

Bone Marrow Mesenchymal Stem Cells Alleviated Bleomycin-Induced Pulmonary Fibrosis in Mice

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Abstract: *Objective:* The purpose of this study was to investigate the effect of bone marrow mesenchymal stem cells (BMSCs) on bleomycin-induced pulmonary fibrosis in mice. *Methods:* Pulmonary fibrosis model in mice was established by bleomycin (BLM) induction. This study was divided into 7 groups, bone marrow mesenchymal stem cells (BMSCs) as the treatment measure in 4 groups, the saline and pirfenidone as other 2 groups. The body weight of mice after BLM modeling was measured. The content of hydroxyproline (HYP) and collagen 1 (COL1) in lung tissue were determined by kits. Pathological changes of lung tissue were observed by hematoxylin-eosin (HE) staining. The levels of cytokines in serum and lung tissue of mice were detected by Enzyme-Linked Immunosorbent Assay (ELISA) kits. Immunohistochemistry was used to detect the expression of collagen-1 and α -SMA protein in lung tissue of mice. The levels of TGF- β /smad-3 and NLRP3/NF- κ B signal pathway in lung was detected by western blotting. *Results:* BMSCs significantly decreased the content of HYP and COL1 in lung tissue of mice. BMSCs significantly decreased cytokines in serum and lung tissue. Immunohistochemistry results shown BMSCs significantly decreased the levels of collagen-1 and α -SMA in lung tissue. In addition, BMSCs significantly inhibited TGF- β /smad-3 and NLRP3/NF- κ B signal pathway in lung tissue. *Conclusions:* BMSCs effectively inhibited bleomycin-induced pulmonary fibrosis in mice, and its mechanism may be related to inhibiting the activation of TGF- β /smad-3 and NLRP3/NF- κ B signal pathway.

Keywords: BMSCs, Bleomycin, TGF- β /smad-3, NLRP3/NF- κ B

1. Introduction

Pulmonary fibrosis is a common group of destructive diseases of lung tissue caused by various etiologies [1]. It is characterized by disordered arrangement of airway and alveolar structure and massive deposition of collagen. At present, there is no ideal treatment for pulmonary fibrosis [2, 3]. At present, lung transplantation is the most effective treatment for lung fiber [4, 5]. Therefore, it has become more urgent to develop new treatment strategies. Clinical anticancer agents including bleomycin (BLM) caused pulmonary fibrosis, so BLM was often used to produce inflammatory response and oxidative stress in pulmonary fibrotic lesions in mice or rat models. Inflammation is one of the most critical aspects of host defense in BLM-induced dysfunction. Excessive levels of inflammatory mediators (including tumor necrosis factor- α , interleukin-1 β and

interleukin-6) caused lung fibrosis. It was also proposed that BLM challenged the promotion of fibroblast proliferation by releasing TGF- β [6]. TGF- β binds to receptors on the cell membrane and activates the Smad complex during fibrosis [7]. The matrix metalloproteinase family is related to the degradation of basement membrane and the peroxidation of matrix lipids caused by oxidants [8].

Bone marrow mesenchymal stem cells (BMSCs) can be isolated from the marrow of bones in large numbers. During the few past decades, the bone marrow mesenchymal stem cells (BMSCs)-based cell therapy has been considered as an innovative treatment strategy. The BMSCs was a population of multipotent stem cells which induce tissue regeneration. It was reported that BMSCs transplantation exhibited anti-fibrotic property in lung injury [9, 10]. However, the underlying pathogenesis by which BMSCs treatment exerted protective effect on BLM-induced pulmonary fibrosis remains not fully understood. Therefore, the present study was

conducted to further explore its potential mechanism in BLM-induced pulmonary fibrosis in mice.

2. Materials and Methods

2.1. Chemicals and Regents

Pifeninone (PFD) was purchased from Biotech Co., Ltd. (batch number: BP0918). Hydroxyproline (HYP) was purchased from Nanjing Jiancheng Institute of Bioengineering (batch number: SNM168), bleomycin sulfate was purchased from source leaf biology (batch number: S17100-10mg), and ELISA of IL-1 β , IL-6 and TNF- α kits were purchased from Elabscience Company (batch number: Ex201905). TGF- β (#3709), p-smad-3 (#9520), smad-3 (#9523), NLRP3 (#15101), ASC (#13833), IL- β (#12703), NF- κ Bp65 (#8801) and GAPDH (#2118) were purchased from Cell Signaling Technology Company.

2.2. Experimental Animal

140 male mice (8 weeks old, 18-22g) were purchased from Cavens Experimental Animal Co., Ltd., with the certificate number of 202011741 and the license number of SCXK (Su) 02016-0010. All animal experiments were conducted in accordance with the "Guidelines for Animal Operation in Pharmacological Experiments".

2.3. Experimental Method

Mice was injected with avertin at a dose of 20 mg/ml to anesthetize the mice. Except the blank group mice, bleomycin (BLM at a dose of 3mg/kg, injection volume of 50ul) was used in other groups. After that, the survival and respiratory reaction of mice were observed daily. On the 14th day after BLM injection, the model mice were divided into six groups: saline group (NS), 14d low dose stem cell treatment group (BMSCs +14d^{low}), 14d high dose stem cell treatment group (BMSCs +14d^{high}), 21d low dose stem cell treatment group (BMSCs +21d^{low}), 21d high dose stem cell treatment group (BMSCs + 21d^{high}) and pirfenidone group (PFD, 400 mg/kg/d). The whole treatment experiment cycle is 21 days. Experimental groups and experimental process were seen in Table and Figure 1.

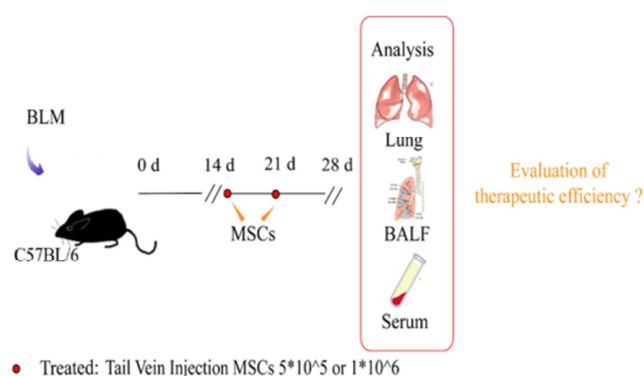


Figure 1. Laboratory Animal Grouping and Drug Treatment Description.

Table 1. Laboratory Animal Grouping and Drug Treatment Description.

Group	Number	Intervene
Normal	19	-
NS	17	BLE
BMSCs ^{low} +14d	18	BMSCs 5*10 ⁵
BMSCs ^{high} +14d	17	BMSCs 1*10 ⁶
BMSCs ^{low} +21d	16	BMSCs 5*10 ⁵
BMSCs ^{high} +21d	17	BMSCs 1*10 ⁶
PFD	16	400 mg/kg/d

2.4. Extraction of Bmscs

Mice BMSCs with multiple differentiation potentials were obtained by whole bone marrow attachment and gradient density centrifugation, and cell surface antigen molecule identification by flow cytometry analysis.

2.5. Hematoxylin-Eosin (HE) Staining

Lung tissues were taken out for histological evaluation, and the lungs were fixed in 10% neutral buffered formalin for 48 hours. In short, lung tissue was dehydrated in graded alcohol and dewaxed with xylene. After paraffin embedding, the sections were stained with hematoxylin and eosin. Histopathological observation was carried out by two pathologists under an optical microscope.

2.6. Measurement of Body Weight

All experimental mice were measured at set time points in each groups with experimental scales being killed.

Measurements of inflammatory cytokine in serum and lung.

IL-1 β , IL-6 and TNF- α levels in serum and lung were measured by ELISA kits according to the manufacturer's instructions.

2.7. Hyp and Col1 Detection

Lung HYP and COL1 levels were measured using the ELISA kits according to the manufacturer's instructions.

2.8. Western Bolt Analysis

TGF- β /smad-3 and NLRP3/NF- κ B signal pathway were measured by western bolt. Lung tissue was homogenized, washed with PBS, and lysed in lysis buffer for half an hour to extract total protein. The concentration of lung protein was quantified by BCA protein detection kit. The extracted lung protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. They were sealed with 5% skimmed milk powder for 2 h, and then incubated with corresponding primary antibody (mouse anti-TGF- β , p-smad-3 and mouse anti-NLRP3, ASC, IL- β , Caspase-1 and p-NF- κ B) at 4°C overnight. The strips were washed and incubated with the corresponding horseradish peroxidase-bound secondary antibody at room temperature for 2 hours. Protein content was normalized using GAPDH monoclonal antibody as an internal control for each data point. The image was scanned with a GS800 Densitometer Scanner, and absorbance values were analyzed using PDQuest 9.2.0

software. The strips were developed by ECL KeyGEN test kit and Tianneng exposure instrument.

2.9. Statistical Analysis

SPSS23.0 statistical software was used to analyze the data, and the measurement data were expressed by mean values \pm SDs, T test was used for comparison between the two groups, and nonparametric test was used for nerve injury score analysis. The difference was statistically significant with $p < 0.05$.

3. Results

3.1. Death of Animals in Each Group

Up to the 28th day of modeling, that is, at the end of the experiment, 20/140 animals died in the model group. One died in Normal control group, 3 in NS group, 2 in BMSCs low+14d, 3 in BMSCs high+14d, 4 in BMSCs low+21d, 3 in BMSCs high+21d and 4 in pirfenidone group (Table 2).

Table 2. Number of Dead Animals.

Group	Number of deaths
Normal	1
NS	3
BMSCs ^{low} +14d	2
BMSCs ^{high} +14d	3
BMSCs ^{low} +21d	4
BMSCs ^{high} +21d	3
PFD	4

3.2. Experimental Animal Observation

In the stage of pulmonary fibrosis modeling after administration of BLM, animals showed the phenomena of decreased activity, hair standing, body curled up, and many animals reunited, and at the same time, their diet and drinking water decreased. The weight of mice in each group increased slowly, especially in the model group.

3.3. Effect of BMSCs on Body Weight of Fibrotic Mice

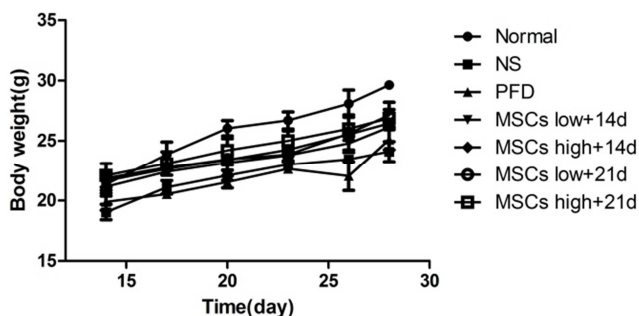


Figure 2. Effect of BMSCs on Body Weight of Fibrotic Mice.

Values are expressed as means \pm SDs. Compared with Normal: # $P < 0.05$, ## $P < 0.01$; Compared with NS: * $P < 0.05$, ** $P < 0.01$

On the whole, the body weight of mice after BLM modeling was lower than Normal, and compared with normal saline (NS), the body weight of mice treated with stem cells increased slowly before and after treatment. However, the

weight gain of mice treated with PFD for 28 days was decreased (Figure 2).

3.4. Effect of Bmscs on Hyp Content in Lung Tissue

Compared with NS group, the content of HYP in BMSCs +14d^{low} did not decrease, while the content of HYP in BMSCs +14d^{high} was decreased. The content of HYP in lung of BMSCs +14d^{low} and BMSCs +21d^{high} group were decreased (Figure 3).

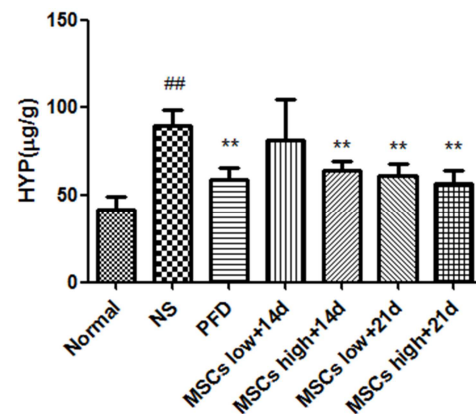


Figure 3. Effect of BMSCs on HYP Content in Lung Tissue of Fibrotic Mice.

Values are expressed as means \pm SDs. Compared with Normal: # $P < 0.05$, ## $P < 0.01$; Compared with NS: * $P < 0.05$, ** $P < 0.01$

3.5. Effect of Bmscs on Col1 Content in Lung Tissue of Fibrotic Mice

Compared with NS group, the content of COL1 in BMSCs +14d^{low} did not decrease, while the content of COL1 in BMSCs +14d^{high} was decreased. The content of COL1 in lung of BMSCs +14d^{low} and BMSCs +21d^{high} groups were decreased (Figure 4).

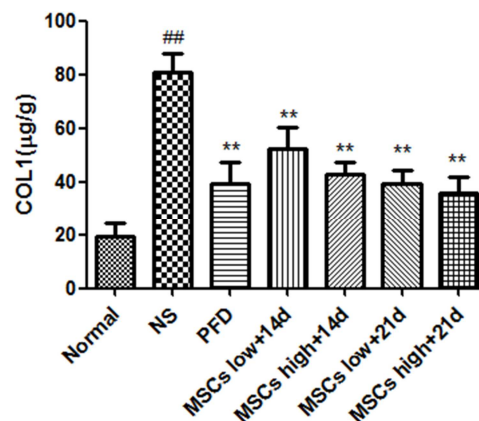


Figure 4. Effect of BMSCs on COL1 Content in Lung Tissue of Fibrotic Mice.

Values are expressed as means \pm SDs. Compared with Normal: # $P < 0.05$, ## $P < 0.01$; Compared with NS: * $P < 0.05$, ** $P < 0.01$

3.6. Effect of Bmscs on Serum Cytokine Content in Mice with Fibrosis

Compared with Normal group, the levels of IL-1 β , IL-6 and

TNF- α in serum of NS group were increased. The levels of IL-1 β , IL-6 and TNF- α in serum of BMSCs +14d^{low} and BMSCs +14d^{high} groups were not decrease. The levels of IL-1 β , IL-6 and TNF- α in serum of BMSCs +14d^{low} and BMSCs +21d^{high} were decreased (Figure 5).

3.7. Effect of Bmscs on Cytokine Content in Lung Tissue of Mice with Fibrosis

Compared with Normal group, the levels of IL-1 β , IL-6 and TNF- α in lung tissue of NS group were increased. The levels of IL-1 β , IL-6 and TNF- α in lung tissue of BMSCs +14d^{low} and BMSCs +14d^{high} groups were not decrease. The levels of IL-1 β , IL-6 and TNF- α in lung tissue of BMSCs +14d^{low} and BMSCs +21d^{high} were decreased (Figure 6).

3.8. Effect of Bmscs on Lung Histopathology in Mice with Fibrosis

Compared with Normal, the pathological results of lung tissue in NS group showed dilatation of lumen, dysplasia of epithelium, deposition of collagen fibers and increase of lymphocyte infiltration. In BMSCs and PFD treatment group, collagen fiber deposition in lung tissue decreased and lymphocyte infiltration decreased (Figure 7).

Effect of BMSCs on immunohistochemistry of collagen-1 and α -SMA in lung tissue of mice with fibrosis.

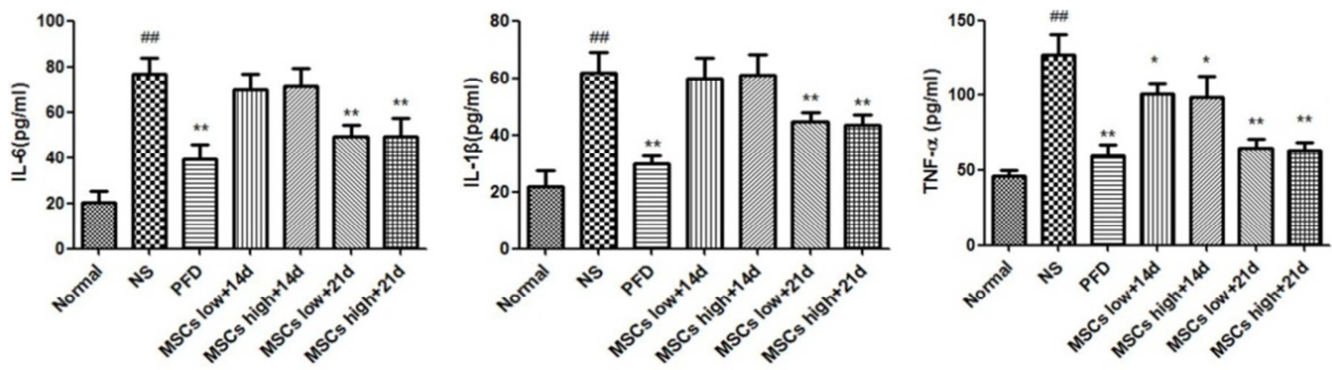
The contents of collagen-1 and α -SMA were analyzed by immunohistochemistry. Compared with Normal, the expression of collagen-1 and α -SMA in NS group were significantly increased, but decreased after BMSCs and PFD treatment (Figure 8).

3.9. Effect of Bmscs on Tgf-B/Smad-3 Signal Pathway in Lung Tissue

The levels of TGF- β and p-smad-3 in lung tissue were analyzed by western blot. Compared with Normal, the expressions of fibrosis-related proteins TGF- β and p-smad-3 significantly increased in NS, but decreased after BMSCs and PFD treatment (Figure 9).

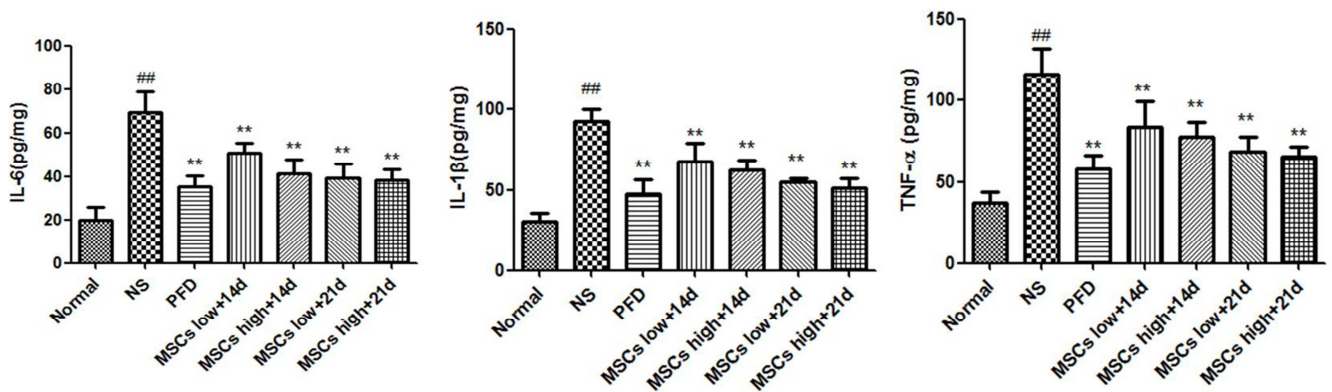
3.10. Effect of Bmscs on Nlrp3/Nf-Kb Signal in Lung Tissue

The levels of NLRP3, ASC, IL- β , Caspase-1 and p-NF- κ B were analyzed by western blot. Compared with Normal group, the expression of fibrosis-related proteins NLRP3, ASC, IL- β , Caspase-1 and p-NF- κ B increased significantly in NS group, but decreased after BMSCs and PFD treatment (Figure 10).



Values are expressed as means \pm SDs. Compared with Normal: #P<0.05, ##P<0.01; Compared with NS: *P<0.05, **P<0.01

Figure 5. Effect of BMSCs on Serum Cytokine Content in mice with Fibrosis.



Values are expressed as means \pm SDs. Compared with Normal: #P<0.05, ##P<0.01; Compared with NS: *P<0.05, **P<0.01

Figure 6. Effect of BMSCs on Cytokine Content in lung Tissue of Mice with Fibrosis.

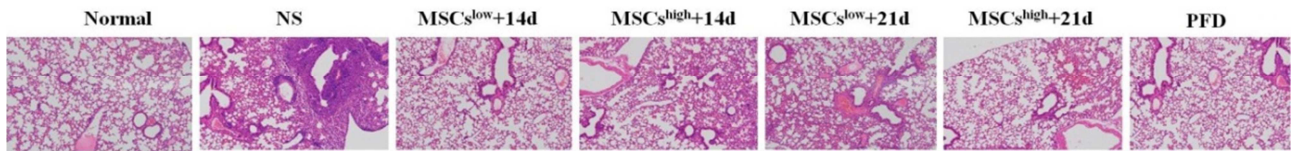


Figure 7. Effect of BMSCs on Lung Histopathology in Mice with Fibrosis (x200).

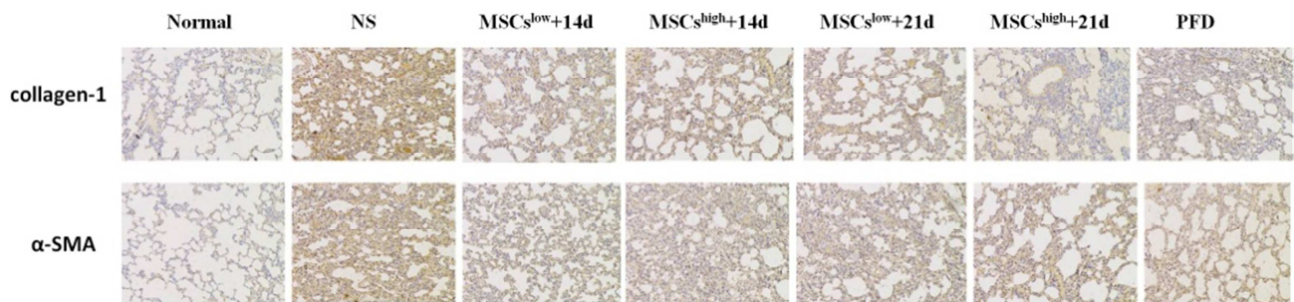


Figure 8. Effect of BMSCs on Immunohistochemistry of Collagen-1 and α -SMA in lung Tissue of Rat with Fibrosis (x200).

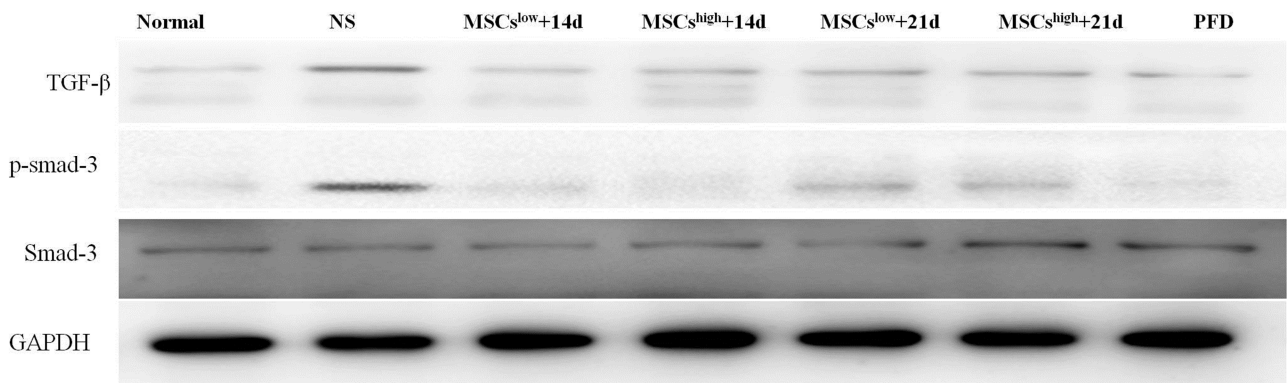


Figure 9. Effect of BMSCs on TGF- β /smad-3 Signal Pathway in lung tissue of Fibrosis Rat.

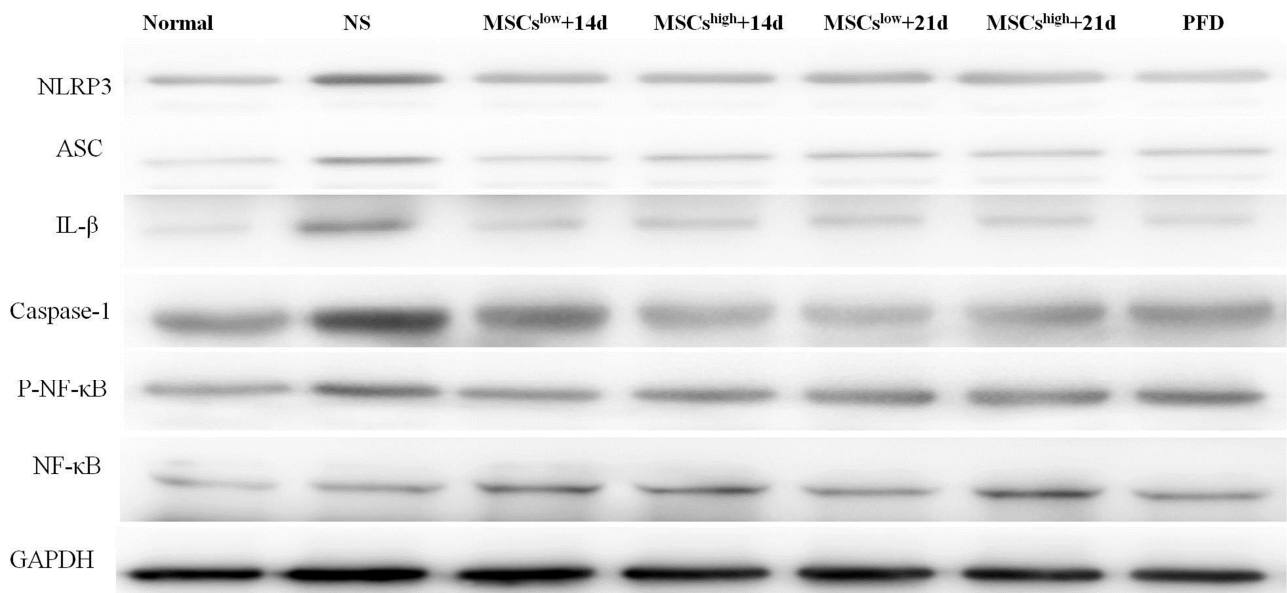


Figure 10. Effect of BMSCs on NLRP3/NF- κ B signal in lung Tissue of Fibrosis Rat.

4. Discussion

Pulmonary fibrosis (PF) is a chronic respiratory disease,

which has a low cure rate, seriously damages the respiratory function of the lungs and eventually dies due to respiratory failure. Clinically, the drugs used to treat PF mainly include immunosuppressants, corticosteroids and new drugs such as

nidanib and pirfenidone. However, due to its complicated etiology, the pathogenesis was still unclear. Therefore, finding new drugs to effectively treat PF has become a hot and difficult point at present.

The pathogenesis of fibrosis has not yet been fully elucidated, but the inflammatory reaction has always dominated [11]. In the early stage of pulmonary fibrosis, persistent abnormal stimuli lead to inflammatory reactions in the alveoli. BMSCs have a tendency towards inflammation or injury sites and exhibit strong migration ability, homing to damaged lung tissue [12-15]. Chen *et al.* found that BMSCs transplantation can inhibit tumor necrosis factor in lung tissue of paraquat poisoned mice (α (TNF- α), Interleukin-1 β (IL-1 β), The production of pro-inflammatory cytokines such as interleukin-6 (IL-6) ultimately reduces pulmonary fibrosis [16]. This is consistent with the experimental results of Gad *et al.* [17]. Wei *et al.* showed that BMSCs can inhibit the inflammatory cytokine TNF induced by silica particles in rat lung tissue and bronchoalveolar lavage fluid- α , IL-1 β The expression of IL-6 and IL-6 plays a role in inhibiting inflammatory response and alleviating alveolar epithelial damage [18]. Recent studies have found that the activation of NLRP3 inflammatory corpuscles was the key molecular mechanism of lung injury and PF formation [19]. In this study, compared with Normal, the expression of fibrosis-related proteins NLRP3, ASC, IL- β , Caspase-1 and p-NF- κ B significantly increased in NS, but decreased after BMSCs and PFD treatment.

Transforming growth factor- β (TGF- β) is a large family composed of many signal molecules with common biological characteristics, which are collectively called transforming growth factor- β superfamily, and TGF- β 1 is one of the important members. As a multifunctional cytokine, TGF- β 1 controls cell growth, differentiation and extracellular matrix deposition, mainly participate in organ fibrosis, activates fibroblasts and accelerate wound healing, but its over-expression will lead to tissue and organ fibrosis [20]. TGF- β 1 is recognized as the strongest fibrogenic cytokine [21]. TGF- β is the most effective pro-fibrotic mediator to regulate pulmonary fibrosis by recruiting and activating monocytes and fibroblasts and inducing ECM production [22]. TGF- β 1 was recognized as the strongest fibrogenic cytokine. TGF- β 1 plays a pivotal role in the formation and development of pulmonary fibrosis, which mainly acts as a starting signal in the formation of pulmonary fibrosis by inducing and promoting the activation, proliferation and transformation of pulmonary fibroblasts into myofibroblasts, regulating the synthesis of extracellular matrix such as collagen and inhibiting its degradation. At TGF- β Mediated by the loss of specific endothelial cell markers by alveolar epithelial cells, they become labeled with interstitial markers, such as α -Smooth muscle actin (α - SMA myofibroblasts aggregate at the damaged alveolar basement membrane, forming a "fibroblast lesion" and promoting excessive secretion of extracellular matrix such as collagen, thereby promoting the occurrence and development of pulmonary fibrosis [22, 23]. It can also chemotactic inflammatory cells and synthesize and release inflammatory cytokines, which plays an indispensable

role in the occurrence and development of pulmonary fibrosis [23]. In the pathological process of pulmonary fibrosis, there are abnormal hyperplasia of connective tissue, increased synthesis of extracellular matrix and pathological accumulation. Extracellular matrix mainly comes from lung long fiber cells, which is the material basis of cell adhesion, proliferation and differentiation. TGF- β 1 plays an important regulatory role in abnormal synthesis of extracellular matrix in pulmonary fibrosis. Animal experiments showed that the expression of TGF- β 1 increased in pulmonary fibrosis caused by bleomycin and radiation [24]. In this study, compared with normal, the expressions of fibrosis-related proteins TGF- β and p-smad-3 significantly increased in NS, but decreased after BMSCs and PFD treatment.

In this study, We could find that BMSCs were effective in pulmonary fibrosis, but no clear advantage was found in the comparison with pirfenidone. BMSCs showed different inhibitory effects on pulmonary fibrosis at different doses, and the effects were better in the high-dose group, and changed increased over time. The TGF- β /smad-3 and NLRP3/NF- κ B signal pathways were decreased obviously with the high dose and long-time treatment of BMSCs, part of the same as some past studies [25-27]. There were indications of TGF- β as the initiator act through these two pathways, and may have other unknown pathways and have a additional inhibitory effect on pulmonary fibrosis [28], which needs to be verified in further studies.

5. Conclusion

To sum up, BMSCs alleviated PF induced by bleomycin and its mechanism may be related to the inhibition of TGF- β /smad-3 and NLRP3/NF- κ B signal pathway.

Ethical Approval and Consent to Participate

All animal experiments were conducted in accordance with the Jilin University.

Consent for Publication

No available.

Availability .of Supporting Data

The data used to support the findings of this study are available from the corresponding author upon request.

Competing Interests

The authors declare that they have no competing interests.

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Authors' Contributions

Chunmei Zhang, Zhongyan Zhao write the manuscript; Chunmei Zhang, Chenguang Li finished the animal experiments and data analysis; and Zhongyan Zhao designed experiment.

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