**

Probability of Survival %

Survival time (Days)

\[ \chi^2 = 15.16 \]

P = 0.019