

ORIGINAL ARTICLE

# Inhibitory Effects of Astragaloside IV on Bleomycin-Induced Pulmonary Fibrosis in Rats Via Attenuation of Oxidative Stress and Inflammation

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**Abstract**—In this study, we investigated the effects of astragaloside IV (As-IV) on pulmonary fibrosis and its mechanisms of action. Sprague-Dawley rats were used in a model of pulmonary fibrosis induced by an intratracheal instillation of bleomycin (BLM). Rats were intraperitoneally injected with As-IV (10, 20, 50 mg/kg) daily for 28 days, while the rats in control and BLM groups were injected with a saline solution. The effects of As-IV treatment on pulmonary injury were evaluated with the lung wet/dry weight ratios, cell counts, and histopathologic. Oxidative stress was evaluated by detecting the levels of malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and reactive oxygen species (ROS) in lung tissue. Inflammation was assessed by measuring the levels of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 in bronchoalveolar lavage fluid (BALF). The results indicated that As-IV treatment remarkably ameliorated BLM-induced pulmonary fibrosis and attenuated BLM-induced oxidative stress and inflammation. Our findings indicate that As-IV-mediated suppression of fibroproliferation may contribute to the anti-fibrotic effect against BLM-induced pulmonary fibrosis. Its mechanisms of action are associated with inhibiting oxidative stress and inflammatory response. In summary, our study suggests a therapeutic potential of As-IV in the treatment of pulmonary fibrosis.

**KEY WORDS:** astragaloside IV; pulmonary fibrosis; oxidative stress; inflammation.

## INTRODUCTION

Pulmonary fibrosis, which is characterized by accumulation of extracellular matrix and loss of pulmonary function, is the most common form of interstitial lung disease [1, 2]. The studies found that a number of key factors have been implicated in the pathogenesis of pulmonary fibrosis, such as inflammation, apoptosis, oxidative stress, and hyperplasia [3–6]. Inflammation is the initial response following lung injury. In the

lower airways, the inflammatory cells accumulate and release some pro-inflammatory cytokines, growth factors, and so on, which regulate the proliferation and secretory activity of alveolar fibroblasts [7, 8]. There is increasing evidence that oxidative stress is involved in the pathogenesis of pulmonary fibrosis. Markers of oxidative stress have been identified in patients and aberrant antioxidant activity has been demonstrated to exacerbate pulmonary fibrosis in animal models [9, 10]. Therefore, anti-inflammation and antioxidant treatment strategies are being developed to treat pulmonary fibrosis.

Astragaloside IV (As-IV; 3-O- $\beta$ -D-xylopyranosyl-6-O- $\beta$ -D-glucopyranosyl cycloastragenol) is a natural saponin purified from *Astragalus membranaceus* with antioxidant, anti-inflammatory, and anti-apoptotic effects [11–14]. It has demonstrated the anti-inflammatory effects of

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As-IV through inhibition of NF- $\kappa$ B-mediated inflammatory genes expression [15]. Moreover, As-IV exhibits antioxidant effects through the inhibition of reactive oxygen species (ROS) production, the reduction of lipid peroxidation, and the stimulation of antioxidant enzymes [16]. As we mentioned before, anti-inflammation and antioxidant treatment strategies are being developed to treat pulmonary fibrosis. Therefore, these researches suggest As-IV may have a protective effect on fibrotic diseases. Its mechanisms of action may be associated with inhibiting oxidative stress and inflammatory response. However, there is a lack of data regarding the effects of As-IV on fibrotic lung disease.

Therefore, this study intends to investigate the effects of As-IV on pulmonary fibrosis in a bleomycin (BLM)-induced pulmonary fibrosis rat model. The underlying regulatory mechanisms associated with the potential anti-inflammatory and antioxidant effects of As-IV were also investigated.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats (around 250 g, 8 weeks old) were obtained from the Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (SPF grade, Certificate No. SCXK20140007). The rats were maintained at a constant room temperature ( $22 \pm 2$  °C) and humidity ( $50 \pm 10$  %) with 12 h light/dark cycle and free access to water and food.

### Establishment of BLM-Induced Pulmonary Fibrosis in Rats and As-IV Treatment

The rats were randomly divided into five groups ( $n = 10$ ): control, BLM, BLM + As-IV<sub>10</sub>, BLM + As-IV<sub>20</sub>, BLM + As-IV<sub>50</sub>. Induction of pulmonary fibrosis with BLM was conducted according to a previously described method [17]. Briefly, all rats were anesthetized with 10 % chloral hydrate (3.5 ml/kg body weight; Sinopharm, Shanghai, China). Then, the rats were administered a single intratracheal instillation of BLM dissolved in saline (5 mg/kg body weight; Melone Pharmaceutical Co., Ltd, Dalian, China), while an equal volume of saline was injected into the rats from the control group.

The rats from the BLM + As-IV groups received an intraperitoneal injection of As-IV (99.2 % purity; National Institute for the Control of Pharmaceutical

and Biological Products, Shanghai, China) at a dose of 10, 20, and 50 mg/kg bw, respectively, every day for a total of 28 days, and the rats in control and BLM groups were injected with a saline solution. On day 28 after BLM induction, the rats were sacrificed by intraperitoneal injection of 10 % chloral hydrate (5 ml/kg body weight). The bronchoalveolar lavage fluids (BALFs) were collected and the lungs were excised for further analysis.

### Lung Wet/Dry Weight Ratios

The lungs of the rats were removed and weighed immediately, and following drying in an incubator at 60 °C for 72 h to obtain the dry weight. The pulmonary edema was calculated as a wet/dry weight ratio.

### Cell Counting in Bronchoalveolar Lavage Fluid (BALF)

The BALF was centrifuged at  $1000 \times g$  for 10 min at 4 °C, and the supernatant was stored immediately at  $-80$  °C until analysis. The sediment cells were resuspended in 50  $\mu$ l PBS and 10  $\mu$ l of the cell suspension was made into the smear on a glass slide. The cell smear was air-dried, fixed in methanol for 15 min, and stained with Giemsa solution (Jiancheng Bioengineering Institute, Nanjing, China). Then, total cells, neutrophils, macrophages, and lymphocytes were counted with a hemocytometer (Hausser Scientific, Horsham, PA, USA).

### BALF Biochemical Analysis

The levels of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 in BALF were measured by Enzyme-Linked Immunosorbent Assay (ELISA) kits (Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions.

### Measurement of Oxidative Stress

The lung homogenates were centrifuged for 30 min at  $3000 \times g$  at 4 °C. The serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and reactive oxygen species (ROS) in lung tissue were measured according to the instructions of detection kits (Jiancheng Bioengineering Institute, Nanjing, China).

### Histopathological Examination

The lungs were fixed, paraffin embedded, sectioned into 5- $\mu$ m slices, and then stained with hematoxylin and eosin (H&E), and examined under a light microscope (DP73; Olympus).

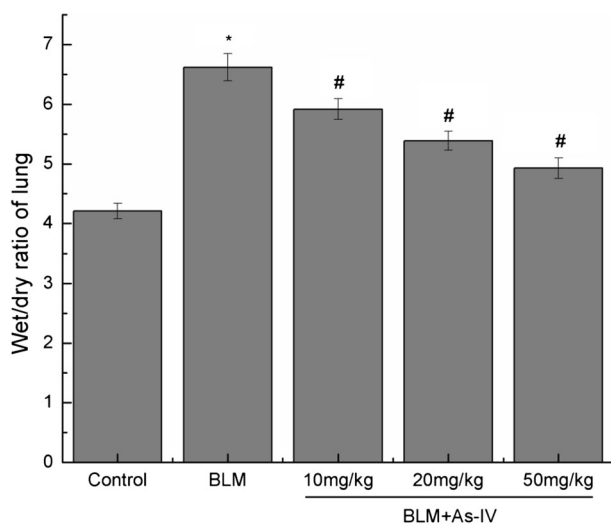
### Statistical Analysis

SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to perform all statistical analysis. The present data were expressed as the mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences among multiple groups, followed by Bonferroni post hoc test for comparisons between two groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

### Wet/Dry Weight Ratio

The lung wet/dry ratio was evaluated to indicate the pulmonary edema (Fig. 1). Compared to the control group, the lung wet/dry ratio significantly increased in the BLM-induced pulmonary fibrosis models (from  $4.21 \pm 0.13$  to  $6.62 \pm 0.23$ ,  $P < 0.05$ ), and As-IV treatment significantly decreased the lung wet/dry ratios in BLM-induced models (from  $6.62 \pm 0.23$  to  $4.93 \pm 0.17$ ,  $P < 0.05$ ).



**Fig. 1.** As-IV ameliorates BLM-induced wet/dry ratio changes in the lung. Data were shown as mean  $\pm$  SD. \* $P < 0.05$  vs. control group, # $P < 0.05$  vs. BLM group.

### As-IV Mitigated the Inflammatory Responses in BLM-Induced Pulmonary Fibrosis

To determine the effect of As-IV on BLM-induced pulmonary inflammation responses in rats, the inflammatory cell counts in BALF were assayed. As shown in Table 1, the number of total cells, neutrophils, macrophages, and lymphocytes in BALF were significantly increased after BLM injection. As-IV administration dose-dependently reduced the increases of inflammatory cell counts in BALF in BLM-stimulated rats. In addition, the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF were subsequently detected. It was noted that As-IV significantly prevented BLM-induced elevation of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in a dose-dependent manner (Fig. 2).

### As-IV Attenuated Oxidative Stress in BLM-Induced Pulmonary Fibrosis

Oxidative stress was evaluated by detecting the levels of MDA, SOD, T-AOC, and ROS in pulmonary tissue (Fig. 3). In the BLM group, MDA and ROS levels were significantly elevated while SOD and T-AOC levels were reduced. In contrast, treatment with As-IV remarkably decreased the levels of MDA and ROS, simultaneously increased the levels of SOD and T-AOC.

### Histopathological Evaluation (HE)

The HE images of lung tissue are shown in Fig. 4. The results shown that the control group displayed normal structure and no inflammatory or fibrotic lesions (Fig. 4a). In BLM group, the lung structure was severely damaged and the lung interstitium was evidently thickened (Fig. 4b). On the contrary, these pathological changes were attenuated by the administration of As-IV (Fig. 4c-e).

## DISCUSSION

BLM-induced pulmonary fibrosis is one of the most commonly used experimental models in the study of pulmonary fibrosis. In the present study, we established a rat model to investigate the protective effects of As-IV against pulmonary fibrosis induced by intratracheal instillation of BLM. Our findings indicated that administration of As-IV markedly ameliorated BLM-induced pulmonary edema, oxidative stress, and inflammatory responses. Given these results, we demonstrated that As-IV treatment significantly ameliorates BLM-induced pulmonary fibrosis. Our data

**Table 1.** Effect of As-IV on BLM-Induced Changes in Total and Differential Cell Counts in the BALF of Rats

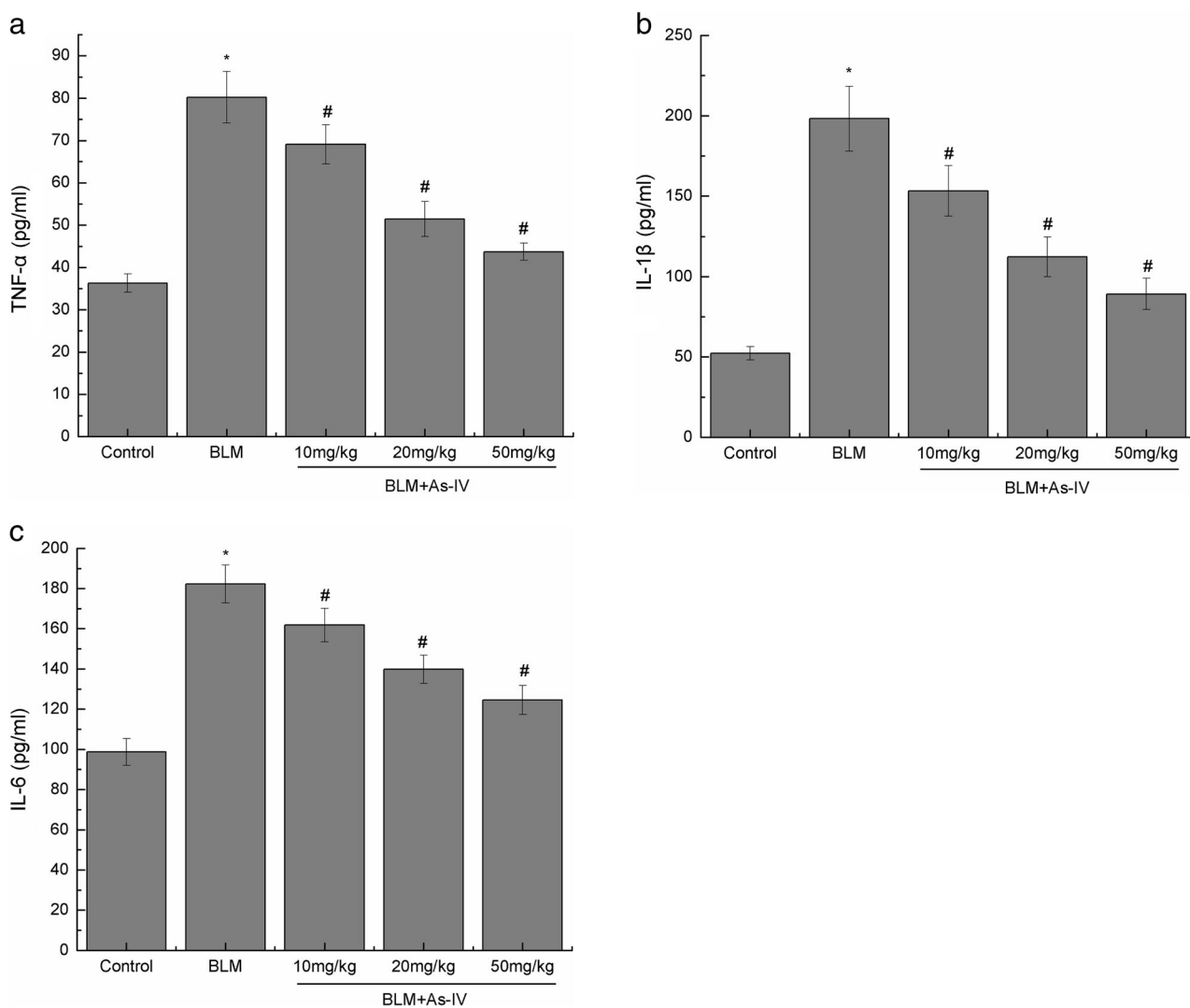
Group	Total cells ( $\times 10^5/\text{ml}$ )	Neutrophils ( $\times 10^4/\text{ml}$ )	Macrophages ( $\times 10^5/\text{ml}$ )	Lymphocytes ( $\times 10^4/\text{ml}$ )
Control	2.05 $\pm$ 0.61	4.61 $\pm$ 0.62	1.34 $\pm$ 0.39	2.51 $\pm$ 0.74
BLM	11.50 $\pm$ 1.73*	12.41 $\pm$ 1.81*	8.51 $\pm$ 1.27*	17.49 $\pm$ 2.43*
BLM + As-IV <sub>10</sub>	9.53 $\pm$ 1.22 <sup>#</sup>	9.24 $\pm$ 1.01 <sup>#</sup>	7.03 $\pm$ 1.14 <sup>#</sup>	15.76 $\pm$ 2.26 <sup>#</sup>
BLM + As-IV <sub>20</sub>	7.12 $\pm$ 0.94 <sup>#</sup>	8.03 $\pm$ 1.11 <sup>#</sup>	5.31 $\pm$ 0.67 <sup>#</sup>	10.07 $\pm$ 1.33 <sup>#</sup>
BLM + As-IV <sub>50</sub>	6.04 $\pm$ 0.83 <sup>#</sup>	7.16 $\pm$ 0.98 <sup>#</sup>	4.39 $\pm$ 0.42 <sup>#</sup>	9.34 $\pm$ 1.18 <sup>#</sup>

Values are mean  $\pm$  SD

\* $P < 0.05$  vs. Control group; <sup>#</sup> $P < 0.05$  vs. BLM group

reveal that As-IV has potential for treatments of BLM-induced pulmonary fibrosis.

Previous researches have shown that inflammation and oxidative stress are both involved in the pathogen-



**Fig. 2.** Effects of As-IV on TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in BALF. Data were shown as mean  $\pm$  SD. \* $P < 0.05$  vs. control group, <sup>#</sup> $P < 0.05$  vs. BLM group.

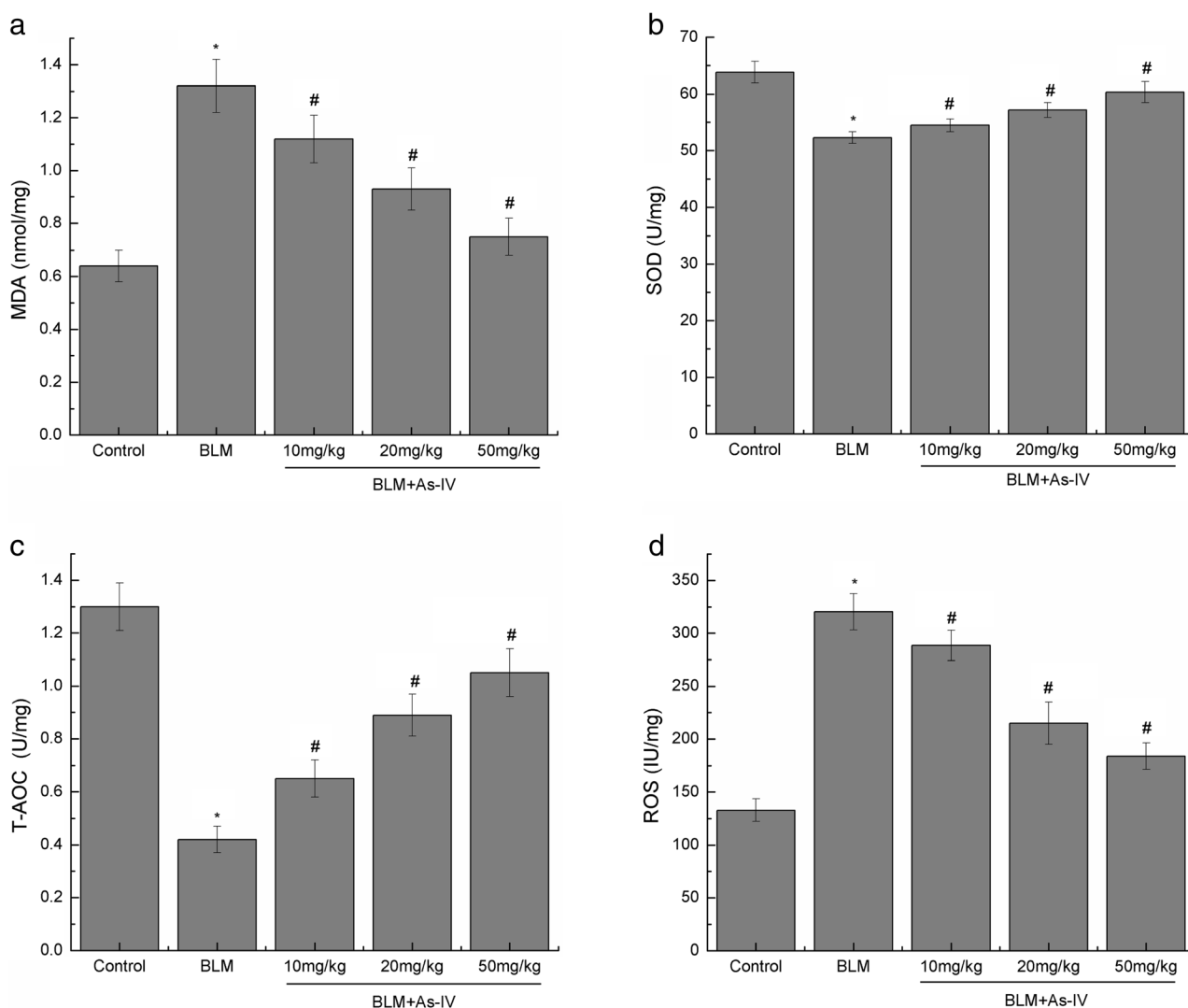
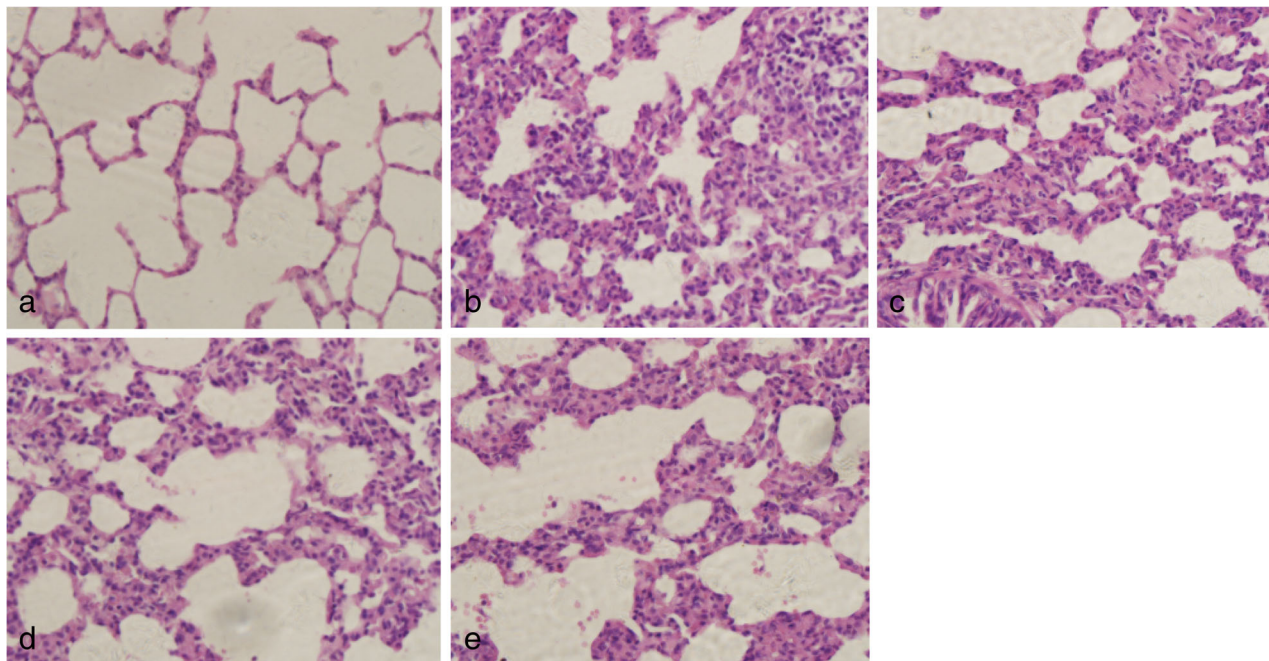


Fig. 3. Effects of As-IV on oxidative stress induced by BLM. Data were shown as mean  $\pm$  SD. \* $P$  < 0.05 vs. control group, # $P$  < 0.05 vs. BLM group.

esis of pulmonary fibrosis [18–21]. Following BLM administration, inflammatory responses were stimulated. Our data showed that total cells, macrophages, and lymphocytes in BALF were significantly raised after BLM injection. These inflammatory cells stimulate the proliferation of fibroblasts and the activation of myofibroblasts gradually resulting in pulmonary fibrosis [22]. We also found that As-IV reduced these cells counts in BALF in a dose-dependent manner. In addition, the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in BALF were enhanced following BLM treatment. TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 play important roles in pulmonary fibrosis, which induces stimulates the proliferation of

fibroblasts, increasing collagen aggregation and synthesis of other cytokines [23, 24]. Our study noted that As-IV significantly prevented BLM-induced elevation of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in a dose-dependent manner. Accumulating evidence has indicated that oxidative stress is a possible molecular mechanism underlying fibrosis in a variety of organs [25], and it is an imbalance between the generation of ROS and the capacity to detoxify these intermediates [26]. Accordingly, in the present study, oxidative stress was evaluated by detecting MDA, SOD, T-AOC, and ROS levels in lung tissue. MDA level reflects the degree of organic lipid peroxidation, which indicated the severity of





**Fig. 4.** Effects of As-IV against BLM-induced pathological changes. **a** Control group; **b** BLM group; **c** BLM + As-IV<sub>10</sub> group; **d** BLM + As-IV<sub>20</sub> group; **e** BLM + As-IV<sub>50</sub> group (He×200).

damage to cell membranes [27]. SOD is an important component of the antioxidant enzymatic defense system and T-AOC reflects the overall cellular endogenous antioxidative capability [28]. It was shown that in the BLM group, MDA and ROS levels were significantly elevated while SOD and T-AOC levels were reduced. In contrast, treatment with As-IV remarkably decreased the levels of MDA and ROS, and simultaneously increased the levels of SOD and T-AOC. Those results suggested that As-IV could significantly decrease BLM-induced inflammation and oxidative stress, which might be important protective mechanisms against pulmonary fibrosis induced by BLM.

There are some limitations of this study. Further studies are required to investigate alternative and additional mechanisms.

## CONCLUSIONS

The present study has shown that As-IV inhibited BLM-induced pulmonary fibrosis in rats. This protection is associated with anti-inflammatory and antioxidative properties of As-IV. The present findings suggest a potential therapeutic usefulness of As-IV on pulmonary fibrosis.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Ethics Statements.** All animal use procedures were approved by the Committee on the Ethics of Animal Experiments of Yantai Hospital of Traditional Chinese Medicine. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Conflict of Interest.** The author declares no conflict of interest.

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