



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Neferine, a bisbenzylisoquinoline alkaloid attenuates bleomycin-induced pulmonary fibrosis

Libo Zhao, Xiaomin Wang, Qing Chang, Junying Xu, Ying Huang, Qiuyun Guo, Shaohui Zhang, Wenquan Wang, Xiujuan Chen, Jialing Wang*

Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

In this study, we evaluated the potential anti-fibrotic property of neferine, a bisbenzylisoquinoline alkaloid extracted from the seed embryo of *Nelumbo mucifera* Gaertn. Intratracheal bleomycin administration resulted in pulmonary fibrosis 14 and 21 days posttreatment, as evidenced by increased hydroxyproline content in bleomycin group ($255.77 \pm 97.17 \mu\text{g}/\text{lung}$ and $269.74 \pm 40.92 \mu\text{g}/\text{lung}$) compared to sham group ($170.78 \pm 76.46 \mu\text{g}/\text{lung}$ and $191.24 \pm 60.45 \mu\text{g}/\text{lung}$), and the hydroxyproline was significantly suppressed ($193.07 \pm 39.55 \mu\text{g}/\text{lung}$ and $201.08 \pm 71.74 \mu\text{g}/\text{lung}$) by neferine administration (20 mg/kg, b.i.d.). The attenuated-fibrosis condition was also validated by histological observations. Biochemical measurements revealed that bleomycin caused a significant decrease in lung superoxide dismutase (SOD) activity, which was accompanied with a significant increase in malondialdehyde (MDA) levels and myeloperoxidase (MPO) activity on the 7th and 14th days. However, neferine reversed the decrease in SOD activity as well as the increase in MDA and MPO activity. Enzyme-linked immunosorbent assay and radio-immunity assay showed that treatment with neferine alleviated bleomycin-induced increase of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , interleukin (IL)-6 and endothelin-1 in plasma or in tissue. Additionally, neferine blocked bleomycin-induced increases of NF- κ B in nuclear extracts and TGF- β ₁ in total protein extracts of murine RAW264.7 macrophages. In summary, neferine attenuates bleomycin-induced pulmonary fibrosis *in vitro* and *in vivo*. The beneficial effect of neferine might be associated with its activities of anti-inflammation, antioxidation, cytokine and NF- κ B inhibition.

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1. Introduction

Pulmonary fibrosis is a chronic and progressive lung disease with an average survival of 3 years from the onset of dyspnoea (Douglas et al., 2000; Ota, 2007). The disease can be idiopathic or developed as a complication of many respiratory and systemic diseases and be characterized by excessive deposition of extracellular matrix proteins within the pulmonary interstitium, leading to impaired gas transfer and respiratory failure. Although the etiology of pulmonary fibrosis has not been clearly clarified yet, inflammation, oxidative stress and injuries from cytokines are definitely involved in pathogenesis (Carter and Driscoll, 2001; Mastruzzo et al., 2002; Fubini and Hubbard, 2003; Hoshino et al., 2003; Hartl et al., 2005; Mizuguchi et al., 2006; Cuzzocrea et al., 2007). Inflammation is the initial response following injury. Activated inflammatory cells such as neutrophils and macrophages accumulate in the lower airways, releasing harmful amounts of reactive oxygen species and a variety of harmful cytokines and growth factors that regulate the proliferation, chemotactism, and

secretory activity of alveolar fibroblasts in alveolar wall. The activated fibroblasts then produce increasing amounts of matrix proteins, distorting the normal lung architecture. On the basis of these concepts, targeted inhibition of inflammation, oxidative stress and cytokine release represent possible strategic points for therapeutic intervention.

Up to now there has been no satisfactory treatment for pulmonary fibrosis. Corticosteroids continue to be the primary mode of treatment recommendations for this disorder (Selman et al., 2005; Daniels and Ryu, 2006; Dury, 2006; Raghu, 2006). Despite the potentially favorable effects of these drugs on several inflammatory processes, only 15–30% of patients with pulmonary fibrosis could benefit from them. Thus the development of effective agents to ameliorate pulmonary fibrosis is urgently needed (Daba et al., 2004). Intratracheal administration of bleomycin is the most widely used experimental model of lung fibrosis, since the pathology in rodents is very similar to human. Recently, many agents, for example, pirfenidone (Antoniu, 2006), PG490-88 (Krishna et al., 2001), LLDT-8 (Ren et al., 2007), taurine and niacin (Giri, 2003) were investigated with this model. The origins of these agents are different but they exert similar anti-fibrotic effects due to their similar antioxidant activity and inhibitory effect of harmful cytokine.

* Corresponding author.

E-mail address: wangtjmu@hotmail.com (J. Wang).

Our laboratory has consistently investigated the protective effects of traditional Chinese medicine on pulmonary fibrosis and demonstrated that isoliensinine, a bisbenzylisoquinoline alkaloid isolated from the seed embryo of *Nelumbo mucifera* Gaertn, abates the accumulation of collagen of the lung in bleomycin- or paraquat-induced pulmonary fibrosis models (Xiao et al., 2005). Neferine is another bisbenzylisoquinoline alkaloid (Fig. 1) extracted from the same seed embryo, *N. mucifera* Gaertn, and the amount of neferine is three times of isoliensinine in the seed. Previous studies have documented that neferine has extensive pharmacological effects in the cardiovascular system (Li et al., 1988; Yu and Hu, 1997; GuoZhibin and CaoHongyu, 2002; Qian, 2002; Wei et al., 2005). In recent years, it has been reported that neferine inhibits the proliferation of vascular smooth muscle cells (VSMCs) (Caiying and Qutang, 2006) and hypertrophic scar fibroblasts (Lizhong and Guohui, 2002). Moreover, when neferine is locally injected into the nude mice with scars, it could help reduce the volume of the scars, decrease the contents of collagen and acidic mucopolysaccharide (Lizhong et al., 2003). A pharmacokinetic study showed that the distribution to the lung is the highest of all the tissues after an oral administration of 50 mg/kg of neferine to rats (Huang et al., 2007). Thus the present investigation was to test whether neferine has an effect on bleomycin-induced fibrosis, which is associated with the attenuation of inflammation, oxidative stress and injuries from cytokines.

2. Materials and methods

2.1. Animals and drugs

With approval by the Institutional Care Investigation Committee, about 120 Kunming mice weighing 20 to 25 g provided by the Department of Experiment Animals of Tongji Medical College of Huazhong University of Science and Technology were housed in the animal laboratory of the Department of Pharmacology. A 12 h light/dark cycle was maintained and the temperature was controlled 23 ± 2 °C. All mice were acclimatized to their new surroundings for 1 week prior to experimental procedures.

Bleomycin Hydrochloride purchased from Nippon Kayaku Co., Ltd was dissolved in sterile 0.9% saline on the day of intratracheal instillation at a dose of 0.1 mg in 0.05 mL solution per mouse. Neferine was extracted from the seed embryo of *N. mucifera* Gaertn by the Phytochemistry Laboratory of the Department of Pharmacology, Tongji Medical College of Huazhong University of Science and Technology. The quality of neferine was tallied with monomer standard stipulated by the National Pharmacopoeia of China (FW = 625, purity >98% by HPLC).

2.2. Animal model of bleomycin-induced pulmonary fibrosis

A single-dose bleomycin-mouse model of acute lung injury that eventuates into fibrosis has been previously established in our laboratory and the same model was used in the present study. Briefly, after body weights were recorded, mice were anesthetized via intraperitoneal injection of 40 mg/kg pentobarbital sodium solution. The skin and subcutaneous tissue overlying the proximal portion of the trachea were exposed by a 5 mm transversal incision to allow

insertion into the trachea of a needle containing the instilled solution. The mice were shaken to facilitate distribution of the bleomycin solution or saline throughout the lung. The incision was closed by a single suture. Then the mice were randomized into 4 groups as sham (treated with saline), bleomycin, neferine (20 mg/kg, b.i.d), and pirfenidone (100 mg/kg, b.i.d). Saline, neferine or pirfenidone was administered with intragastric administration on the same day. Here, pirfenidone, a generally accepted anti-fibrotic agent was used as a positive control. We chose these doses of neferine and pirfenidone according to the results of preliminary experiments or therapeutic dose in clinical trial (Iyer et al., 2000; Card et al., 2003a; Kakugawa et al., 2004; Tian et al., 2006). For each of the forgoing three groups (about 36 mice in each group), animals were divided into three subgroups, which were sacrificed on the 7th, 14th or 21st day after intratracheal treatment, individually. For the pirfenidone group (18 mice), animals were sacrificed on the 21st day posttreatment. Plasma and lungs were isolated and prepared for various measurements.

2.3. Pathological examinations

The lungs were fixed in 4% paraformaldehyde for 24 h and then processed for paraffin embedding. Sequential 4 μ m sections of the lungs were stained with routine hematoxylin–eosin for general morphology and stained with Masson's trichrome for evaluation of fibrosis under a photomicroscope (Olympus BX51 Tokyo, Japan). Each successive field was individually assessed for the severity of interstitial fibrosis using the semiquantitative grading system described by Ashcroft et al. (1988). The grade of pulmonary fibrosis was scored in a blinded fashion by examining 30 randomly chosen regions per sample at a magnification of 100 \times . A score ranging from 0 (normal lung) to 8 (total fibrosis) was assigned. The major criteria for grading pulmonary fibrosis included inflammatory cell infiltration, edema, interstitial thickening of alveolar or bronchiolar walls, and collagen deposition, as follows. Grade 0 = normal lung; Grade 1 = minimal fibrous thickening of alveolar or bronchial walls; Grades 2–3 = moderate thickening of walls without obvious damage to lung architecture; Grades 4–5 = increased fibrosis with definite damage to lung architecture and formation of fibrous bands or small fibrous mass; Grades 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 mean score of all fields was taken as the fibrosis score of that lung section.

2.4. Hydroxyproline assay

To estimate the total amount of collagen as an indicator of pulmonary fibrosis, the hydroxyproline content of the lungs was measured by a spectrophotometric assay according to the procedure described by Woessner (JF, 1961). Briefly, lungs were homogenized in saline with a polytron homogenizer at a ratio of 1:9 (weight:volume). Each sample (0.5 mL) was then digested in 3 mL of 6 mol/L HCl for 18 h at 110 °C. The samples were neutralized to pH 7.0 with NaOH. Then 1 mL of 0.5 mol/L chloramine T reagent was added in the sample and the samples were left at 37 °C for 20 min. Next, 1 mL of 3.15 mol/L perchloric acid and P-dimethylaminobenzaldehyde were added to each sample, and the samples were incubated for 10 min at 80 °C. Finally, the samples were cooled for 10 min and read at 560 nm on a spectrophotometer. Trans-4-hydroxy-L-proline (10 μ g/mL) was used as a standard solution. The content of hydroxyproline was expressed as μ g/lung.

2.5. Assay of MPO, SOD and MDA

Lung tissue samples were homogenized in cold saline at 4 °C. The tissue homogenate was 10% (w/v). Samples were centrifuged at 800 \times g for 10 min at 4 °C, and the supernatant was used to biochemical measurements.

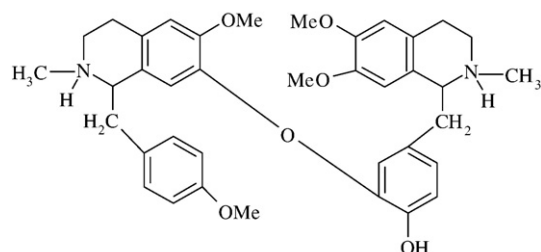


Fig. 1. Chemical structure of neferine.

Myeloperoxidase (MPO) activity, total (Cu–Zn and Mn) superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels in lung tissue were determined by the test kits provided by Nanjing Jiancheng Bioengineering Institute. MPO activity was determined using an *o*-dianisidine solution as the substrate for MPO-mediated oxidation by H₂O₂ and changes in absorbance at 460 nm were recorded with a micro plate reader (Bio-Tek ELX800uv, Bio-Tek Instrument Inc, Winooski, VT, USA). Data were presented as U/g tissue. For SOD, the principle of the method is based on the inhibition of nitro blue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as U/mg protein. For MDA, the principle of the method is based on the reaction of MDA with thiobarbituric acid at 90–100 °C. Levels of MDA were expressed as nmol/mg protein.

2.6. Assay of TNF- α , IL-6 and endothelin-1

TNF- α and IL-6 in plasma were assayed by specific enzyme-linked immunosorbent assay using commercially available ELISA test kits (Jingmei Biotech, China). The kit contains a TNF- α or IL-6 coat monoclonal antibody for a 96-well micro titer plate coating and immunomobilized mouse polyclonal antibody to TNF- α or IL-6. The representative standard curve was generated using the TNF- α or IL-6 standard with the kit. According to the manufacturer's data, the lower limit of detection for TNF- α and IL-6 with these assay system was 4 pg/mL.

Endothelin-1 in lung tissue was assayed by radio-immunity assay using the test kit provided by Dongya Institute of Immune Technology (Beijing, China). The sensitivity of the assay was 5 pg/mL.

2.7. Culture of murine RAW264.7 macrophages

The murine RAW264.7 macrophages were obtained from the cell repository of Wuhan University (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cell culture was performed in plastic tissue culture flasks and passaged by trypsin treatment, split 1:3 once or twice weekly, and used between 10 and 15 passages.

2.8. Assay of cell viability

Cell viability was evaluated using a colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), an indicator of mitochondrial respiratory chain activity. Briefly, the murine RAW264.7 macrophages were seeded onto a 96-well collagen-coated plate at a density of 5×10^4 cells per well. When the cells were nearly confluent (70%), the medium was replaced with DMEM containing 2% FBS. The cells were treated with 1 μ g/ μ L bleomycin and various concentrations of neferine (0.1, 0.3, 1, 3, 10, 30 and 100 μ mol/L), and then incubated for 24 h. After that 20 μ L of MTT (5 mg/mL) was added per well and incubated for 4 h. Finally the medium was removed and the formazan crystals were solubilized with 150 μ L dimethyl sulphoxide (DMSO). The optical density of each well was measured at 490 nm. The results are based on the cleavage of tetrazolium salt by viable cells that were proportional to the number of living cells in the wells.

2.9. Western blot assay

The murine RAW264.7 macrophages were treated with various concentrations of neferine (1, 3 and 10 μ mol/L) and bleomycin (1 μ g/

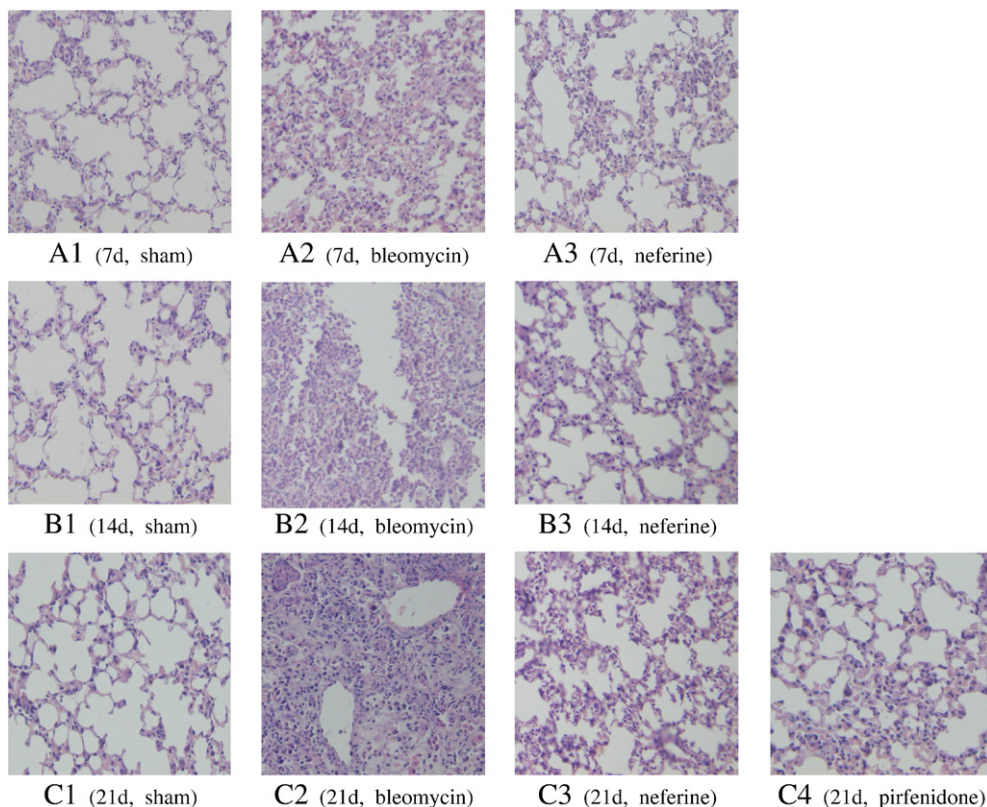


Fig. 2. Effect of neferine on histological change of lung tissue in mice induced by bleomycin. See Animal model of bleomycin-induced pulmonary fibrosis for details. Stained with hematoxylin–eosin, 100 \times .

μL), and then incubated for 24 h. For total protein extracts, 1×10^7 cells were washed twice in cold PBS, lysed with ice-cold whole cell lysis buffer (mmol/L): 150 NaCl, 1 EGTA, 1 NaF, 50 Tris-HCl (pH 7.4), 1% NP-40, 0.1 NaN_3 , 0.5 $\mu\text{g/mL}$ Leupeptin, 0.7 $\mu\text{g/mL}$ Pepstatin A, 50 $\mu\text{g/mL}$ PMSF, and 2.2 $\mu\text{g/mL}$ aprotinin. Lysates were centrifuged at $12,000 \times g$ at 4°C for 15 min, and the supernatant were stored at -20°C until use. For nuclear protein extracts, 1×10^7 cells were pelleted and resuspended in 100 μL cold hypotonic buffer (m mol/L): 10 HEPES, pH 7.9, 10 KCl, 0.1 EDTA, 1.5 MgCl_2 , 1 DTT, 0.6% NP-40, 0.5 PMSF and 1 mg/L A.P.L. Following 15 min incubation on ice, the cells were lysed by adding 25 μL of 10% NP-40 and centrifuged at $7200 \times g$ for 5 min at 4°C . The pellets were resuspended in 50 μL cold hypertonic buffer (m mol/L): 20 HEPES, pH 7.9, 420 NaCl, 1.2 MgCl_2 , 1 EDTA, 1 DTT, 1 PMSF, 1 mg/L A.P.L. and 25% glycerol, and stirred gently at 4°C for 30 min and centrifuged at $15,800 \times g$ for 10 min at 4°C . The supernatant was stored at -20°C until used. Protein concentrations were determined with BCA method (Beyotime Institute of biotechnology, China). 50 μg nuclear extracts or total extracts were separated on 10% SDS-poly-acrylamide gels and transferred to nitrocellulose (NC) membranes. The membranes were immediately blocked with 5% nonfat milk protein in TBST buffer for 1 h at room temperature, and then incubated with primary antibodies against NF- κB and TGF- β_1 (BioVision, USA) at a dilution of 1:200 at 4°C overnight and then incubated for 1 h at room temperature with

sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies (Protein Tech Group, Inc.) at a dilution of 1:5000. The membranes were visualized by a chemiluminescence reagent (ECL, Pierce Biotechnology, Inc.), and the image was obtained by exposure to X-ray films. β -Actin or lamin B1 (Santa Cruz Biotechnology, Inc) was used to normalize the quantity of the protein on the blot.

2.10. Statistical analysis

Data are expressed as means \pm S.D. Statistical analysis was carried out by analysis of variance (ANOVA) followed by appropriate post hoc tests including multiple comparison tests (LSD). All analyses were made using SPSS 11.5 statistical software package and probability values of less than 0.05 were considered statistically significant.

3. Results

3.1. General observation and histopathologic findings

Administration of bleomycin (0.1 mg, intratracheally) affected neither body and lung weights nor lung weight/body weight ratio of treated mice. Similarly, treatment with neferine (for 7, 14 or 21 days) or pirfenidone (for 21 days) did not significantly alter the aforementioned parameters.

Lung tissue sections from the sham group (Figs. 2 A1, B1, C1 and 3 A) displayed normal structure and no pathologic changes under a light microscope. On the 7th and 14th day posttreatment, severe edema and large numbers of inflammatory cells infiltration were found, and small fibrous areas emerged in the bleomycin group mice (Fig. 2 A2 and B2), while mice of the neferine group (Fig. 2 A3 and B3) showed less inflammation and less fibrosis at the two time points. On the 21st day, the bleomycin group (Figs. 2 C2 and 3 B) showed marked histopathologic changes, such as large fibrous areas and collapsed alveolar spaces. Although fibrotic lesions were observed in the neferine (Figs. 2 C3 and 3 C) and pirfenidone (Figs. 2 C4 and 3 D) groups, the extent of fibrosis was much less severe than that of the bleomycin group (Figs. 2 C2 and 3 B).

3.2. Effect on hydroxyproline content

The accumulation of extracellular collagen is the hallmark of pulmonary fibrosis. The quantity of hydroxyproline is an efficient index of collagen deposition, since collagen contains significant amount of this amino acid. The effect of neferine on the hydroxyproline content of lung homogenate is present in Fig. 4. On the 7th day, tissue hydroxyproline in the bleomycin group mice was higher than that in the sham group mice, but this difference did not reach a statistically significant level. After 14 and 21 days, the hydroxyproline content in the

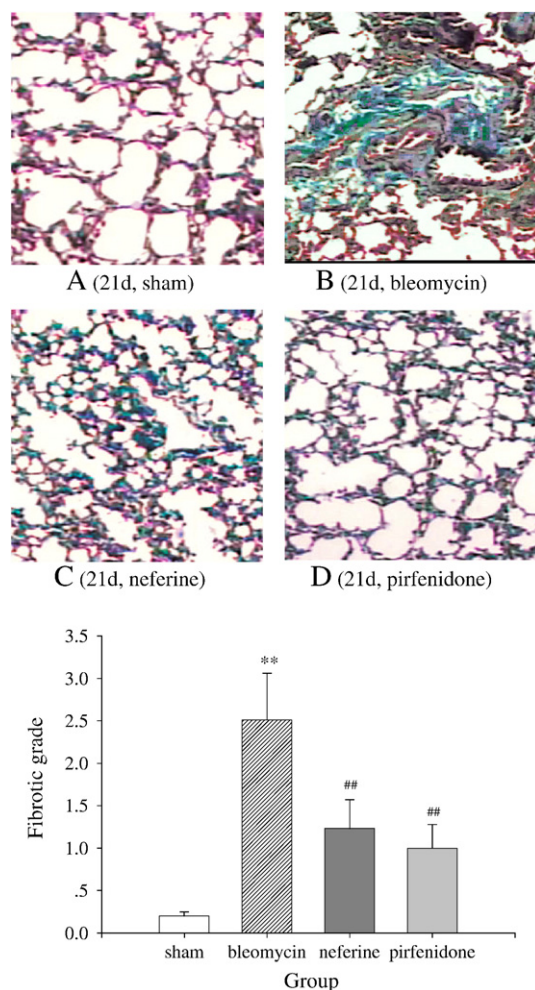


Fig. 3. Effect of neferine on histological change of lung tissue in mice induced by bleomycin. See Animal model of bleomycin-induced pulmonary fibrosis for details. Stained with Masson's trichrome, 100 \times . The Ashcroft scale was used for quantitative analysis of fibrotic grades induced by bleomycin. Results are presented as means \pm S.D. $n = 11$ –18. ** $P < 0.01$, compared with the sham group; ## $P < 0.01$, compared with the bleomycin group.

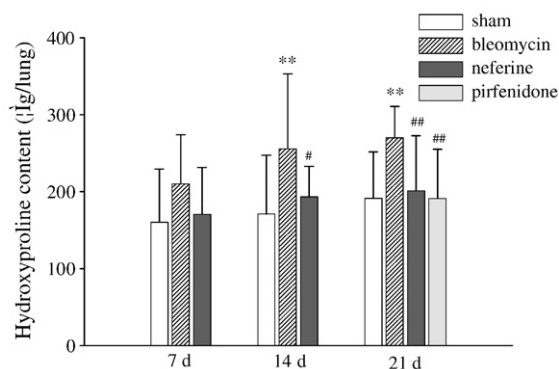


Fig. 4. Effect of neferine on hydroxyproline contents of lung tissue in mice induced by bleomycin or saline. See Animal model of bleomycin-induced pulmonary fibrosis for details. Results are presented as means \pm S.D. For each group, $n = 10$ –18. ** $P < 0.01$ compared with the sham group; # $P < 0.05$, ## $P < 0.01$ compared with the bleomycin group.

lungs increased from $170.78 \pm 76.46 \mu\text{g}/\text{lung}$ and $191.24 \pm 60.45 \mu\text{g}/\text{lung}$ in the sham groups to $255.77 \pm 97.17 \mu\text{g}/\text{lung}$ and $269.74 \pm 40.92 \mu\text{g}/\text{lung}$ in the bleomycin groups ($P < 0.01$). Lung hydroxyproline content in the pirfenidone group was significantly decreased to $191.13 \pm 63.85 \mu\text{g}/\text{lung}$ on the 21st day ($P < 0.01$, versus the bleomycin group). Similarly, lung hydroxyproline content in the neferine groups was reduced to $193.07 \pm 39.55 \mu\text{g}/\text{lung}$ and $201.08 \pm 71.74 \mu\text{g}/\text{lung}$ ($P < 0.05$ or $P < 0.01$, versus the bleomycin group), indicating beneficial effect of neferine against bleomycin-induced pulmonary fibrosis.

3.3. Influence on inflammation and oxidant stress

As shown in Fig 5, MPO content, a marker of neutrophil influx in tissue, was increased significantly in mice induced by bleomycin alone, which was 2.7, 2.2 and 1.8 times of that in the sham groups on the 7th, 14th and 21st days respectively. The increase of MPO activity in lung tissue produced by bleomycin was significantly prevented by neferine (20 mg/kg, b.i.d) on the 7th and 14th days ($P < 0.05$ or $P < 0.01$). There was also a decrease of MPO activity on the 21st day

posttreatment in the mice of the neferine group, but the difference did not reach statistical significance.

Intratracheal instillation of bleomycin produced a significant decrease in the SOD activity in the lung tissue after 7 and 14 days intratracheal treatment (75.18 ± 13.89 and 85.28 ± 20.80 U/mg protein) when compared with the sham groups (101.36 ± 16.31 and 114.58 ± 34.78 U/mg protein) respectively ($P < 0.05$ or $P < 0.01$). Neferine treatment significantly prevented the depletion in SOD induced by bleomycin (102.62 ± 27.44 and 116.27 ± 42.71 U/mg protein, $P < 0.05$ or $P < 0.01$). On the 21st day, the SOD activity in bleomycin group was reverted to the level of sham group.

Bleomycin intratracheal administration also produced a significant increase in the lung MDA levels (1.99 ± 0.40 and 2.15 ± 0.56 nmol/mg protein) on the 7th and 14th day post intratracheal treatment when compared with the sham groups (1.44 ± 0.40 and 1.63 ± 0.55 nmol/mg protein, $P < 0.05$). Neferine treatment significantly prevented the increase induced by bleomycin on the 7th day (1.50 ± 0.63 nmol/mg protein, $P < 0.05$). There was a tendency of decrease on the 14th day in the neferine group, but there was no statistical significance versus the

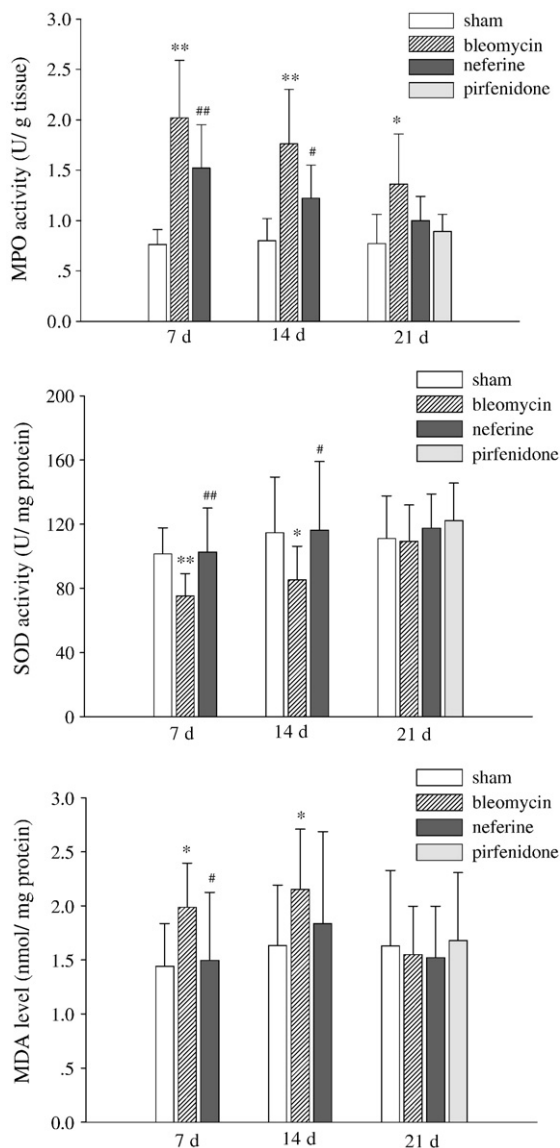


Fig. 5. Effect of neferine on MPO activity, SOD activity and MDA levels in lung tissue in mice induced by bleomycin or saline. See Animal model of bleomycin-induced pulmonary fibrosis for details. Results are presented as means \pm S.D. For each group, $n = 10$ –18. * $P < 0.05$, ** $P < 0.01$ compared with the sham group; # $P < 0.05$, ## $P < 0.01$ compared with the bleomycin group.

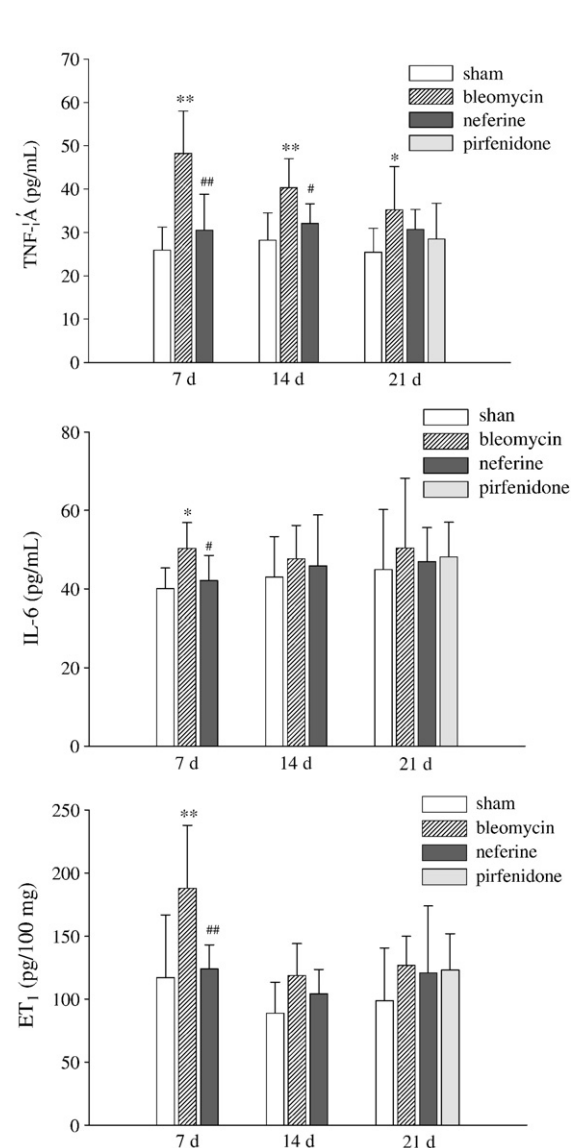


Fig. 6. Effects of neferine on TNF- α , IL-6 in plasma and ET₁ levels in lung tissue in mice treated with bleomycin or saline. See Animal model of bleomycin-induced pulmonary fibrosis for details. Results are presented as means \pm S.D. For each group, $n = 10$ –18. * $P < 0.05$, ** $P < 0.01$ compared with the sham group; # $P < 0.05$, ## $P < 0.01$ compared with the bleomycin group.

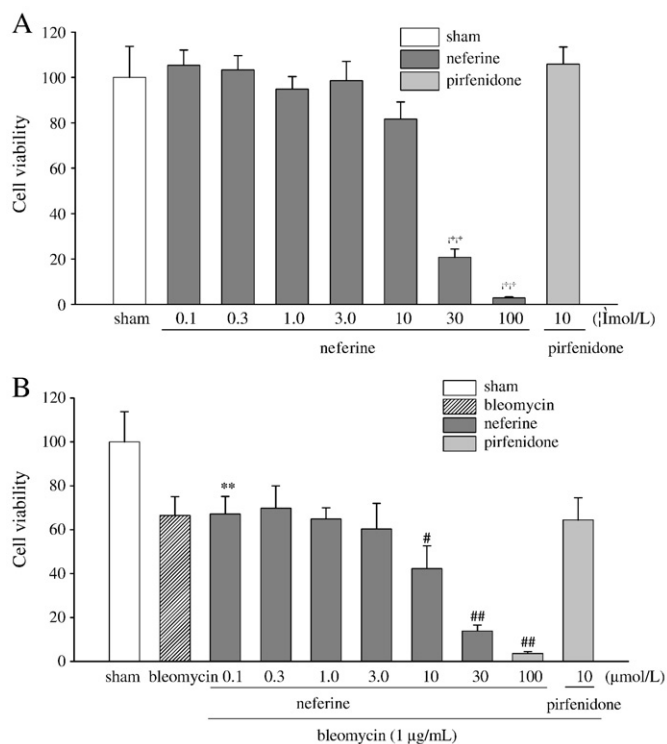


Fig. 7. Effect of neferine on the viability of murine RAW264.7 macrophages after 24 h incubation, A, without bleomycin; B, bleomycin was treated with bleomycin simultaneously except the sham group. See Assay of cell viability for details. Results are presented as means \pm S.D. For each group, $n = 12$. $\Delta\Delta P < 0.01$, $**P < 0.01$, compared with the sham group; $#P < 0.05$, $##P < 0.01$, compared with the bleomycin group.

bleomycin group. Just like SOD activity, the MDA level in bleomycin group came back to the basal level of the sham group after 21 days.

3.4. Effect on levels of TNF- α , IL-6 and endothelin-1

The TNF- α protein levels in plasma from mice in bleomycin groups remained elevated persistently from the 7th to 21st day compared with the sham groups, and significant increases occurred at all the three time points ($P < 0.05$ or $P < 0.01$). Treatment with neferine decreased bleomycin-induced increases of TNF- α levels at all time points but significant decreases occurred on the 7th and 14th days compared with the bleomycin groups at the corresponding time points ($P < 0.05$ or $P < 0.01$). Compared with the sham groups, the IL-6 protein levels in plasma were increased in bleomycin groups after intratracheal instillation of bleomycin but significant increases occurred only on the 7th day ($P < 0.05$) and treatment with neferine decreased the increase of IL-6 at that time point ($P < 0.05$).

As shown in Fig. 6, endothelin-1 expression in the bleomycin group mice on the 7th day was significantly enhanced (187.97 ± 49.90 pg/100 mg protein), compared with that in the sham group (117.01 ± 49.91 pg/100 mg protein, $P < 0.01$). Endothelin-1 level in neferine group (20 mg/kg) showed obvious decrease (123.98 ± 18.91 pg/100 mg protein), compared with that in the bleomycin group ($P < 0.01$), indicating beneficial effect of neferine on lung injury.

At other time points, there was no difference on the endothelin-1 levels between the bleomycin group and the sham group.

3.5. Effect on cell proliferation

Shown in Fig. 7, bleomycin (1 μ g/mL) exhibited significant cell toxicity after 24 h incubation. However, neferine (0.1–10 μ mol/L) alone did not decrease the cell viability, and neferine (0.1–3 μ mol/L) did not increase the toxicity caused by bleomycin. Based on these screening

results, we selected concentrations (1–10 μ mol/L) of neferine that did not cause significant effects on cell viability for Western blot analysis.

3.6. Effect of neferine on NF- κ B and TGF- β ₁ expressions

NF- κ B is known to be activated in constitutive manner during lung fibrosis. To detect whether the anti-fibrosis effect of neferine was due to the alteration of NF- κ B expression, Western blot was carried out to detect the change of the NF- κ B protein levels. Because NF- κ B functions in nucleus, nuclear extracts from treated and untreated murine RAW264.7 macrophages were used for the analysis. As can be seen in Fig. 8A, NF- κ B showed a 65 kDa band, and NF- κ B expression was

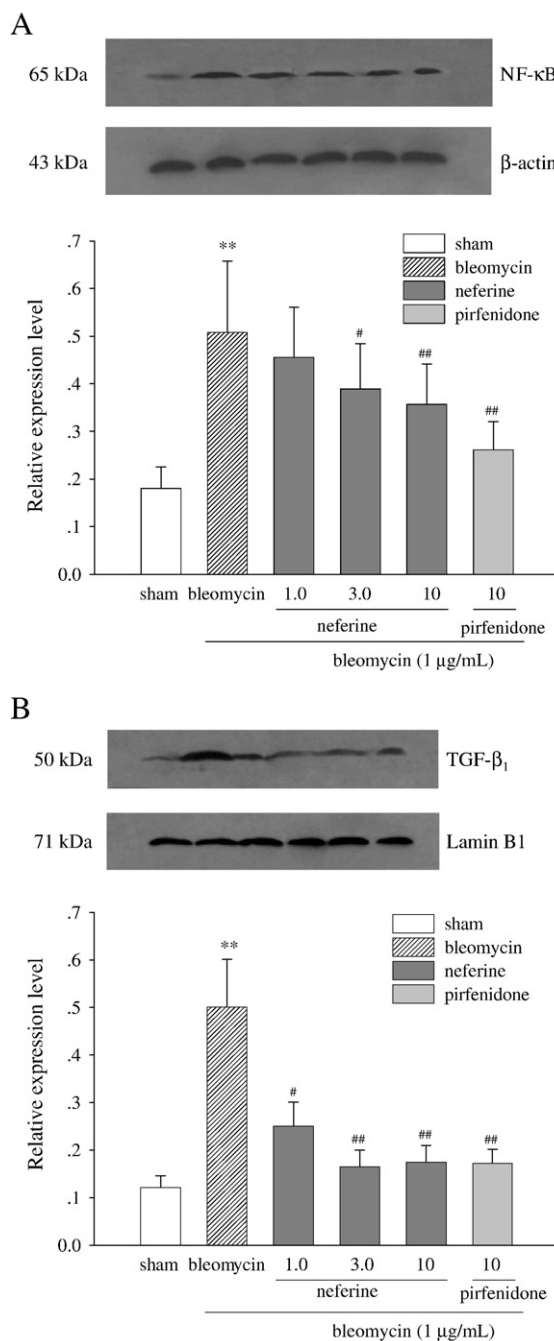


Fig. 8. Inhibitory effect of neferine on the expression of NF- κ B and TGF- β ₁ in murine RAW264.7 macrophages (A for the NF- κ B, and B for TGF- β). See Western blot assay for details. Results are presented as means \pm S.D. For each group, $n = 3$. $**P < 0.01$, compared with the sham group; $#P < 0.05$, $##P < 0.01$, compared with the bleomycin group.

increased by bleomycin significantly after incubation for 24 h ($P < 0.01$). However, the enhanced effect of bleomycin on the expression of NF- κ B can be attenuated by neferine in a dose-dependent manner ($P < 0.05$ or $P < 0.01$). Pirfenidone at 10 μ mol/L produced a similar inhibitory effect ($P < 0.01$).

TGF- β_1 is known to play a central role in the pathogenesis of pulmonary fibrosis. To determine the effect of neferine on TGF- β_1 , we assayed TGF- β_1 activity in bleomycin-treated murine RAW264.7 macrophages. The results in Fig. 8B show that TGF- β_1 expression was significantly increased by bleomycin ($P < 0.01$), and neferine suppressed the expression in a dose-dependent manner ($P < 0.05$ or $P < 0.01$), while pirfenidone at 10 μ mol/L inhibited such change ($P < 0.01$).

4. Discussion

It is reported that the histological hallmarks, such as inflammatory response and excessive deposition of extracellular matrix proteins within the pulmonary interstitium are present in bleomycin-treated animals similar to idiopathic pulmonary fibrosis patients (Usuki, 1995). This observation has led to the assumption, that bleomycin reproduces typical features of the human disease. Moreover, the advantage of bleomycin model is quite easy to perform, and therefore fulfills important criteria expected from a good animal model. Hence, the use of this model has become very popular. Recently, Chaudhary *et al.* reported that the “switch” between inflammation and fibrosis appears to occur around day 9 after bleomycin intratracheal administration (Chaudhary *et al.*, 2006). Thus this study selected three time points (7, 14 and 21 days post bleomycin administration) in bleomycin-induced fibrosis model to assess the potential anti-fibrotic effect of neferine.

Inflammatory response is the initial response following injury challenge of bleomycin. The inflammatory response is mediated partially by endogenous and migrating leukocytes. These activated leukocytes can synthesize and secrete various cytokines, chemokines, reactive oxygen species, and proteases, sustaining the injury/repair processes and leading to proliferation of mesenchymal cells and deposition of extracellular matrix protein. In the present study, administration of neferine to the mice significantly attenuated the bleomycin-induced histological changes such as neutrophils and other inflammatory cell infiltrations in the inflamed region. The decreased activity of MPO in neferine treated mice also supported these findings. All of these observations suggest that inhibitory effect of neferine on inflammation response may be one of the reasons of its anti-fibrotic effect.

A balance between intracellular and extracellular oxidants and antioxidants is a prerequisite for normal lung homeostasis. Impaired induction or inactivation/clearance of antioxidant enzymes may result in injury to the lung cells and matrix (Kinnula *et al.*, 2005; Reid *et al.*, 2007). Bleomycin is known to bind to DNA/Fe²⁺ and form a complex. This DNA/Fe²⁺/bleomycin complex undergoes redox cycling and generates reactive oxygen species such as superoxide and hydroxy radicals (Li *et al.*, 2002), causing directly injury to lung cells and matrix. Some antioxidants including N-acetylcysteine (Mata *et al.*, 2003; Yildirim *et al.*, 2005), erdosteine (Yildirim *et al.*, 2004; Yildirim *et al.*, 2005; Boyaci *et al.*, 2006), vitamin E (Bolt *et al.*, 2001; Card *et al.*, 2003b) and SOD can decrease collagen deposition and protect the lungs from oxidative damage in a variety of animal models or even in clinical trials. In this experiment, neferine served as a free-radical scavenger, enhancing SOD activity and inhibiting lipid peroxidation. We suppose that neferine has antioxidant activity because there is hydroxyl group in its structure. In a previous study (Huang *et al.*, 2007) we found that neferine is converted partially into liensinine, isoliensinine, desmethyl-liensinine and desmethyl-isoliensinine *in vivo*. Neferine and all the metabolites have one to three hydroxyl groups on their structure. In addition, hydrophobicity of the compound may be one of the interpretations.

In addition to direct injury, oxidants may also contribute to the development of pulmonary fibrosis by their effects on cytokines and

growth factors. One such mediator, TGF- β_1 is an important mediator and has a broad spectrum of activities in pulmonary inflammation, tissue repair, and fibrosis. TGF- β_1 can serve as a chemoattractant for fibroblasts and monocytes/macrophages and stimulate these cells to synthesize a number of pro-inflammatory and fibrogenic cytokines such as TNF- α , IL-6, and TGF- β_1 itself. Furthermore, TGF- β_1 is one of the most potent inducers of extracellular matrix production. At the same time, TGF- β_1 reduces the breakdown of collagen and other matrix proteins by inhibiting the generation of plasminogen activators, matrix metalloproteinase, and elastase, as well as by enhancing the expression of tissue inhibitors of metalloproteinases, plasminogen activator inhibitor-1 and -2. In this study, TGF- β_1 production in RAW 264.7 cells elevated after bleomycin treatment, as was consistent with previous investigations (Ogawa *et al.*, 2004; Wang *et al.*, 2006). While the increased TGF- β_1 production was potently suppressed by neferine in a dose-dependent manner. We infer that the reduced hydroxyproline production and lung injury by neferine was at least partially attributed to its inhibition of TGF- β_1 production.

TNF- α is another key cytokine in pulmonary fibrosis, which induces adhesion molecule expression, intensifies the recruitment of inflammatory cells into the lungs and augments synthesis of fibronectin, prostaglandin, and TGF- β_1 . Administration of the anti-TNF- α antibody is demonstrated to be beneficial to suppressing bleomycin-induced lung injury (Yara *et al.*, 2001), and now a phase II clinical trial of soluble TNF- α receptor (etanercept) is under way (Ask *et al.*, 2006; Walter *et al.*, 2006). In our study, neferine markedly inhibited TNF- α production, providing one possible mechanism of its protective effect against lung injury.

It is generally accepted that an aberrant host response to wound healing following alveolar epithelial cell injury is involved in pulmonary fibrosis. Endothelin-1 and IL-6 are thought to play profibrotic role in the wound healing (Sofia *et al.*, 1995; Ugucioni *et al.*, 1995; Morelli and Ferri, 1996). Further, a growing body of evidence has supported a mitogenic effect of endothelin on fibroblasts and demonstrated that endothelin can reduce collagen breakdown and induce the synthesis of extracellular matrix components, all contributing to fibrosis (Bois, 2007). In our study, neferine can decrease the increase of endothelin-1 and IL-6 induced by bleomycin, thus may be helpful to facilitate the wound healing following alveolar epithelial cell injury and alleviate pulmonary fibrosis.

Studies indicate that the regulation of harmful cytokines is mediated, at least partly by NF- κ B and its importance in the development and progression of pulmonary fibrosis has recently become widely recognized. The regulation of NF- κ B and its degradation are topics of contemporary interest, as many inducible genes that encode cytokines (such as TNF- α , TGF- β_1), chemokines, adhesion molecules, growth factors, enzymes, and transcription factors contain binding sites for NF- κ B within their promoter or enhancer regions. During lung injury, NF- κ B is activated persistently by inflammatory cells and reactive oxygen species, stimulating the transcription of a lot of harmful cytokines mentioned above, and ultimately promotes the proliferation of fibroblast. Therefore, NF- κ B can be taken as a potential target for the therapy of lung fibrosis. Since macrophage is one the fundamental cytokine secretion cells, the present study was undertaken to ascertain the anti-fibrotic effect of neferine on the RAW 264.7 cells, a murine macrophage line, taking NF- κ B as target. As described in the Results section, neferine decreased the NF- κ B protein levels in RAW 264.7 cell nucleus, i.e., neferine inhibited the nuclear translocation of NF- κ B, thus led to suppression of harmful cytokines and growth factors, therefore exerted its action of inhibiting the proliferation of fibroblasts. Our findings are in line with the findings of other *in vitro* or *in vivo* studies (Gunella *et al.*, 2006; Hsu *et al.*, 2006; Inayama *et al.*, 2006; Saadane *et al.*, 2007; Shishodia *et al.*, 2007; Zhang *et al.*, 2007) in which suppression of constitutive NF- κ B activation by certain herbal medicines or by genetic manipulation can inhibit growth, induce apoptosis, and enhance chemosensitization, therefore potentially be useful in the prevention

or treatment of pulmonary fibrosis. Our findings are also consistent with the prevailing theory that NF- κ B is an oxidant-sensitive transcription factor and its activation can often be prevented by antioxidants (Sun and Oberley, 1996).

In summary, the present study demonstrated that neferine could significantly prevent bleomycin-induced pulmonary fibrosis in mice due to its activities of anti-inflammation, antioxidation, cytokine and NF- κ B inhibition. Based on the findings we guess that neferine may be a promising candidate in the treatment of idiopathic pulmonary fibrosis.

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