# Role of bone marrow mesenchymal stem cells in the development of PQ-induced pulmonary fibrosis

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**Abstract.** Paraquat (PQ) poisoning-induced pulmonary fibrosis is one of the primary causes of mortality in patients with PQ poisoning. The potential mechanism of PQ-induced pulmonary fibrosis was thought to be mediated by inflammation. Recently, bone marrow-derived mesenchymal stem cells (BMSCs) have been considered as a potential strategy for the treatment of fibrotic disease due to their anti-inflammatory and immunosuppressive effects. In the present study, an increased accumulation of BMSCs in a mouse model of PQ-induced pulmonary fibrosis following their transplantation, markedly improving the survival rate of mice with PQ poisoning. In addition, the results indicated that BMSC transplantation may inhibit the production of pro-inflammatory cytokines, including tumor necrosis factor-α interleukin (IL)-1β, IL-6 and IL-10 in the lung tissues of PQ-poisoned mice, and ultimately attenuate the pulmonary fibrosis. *In vitro*, BMSCs may suppress PQ-induced epithelial-to-mesenchymal transition and protect pulmonary epithelial cells from PQ-induced apoptosis. These findings suggest that BMSC transplantation may be a promising treatment for pulmonary fibrosis induced by PQ poisoning.

## Introduction

Paraquat (PQ), a widely used herbicide, is highly toxic to humans and animals (1-3). Acute PQ poisoning by accidental or voluntary ingestion has toxic effects on various organs, including the liver, lungs, kidneys and central nervous system (4-7). Studies have reported that the lungs are the primary target organ in PQ poisoning, where PQ may accumulate and cause pulmonary edema, hemorrhage, interstitial inflammation and fibrosis (8,9). Approximately 90% of ingested PQ accumulates in the lung tissue within 4-6 h after poisoning, since pulmonary epithelial cells, particularly type I and II alveolar epithelial cells, may actively absorb PQ through an endogenous transport system. Moreover, the concentration of PQ in lung tissue may be 6-10 times higher than that in plasma (4). Accumulated PQ also damages the alveolar epithelium, followed by inflammatory cell stimulation, fibroblast activation, excessive accumulation and production of extracellular matrix (ECM) and, finally, progressive and inexorable pulmonary fibrogenesis (8). Therefore, the most common cause of PQ-induced mortality is respiratory failure from pulmonary fibrosis (10). However, the underlying mechanisms of PQ-induced pulmonary fibrosis remain unclear, and there are no effective drugs or measures for the treatment of PQ-poisoned patients.

Pulmonary fibrosis is an interstitial lung disease characterized by the accumulation of excess fibrous connective tissue and lung scarring (11). The most frequent histopathological patterns of pulmonary fibrosis is interstitial pneumonia (12,13). Clinically, steroids and immunosuppressants have been used for the treatment of lung inflammation and fibrosis (14-17). However, numerous cases are treatment-resistant and the outcomes are poor. Therefore, the development of new strategies for the therapy of pulmonary fibrosis is necessary.

Recently, research on mesenchymal stem cells (MSCs) derived from different tissues, including bone marrow, skeletal muscle, cord blood, placenta and adipose tissue, has been performed in the field of regenerative medicine research (18-20). Due to the multiple differentiation potential of MSCs, studies on MSC transplantation have been performed, aiming at potential clinical applications (21,22). Moreover, it was demonstrated that MSCs may modulate the immune response and adjust the microenvironment of the engraftment sites, improving their efficacy against inflammatory and autoimmune responses (18,23-25). Systemic administration of bone marrow-derived MSCs (BMSCs) may reduce bleomycin-induced inflammatory cell infiltration and lung fibrosis via the accumulation of MSCs in the pulmonary parenchyma and large airway (26-30). Therefore, direct delivery of BMSCs to the lung appears to constitute effective protection against PQ-induced pulmonary fibrosis.

In the present study, given the unique characteristics of BMSCs, the therapeutic effects and possible mechanism of intravenous BMSC transplantation on PQ-poisoned mice was assessed. Meanwhile, the accumulation and retention of BMSCs

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in lung tissues was evaluated. This procedure attenuated lung inflammation and cell apoptosis following PQ poisoning, indicating that BMSC transplantation may be a potential strategy for the treatment of PQ-induced pulmonary fibrosis.

## Materials and methods

*Ethics statement*. The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and all experimental protocols were approved under the animal protocol no. SYXK (Su) 2015-0019 by the Animal Care and Use Committee of Medical School of Nanjing University (Nanjing, China).

*Cell culture*. Mouse lung epithelial cells (MLE-12) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (both purchased from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). The cells were grown at 37˚C in a 5% $CO_2$ incubator, and were passaged following trypsinization.

*Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was extracted from mouse lung tissues and MLE-12 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RT-qPCR was used to confirm the expression levels of mRNAs. cDNA was produced according to the protocol for PrimeScript™ RT Reagent (Takara Bio, Inc., Otsu, Japan), at 37˚C for 15 min and 85˚C for 5 sec. RT-qPCR was performed as described in the method for the FastStart Universal SYBR Green Master mix (Roche Molecular Systems, Inc., Pleasanton, CA, USA) on a 7300 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Each sample was run in triplicate, and the data were analyzed with the 7300 Sequence Detection Software version 1.4.0.25 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers for mRNAs tested are listed in Table I. The thermocycling conditions were as follows: 1 cycle of initial denaturation (95˚C for 10 min); 40 cycles of denaturation (95˚C for 10 sec), annealing and elongation (60˚C for 30 sec). The Cq values were analyzed using the ΔΔCq method (31), and relative changes in mRNA levels were calculated by normalization to GAPDH relative to the control.

*Western blot analysis*. Cells and lung tissues were lysed in ice-cold radioimmunoprecipitation assay extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS) containing a protease inhibitor cocktail (Roche Molecular Systems, Inc.) for 30 min. The whole lysates were then centrifuged at 12,000 x g for 30 min at 4˚C and the protein concentration in the supernatant was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The protein samples were boiled for 10 min and 30 $\mu$g aliquots were then subjected to SDS-PAGE (12% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.) at 37˚C for 1 h, and incubated with the following primary antibodies at 4˚C overnight: Rabbit anti-α-SMA (at a 1:3,000 dilution; cat. no. ab5694), anti-E-cadherin (at a 1:1,000 dilution, cat. no. ab76055), rabbit anti-Vimentin (at a 1:5,000 dilution, cat. no. ab92547), and mouse anti-GAPDH (at a 1:3,000 dilution; cat. no. ab9485). All primary antibodies were purchased from Abcam, Cambridge, UK. Next, the membrane was further incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse immunoglobulin G (Boster Biological Technology, Pleasanton, CA, USA) at 37˚C for 1 h. GAPDH was used as the reference protein. Immunoreactive protein bands were visualized with an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA) and detected using an Odyssey Scanning System (LI-COR Biosciences, Lincoln, NE, USA). The expression levels were quantified with ImageJ (version 1.48, National Institutes of Health, Bethesda, MD, USA).

*PQ-induced pulmonary fibrosis model and BMSC recruitment detection*. The recombinant adenovirus vectors carrying a green fluorescent protein (GFP) reporter gene (Ad-GFP) were obtained from Cyagen Biosciences, Inc. (cat. no. mubmx-01101; Santa Clara, CA, USA). BMSCs, purchased from Cyagen Biosciences (cat. no. mubmx-01101), were treated with 100 $\mu$l Ad-GFP at a concentration of $1x10^9$ TU/ml for 16 h. Following this step, stem cell culture medium (cat. no. MUBMX-90011) containing adenoviral particles was replaced with fresh medium. After 48 h, BMSCs were prepared for transplantation.

C57BL/6 mice (male; 8 weeks old; 20 g) were randomly divided into four groups (n=10 for each group). Animals were maintained at 19-24˚C, 40-50% humidity, with a 12 h light/dark cycle and were fed a chow diet with free access to drinking water in the animal facilities of the Medical School of Nanjing University. Pulmonary fibrosis was induced by intraperitoneal administration of PQ at the concentration of 20 mg/kg. BMSCs ($2x10^6$ cells) were injected via the tail vein on day 7. Mice receiving the same volume of saline without BMSCs were used for control. The two controls of mice injected with saline and mice injected saline and transplanted with BMSCs-Ad-GFP cells. The two PQ experimental groups consisted of mice injected with PQ, and another injected with PQ and with BMSCs-Ad-GFP cells. The mice (n=6) were sacrificed at day 7 and 14 after PQ treatment, and the lungs were harvested for histological analysis.

*Histology*. Lung tissue was fixed overnight with 4% paraformaldehyde at 4˚C, dehydrated in an ascending alcohol gradient, embedded in paraffin and cut into 5 $\mu$m sections. The sections were stained with hematoxylin and eosin (H&E), and Masson's trichrome (Solarbio, Beijing, China) were performed according to standard procedures. Paraffin-embedded sections (5 $\mu$m) were deparaffinized with xylene (room temperature, twice for 5 min each) before being rehydrated in water using an ethanol gradient. For H&E staining, lung sections were stained with hematoxylin (1 min) and eosin (2 min) at room temperature. For Masson's trichrome staining, lung sections were stained with hematoxylin (1 min), Ponceau S (5 min) and Aniline blue (5 min) at room temperature. Pathological changes in the lungs were observed under a light microscope (Olympus

Table I. Reverse-transcription quantitative polymerase chain reaction primers and products.

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| α-SMA | CCCAGATTATGTTTGAGACCTTC | ATCTCCAGAGTCCAGCACAATAC |
| Vimentin | CCTGGAGTCACTTCCTCTGGTTG | TCTTGCTGGTACTGCACTGTTGC |
| Col1a1 | CTTCTGGTCCTCGTGGTCTCCCT | AAGCCTCGGTGTCCCTTCATTCC |
| Fibronectin | GCACCGACGAAGAGCCCTTACAG | GCACCATTCAGCGTTGCCCACAG |
| GAPDH | AGGTCGGTGTGAACGGATTTG | TGTAGACCATGTAGTTGAGGTCA |

α-SMA, α-smooth muscle actin; Col1a1, collagen type 1 α1 chain.

Corporation, Tokyo, Japan). To visualize the efficacy of BMSC transplantation, the lung sections were washed with PBS and stained with 1 $\mu$g/ml DAPI at 37˚C for 6 min (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The fluorescence images were captured using a confocal fluorescence microscope (Olympus Corporation).

*Hydroxyproline (HDP) content assay*. Lung tissue weighing 50 mg was homogenized with 1 ml $ddH_2O$, and mixed with 1 ml NaOH (10 N) in a pressure-tight, screw-capped polypropylene vial that was heated at 120˚C for 1 h. The supernatant was centrifuged at 10,000 x g for 10 min at 4˚C according to the instructions of the hydroxyproline assay kit (cat. no. K226; BioVision, Inc., Milpitas, CA, USA). The absorbance at 550 nm was measured by a spectrophotometer, and the HDP content of each lung tissue was calculated.

*ELISA*. Cytokines in bronchoalveolar lavage fluid (BALF) were detected by using ELISA kits for mouse tumor necrosis factor-α (TNF-α; cat. no. MTA00B), interleukin (IL)-1β (cat. no. MLB00C), IL-6 (cat. no. D6050) and IL-10 (cat. no. DY417; R&D Systems China Co., Ltd., Shanghai, China), according to the manufacturer's instructions (32). Briefly, a total of 100 $\mu$l BALF supernatant was added into a 96-well plate and incubated for 1 h. Then, 100 $\mu$l enzyme-linked antibodies were added for 30 min at 37˚C. Following three washes with washing buffer, the chromogenic reagent was added and incubated for 30 min. To terminate the reaction, 50 $\mu$l 2 M $H_2SO_4$ was added to each well. Absorbance was determined at 450 nm.

*Flow cytometry analysis*. To determine the numbers of BMSCs that translocated into the lung tissues, mouse lung parenchyma tissues were digested after cutting with a razor blade, as described previously (33). The digested material was filtered and depleted of red blood cells using Ammonium Chloride Potassium buffer (cat. no. A1049201; Thermo Fisher Scientific, Inc.). GFP positive cells were detected by flow cytometry and analyzed by FlowJo software version 7.6.1 (Tree Star, Inc., Ashland, OR, USA).

Cell apoptosis was measured by an Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. MLE-12 cells from different groups were collected following centrifugation at 1,000 x g at 4˚C for 5 min. Cells were washed twice with cold PBS and re-suspended in 500 $\mu$l binding buffer. Each cell sample was stained with 5 $\mu$l Annexin V-FITC and 5 $\mu$l PI, and incubated in the dark at 25˚C for 15 min. Flow cytometry was performed on a FACSCalibur™ flow cytometer (BD Biosciences, Franklin, Lakes, NJ, USA) and the data were analyzed with FlowJo software 7.6.1 (Tree Star, Inc., Ashland, OR, USA).

*Statistical analysis*. Statistical analysis was performed using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean ± standard deviation (n=3). Differences were analyzed with one-way analysis of variance, using a Tukey-Kramer t-test to perform multiple comparisons among different groups. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*PQ induces pulmonary fibrosis*. The development of pulmonary fibrosis in mice treated with 20 mg/kg PQ was assessed by H&E (Fig. 1A) and Masson's trichrome staining (Fig. 1B), and the HDP content assay for collagen (Fig. 1C). Alterations in lung structure are visible in Fig. 1A. Lung tissue sections from the control group exhibited no evidence of inflammation or epithelial damage. As expected, lung tissue sections from mice treated with PQ displayed extensive cellular thickening of the interalveolar septa, interstitial edema, inflammatory cell infiltration, increased interstitial cells with a fibroblastic appearance, and fibrogenesis. The results demonstrated that the alveolar structure was damaged, with extensive collagen deposition between days 7 and 14. The results of the Masson's trichrome staining and HDP content assay also indicated that PQ may have induced excessive collagen deposition in lung tissue. The expression of fibrosis-associated genes, including α-smooth muscle actin (α-SMA), collagen type 1 α1 chain (Col1a1), vimentin and fibronectin, was significantly increased following PQ administration compared with the control group (Fig. 1D).

*BMSCs were recruited to lung tissues*. In order to confirm whether BMSCs may be recruited to the lung tissues during the development of PQ-induced pulmonary fibrosis, BMSCs were transfected with adenovirus vectors carrying the GFP reporter gene (Ad-GFP). These cells were subsequently transplanted into mice via injection through the tail vein on day 7 following either saline or PQ injection. A number of GFP-positive BMSCs were observed in the lung tissues (Fig. 2A). Compared with the mice injected with saline, the number of BMSCs recruited

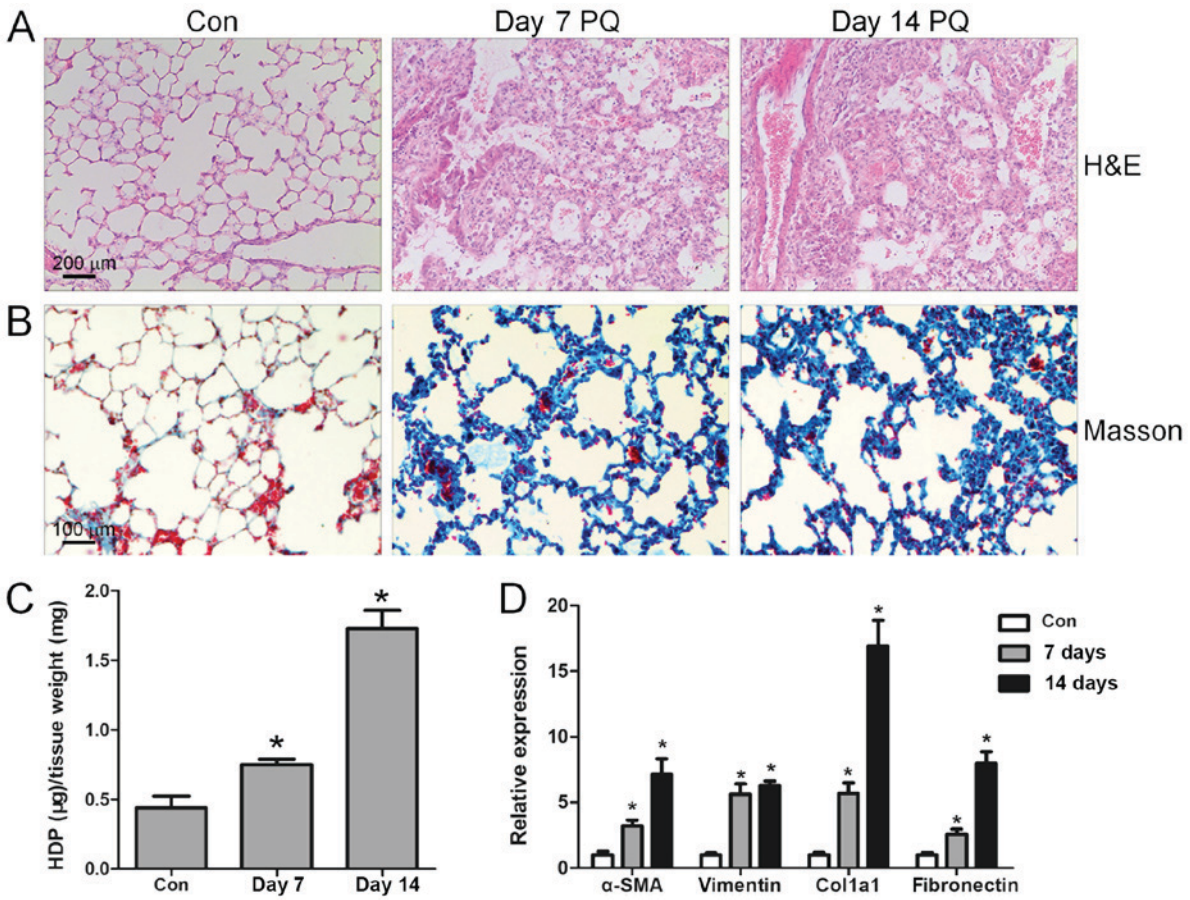


Figure 1. Pulmonary fibrosis was induced in PQ-treated mice. (A) Mice received either saline or PQ (25 mg/kg) intraperitoneally. Pulmonary fibrosis was determined by H&E staining. (B) Collagen deposition was revealed by Masson's trichrome staining. (C) A portion of lung isolated from animals in each group was hydrolyzed and analyzed for HDP content. (D) The expression of α-SMA, Col1a1, vimentin and fibronectin were assessed by reverse-transcription quantitative polymerase chain reaction. *P<0.05 vs. respective control group. PQ, paraquat; H&E, hematoxylin and eosin; HDP, hydroxyproline; Con, control; α-SMA, α-smooth muscle actin; Col1a1, collagen type 1 α1 chain.

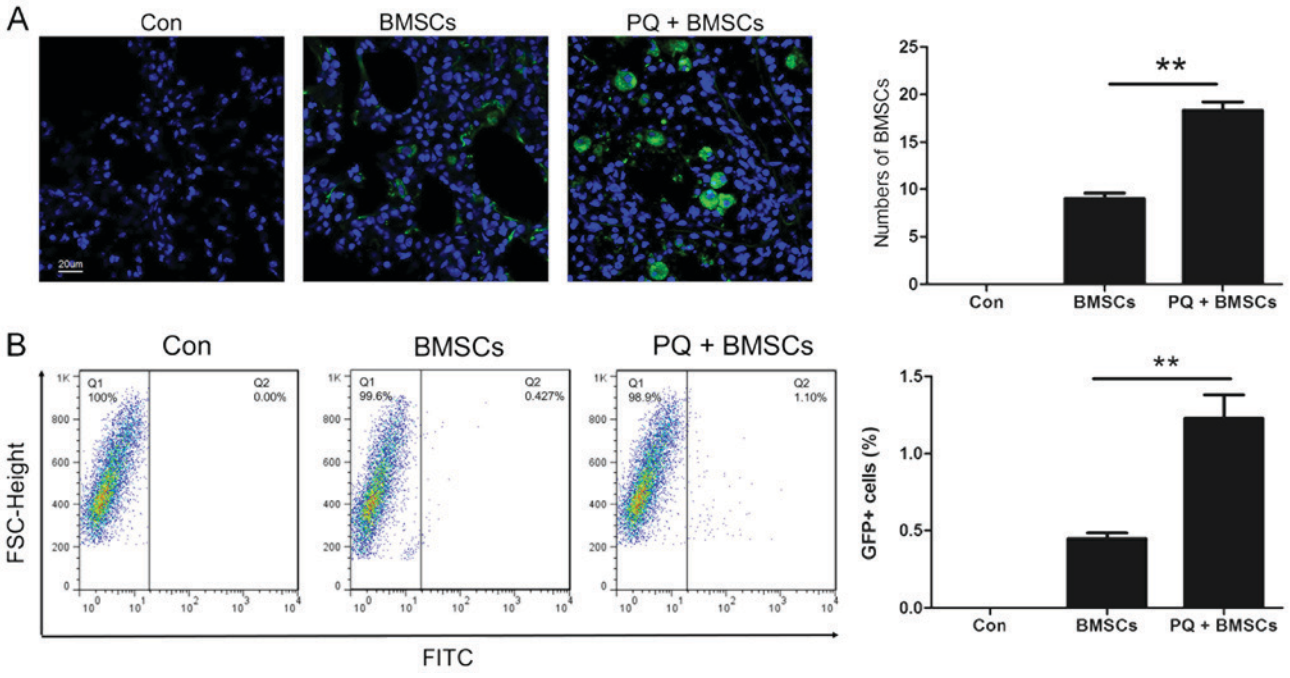


Figure 2. BMSCs were recruited to the lung tissues during treatment with PQ. (A) Engraftment of BMSCs transfected with adenovirus vectors carrying the GFP reporter gene in lung tissues was measured by immunofluorescence. (B) The number of GFP-positive cells in lung tissues was further analyzed via flow cytometry. **P<0.01. BMSCs, bone marrow-derived mesenchymal stem cells; PQ, paraquat; GFP, green fluorescent protein; Con, control; FITC, fluorescein isothiocyanate; FSC, forward scatter.

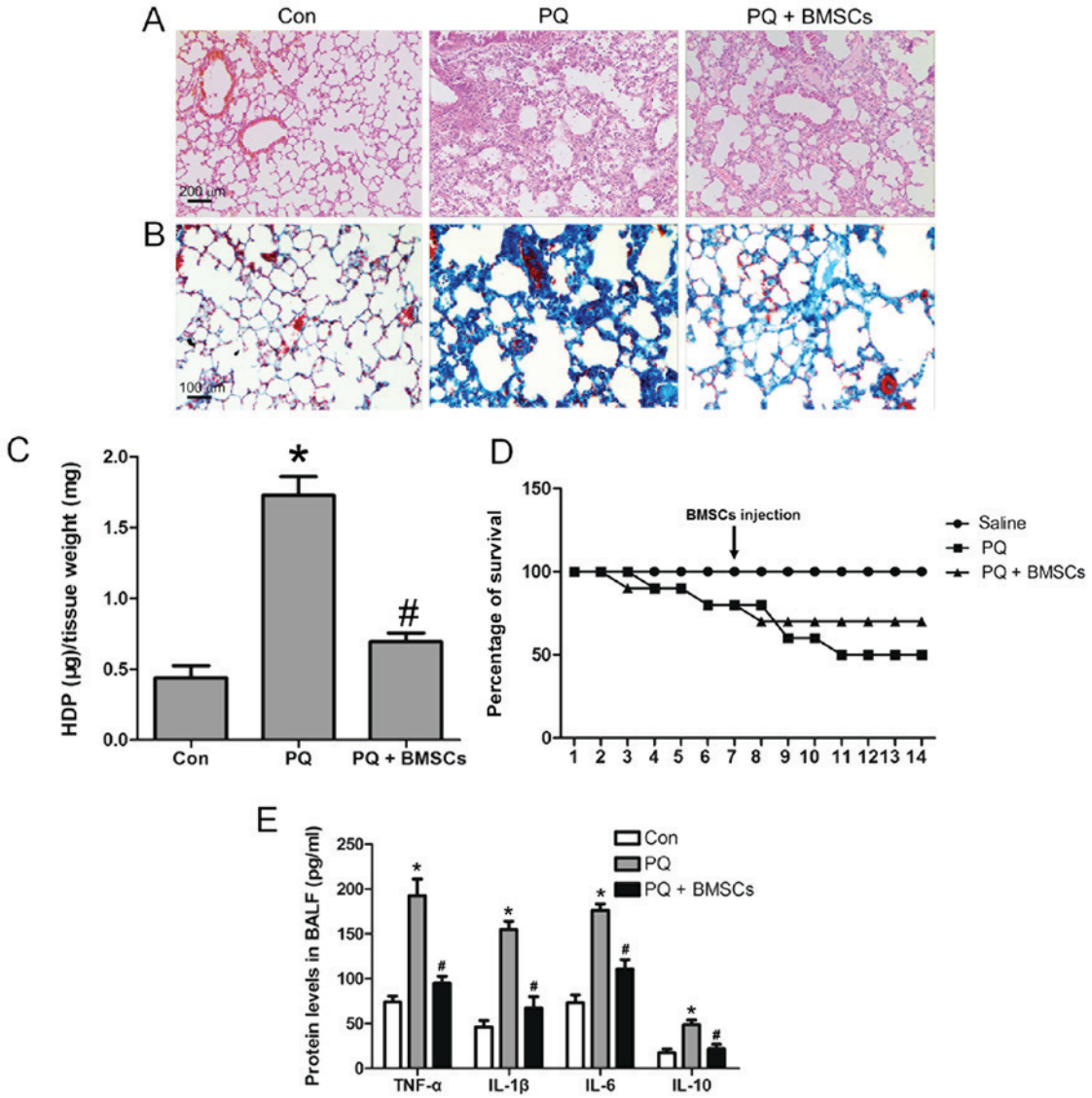


Figure 3. BMSC transplantation suppresses the development of pulmonary fibrosis in PQ-treated mice by decreasing lung inflammation. BMSCs were administered via the tail vein at day 7 after PQ treatment. (A) Pulmonary fibrosis lesions were determined by H&E staining. (B) Collagen deposition was revealed by Masson's trichrome staining. (C) A portion of lung isolated from animals in each group was hydrolyzed and analyzed for HDP content. (D) Survival of PQ-treated mice following treatment with BMSCs was recorded. (E) The expression of TNF-α, IL-1β, IL-6 and IL-10 in bronchoalveolar lavage fluid was measured by ELISA. *P<0.05 vs. respective control group; #P<0.05 vs. respective PQ treatment group. BMSCs, bone marrow-derived mesenchymal stem cells; PQ, paraquat; H&E, hematoxylin and eosin; HDP, hydroxyproline; Con, control; TNF-α, tumor necrosis factor-α; IL, interleukin.

in the lung tissues of PQ-poisoned mice was significantly elevated, indicating that PQ poisoning may have promoted the recruitment of BMSCs to injured lung tissues. This result was further confirmed by flow cytometry (Fig. 2B).

*BMSCs suppress PQ-induced pulmonary fibrosis by reducing lung inflammation*. In order to determine the potential therapeutic effects of BMSCs on PQ-induced pulmonary fibrosis, this model was reproduced and it was demonstrated that BMSC transplantation significantly reduced the severity of pulmonary fibrosis, as assessed by H&E, Masson staining and HDP content assay (Fig. 3A-C). BMSC injection may have effectively increased the survival rate of mice poisoned with PQ (Fig. 3D). In addition, it was observed that BMSC treatment may have attenuated inflammatory responses by decreasing the expression of pro-inflammatory factors, including TNF-α, IL-1β, IL-6 and IL-10 (Fig. 3E). These results suggested that BMSCs may prevent PQ-induced pulmonary fibrosis through inflammatory response suppression.

*BMSCs protect pulmonary epithelial cells from PQ-induced epithelial-mesenchymal transition (EMT) and apoptosis*. It has been reported that EMT of pulmonary epithelial cells serves a critical role in the development of pulmonary fibrosis (34). In the present study, MLE-12 cells were co-cultured with or without BMSCs, followed by PQ treatment. As demonstrated in Fig. 4A, PQ may have induced the expression of α-SMA and vimentin, which was decreased when MLE-12 cells were co-cultured with BMSCs. Conversely, the epithelial marker E-cadherin was decreased with PQ treatment, which was increased when MLE-12 cells were co-cultured with BMSCs. In addition, co-culture of BMSCs with MLE-12 cells may have suppressed PQ-induced epithelial cell apoptosis (Fig. 4B). These results indicated that the therapeutic effects of BMSCs

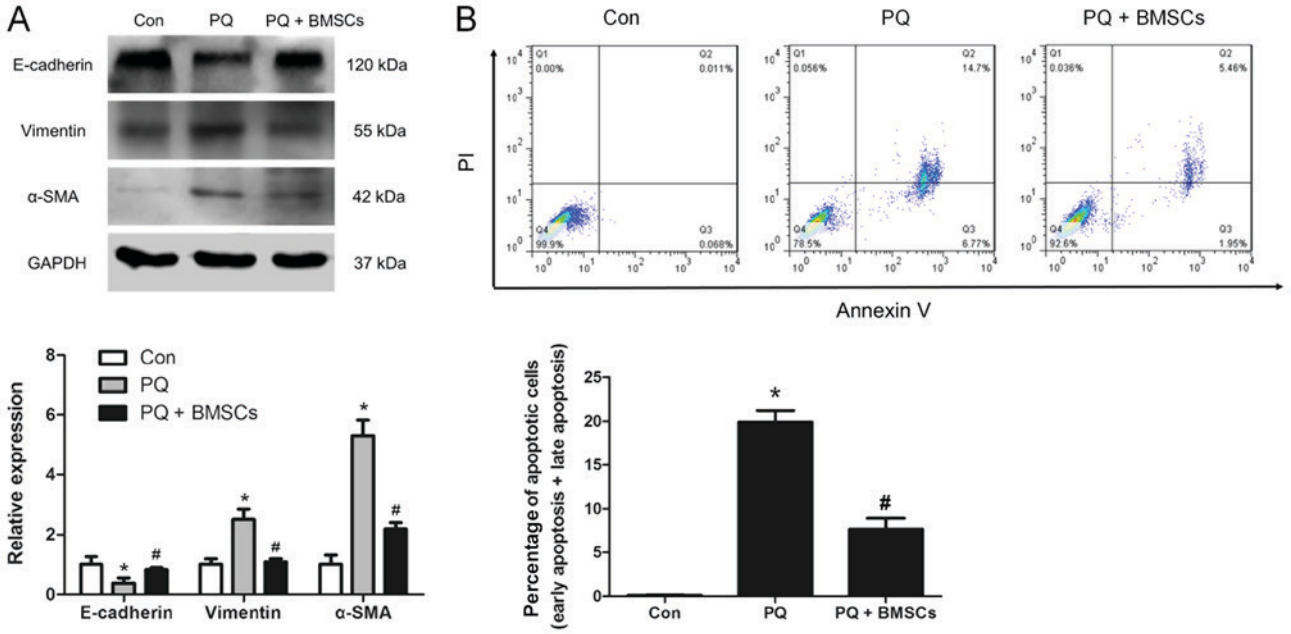


Figure 4. BMSCs impacted PQ-induced epithelial-mesenchymal transition and apoptosis of pulmonary epithelial cells. BMSCs were co-cultured with MLE-12 cells in the presence of PQ. (A) The expression of α-SMA, Vimentin and E-cadherin were measured by western blot. The expression levels were quantified with ImageJ (right panels; n=3). (B) Cell apoptosis was evaluated by flow cytometry by measuring Annexin V and PI expression. *P<0.05 vs. respective control group; #P<0.05 vs. respective PQ treatment group. BMSCs, bone marrow-derived mesenchymal stem cells; PQ, paraquat; MLE-12, mouse lung epithelial cells; α-SMA, α-smooth muscle actin; PI, propidium iodide.

on PQ-induced pulmonary fibrosis may be mediated by inhibition of apoptosis and the EMT process of epithelial cells.

## Discussion

PQ is an herbicide with high toxicity in human lung tissues, and causes mortality by inducing pulmonary fibrosis. It has been reported that the clinical course of PQ-induced pulmonary fibrosis may be divided into three phases: The early inflammatory phase, the proliferative phase characterized by fibroblast proliferation, and the phase of collagen deposition (35,36). Among these phases, the inflammatory response serves a critical role in the pathogenesis of pulmonary fibrosis. A large number of inflammatory cytokines, particularly TNF-α, IL-1β, IL-6, transforming growth factor β1 (TGF-β1) and platelet-derived growth factor (PDGF) are known to be associated with the initiation and development of pulmonary fibrosis (37,38). TGF-β1, IL-1β and TNF-α may inhibit lung injury repair, promote lung cell apoptosis and finally induce pulmonary fibrosis (38,39). Reduced pulmonary inflammation may therefore contribute to the recovery from lung injury (40,41).

Optimal therapies for pulmonary fibrosis remain controversial. There is no effective agent that improves the survival of patients with pulmonary fibrosis (42). To date, lung transplantation is the only effective treatment for pulmonary fibrosis (43). However, lung transplantation also has its own disadvantages, namely the shortage of organs and complications associated with long-term immunosuppression (44). Therefore, exploring new effective therapies to suppress or reverse pulmonary fibrosis is essential for decreasing the morbidity and mortality of patients with pulmonary fibrosis. Recently, it was reported that cells derived from bone marrow, particularly MSCs, may repopulate the lung and repair the injured lung tissues, which is a promising treatment for lung injury (45,46). MSCs are multipotent cells capable of differentiating into different cell lines, and display anti-proliferative, immunomodulatory and anti-inflammatory effects (47). The protective roles of BMSCs have been attributed to different mechanisms. Tracking of labeled cells has demonstrated that MSCs localize primarily to the lung following intravenous administration (48), engrafting into the injured lung and trans-differentiating into lung epithelium (26,49). In the present study, it was demonstrated that BMSCs may engraft into injured lung tissue and reduce the lung epithelium damage, which is beneficial for lung repair following PQ exposure. Although the precise mechanism of action of MSCs remains elusive (25), in a number of clinical trials (50,51) it was demonstrated that MSCs may have the ability to produce paracrine factors that may mitigate tissue damage (52). The paracrine factors released by MSCs may alter the microenvironment and contribute to a repairing effect in pulmonary fibrosis (50). The present results demonstrated that BMSCs may have attenuated lung injury by inhibiting the production of inflammatory cytokines in lung tissue, and also by reducing lung epithelial cells apoptosis *in vitro*. Therefore, these findings illustrate that BMSC-based therapies for PQ-induced lung injury may be clinically relevant, as these cells target inflammatory pathways and reduce epithelium damage.

In conclusion, the present observations provided evidence that intravenous BMSC transplantation elevated the survival rate and suppressed the development of PQ-induced pulmonary fibrosis, by accumulating in the lung interstitium and reducing pulmonary inflammation. BMSCs retarded the secretion of pro-inflammatory cytokines, namely TNF-α and

IL-1β, in PQ-poisoned lung tissues, and protected pulmonary epithelial cells from PQ-induced apoptosis. Taken together, the present study provides a novel strategy for the treatment of PQ-induced pulmonary fibrosis.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

JC performed the majority of the experiments; LS and LZ provided the statistical analysis, interpretation of data and contributed to some experiments. JC and YD contributed to the manuscript drafting and revision, designed the study and analyzed the data. All the authors contributed to the manuscript preparation and gave final approval of the submitted manuscript.

**Ethics approval and consent to participate**

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and all experimental protocols were approved under the animal protocol number SYXK (Su) 2015-0019 by the Animal Care and Use Committee of Medical School of Nanjing University (Nanjing, China).

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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