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Treatment with Number 2 Feibi Recipe, a Compound Traditional Chinese Medicine Reduces Bleomycin-induced Pulmonary Fibrosis in Mice

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Abstract: Objective: Oxidative stress with reactive oxygen species (ROS) generated from exogenous oxidants and pollutants exposure is involved in the pathogenesis of Idiopathic pulmonary fibrosis (IPF). Number 2 Feibi Recipe (Number 2 FBR) is a traditional Chinese herbal formula which can attenuate the lung injury induced by PM2.5. The present study is to explore the effect and mechanism of Number 2 FBR on bleomycin (BLM)-induced pulmonary fibrosis in C57BL/6Cnc mice. Method: Bleomycin-induced C57BL/6Cnc mice were treated with Number 2 FBR and Sulforphane for two weeks. HE and Masson trichrome staining were performed to evaluate pathological changes in lung tissues. The extent of lung fibrosis was evaluated with fibrosis scores, collagen volume fraction, and hydroxyproline concentration. Levels of SOD and 8-iso-PGF2α in lung tissues were measured by using commercial assay kits. The levels of Nrf2, SOD, GSH-Px, and TGF-β1 relative protein and mRNA in lung tissues were measured by real time PCR and Western blot respectively. Results: The results showed that Number 2 FBR ameliorated bleomycin-induced pathological changes, collagen deposition and significantly decreased fibrosis scores, collagen volume fraction, and hydroxyproline concentration in the mice lungs. Additionally, Number 2 FBR inhibited the expression of 8-iso-prostaglandin F2α (8-iso-PGF2α) and transforming growth factor beta1 (TGF-β1), and increased the expression of Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2), superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) in lung tissues. Conclusion: Number 2 FBR has an effect of anti-fibrosis by regulating the lung oxidants and antioxidants balance.

Keywords: Number 2 Feibi Recipe, Chinese Medicine, Pulmonary Fibrosis, Nrf2, Bleomycin, Mice

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fatal, fibrotic interstitial lung disease with a median survival time between 3-5 years from diagnosis and limited therapeutic options [1]. Although the pathogenesis is not fully understood, a growing body of evidence has demonstrated that oxidative stress plays a significant role in IPF [2]. Oxidative stress is defined as an imbalance between oxidant production and antioxidant defence in favour of oxidants, that leads to cellular dysfunction and tissue damage [3]. Cigarette smoke [4] and multiple environmental insults such as silica [5], coal dust [6], asbestos fibers [7], radiation [8], and certain chemotherapeutic agents [9], which are considered as risk factors for pulmonary fibrosis, have also been found to trigger the production of the reactive oxygen species (ROS) include hydroxyl radical, hydrogen peroxide, and superoxide radical [10]. The excess ROS overwhelm antioxidant capacity to perturb the balance in this

reduction-oxidation equilibrium, and eventually lead to oxidative stress of cells and tissues.

The promotion of pulmonary fibrosis by oxidative stress involves multiple mechanisms that include (i) causing extensive modifications and damage to macromolecules (e.g., DNA, lipids, proteins), and trigger the generation of peroxidation products (e.g., DNA adducts, lipid peroxides) further promoting the cycle of alveolar damage and abnormal repair [11-13], (ii) results in an acute accumulation of inflammatory cells in the alveolar spaces and interstitium through the activation of nuclear factor-kappa B (NF-κB) [14, 15], (iii) promote alveolar epithelial cell apoptosis through mitochondrial-mediated intrinsic pathways or death receptor-mediated extrinsic pathways [16], (iv) directly contribute to profibrotic activation of TGF-β, initiating a positive feedback loop of TGF-β1 activation with resultant persistent fibrosis [17, 18], (v) promote apoptosis resistance of myofibroblasts leading to their accumulation [19], (vi) activate matrix metalloproteinases (MMPs), increasing their transcription, deactivating proteases, and subsequent collagen deposition [20, 21].

Lung protection against oxidants is guaranteed by protective antioxidants such as intracellular and extracellular superoxide dismutases (SODs) and detoxification enzyme systems (e.,g., glutathione-S-transferases) to restore systemic redox balance and maintain homeostasis [22]. The induction of antioxidant enzymes and related proteins after exposure to insults may protect the lung and promote damage repair. Conversely, reduced induction or inactivation of antioxidant enzymes may result in a continuous redox imbalance, that may contribute to the progression of pulmonary fibrosis [23].

Nuclear factor erythroid-derived 2-like2 protein (Nrf2) is a key transcriptional regulator for antioxidant response element (ARE) -mediated induction of cellular antioxidant enzymes and detoxifying enzymes, which protects against the pathogenesis of pulmonary fibrosis [24, 25]. Indeed, deficiency of Nrf2 significantly enhanced bleomycin-induced pulmonary fibrosis in mice [26, 27]. Moreover, in IPF patients, compensatory increased pulmonary Nrf2 expression as well as elevated antioxidant levels in the broncho-alveolar fluid are reported [28]. Observations from these studies implicated that the Nrf2-antioxidant system may provide potential therapeutic strategies for IPF.

Traditional Chinese medicine (TCM) has more than two thousand year clinical practice history. Nowadays, TCM formulations are still widely used in pulmonary disorders in China. Number 2 Feibi Recipe (Number 2 FBR) is a traditional Chinese medicine formula according to Professor Ping'an Zhou's more than 50 years' clinical experience for treating pulmonary fibrosis. Former research approved that Number 2 FBR could decrease the expression of TGF-β1, IL-6 in lung tissue, downregulate the expression of IL-6, IL-13, IL-17, MCP-1, TNF-α, and upregulate GSH-Px in serum [29]. It suggested that the Number 2 FBR can inhibit fibrosis and immunoinflammatory injury, meanwhile enhancing protective antioxidant. Nevertheless, the mechanisms by which Number 2 FBR attenuate pulmonary fibrotic effects have not been fully elucidated. Based on our previous studies, we designed this study to evaluate the antifibrotic properties of Number 2 FBR and focused on exploring whether it enhances the Nrf2-antioxidant system to attenuate BLM-induced PF in mice.

2. Materials and Methods

2.1. Reagents and Materials

Bleomycin (lot No 6115292) was purchased from Fresenius Kabi. Sulforphane(lot No C4733) was purchased from APExBIO. Hydroxyproline (HYP) kit (lot No A030-2-1) was purchased from Nanjing Jiancheng Biochemical Institute. Modified masson's trichrome stain kit (lot No 20191108) was purchased from Beijing Solarbio Science technology Co. Ltd. Mouse 8-iso-PGF2α ELISA Kit (lot No DG94697Q-96T), Mouse SOD ELISA Kit (lot No DG30430M-96T) were purchased from Dogesce. Nrf2 Antibody (Cat No bs-1074r), GSH-Px Antibody (Cat No bs-3882r), TGF-β1 Antibody (Cat No bs-0086r) were purchased from Bioss.

2.2. Preparation of No. 2 FBR

Number 2 FBR was purchased from Beijing Tong Ren Tang, the Pharmaceutical Industry Co. The components of it are Radix Astragali, Rhodiolae Crenulatae Radix et Rhizoma, Flos Lonicerae Japonicae, Radix Scutellariae, Radix et Rhizoma Salviae Miltiorrhizae, Radix et Rhizoma Glycyrrhizae.Total net weight is 140g.

2.3. Animals

All animal experiments were approved by the Animal Research Ethics Board at Dongfang Hospital Affiliated to Beijing University of Chinese Medicine. A total of 40 C57BL/6Cnc male mice, weighing 20–24g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. After 7 days adaptive feeding in community cages with 12 h periods of light and dark cycles and maintained on standard rodent chow with access to water ad libitum, all mice were randomly assigned into four groups (n=10 per group): Normal control group, Model group, Sulforphane group, and Number 2 FBR group.

The mice were inhalation anesthetized by propylene glycol isoflurane 2:3 mixture. Hung the teeth of anesthesia mice rapidly on a suspension line, kept the body erect, dragged out the tongue with curved pliers, dripped bleomycin (5 mg/kg, dissolved in sterile normal saline) quickly into the trachea, then rotated the mice to the left and right uprightly 3-5 times to make the bleomycin suspension evenly distributed in the lungs. Groups except for the normal control group were dripped bleomycin into the trachea. The normal control group was given equal amounts of normal saline.

Considered of the body surface area between human beings and mice, starting from the next day of model making, we gave Number 2 FBR (10ml/kg) with the concentration of 2.102g/ml to the Number 2 FBR group by gavage for 14 days. Mice in Sulforphane group were intraperitoneally injected with Sulforphane (10ml/kg) with the concentration of

2.5mg/ml for 14 days. Normal control group and the model group were taken equal amounts of normal saline by gavage for 14 days.

2.4. Sample Collection

On the fifteenth day, the blood of the mice was drawn from the abdominal aorta under pentobarbital anesthesia and the lungs were removed. The left lungs were fixed by polyoxymethylene for HE staining, Masson's trichrome staining, and immunohistochemical staining, and the right lungs were frozen in liquid nitrogen for Western Blot and Real Time PCR.

2.5. General Histological Staining

The left lung was fixed in 10% formalin for more than 24h and then dehydrated and embedded in paraffin. Coronal sections (5 μm) of the upper, upper-mid, lower-mid, and lower part of the left lungs were deparaffinized and stained with HE and Masson's trichrome. Three consecutive but not repeated microscopic fields of each section were microscopically photographed with a 20-fold magnification by a technician blinded to the results of animal grouping (twelve photographs per mouse). All photographs photographed sharply, >95% of photographs had to be covered with lung tissue to ensure that the frequency of each grade of fibrosis was similar to avoid a bias to certain fibrotic grades.

One observer evaluated the HE staining photographs independently using the modified Ashcroft scale [45]. Areas with dominating tracheal or bronchial tissue were omitted. The grades were summarized to obtain a fibrotic index for the lung.

Masson's trichrome staining photographs were evaluated by Image J 1.52i (Wayne Rasband, NIH, USA). The gray-scale threshold count method was used to quantify the area occupied by collagen staining blue colour within each photograph. Collagen volume fraction (CVF) was calculated based on the percent of blue collagen staining quantified within a tissue section. (Collagen volume fraction (%) = area of collagen fiber/area of total tissue) [46].

2.6. Measurement of Content of HYP, SOD, and 8-iso-PGF2α in Lung Tissues

Hydroxyproline (HYP) content of lung tissue was measured by using hydroxyproline kits. SOD and 8-iso-PGF2α levels in lung tissues were measured by ELISA kits. All procedures were performed in accordance with the manufacturer's instructions.

2.7. Western Blot Was Used in Analysis of Protein in Lung Tissue

Levels of Nrf2, GSH-Px, and TGF-β1 expression in full thickness were analyzed. The tissue was homogenized in RIPA lysis buffer. The homogenate was centrifuged at 12000r/min (the centrifuge from THERMO, legend micro 21r), for 10min, and the supernatant was collected. The protein samples were denatured by boiling for 5 minutes in SDS sample buffer (MDL). Afterward, equal quantities of protein were separated with 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF). The membranes were blocked with 5% skim milk for 2 hours and then incubated with primary antibodies (Nrf2, GSH-Px, and TGF-β1) overnight at 4°C. After exposure to secondary antibodies for 60 minutes at 37°C, the membranes were analyzed with ECL reagents. All assays were performed independently and repeated three times.

2.8. Real Time PCR

The tissue was homogenized in liquid nitrogen. Total RNA was extracted using a Transzol reagent. The total RNA concentration and purity were determined by UV–Vis spectroscopy using the Bio-Rad SmartSpec 5000 system (Bio-Rad, Hercules, CA, USA). cDNA reverse transcribe was according to the instructions of Super Script III RT Reverse Transcription kit (ABI-Invitrogen). The primers were designed (Table 1). β-actin was used as an endogenous control. The mRNA levels were examined by real-time PCR. A single melting curve peak confirmed the presence of a single product. The mRNA level was quantified relative to β-actin using the 2-ΔΔCT method.

Table 1. *Sequences of primers used for the real-time PCR analysis.*

Gene	Oligonucleotide primer sequences (5′–3′)
Nrf2	Forward: TCCCAGCAGGACATGGATTT Reverse: GGCCTTCTCCTGTTCCTTCT
SOD	Forward: CCCAGACCTGCCTTACGACT Reverse: TACTTCTCCTCGGTGGCGTT
GSH-Px	Forward: AACCTACGAGGGAGGAACAC Reverse: CTGGCAGAGACTGGGATCAA
TGF-β1	Forward: TTGCTTCAGCTCCACAGAGA Reverse: CAGAAGTTGGCATGGTAGCC
actin	Forward: CTCCTGAGCGCAAGTACTCT Reverse: TACTCCTGCTTGCTGATCCAC

2.9. Statistical Analysis

The data are expressed as mean±SD. Statistical testing was performed with SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA). Data distribution was assessed using the D'Agostino-Pearson normality test. Parametric data was analyzed with a t-test for comparison of two variables and ANOVA with Tukey-Kramer post-test analysis for multiple comparisons. If the data did not satisfy the criteria for nonparametric homogeneity and normality, the Kruskal-Wallis test was used for comparison of two variables and Dunn's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Histologically Evaluating of Pulmonary Fibrosis

HE (Figure 1a) and Masson trichrome (Figure 1b) staining were performed to evaluate pathological changes in lung tissues. Compared with the normal control group, obvious alveolar structural disorder, abnormal thickening of the

alveolar wall, massive deposition of matrix, amount of blue collagen fibers, and fibroblasts appeared in the model group after the BLM intratracheal instillation. Compared with the model group, the lung injury and fibrosis induced by BLM were reduced to varying degrees in both sulforphane and Number 2 FBR groups.

We performed a quantitative visual assessment of the degree of pulmonary fibrosis on HE staining photographs by the modified Ashcroft scale. The scores of fibrotic lesions were significantly increased 14 days after bleomycin administration, compared with those in the normal control group. However, after 14 days treatment of sulforphane or Number 2 FBR, scores were decreased compared with the model group. And the effect was more significant when treated with Number 2 FBR ($P < 0.05$) (Figure 1c)

We next performed a quantitative assessment of the degree of pulmonary fibrosis on Masson trichrome staining photographs. CVF was the parameter to be measured. Compared with the normal control group, CVFs were significantly higher in the model, suiforphane, and Number 2 FBR groups. However, CVFs in suiforphane and Number 2 FBR groups were decreased compared with the model group. And compared with model or suiforphane group, CVFs in Number 2 FBR group were lower with the statistical difference ($P < 0.05$). (Figure 1d)

We further assessed the degree of pulmonary fibrosis by measuring the lung hydroxyproline content, which we found to be significantly increased 14 days after bleomycin administration compared with the normal control group. The concentration of hydroxyproline in the suiforphane and Number 2 FBR groups, however, were reduced compared with the model group. And compared with model or suiforphane group, the concentrations of hydroxyproline in Number 2 FBR group were lower with the statistical difference ($P < 0.05$) (Figure 1e).

3.2. Expression of Nrf2, SOD, GSH-Px, and TGF-β1 mRNAs in Lung Tissues

Compared with the normal control group, the levels of Nrf2, SOD, GSH-Px, and TGF-β1 mRNAs expression increased in the lung tissues after BLM administration. The levels of Nrf2, SOD, and GSH-Px mRNAs expression in the suiforphane group were significantly greater than the model group ($P < 0.05$). The levels of SOD mRNA expression in Number 2 FBR group were significantly greater than the model group ($P < 0.05$). The levels of TGF-β1 mRNA expression in Number 2 FBR group were significantly decreased compared with the model group ($P < 0.05$) (Figure 2).

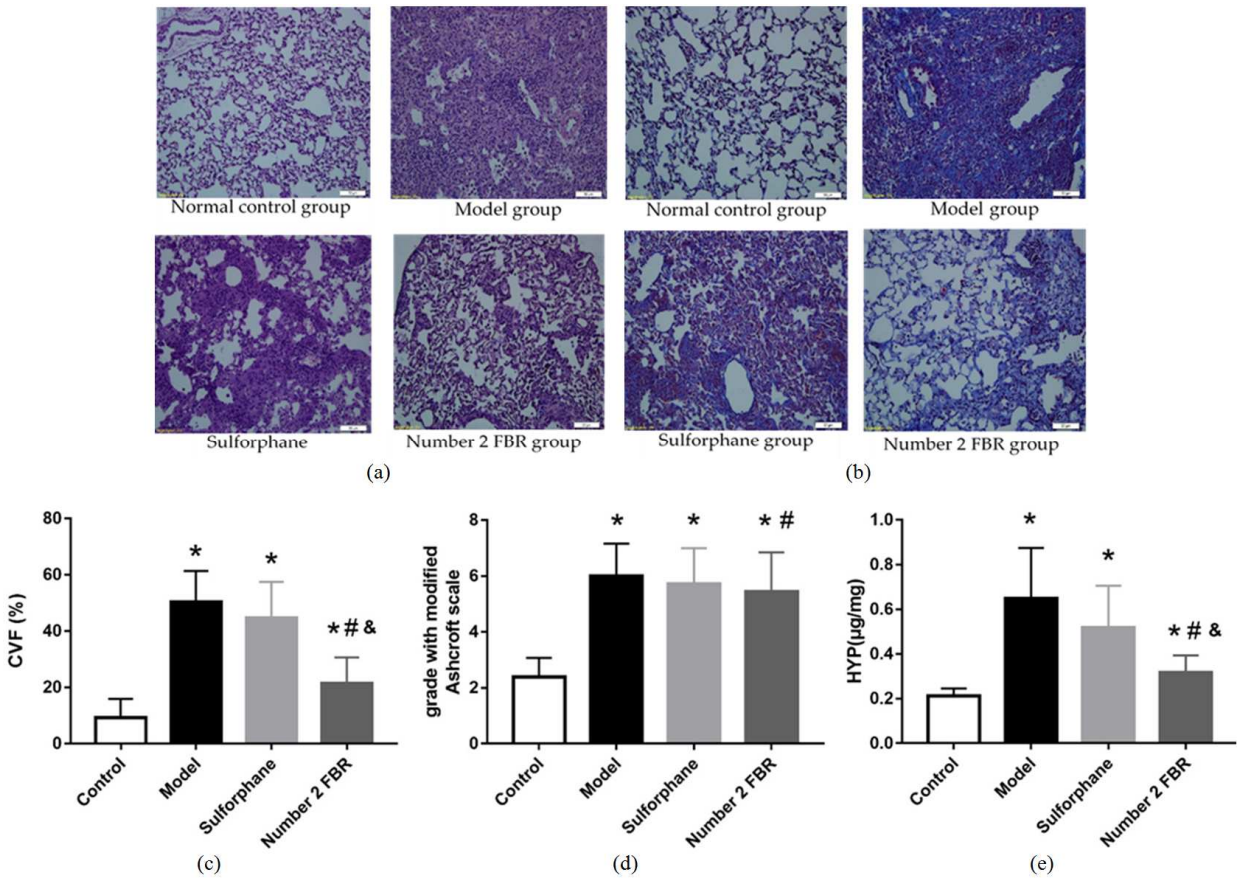

Figure 1. *Effect of Number 2 FBR on histopathologic in BLM-induced pulmonary fibrosis mice. Representative photographs of sections stained with HE(a) and Masson trichrome(b) are shown at the same magnification (200×). Quantitative assessment of pulmonary fibrosis with HE staining photographs (c). n =6 in each group. quantitative assessment of Pulmonary fibrosis with Masson trichrome staining photographs (d). n =6 in each group. Hydroxyproline (HYP) content of lung tissues(e). n =6 in each group. Data were expressed as mean ± S.D. * $P < 0.05$ compared with the normal control group. # $P < 0.05$ compared with the model group. & $P < 0.05$ compared with the sulforphane group.*

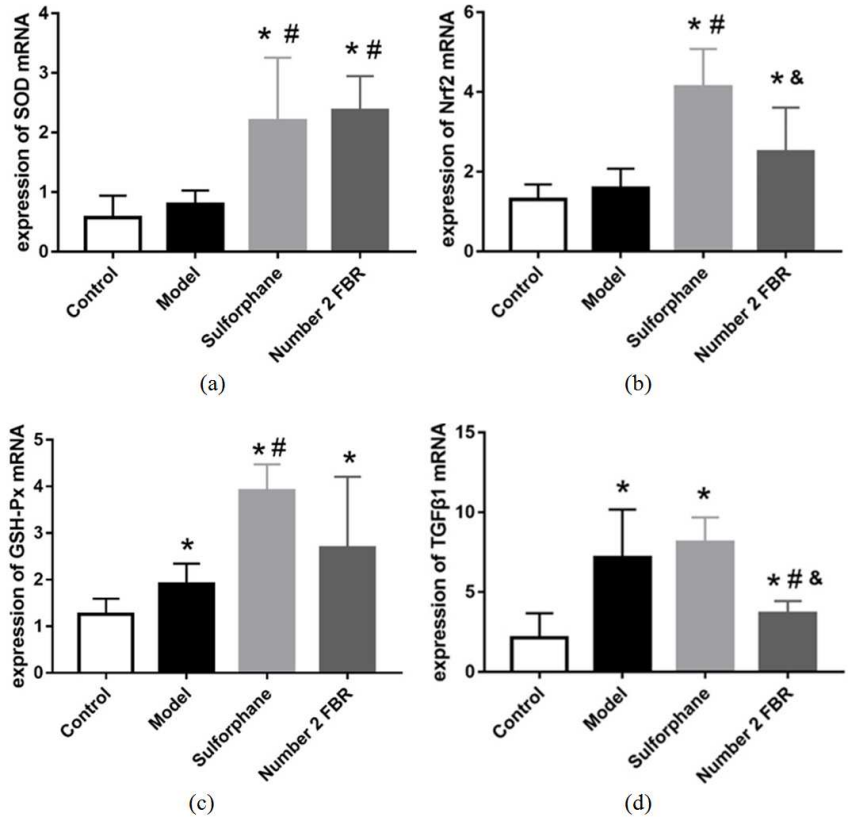

Figure 2. *Effect of Number 2 FBR on the mRNA expressions of Nrf2, SOD, GSH-Px, and TGF-β1 in the lung tissues of BLM-induced pulmonary fibrosis mice. The semiquantitative results of Nrf2(a), SOD(b), GSH-Px(c) and TGF-β1(d) mRNA expression in the lung tissues. n =3 in each group. Data were expressed as mean ± S.D. * P < 0.05 compared with the normal control group. # P < 0.05 compared with the model group. & P < 0.05 compared with the sulforphane group.*

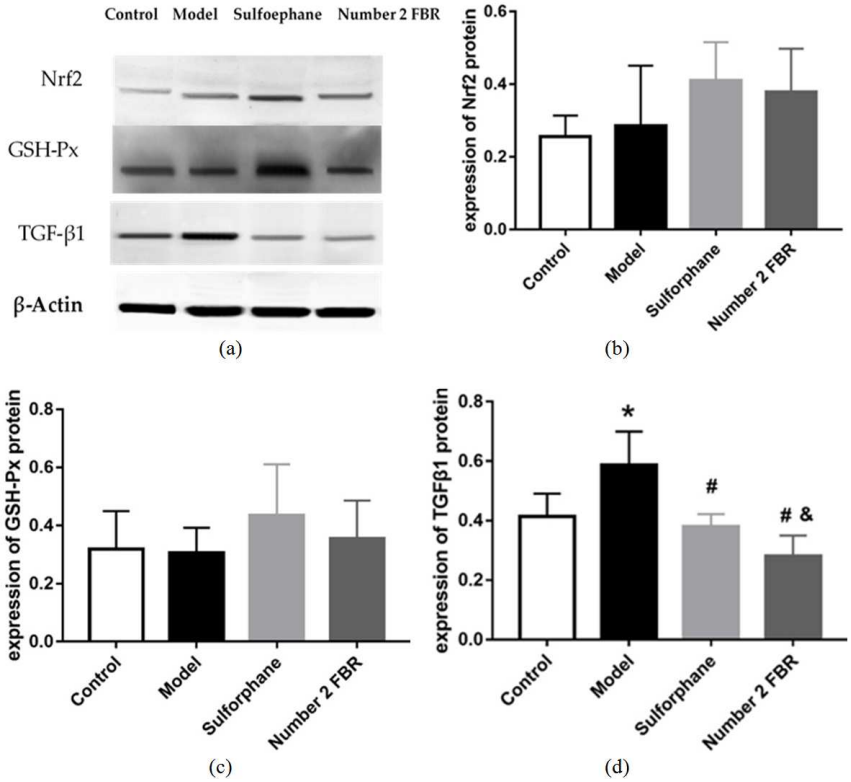

Figure 3. *Effect of Number 2 FBR on the protein expressions of Nrf2, GSH-Px, and TGF-β1 in the lung tissues of BLM-induced pulmonary fibrosis mice. The Nrf2, GSH-Px, and TGF-β1 protein expressions in the lung tissues were detected by Western blot analysis(a). The quantitative results of the Nrf2(b), GSH-Px(c) and TGF-β1(d) protein expressions. n =3 in each group. Data were expressed as mean ± S.D. * P < 0.05 compared with the normal control group. # P < 0.05 compared with the model group. & P < 0.05 compared with the sulforphane group.*

3.3. Effect of Number 2 FBR on Levels of the Protein of Nrf2, GSH-Px, and TGF-β1 in Lung Tissues

Compared with the normal control group, the level of TGF-β1 protein expression significantly increased in the model group after BLM administration ($P < 0.05$). However, after 14 days treatment of sulforphane or Number 2 FBR, the level of TGF-β1 protein significantly decreased compared with the model group ($P < 0.05$). Furthermore, there was no statistical significance comparing the level of Nrf2 and GSH-Px in the 4 groups ($P > 0.05$). However, Number 2 FBR still had the trend to increase the level of Nrf2 and GSH-Px protein expression. The content of SOD and 8-iso-PGF2α in the lung tissues were measured by ELISA analysis (Figure 3).

3.4. Effect of Number 2 FBR on Levels of SOD and 8-iso-PGF2α in Lung Tissues

The content of SOD and 8-iso-PGF2α in the lung tissues were measured by ELISA analysis. Compared with the normal control group, the levels of SOD were lower, and 8-iso-PGF2α were higher in the model group. However, after 14 days treatment of Number 2 FBR, the content of SOD were significantly increased, and 8-iso-PGF2α were significantly reduced compared with the model group ($P < 0.05$) (Figure 4).

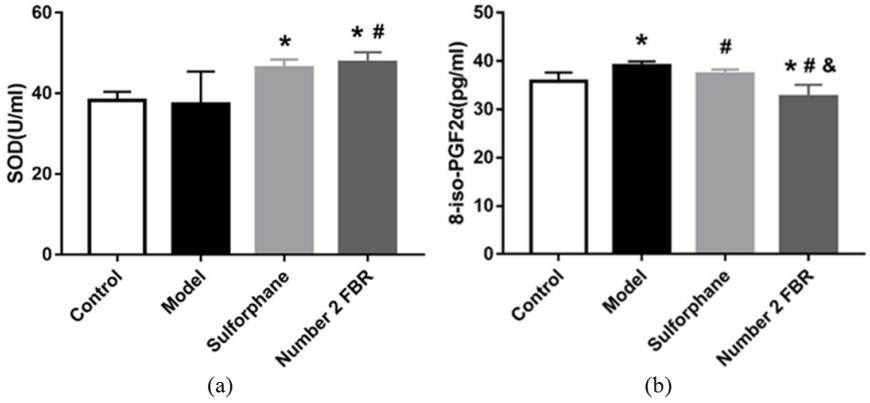

Figure 4. *Effect of Number 2 FBR on the content of SOD and 8-iso-PGF2α in the lung tissues of BLM-induced pulmonary fibrosis mice. SOD (a) and 8-iso-PGF2α (b) content in the lung tissues detected by ELISA. n =6 in each group. Data were expressed as mean ± S.D. * $P < 0.05$ compared with the normal control group. # $P < 0.05$ compared with the model group. & $P < 0.05$ compared with the sulforphane group.*

4. Discussion

In this study, we demonstrated that the Number 2 FBR has the effect of alleviating BLM-induced pulmonary fibrosis. A reduction was observed after the treatment of Number 2 FBR in hydroxyproline content of lung tissues, consistent with the decrease of visual assessment scores and collagen volume fraction in histological changes induced by bleomycin. The increase in the expression of SOD and GSH-Px in mRNA and protein levels, and the decrease of the concentration of 8-iso-PGF2α in lung tissues after Number 2 FBR administration indicates the beneficial role of Number 2 FBR to restore the redox imbalance induced by bleomycin. Furthermore, Number 2 FBR upregulated Nrf2 and decreased the expression of TGF-β1 in mRNA and protein levels, suggesting the involvement of Nrf2 and TGF-β1 in the signaling pathway for Number 2 FBR action.

Oxidative stress presents as a critical scenario in the progress of IPF. However, reactive oxygen species with a half-life of only milliseconds are almost unable to be detected directly in vivo [32]. In this present study, 8-iso-PGF2α, a widely accepted biomarker of oxidative stress [33], was detected to assess the extent of oxidative stress, and it was found that the concentration of 8-iso-PGF2α in lung tissue was significantly increased, the TGF-β1 expression levels were elevated as well, and the fibrosis progressed, indicating that oxidative stress and persistent pulmonary fibrosis was induced as expected after bleomycin administration.

In the presence of bleomycin, the expression of Nrf2, SOD and GSH-Px in the lung tissues increased compensatively in mRNA and protein levels, but it was not sufficient to eliminate oxidative damage. After the treatment of Number 2 FBR, a clear reduction of 8-iso-PGF2α concentration and the significantly increasing of the SOD mRNA and protein expression were observed in lung tissues. In line with SOD and GSH-Px, Nrf2, the upstream regulator for antioxidant enzymes were also elevated, indicating that Number 2 FBR could confer protection against bleomycin induced oxidative stress through activating Nrf2 signaling pathway to promote the expression of antioxidant enzymes. In addition, the inappropriate activation of TGF-β1 and subsequent fibrosis progression, which has been proved to be initiated by ROS [34], were attenuated in lung tissues of Number 2 FBR-treated mice, as evidenced by significantly reduced TGF-β1 expression, hydroxyproline content, collagen volume fraction, and histopathological scores.

In this study, sulforaphane, a strong Nrf2 activator which has been shown to activate antioxidant response [35], was used as positive control. After 14 days intraperitoneally injection of sulforaphane, the expression of Nrf2, SOD, GSH-Px in lung tissues were upregulated, 8-iso-PGF2α concentration of lung tissues were decreased, which indicates the effect of sulforaphane on the Nrf2 signaling pathway

activation and the induction of downstream antioxidant enzymes to reduce oxidative stress. But the hydroxyproline content, collagen volume fraction, and histopathological scores were only have a reduced trend but without statistically significant after sulforaphane treatment, which shows that the anti-fibrosis effect of sulforaphane was not satisfactory.

Table 2. *Pulmonary fibrosis related biological functions of ingredients isolated from Number 2 FBR components.*

Components	ingredients	Biological Functions associated with PF	References
	Polysaccharides	Inhibit EMT and NF-κB pathway	[47]
		Inhibit TGF-β1/Smad3 pathway	[37]
Radix	Astragaloside IV	Attenuate ECM deposition	[36]
Astragali		Inhibit EMT	[38, 39]
		Anti-oxidative, anti-inflammatory	[48]
Flos Lonicerae Japonicae	Chlorogenic Acids	Anti-oxidative, anti-inflammatory	[49, 50]
Radix	Baicalein	inhibit miR-21	[45]
Scutellariae	Baicalin	alleviates fibroblast proliferation	[44]
		upregulate A2aR	[43]
Rhodiolae	Gallic acid	Anti-oxidative	[51]
Crenulatae		Inhibit EMT	[52]
Radix et		Anti-inflammation	[53]
Rhizoma	Rosavin	Inhibit NF-κB p65, TGF-β1 and α-SMA	[54]
		Inhibit TGF-β pathway	[55]
	Salvianolic acid B	Inhibit myofibroblast trans differentiation	[56]
		anti-inflammatory	[57]
Radix et	caffeic acid	decrease type I collagen, modulate Interferon-gamma	[58]
Rhizoma		decrease TNF-α and TGF-β1, increase PGE2	[59]
Salviae		Anti-oxidative, anti-inflammatory	[57, 60-63]
Miltiorrhizae	Tanshinone IIA	inhibit TGFβ-dependent EMT	[41]
		inhibit TGF-β1-Smad pathway	[40, 62]
		modulate ACE-2/ANG-(1-7) axis	[42]
	cryptotanshinone	inhibit Smad and STAT3 pathways	[64, 65]
	Protocatechuic aldehyde	modulate HMGB1/RAGE pathway	[66]
Radix et		Inhibit TGFβ，EMT and fibroblast proliferation	[67]
Rhizoma Glycyrrhizae	Glycyrrhizic acid	anti-inflammatory, anti-oxidative, inhibit TGFβ1/Smad2 pathway and NOX4	[68]

Interestingly, compared with sulforaphane, Number 2 FBR activated Nrf2 single signaling pathway to lesser extents, but showed obvious advantages in terms of the effective in suppressing TGF-β1 activation and fibrosis progression. Combined with the anti-inflammatory effect of Number 2 FBR have been found in our previous experiments, it was hypothesized that the effect of Number 2 FBR for ameliorating pulmonary fibrosis was involved in some other mechanisms besides improving oxidative stress. The results of pharmacological studies confirmed this hypothesis by that the active ingredients of Number 2 FBR components attenuate pulmonary fibrosis through multiple mechanisms (Table 2). For example, in addition to the antioxidant function, Astragaloside IV extracted from Radix Astragali was shown to inhibit epithelial-mesenchymal transition (EMT) and TGF-β1/Smad3 pathway and attenuating extracellular matrix (ECM) deposition [36-39], and Tanshinone IIA from Radix et Rhizoma Salviae Miltiorrhizae was shown to inhibit TGF-β1-Smad pathway and EMT, and modulating ACE-2/ANG-(1-7) axis [40-42]. Moreover, the main active ingredients of Radix Scutellariae, Baicalin and Baicalein, could alleviate fibroblast proliferation, prevent pulmonary fibrosis through inhibiting miR-21 and upregulating Adenosine A2a receptor (A2aR) [43-45].

IPF is a highly complex disease and involves multiple genes and processes. Previous studies have shown that purely oxidant scavenging has not been enough to completely cure or prevent IPF [46], and our findings of the sulforaphane group in this study are consistent with this concept. In contrast, as a compound preparation, Number 2 FBR alleviate the fibrosis process in a more delicate way by acting on a variety of fibrosis related targets which including but not limited to oxidative stress might be more effective to exert beneficial effects on pulmonary fibrosis.

Number 2 FBR consists of six different components, each of them contains numerous bioactive ingredients，and the mechanisms of its mitigation of pulmonary fibrosis is far from fully understood. The present study focused on the effect of Number 2 FBR as a whole on the BLM-induced pulmonary fibrosis and its antioxidant stress mechanism. Much more work is required to elucidate other underlying mechanisms and the role of any individual ingredient in Number 2 FBR in the effect observed.

5. Conclusions

This study demonstrated that Number 2 FBR has an effect on protecting against the development of pulmonary fibrosis and might be employed as a therapeutic candidate agent for attenuating pulmonary fibrosis. An involvement of Nrf2 in the signaling pathway responsible for the protective effect of Number 2 FBR on the oxidative and antioxidant balance of bleomycin-induced pulmonary fibrosis.

The evidence from the present study also suggested Number 2 FBR alleviate the fibrosis process by acting on a variety of fibrosis related targets. There are some underlying mechanisms required to elucidate.

Abbreviations

ROS	reactive oxygen species
IPF	idiopathic pulmonary fibrosis
Number 2 FBR	Number 2 Feibi Recipe
BLM	bleomycin
8-iso-PGF2α	8-iso-prostaglandin F2α
TGF-β1	transforming growth factor beta1
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
SOD	superoxide dismutase
GSH-Px	glutathione peroxidase
NF-κB	nuclear factor-kappa B
MMP	matrix metalloproteinase
ARE	antioxidant response element
TCM	traditional Chinese medicine
MCP	monocyte chemoattractant protein
IL-6	interleukin-6
IL-13	interleukin-13
IL-17	interleukin-17
TNF-α	tumor necrosis factor-α
HYP	hydroxyproline
CVF	collagen volume fraction
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
PF	pulmonary fibrosis
A2aR	adenosine A2a receptor
PGE2	prostaglandin E2
ACE-2	angiotensin-converting enzyme 2
ANG-(1-7)	angiotensin-(1-7)
HMGB1	high mobility group box 1
RAGE	receptor for advanced glycation end-product
STAT3	signal transducer and activator of transcription
NOX4	NADPH oxidase 4

Author Contributions

Qi Long and Yang Jiao conceived and designed the research; Qi Long, Haoge Liu, Xiaofeng Gu, and Qinglu Pang performed the experiments; Qi Long and Zhaoheng Liu analyzed the data; Jie Niu, and Fang Cao contributed reagents/materials/analysis tools; Qi Long wrote the first draft of the manuscript and Yang Jiao participated in revision. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

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