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Effect of peimisine on pulmonary interstitial fibrosis induced by bleomycin in mouse pulmonary fibrosis

Haoyan Jiao[1]*, Shuyu Li[2] and Xue Xue[1]
[1]School of Pharmacy, Guangdong Food and Drug Vocational College, China
[2]School of Traditional Chinese Medicine, Southern Medical University, China

Abstract: Peimisine has therapeutic effects on cough, asthma, acute lung injury and liver fibrosis. However, it has not been reported whether peimisine has an inhibitory effect on pulmonary fibrosis. In current study, a mouse pulmonary interstitial fibrosis model was established to investigate the efficacy of peimisine. Mice were categorized into six groups: Control, model, pirfenidone and three peimisine multi-dose groups. After the modelling, each group was given drugs for 21 days. Mice were euthanized and the histopathology changes of the lung were compared. The contents of cytokines in serum were determined. The mRNA expression levels of related genes in the lung tissue were detected. The contents of macrophages and neutrophils in the bronchoalveolar lavage fluid (BALF) were detected. The antifibrotic effect of peimisine was validated by using MRC-5 cells. The results demonstrated that peimisine could alleviate the destruction of alveolar structure and reduce the aggregation of inflammatory cells. Peimisine could reduce the protein expression levels of cytokines in serum. The mRNA levels of related genes were regulated. The contents of macrophages and neutrophils were decreased. Peimisine had a regulatory effect on the abnormal proliferation of MRC-5 cells. The mechanism was related to regulating extra cellular matrix (ECM) and epithelial-mesenchymal transition (EMT).

Keywords: Pulmonary fibrosis, peimisine, inflammation, TGF-β1.

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INTRODUCTION

The main pathological change of tissue fibrosis is a proliferation of extra cellular matrix (ECM) to repair defect tissue. Still, the proliferation does not have the structure and function of parenchymal cells (Koudstaal *et al.,* 2023). The continuous progress of this fibrosis will cause structural damage and loss of function of organs. Pulmonary fibrosis is a chronic, age-related lung disease. Clinical examination showed: Amages to lung epithelial cells, abnormal proliferation of pulmonary fibroblasts, deposition of ECM, devastation of lung tissue structure, lead to lung failure. The pathogenesis of this disease is complex and the medications used in clinical practice have poor efficacy (Li and Kan 2017; Wong *et al.,* 2020).

Fritillaria is the dry bulb of the perennial herb Fritillaria (Liliaceae). It is one of the essential traditional Chinese medicines in China. Fritillaria is widely used to treat cough, asthma, chronic pharyngitis, bronchitis and other respiratory diseases (Xiao *et al.,* 2007). The legal medicinal Fritillaria collected in the 2020 edition of "Chinese Pharmacopoeia" comes from different regions of China, including *Fritillariae cirrhosae, Fritillariae thunbergii, Fritillariae pallidiflorae, Fritillariae ussuriensis* and *Fritillariae hupehensis*. The study on the chemical components of Fritillaria from different regions shows that Fritillaria contains a class of common and particular chemical components: Isosteroidal alkaloids. Isosteroidal alkaloids are the main active ingredients of Fritillaria (Wang and Liu 2022).

The isosteroidal alkaloids in Fritillaria mainly include imperialine, peimine, peiminine, peimisine, verticinone, zhebeinone, isoverticine and so on. Peimisine is mainly found in *Fritillariae thunbergii* (Jiang *et al.,* 2006). Peimisine could relax tracheal smooth muscle and alleviate asthma by acting on M receptors, excitatory beta receptors, inhibiting calcium release in the body, and promoting nitric oxide release (Zhao *et al.,* 2009). Peimisine could inhibit NF-κB signaling pathway to prevent the acute lung injury (Jin *et al.,* 2022). Peimisine had an excellent protective effect on liver fibrosis induced by CCl_4 in rats. The mechanism may be related to inhibiting collagen synthesis, reducing free radical production and reducing lipid peroxidation (Liu *et al.,* 2013a). Peimisine could reduce LPS-induced lung injury and mucus hypersecretion. Its mechanism is related to deregulating MUC5AC mRNA (Cui *et al.,* 2015). It was observed that peimisine could regulate the expression of α-SMA in rats with hepatic fibrosis tissues (Liu *et al.,* 2013b). The results showed that peimisine could alleviate hepatic fibrosis by reducing activation of hepatic stellate cells. However, it has not been reported whether or not peimisine can inhibit pulmonary fibrosis.

This study established a bleomycin-induced pulmonary interstitial fibrosis model in mice and observed the efficacy of peimisine on pulmonary fibrosis. This study is helpful to the development and utilization of peimisine to provide an available method for clinical application.

**Corresponding authors:* e-mails: jiaohaoyan@sina.com

MATERIALS AND METHODS

Experimental animals

Sixty male C57BL/6 mice, weighing 24-25g, were provided by the experimental animal center of Southern Medical University (certificate number, SCXK (Beijing) 2021-0006). Mice were housed in cages (3-4 per cage) with adaptive feeding, free for food and water for 3 days. This study was allowed by the Experimental Animal Ethics Committee of Southern Medical University with an allowed number (00259256).

Drugs and reagents

Peimisine (purity>99.2%) was purchased from Macklin Biochemical Co., Ltd (Shanghai, China, #H20206841). Pirfenidone (purity>98.9%) was purchased from Continent Pharmaceuticals Inc (Beijing, China, #H20211336). Bleomycin injection was produced by Hanhui Pharmaceutical Co., Ltd (Hangzhou, China, #H20196721). MRC-5 cells were acquired from KeyGEN BioTECH Co., Ltd (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) test kits for estimation of pulmonary levels of interleukin-2 (IL-2) and transforming growth factor-β1 (TGF-β1) were acquired from Elabscience Inc (Wuhan, China). Reverse transcription-polymerase chain reaction (RT-PCR) kits for collagen I (#72068T), α-SMA (#19285T), fibronecrin (#26834S), vimentin (#5771S) and E-cadherin (#3196S) were purchased from Toyobo Co., Ltd (Japan). The Masson staining kit was purchased from Gibco company (USA). 10mg peimisine was fully dissolved with 0.3ml DMSO, diluted with normal saline, fixed to 25ml and the concentration is 0.4mg/ml.

Experimental design

Sixty male C57BL/6 mice were separated into six groups: control, model, pirfenidone and peimisine (low, medium and high) groups. Mice were treated with saline in the control group. Other groups were injected with bleomycin 5mg/kg intratracheal drip on day -2. Starting from day 1, control, model and pifenidone groups were orally given saline, saline and pifenidone (300mg/kg) daily for consecutive 21 days, respectively. At the same time, Peimisine (low, medium and high) groups were intraperitoneally injected with peimisine (2, 4 and 6mg/kg) for 21 days. The dosage of peimisine was determined through preliminary experiments. On the 22nd day, mice were euthanized by excessive CO_2 treatment. fig. 1 shows the flow diagram of the procedure. MRC-5 cells were inoculated into 96 plates and randomly separated into four groups (control, model, SB431542 and peimisine).

Sample collections

0.8 ml blood from the abdominal aorta of euthanized mice was taken, centrifuged at 4000 r/min for 15 minutes. The serum was retained and frozen in a refrigerator at -80ºC. The right lung was taken for molecular biology experiment, while 4% paraformaldehyde was used to fixed the left lung for histopathological analysis. The bronchoalveolar lavage fluid (BALF) was collected to analyse inflammatory cells.

Histopathological analysis

Embedded the fixed left lung in paraffin wax, sectioned, HE stained, Masson stained and sealed. The lung tissue was scored by Ashcroft under the microscope. Ashcroft score evaluation criteria: Grade 0, normal lung tissue; Grade 1, the tiny fibers in alveoli or bronchioles are thickened; Grade 2~3, the alveolar wall is moderately thickened, and the lung tissue structure is not obviously damaged; Grade 4~5, fibrosis is aggravated, lung structure is obviously damaged and fibrous bands or small fibrous masses are formed; Grade 6~7, the lung tissue structure is seriously damaged and the fiber area is large, which may be accompanied by honeycomb lung formation; Grade 8, entire field of vision, fibrous tissue, and whole lung tissue was all occluded and necrotic.

Determination of mRNA expression of related genes by RT-PCR

Trizol was adopted to extract the total RNA, then the RNA concentration was determined. RT reaction was carried out according to the steps in the manual. The cDNA was synthesized and then amplified by PCR. Amplification was carried out according to the instructions of the power SYBR Green PCR Master Mix kit from Toyobo company. The reaction conditions for 40 cycles were as follow: Pre-denaturationat 95ºC (30s), denaturation at 95ºC (5s) and denaturation at 60ºC (34 s). The ΔΔCt values of each group were counted, the RQ values were calculated and then the mRNA expression levels of each group were compared.

Determination of IL-2 and TGF-β1 in serum

The serum under item *'Sample collections'* was thawed in a water bath at room temperature. The levels of IL-2 and TGF-β1 in serum were measured according to the instructions of ELISA kits.

Determination of macrophages and neutrophils in BALF

500ul BALF was centrifuged, precipitated, resuspended with 200ul PBS and placed on ice. Mouse antibodies CD11b, F4/80 and LY6G were dissolved in PBS containing 0.5% BSA. Add 1ul of each antibody to each tube and stain on ice for 20min. Finally, transfer to the flow tube to avoid light and get on the machine as soon as possible.

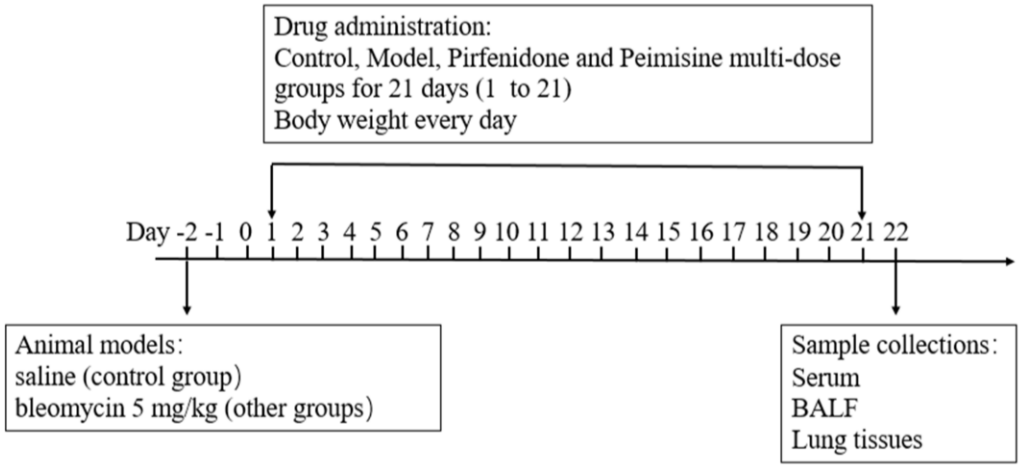

Fig. 1: The flow diagram of the experimental procedure.

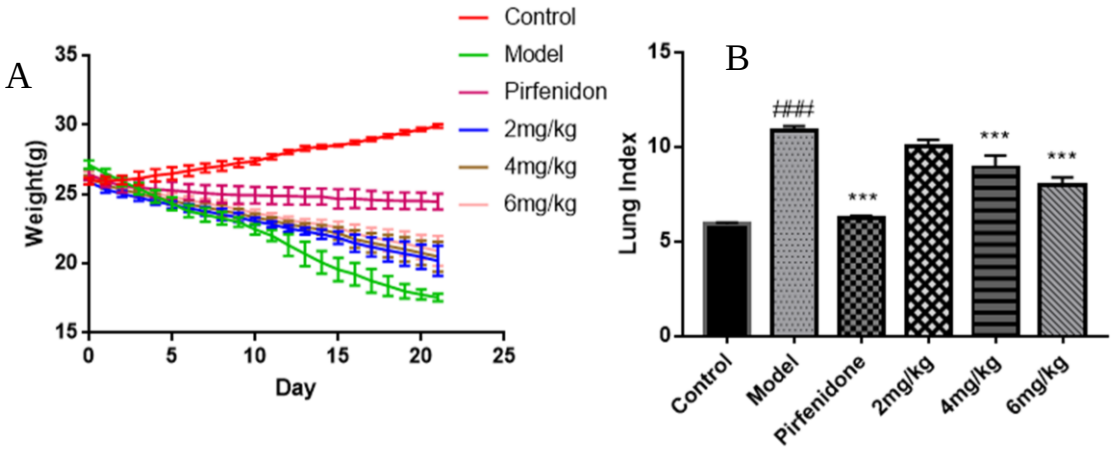

Fig. 2: Peimisine reduced the increase in lung index scores induced by bleomycin. An experimental schedule for evaluation of efficacy of peimisine on bleomycin-induced pulmonary fibrosis as follow. Besides the control group, mice were administered 5mg/kg bleomycin via tracheal instillation. 3 days later, control, model and pifenidone groups were orally given saline, saline and pifenidone (300mg/kg) for 21 days, respectively. Meanwhile, peimisine multi-dose groups were intraperitoneally injected with peimisine (2, 4 and 6 mg/kg) for 21 days. The weight of mice was measured every day. (A) The curves of body weight change in each group for 21 days. (B) The lung index for each group. Lung index is the ratio of lung weight (g) to body weight (kg). One-way ANOVA and LSD-t test were performed for multiple comparisons. Compared with the control group, $^{###}P<0.001$; Compared with the model group, $^{***}P<0.001$.

Detection of MRC-5 cell activity by CCK8

Inoculated MRC-5 cells into 96 plates and cultured at 37ºC for 24 hours. The experimental groups were divided into control group (normal cultured MRC-5 cells), model group (5ng/ml TGF-β1), SB431542 group (5ng/ml TGF-β1, 2μmol/L SB431542) and peimisine group (5ng/ml TGF-β1, 156.25μg/ml peimisine). After cultured for 48h, MRC-5 cells in each group were incubated in CCK-8 solution for 2 hours. The absorbances of each group were measured. Cell survival rate = $[(OD_{treatment\ group}-OD_{control\ group}) / (OD_{model\ group}- OD_{control\ group})] \times 100\%$.

STATISTICAL ANALYSIS

Graphpad Prism 8.3.0 software was adopted for data analysis. Data with normal distribution and homogeneous variance were expressed. One-way ANOVA was used, and the least significant difference method (LSD) - t was used for comparisons between groups. $P<0.05$ is considered to be statistically significant.

RESULTS

Peimisine reduced the increase in lung index scores induced by bleomycin

After the modelling, the lungs of the mice were damaged, the appetite and mental state decreased, and the weight decreased gradually (fig. 2A). The change of lung index is a sign of pulmonary fibrosis. The lung index of mice in the model groups gradually increased, which means the pulmonary fibrosis gradually aggravated. The result also indicates the success of the pulmonary fibrosis model.

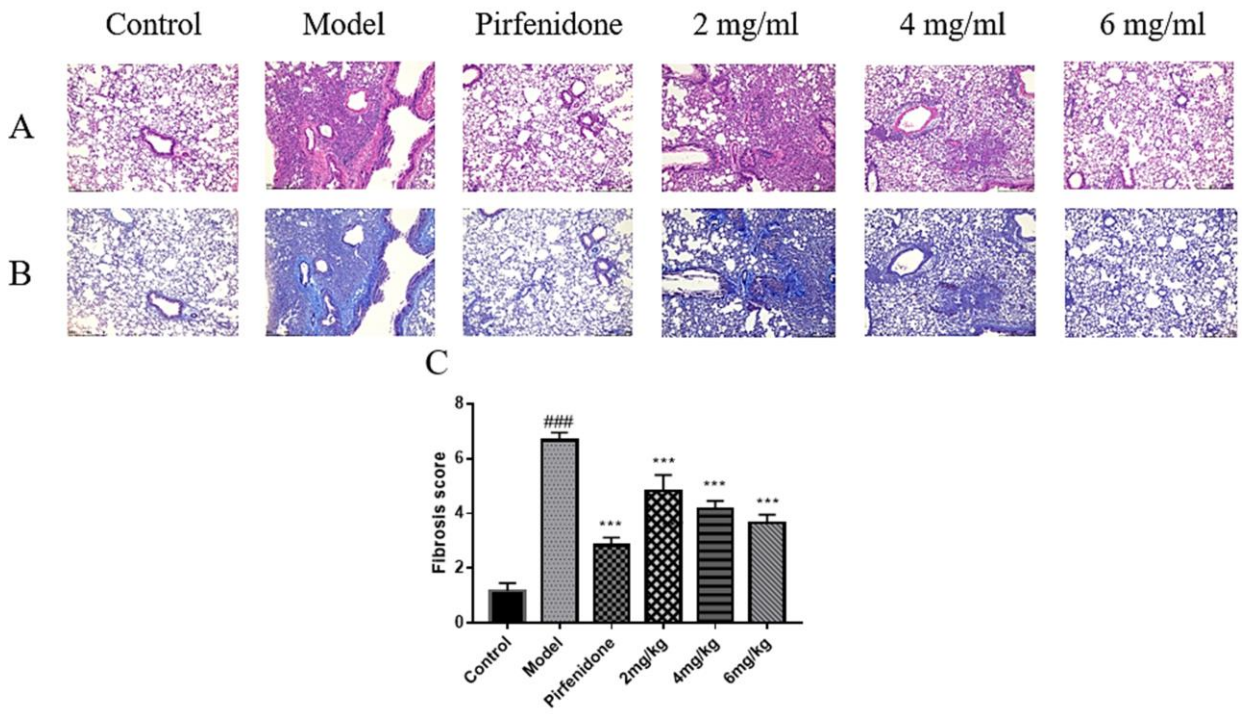

Fig. 3: Peimisine (2, 4 and 6 mg/kg) effectively alleviated bleomycin-induced pulmonary fibrosis and inflammation in lung tissues. Fixed the left lung with 4% paraformaldehyde, then embedded in paraffin wax, sectioned, HE stained, Masson stained and sealed. (A) Representative histological image of mouse lungs stained with HE(×100), (B) Representative histological images of Masson stained mouse lungs (×100), (C) The fibrosis score of lung tissue (n=6 per group) was scored by Ashcroft under the microscope. Statistical analysis was performed. Compared with the control group, $^{###}P<0.001$; Compared with the model group, $^{***}P<0.001$.

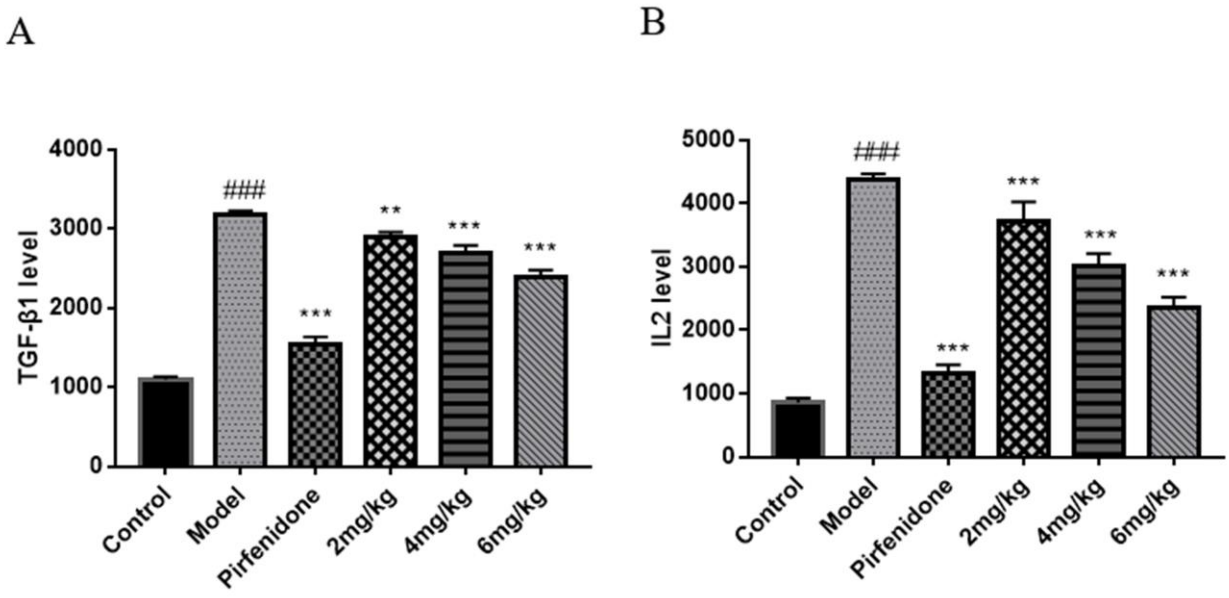

Fig. 4: Peimisine significantly reduced the bleomycin-induced release of inflammatory cytokines in mice. (A) (B) The levels of TGF-β1 and IL-2 in serum were quantified using ELISA, and each group were compared. Compared with the control group, $^{###}P<0.001$; Compared with the model group, $^{**}P<0.01$, $^{***}P<0.001$.

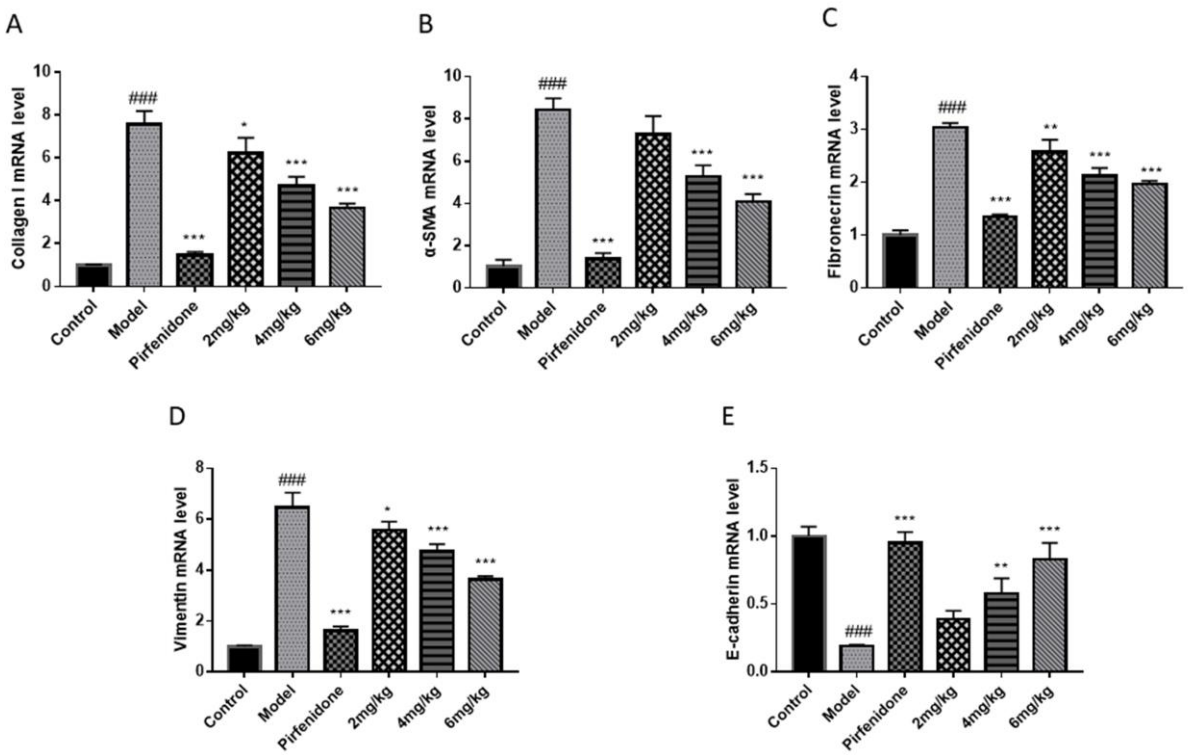

Fig. 5: Peimisine effectively alleviated bleomycin-induced pulmonary fibrosis on fibrosis markers in mice. (A)-(E) The expression levels of collagen I, α- SMA, fibronectin, vimentin and E-cadherin mRNA were measured by RT-PCR and each group were compared. Compared with the control group, $^{\#\#\#}P<0.001$; Compared with the model group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

After administration of peimisine, the lung index decreased in a dose-dependent manner (fig. 2B). There are significant differences (P<0.01) between peimisine (medium, high) groups and the model group. This result indicates that peimisine has an improving effect on pulmonary fibrosis in mice.

Peimisine effectively alleviated pulmonary fibrosis and inflammation induced by bleomycin in lung tissues

The alveolar structure of the control group was normal in HE staining. Lung tissue had good structure and no obvious inflammatory infiltration. The dropsy of the alveolar septal was obviously thickened in the model group when compared with the control group. The structure was significantly damaged and lots of inflammatory cells gathered. The destruction of alveolar structure decreased and aggregation of inflammatory cells was reduced after treated with peimisine. The therapeutic effect of high-dose peimisine was similar to that of pirfenidone (fig. 3A). Masson staining showed that there were lots of blue-stained collagen fibers in the peribronchial, perivascular and alveolar spaces in model group when compared with control group. Area of collagen fibers reduced in a dose-dependent manner after injected with peimisine (fig. 3B). Fibrosis rating (fig. 3C) is shown in fig. 3. Pirfenidone significantly decreased (P <0.001) the fibrosis score when compared with the model group, as did the peimisine multi-dose groups (fig. 3C).

Peimisine significantly reduced the release of inflammatory cytokines in mice

The levels of TGF- β1 and IL-2 of the model group statistically significantly increased (P<0.001) when compared with the control group. After treated with different doses of peimisine, the levels of them reduced. The therapeutic effect of high-dose peimisine is better than that of low-dose and is similar to that of pirfenidone (fig. 4 A-B).

Peimisine effectively alleviated bleomycin-induced pulmonary fibrosis on fibrosis markers in mice

The mRNA levels of collagen I, α- SMA, fibronecrin, and vimentin increased significantly (P<0.001). In contrast, the expression level of E-cadherin mRNA statistically significantly decreased (P<0.001) in the model group, when compared with the control group. After treated with peimisine, the mRNA levels of collagen I, α- SMA, fibronecrin and vimentin statistically significantly reduced (P<0.001) in medium- and high-dose peimisine groups compared with the model group. The therapeutic effect of peimisine in high-dose group was better than the pifenidone group (fig. 5 A-D). The mRNA expression level of E-cadherin increased significantly (P<0.001) in the high-dose group compared with the model group. (fig. 5E). The above results demonstrated that peimisine has anti pulmonary fibrosis effect by regulating ECM and EMT processes in mice.

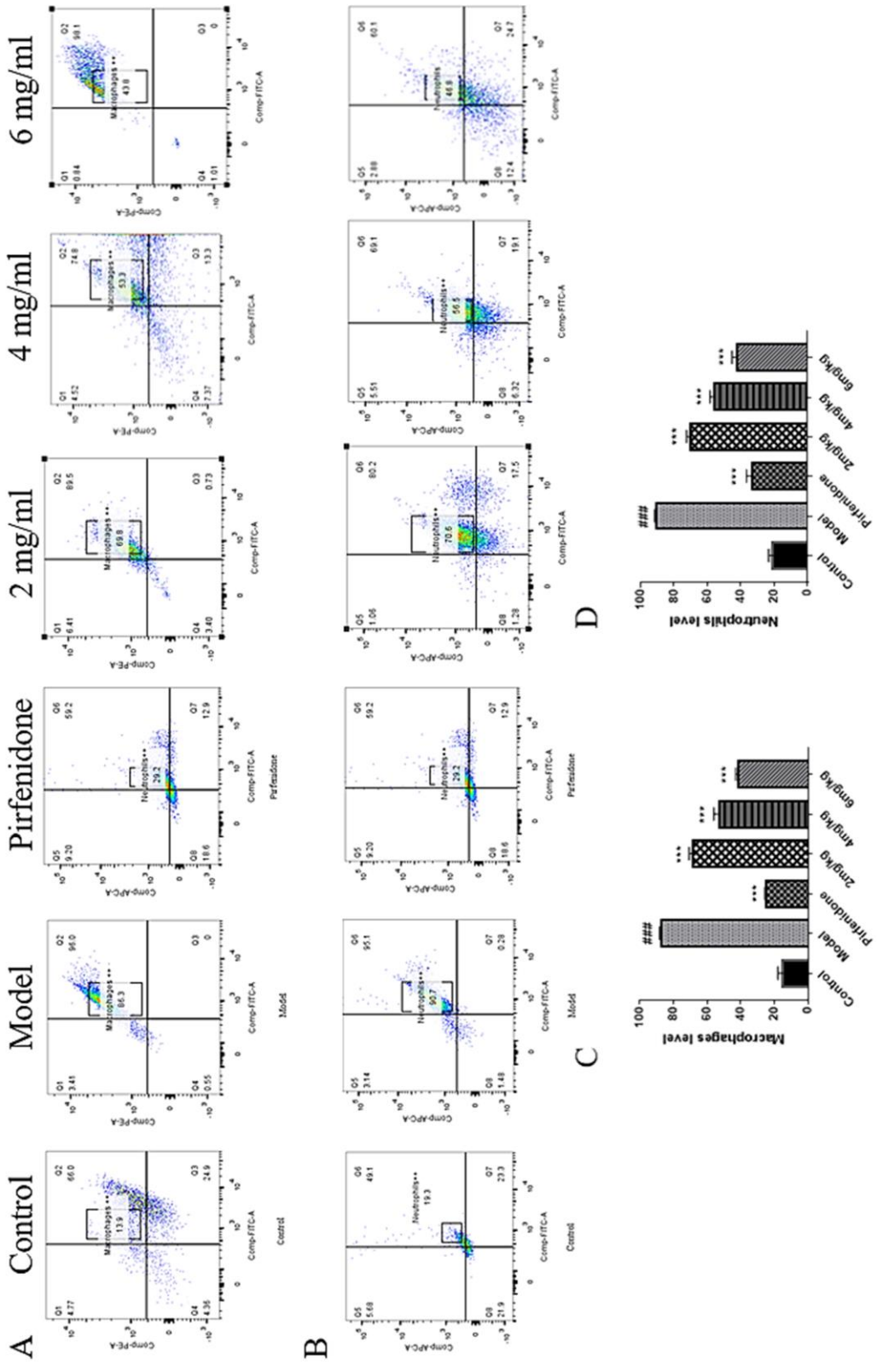

Fig. 6: Peimisine effectively alleviated bleomycin-induced infiltration of macrophages and neutrophils into lung tissue in mice. The levels of macrophages and neutrophils in BALF were measured. (A) (B) Flow cytometry results of macrophage and neutrophils. (C) (D) The levels of macrophages and neutrophils and each group were compared. Compared with the control group, ###$P<0.001$; Compared with the model group, ***$P<0.001$.

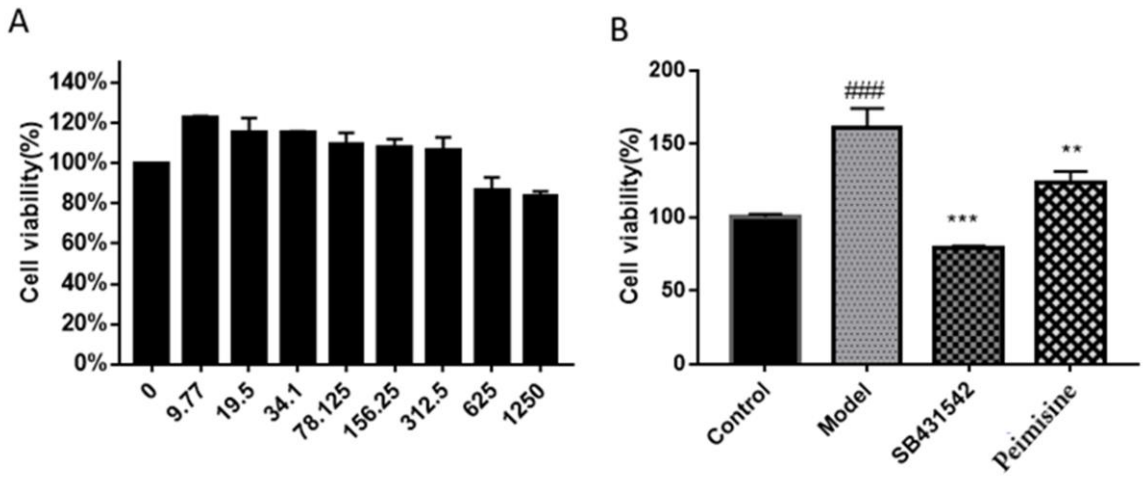

Fig. 7: Peimisine significantly restrained the abnormal proliferation of MRC-5 cells induced by TGF- β1. (A) Cell viability (%) after treatment with multi- doses of peimisine, 156.25ug/ml was selected as the appropriate dose, (B) Cell viability (%) after treatment with SB431542 and peimisine, demonstrated the inhibitory effect of peimisine on myofibroblasts and each group were compared. Compared with the control group, $^{###}P<0.001$; Compared with model group, $^{**}P<0.01$, $^{***}P<0.001$.

Peimisine effectively alleviated bleomycin-induced infiltration of macrophages and neutrophils into lung tissue in mice

Inflammation is a critical pathway that triggers pulmonary fibrosis and inflammatory factors can directly or indirectly promote fibrosis in lung tissue. The contents of macrophages and neutrophils in the model group statistically significantly increased (P<0.001) when compared with the control group. Number of macrophages and neutrophils reduced in a dose-dependent manner after administration with different doses of peimisine (fig. 6 A-D).

Peimisine significantly inhibited TGF- β1- induced abnormal proliferation of MRC-5 cells

First, explored a safe dose of peimisine. The results are shown in fig. 7A. 156.25ug/ml was selected as the dose of peimisine group in the subsequent experiments. Number of cells in the model group increased significantly (P < 0.001) when compared with the control group. After treated with peimisine, compared with the model group, the number of cells in SB431542 (an inhibitor of TGF-β1 signaling) and peimisine groups decreased significantly (P <0.01) (fig. 7B).

The above results indicated that peimisine and SB431542 have significant inhibitory effects on proliferation of MRC-5 cells. This experiment validated the therapeutic effect of peimisine on pulmonary fibrosis *in vitro*.

DISCUSSION

Pulmonary fibrosis is a progressive and common lung disease, which will seriously reduce patient's quality of life. Current study has shown that in the area of pulmonary fibrosis, highly contractile myofibroblasts will over synthesize collagen, resulting in abnormal deposition of ECM and excessive transformation of EMT (Glass *et al.,* 2022). *In vivo* studies, bleomycin is often used to make a pulmonary fibrosis model in mice (Mouratis and Aidinis 2011; Degryse *et al.,* 2010).

In our study, a mouse pulmonary fibrosis model was established using bleomycin. It was found that pulmonary fibrosis mice showed apparent symptoms of pulmonary fibrosis. In the model group, the accumulation of inflammatory factors, increase of collagen content, and obvious damage of alveolar structure are the important pathological characteristics of pulmonary fibrosis. Compared with pulmonary fibrosis mice, the reduce of body weight and rise of lung index were alleviated after administration of peimisine, especially after high-dose peimisine administrated. Peimisine significantly alleviated alveolar structural damage and inflammatory infiltration in model mice. Meanwhile, peimisine had a regulatory effect on inflammatory factors (TGF- β1 and IL-2) in serum. Peimisine exhibited a regulatory effect on neutrophils and macrophages in BALF. It also demonstrated that the antifibrotic effect of peimisine is related to inflammation regulation. RT-PCR studies showed that peimisine significantly regulates the mRNA levels of pulmonary fibrosis marker. Finally, the efficacy of peimisine on pulmonary fibrosis was validated *in vitro* by using MRC-5 cells.

TGF- β1 is considered to be a very critical fibrosis factor. It is closely related to immune response, inflammation and matrix synthesis (Broekelmann *et al.,* 1991; Kim *et al.,* 2006). After bleomycin modelling, numbers of TGF-β1 and IL-2 of mice increased significantly in the model

group. After peimisine treatment, the contents of TGF- β1 and IL-2 decreased and the therapeutic effect of high dose was equivalent to that of pirfenidone. The regulatory effect of peimisine on neutrophils and macrophages in mouse BALF also confirms the antifibrotic effect of peimisine and its association with inflammation.

ECM remodeling and EMT transformation are the main pathological processes of fibrosis (Qian *et al.,* 2020; Loh *et al.,* 2019). α- SMA is the typical marker of myofibroblasts. Vimentin is an essential intermediate fibrin in interstitial cells (Wu *et al.,* 2016; Nielsen *et al.,* 2019). Collagen I is an essential protein in connective tissue (Strieter *et al.,* 2017). α- SMA, vimentin, and collagen I are vital effectors of the ECM process. E-cadherin is involved in mediating the contact, adhesion and propagation between epithelial cells (Mendonsa *et al.,* 2018; Pearson 2019; Lv *et al.,* 2020). Fibronectin is the primary cell adhesion molecule. E-cadherin and fibronectin are both typical EMT epithelial cell markers (Bueno *et al.,* 2020). After intervention with different doses of peimisine, the levels of collagen I. α-SMA, fibronecrin and vimentin decreased, while the mRNA levels of E-cadherin increased. It shows that peimisine can regulate the fibrosis process.

Neutrophils are the largest number of immune cells recruited to the location of infection. Macrophages are indispensable frontier indicators of host defense. Both are important markers of inflammatory phenotype. In the model group, the amounts of neutrophils and macrophages increased significantly. After intervention with different doses of peimisine, the contents of the two kinds of cells decreased in a dose-dependent manner and inflammation was inhibited. *In vitro*, myofibroblasts proliferated very quickly in TGF-β1 group. Peimisine inhibited the transformation of fibroblasts into myofibroblasts.

CONCLUSION

In conclusion, this study demonstrated that peimisine can inhibit pulmonary fibrosis and inflammation in mice by regulating ECM and EMT processes. The abnormal proliferation of myofibroblasts *in vitro* was effectively inhibited by peimisine. We believe that peimisine may provide a therapeutic benefit in patients with pulmonary fibrosis.

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