Feitai Attenuates Bleomycin-Induced Pulmonary Fibrosis in Rats

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Pulmonary fibrosis is a common consequence of numerous pulmonary diseases. The current therapeutic approaches for this condition are unsatisfactory. Feitai, a composite formula consisting of several herbs, is used in China as a folk remedy for treating patients with pulmonary tuberculosis. In this study, we extensively investigate the effects and mechanisms of Feitai on bleomycin (BLM)-induced pulmonary fibrosis in rats. One hundred and twenty male Sprague–Dawley rats were randomly divided into four groups, referred to as the saline–water, saline–Feitai, BLM–water, and BLM–Feitai groups. Following a single instillation of BLM (5 mg/kg) or saline, rats were orally administered Feitai at a dose of 3 g/kg body weight or sterilized distilled water once daily. Rats were killed at 7, 14, or 28 d post-BLM. Inflammatory cell count, protein concentration, and lactate dehydrogenase activity in bronchoalveolar lavage fluid were measured, and myeloperoxidase activity and lipid peroxide content in lung homogenates were analyzed. Treatment with Feitai inhibited lung fibrotic progression induced by BLM, as indicated by the decrease in lung hydroproline content and lung fibrosis score at 28 d post-BLM. This was accompanied by significant amelioration of BLM-induced body weight loss, lung edema, and inflammatory response during the development of lung injury in the acute phase. The results strongly indicate the beneficial effects of Feitai in protecting against BLM-induced pulmonary fibrosis. Furthermore, the inflammatory response and lipid peroxidation were inhibited by Feitai, suggesting that the effect of this formula on BLM-induced lung injury and fibrosis is associated with antiinflammatory and antioxidant properties.

Key words Feitai; pulmonary fibrosis; bleomycin; rat

Pulmonary fibrosis model in rodents induced by bleomycin (BLM) is widely used to study the mechanism of lung fibrosis and antifibrotic effects of numerous drugs.1—11 Following intratracheal administration into the lungs of rats, BLM causes alveolar cell damage, inflammatory response, fibroblast proliferation and subsequent collagen content deposition. Lesions observed in the early stages of lung damage induced by BLM resemble chronic human fibrotic lung disease, both histologically and physiologically.12—20 Interventions designed to limit the consequences of the inflammatory response [such as corticosteroids, anti-tumor necrosis factor (TNF)-α antibody, and anti-transforming growth factor (TGF)-β antibody] and to protect the lung from oxidant damage (such as Ginkgo biloba extract,8 metalloporphyrin,9 and N-acetylcysteine10) are effective in suppressing BLM-induced pulmonary fibrosis. These findings suggest that inhibition of lung inflammation and lipid peroxidation may be employed as therapeutic strategies for pulmonary fibrosis in the clinic.

Feitai, a composite formula comprising the herbs Huangqin (Radix of Scutellaria baicalensis GEORG), Beishashen (Radix of Glehnia littoralis FR. SCHMIDT ex MIQ), Gualou (Fructus of Trichosanthes kirilowii MAXIM), Baibu (Radix of Stemona sussilfolia (MIQ.) MIQ), Pipaye (Folium of Eriobotrya japonica (THUNB.) LINDEL) commonly used in China as a folk remedy for the treatment of patients with pulmonary tuberculosis. The major ingredient of Feitai is Huangqin. The beneficial components of Huangqin are flavonoids that have multiple biological activities, such as antiinflammatory, antibacterial, antiviral, antiallergic, immune-stimulating, and antioxidant properties. The other ingredients of Feitai also have some therapeutic effects, such as antiinflammatory, antibacterial, antitussive, and antipyretic effects. In the present study, we extensively investigated the effects of Feitai on BLM-induced pulmonary fibrosis in rats. Moreover, changes in biochemical and histopathologic parameters following BLM instillation are characterized to elucidate the role of Feitai during the development of pulmonary fibrosis.

MATERIALS AND METHODS

Chemicals and Reagents Feitai, a prescription formula, is a mixture of the extracts of five medical herbs (Table 1). It was supplied by the Botany Department, Shanghai Institute of Materia Medica (batch no. 20030409). Each gram of Feitai is equivalent to 2.56 g of crude drug. For laboratory analyses, authentic plant materials were purchased from a local market and identified at Shanghai Institute of Materia

Table 1. Composition of Feitai

<table>
<thead>
<tr>
<th>Chinese name</th>
<th>Medicinal herb name</th>
<th>Dose amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huangqin</td>
<td>Radix of Scutellaria baicalensis GEORG</td>
<td>50</td>
</tr>
<tr>
<td>Beishashen</td>
<td>Radix of Glehnia littoralis FR. SCHMIDT ex MIQ</td>
<td>15</td>
</tr>
<tr>
<td>Gualou</td>
<td>Fructus of Trichosanthes kirilowii MAXIM</td>
<td>15</td>
</tr>
<tr>
<td>Baibu</td>
<td>Radix of Stemona sussilfolia (MIQ.) MIQ</td>
<td>10</td>
</tr>
<tr>
<td>Pipaye</td>
<td>Folium of Eriobotrya japonica (THUNB.) LINDEL</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
Medica, Shanghai, China. Voucher specimens were deposited in the Herbarium of Shanghai Institute of Materia Medica, Shanghai, China.

Bleomycin A2 hydrochloride was purchased from Tianjin Taihe Pharmaceutical Co. (China). Myeloperoxidase (MPO), lactate dehydrogenase (LDH), total protein, lipid peroxide (LPO), and hydroxyproline kits were obtained from the Nanjing Jiancheng Bioengineering Institute (China).

Animals  Pathogen-free mature male Sprague–Dawley rats (7—8 weeks old) were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China), which was approved by the Shanghai Animal Care and Use Committee (certificate no. SCXK (Shanghai) 2002-0010). Animals were maintained in a specific pathogen-free environment that was temperature-controlled (23±2°C) and humidity controlled (60±10%), under a 12:12-h light–dark cycle. Rats were administered water and a standard diet (Sino-British Sippr BK Lab Animal Ltd) ad libitum. The diet comprised 57.9% carbohydrate, 19—21% protein, 4—4.5% fat, 3—3.5% fiber, 0.9—1.2% calcium, and 0.6—0.8% potassium. All rats were acclimatized to their new surroundings for 1 week prior to experimental procedures. Experiments involving laboratory animals were performed at the Animal Department, Shanghai Institute of Material Medica, which was approved by the Shanghai Animal Care and Use Committee (certificate no. SYXX (Shanghai) 2002-0001).

Experimental Protocols  One hundred and twenty rats weighing 200—250 g were randomly divided into the following four groups: saline—water; saline—Feitai; BLM—water; and BLM—Feitai. The first two control groups were injected intratracheally with saline in a volume of 2 ml/kg body weight and treated with sterilized distilled water or Feitai 3 g/kg body weight orally. Rats in the other two groups were received sterilized distilled water or Feitai 3 g/kg body weight once daily after an intratracheal injection of BLM solution. According to the results from a preliminary dose-response study of Feitai, we selected the dose of 3 g/kg body weight for this experimental protocol.

Intratracheal injection was performed under light chloralhydrate (40 mg/kg body weight) anesthesia. Briefly, the trachea was exposed and punctured with a 27-gauge needle via a small cervical skin incision and separation of the strap muscles. BLM (5 mg/kg body weight, 2 ml/kg body weight) or saline was injected slowly. The day of intratracheal injection with BLM or saline was designated day 0. To investigate the time-course effect of Feitai, rats were euthanized 7, 14, or 28 d after BLM or saline administration. The lung vasculature was perfused free of blood by slowly injecting 50—80 ml of phosphate-buffered saline (PBS) into the right ventricle. The left lungs were removed from the trachea and hilar nodes and weighed. Half of the left lung was fixed in 4% phosphate-buffered paraformaldehyde for histopathologic preparation, while the other half was frozen in liquid nitrogen for homogenate preparation. Bronchoalveolar lavage fluid (BALF) was collected from the right lung as follows: PBS 3 ml was instilled three times and fluid was withdrawn from the right lung via a tracheal cannula. Routine recovery of BALF fluids did not differ significantly between animals with >70% of instilled volume recovered. The body weight of each animal was monitored every other day.

BALF Analysis  The total cell numbers and their proportion in BALF were examined immediately following lavage. The remainder of each sample was centrifuged at 1200×g for 15 min at 4°C. Cell pellets were smeared and stained with Giemsa solution for differential counting. Two hundred cells were counted for each sample and expressed as a percentage of the total cells recovered. The supernatant fractions were used to measure total protein concentration and LDH activity. The total protein concentration was determined using Coomassie brilliant blue G250 staining, and expressed as grams of protein per liter of BALF (g/l). LDH activity was assessed using a test reagent kit and determined as units per liter of BALF (U/l).

Lung Tissue Homogenate Preparation and Analysis  Lung tissue samples were homogenized in cold Tris–HCl buffered saline (pH 7.4, 0.01 mol/l Tris–HCl, 0.0001 mol/l EDTA–2Na, 0.01 mol/l saccharose, 0.8% sodium chloride solution) three times at 4°C with a polytron homogenizer (10 s of homogenization at 10-s intervals). The tissue homogenate was 10% (w/v). After removing 0.1 ml of homogenates for the MPO activity assay, samples were centrifuged at 3000×g for 10 min at 4°C, and the supernatant was used to measure LPO levels and collagen content.

MPO activity in the homogenates was assayed with a microplate reader (BIORAD Model 550) using the MPO test kit. Changes in absorbance at 450 nm were measured with the microplate reader. MPO activity is expressed as units per gram of tissue (U/g). One unit per gram of tissue is defined as MPO degrading 1 μmol peroxide at 37°C per gram of tissue.

LPO content was determined according to the thioarbituric acid method with the malondialdehyde test kit. Results are expressed as nmol LPO per gram of protein of lung tissue (nmol/g).

Collagen content of the lung is presented as the index of hydroxyproline concentration estimated using the test kit. Colored products were measured at 550 nm using the microplate reader. Data are expressed as micrograms of hydroxyproline per gram of protein in lungs (mg/g).

Histopathologic Evaluation  Rat lung tissues were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) and processed for routine paraffin embedding. Serial sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) and a modified Masson trichrome to assess the degree of fibrosis. The severity of alveolitis and fibrosis was blindly assessed semiquantitatively, according to criteria previously described by Szapie et al.21)

Statistical Methods  Data were entered into a database and analyzed using SPSS software. Group mean values and standard deviations were calculated. After homogeneic analysis, homogeneous data were analyzed with one-way analysis of variance and a post hoc test of least significant difference (LSD). To determine intergroup differences, heterogeneous data were analyzed using the independent samples t-test. Cell differential data in BALF and alveolitis and fibrosis scores of lung tissue were evaluated using the Mann-Whitney test. Differences in the severity of lesions between BLM–water and BLM–Feitai treated rats were evaluated for significance using Fisher’s exact test. Differences in the body weight between groups were assessed using the multivariate process. The statistical significance level was set at p<0.05.
RESULTS

Body Weight Changes The body weights of all treated rats were evaluated during the study period. Changes in body weight after BLM treatment are shown in Fig. 1. BLM–Feitai-treated rats displayed less body weight loss than rats treated with BLM alone.

Wet Weights and Relative Weights of Lungs The left lung was weighed and the relative weight (lung weight/body weight ×100%) was calculated for each animal. The increase in wet weight of the lung is one of the indices representing lung edema. As shown in Table 2, the wet and relative weights of the lung increased more than three-fold 7 d after BLM treatment and decreased gradually thereafter. The in-

![Fig. 1. Influence of Feitai on Body Weight Changes in Rats following BLM-Induced Lung Injury](image1)

![Fig. 2. Influence of Feitai on Total and Differential Cell Counts in BALF following BLM-Induced Lung Injury](image2)

Table 2. Effect of Feitai on Wet Weight and Relative Weight of the Left Lung in BLM-Induced Lung Injury and Fibrosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wet weight (g)</th>
<th>Relative weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7** 14 28</td>
<td>7 14 28</td>
</tr>
<tr>
<td>Saline–water</td>
<td>0.41±0.06 0.43±0.05 0.48±0.07</td>
<td>0.172±0.016 0.178±0.026 0.147±0.021</td>
</tr>
<tr>
<td>Saline–Feitai</td>
<td>0.45±0.07 0.46±0.08 0.54±0.12</td>
<td>0.170±0.012 0.178±0.025 0.152±0.024</td>
</tr>
<tr>
<td>BLM–water</td>
<td>1.22±0.21** 0.89±0.123** 0.97±0.41**</td>
<td>0.782±0.183** 0.547±0.164** 0.356±0.144**</td>
</tr>
<tr>
<td>BLM–Feitai</td>
<td>1.00±0.22**# (82%) 0.72±0.22**# (81%) 0.64±0.16* (66%)</td>
<td>0.564±0.133**# (82%) 0.342±0.149**# (81%) 0.217±0.066* (66%)</td>
</tr>
</tbody>
</table>

- **Days after intratracheal instillation of BLM. **p<0.01 vs. the relevant control groups, *p<0.05 vs. the relevant BLM–water-treated group.
- Percentage of the BLM–water-treated group.

Fig. 1. Influence of Feitai on Body Weight Changes in Rats following BLM-Induced Lung Injury

- ⊙, saline–water; ○, saline–Feitai; △, BLM–water; □, BLM–Feitai. **p<0.01 vs. the relevant control groups; *p<0.01 vs. the relevant BLM–water-treated group.

Fig. 2. Influence of Feitai on Total and Differential Cell Counts in BALF following BLM-Induced Lung Injury

- ⊙, saline–water; ○, saline–Feitai; △, BLM–water; □, BLM–Feitai. (A) Number of total cells; (B) percentage of macrophages; (C) percentage of neutrophils; (D) percentage of lymphocytes. **p<0.01 vs. the relevant control groups; *p<0.01 vs. the relevant BLM–water-treated group.
creased wet weight of the lung induced by BLM declined significantly at each time point \((p<0.05)\) following Feitai treatment, indicating that this compound attenuates lung edema induced by BLM.

**BALF Analysis** Total and differential cell counts in BALF at each time point are presented in Fig. 2. The total cell number was much higher in BLM-treated rats, compared with saline-treated rats at 7, 14, and 28 d \((p<0.01)\). A peak appeared at 7 d and was sustained up to 14 d, followed by a decrease. Administration of Feitai reduced the BLM-induced increase in total cell number in BALF during the experimental period. An increase in the percentage of polymorphonuclear leukocytes (neutrophils) and lymphocytes and a decrease in microphages were observed 7 and 14 d after BLM administration. There were no statistical differences in the proportion of cells in BALF between the BLM–water and BLM–Feitai groups at each time point. However, at all times, the absolute numbers of differential inflammatory cells were significantly reduced in the BLM–Feitai group compared with the BLM group (data not shown).

LDH activity in BALF represents the extent of damage to lung cells. LDH activity is a general marker of cell damage. Following BLM instillation, LDH activity was significantly increased in BALF and peaked at 7 d (Fig. 3A). Treatment with Feitai attenuated the BLM-induced increase in LDH activity in BALF, and normal levels were observed at 28 d. The data indicate that Feitai protects lung cells from damage induced by BLM.

Increased protein concentration in BALF is another important marker of alveolar edema in the acute phase. The protein concentration was significantly increased in BALF following BLM treatment. Protein levels peaked 7 d after BLM instillation and declined thereafter. Feitai treatment led to a reduction in the BLM-induced increase in protein content in alveoli, suggesting that the compound ameliorates lung edema (Fig. 3B).

**MPO Activity** MPO activity in lung tissue was measured to determine neutrophil sequestration, which reflects a severe degree of inflammation in the lung after BLM instillation. As shown in Fig. 4A, lung MPO activity rapidly increased following BLM instillation. High levels were observed 7 to 14 d post-BLM. Feitai significantly attenuated the BLM-induced increase in MPO activity at 7 and 14 d. There were no statistical differences at 14 and 28 d in MPO activity between the BLM–Feitai and the control groups. These results showed that treatment with Feitai relieved the early inflammatory response in the lung induced by BLM.

**LPO Content** The LPO content in lung was increased in the BLM–water group at each time point, peaking 7 d post-

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**Fig. 3.** Influence of Feitai on LDH Activity and Protein Content in BALF following BLM-Induced Lung Injury

- \(\square\), saline–water; \(\blacksquare\), saline–Feitai; \(\blacktriangle\), BLM–water; \(\blacksquare\), BLM–Feitai. (A) LDH activity; (B) protein content. \(* p<0.01\) vs. the relevant control groups; \(* p<0.05\) vs. the relevant BLM–water-treated group.

**Fig. 4.** Influence of Feitai on the Index of Lung Homogenates for BLM-Induced Biochemical Parameter Changes in Rats

- \(\square\), saline–water; \(\blacksquare\), saline–Feitai; \(\blacktriangle\), BLM–water; \(\blacksquare\), BLM–Feitai. (A) MPO activity; (B) LPO content; (C) hydroxyproline content. \(* p<0.05\), \(* * p<0.01\) vs. the relevant control groups; \(* p<0.05\) vs. the relevant BLM–water-treated group.
BLM and gradually declining thereafter, without reaching normal levels by the end of the study. Increased lipid peroxidation induced by BLM was consistent with the inflammatory changes in the lung. The LPO level in the BLM–Feitai group was lower than that in the BLM–water group (p < 0.05), as depicted in Fig. 4B.

**Hydroxyproline Content** The hallmark of fibrosis is collagen deposition. The measurement of hydroxyproline is an efficient index of fibrosis, since collagen contains significant amounts of the amino acid. The effect of Feitai on pulmonary fibrosis was assessed by evaluating the hydroxyproline content of lung homogenates 7, 14, and 28 d following BLM intratracheal administration (Fig. 4C). At 28 d, the hydroxyproline content of the lungs in the BLM–water group increased approximately two-fold compared with that in the control groups. Treatment with Feitai led to a significant reduction in the hydroxyproline content compared with that in the BLM–water group (p < 0.05), indicating the beneficial effects of the Feitai formula in protecting against pulmonary fibrosis induced by BLM.

**Histopathologic Findings** To elucidate the histopathologic changes associated with BLM-induced lung fibrosis and the efficacy of Feitai, left lungs from the saline–water, saline–Feitai, BLM–water and BLM–Feitai groups were collected 7, 14, and 28 d after BLM instillation. Sections were stained with H&E and Masson’s trichrome for collagen identification. Sections from the control groups displayed normal structure and no pathologic changes under a light microscope. In the BLM–water group at 7 d, severe edema, large amounts of inflammatory cells including neutrophils and lymphocytes in both alveoli and interstitium, and damaged endothelia and alveolar epithelia cells were observed (data not shown). Treatment with Feitai resulted in significant attenuation of these histopathologic findings. At 14 d post-BLM, foci of collagen deposition and fewer inflammatory changes were observed (data not shown). Furthermore, at 28 d, marked histopathologic changes, such as large fibrous areas, collapsed alveolar spaces, and traction bronchiectasis in the subpleural and peribronchial regions, were seen. Although fibrotic lesions were observed in the BLM–Feitai group, the extent of fibrosis was markedly less severe compared with that in the BLM–water group (Fig. 5).

To confirm the effects of Feitai on the histopathology of BLM-induced lung injury and fibrosis, the overall grades of inflammatory and fibrotic changes of the lungs were estimated by numerical scoring at 7, 14, and 28 d. The scores of alveolitis and fibrosis in the lung sections of the BLM–Feitai group were significantly decreased compared with those in the relevant BLM–water group (p < 0.01; Table 3).

**DISCUSSION**

An ideal therapeutic strategy for pulmonary fibrosis in the clinic has yet to be established. There is a vital need for an agent with antifibrotic properties and acceptable side effects.2–4,22) The acute toxicity data in our laboratory show that the maximum tolerated dose of oral Feitai is greater than 20 g/kg body weight in mice, consistent with the requirement for lower toxicity in long-term clinical treatment for pulmonary tuberculosis. Feitai may have the characteristics of a complex remedy, since the compound is prepared from several Chinese herbs.

Our present studies show that treatment with Feitai inhibits lung fibrotic progression induced by BLM, as determined by
The inflammatory response is the initial response following injury challenges in the lung. The response includes migration and activation of both resident and circulating inflammatory cells and the production of cytokines and growth factors. Chemoattractant agents derived from the injured lung tissue initially recruit inflammatory cells, including neutrophils, macrophages, and lymphocytes, into the alveolar space. Inflammatory cells release cytokines, chemokines and growth factors, such as interleukin (IL)-1 and -4, fibroblast growth factor (FGF)-β, TGF-β, TNF-α, gamma interferon (IFN)-γ, insulin-like growth factor (IGF)-1, heparin binding epidermal-like growth factor (HB-EGF), and platelet-derived growth factor (PDGF), and secrete matrix remodeling proteases, principally the matrix metalloproteinases (MMPs).\(^{23}\)

Meanwhile, injury causes increased permeability of the pulmonary epithelium and endothelium, resulting in extravasation of plasma proteins and ultimately extracellular matrix remodeling and fibrin deposition in the alveolar lumen and interstitium.\(^{11,24}\) Cytokines released by inflammatory cells are important for fibrogenesis (e.g., IL-6, TNF-α, IL-1, PDGF, and TGF-β)\(^{25}\). Those results suggest that inflammatory cell recruitment serves to propagate fibrosis. Similarly, the capacity to ameliorate fibrotic lesions is often associated with attenuation of inflammatory cell recruitment. The first line of therapy, specifically the use of corticosteroids and other immunosuppressive drugs, is based on this hypothesis. In the present animal model, treatment with Feitai significantly decreased BLM-enhanced levels of LPO, suggesting that some antioxidants present in the formula may be involved in the protective mechanism against pulmonary fibrosis.

In summary, our results demonstrate beneficial effects of injured/activated alveolar epithelial cells at the distinguishable early stage of alveolitis are an important factor in the progression of lung fibrosis.\(^{22,31,32}\) In the present analyses, BLM–water-treated rats exhibited sustained cell injury in the lung microenvironment. Treatment with Feitai attenuated cell damage triggered by BLM, as determined by the changes in LDH activity in BALF. Further studies are required to address the possibility that the anti-fibrotic effects of Feitai are mediated, in part, through protecting epithelial cells.

In summary, our results demonstrate beneficial effects of Feitai against pulmonary fibrosis induced by BLM. Furthermore, lipid peroxidation and the inflammatory response are inhibited by Feitai, suggesting that the effect of this formula on BLM-induced lung injury and fibrosis is associated with its antioxidant and anti-inflammatory properties.

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