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Berberine Attenuates Cigarette Smoke Extract-induced Airway Inflammation in Mice: Involvement of TGF-β1/Smads Signaling Pathway*

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Summary: Although several studies confirmed that berberine may attenuate airway inflammation in mice with chronic obstructive pulmonary disease (COPD), its underlying mechanisms were not clear until now. We aimed to establish an experiment mouse model for COPD and to investigate the effects of berberine on airway inflammation and its possible mechanism in COPD model mice induced by cigarette smoke extract (CSE). Twenty SPF C57BL/6 mice were randomly divided into PBS control group, COPD model group, low-dose berberine group and high-dose berberine group, 5 mice in each group. The neutrophils and macrophages were examined by Wright's staining. The levels of inflammatory cytokines TNF-α and IL-6 in bronchoalveolar lavage fluid (BALF) were determined by enzyme-linked immunosorbent assay. The expression levels of TGF-β1, Smad2 and Smad3 mRNA and proteins in lung tissues were respectively detected by quantitative real-time polymerase chain reaction and Western blotting. It was found that CSE increased the number of inflammation cells in BALF, elevated lung inflammation scores, and enhanced the TGF-β1/Smads signaling activity in mice. High-dose berberine restrained the alterations in the COPD mice induced by CSE. It was concluded that high-dose berberine ameliorated CSE-induced airway inflammation in COPD mice. TGF-β1/Smads signaling pathway might be involved in the mechanism. These findings suggested a therapeutic potential of high-dose berberine on the CSE-induced airway inflammation.
Key words: berberine; cigarette smoke extract; chronic obstructive pulmonary disease; TGF-β1/Smads signaling pathway

Chronic obstructive pulmonary disease (COPD) is a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases[1]. Cigarette smoke is the principal risk factor for the development of COPD[2]. Aberrant extracellular matrix (ECM) deposition contributes to both airway remolding and pulmonary emphysema[3]. Some recent studies found that TGF-β1 was locally increased in lung tissues of COPD, and could stimulate EMC production by activating fibroblasts and initiating their differentiation process into myofibrolasts[4]. Fibroblasts can synthesize and secrete collagen, the main component of ECM, and at the same time, ECM can promote fibroblast proliferation and cause abnormal deposition by TGF-β1/Smads signaling pathway, then stimulate the fibroblasts to secrete more TGF-β1, resulting in airway remodeling.

Corticosteroid is one of the major medical therapies for COPD, but many clinical trails and observational studies suggested that inhaled corticosteroids might have the potential adverse effects, such as dysphonia, upper airway thrushosteoporosis, pneumonia, hyperglycaemia and cataracts, which are of particular concern for older patients that cause intolerance[5–7]. Thus, for these patients, there is a need for alternative or adjuvant drugs targeting the airway inflammation.

Berberine is the main effective ingredients extracted from herbal medicine Coptis chinensis, widely distributed in China[8]. Berberine, as a non prescription drug used in clinical treatment of bacteria-induced diarrhea for a long time in China, not only has the advantages of low price, less adverse reaction, also has broad-spectrum antibacterial effect[9]. In addition, berberine has multiple pharmacological activities including anti-inflammation, antioxidation, and immune regulation[10–12]. Studies demonstrated that berberine can attenuate cigarette smoke extract (CSE)-induced airway inflammation in mice[13, 14]. However,

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its underlying mechanisms were not clear until now. Thus, we proposed the hypothesis that berberine may attenuate the airway inflammation through down-regulating the TGF-β1/Smads signaling pathway. We will examine the airway inflammation and TGF-β1/Smads mRNA and protein expression in different treatment groups of CSE-induced COPD mice model to verify this hypothesis.

1 MATERIALS AND METHODS

1.1 Preparation of CSE

CSE was prepared by a modification of a previously published method[15, 16]. We used a thoracic puncture bag with negative pressure to replace a vacuum pump. Briefly, the smoke from one cigarette was passed through a cigarette filter into thoracic puncture bag containing phosphate-buffered saline (PBS) (10 mL per 4 cigarettes, China Tobacoo Guangdong Industrial Co., Ltd., China; Tar: 11 mg, Nicotine: 1.1 mg, carbon monoxide: 13 mg). The CSE solution was adjusted to a pH of 7.2 to 7.4 and immediately filtered through a 0.22-μm filter. The CSE-PBS solution was prepared fresh for each set of experiments.

1.2 Animals

Twenty-six to twenty-eight-week-old male C57BL/6 mice were purchased from the Slack King Company of Hunan Province (China), weighing 18±20 g. These animals were housed in a temperature- and humidity-controlled condition and kept on a 12 h light/dark cycle, with free access to laboratory food and water. All mice were randomly divided into four groups (according to the computer-generated randomisation list).

The following four groups were set up: control group (Con group), which was intraperitoneally injected with PBS, CSE-induced group (CSE group), which was intraperitoneally injected with 0.3 mL CSE, but not given oral berberine, CSE-induced low-dose berberine group [CSE+Ber (L) group], which received oral berberine (25 mg/kg) and was subsequently intraperitoneally injected with 0.3 mL CSE, and CSE-induced high-dose berberine group [CSE+Ber (H) group], which received oral berberine (50 mg/kg) and was subsequentlly intraperitonealy injected with 0.3 mL CSE. The animals in CSE+Ber (L) and CSE+Ber (H) groups received oral berberine at 9:30 am every day, 6 days per week. They were intraperitoneally injected with 0.3 mL CSE on the day 1, 11, 21, 31, 41 and 51. One the day 61, all the mice were sacrificed by intraperitoneal injection of 10% chloral hydrate. The preparation and treatment of all animals were handled according to the laboratory animal care guidelines of Renmin Hospital of Wuhan University. The experimental protocols were approved by the Committee for Laboratory Animal Welfare and Ethics of Hubei University.

1.3 Inflammatory Cell Counting in Bronchoalveolar Lavage Fluid (BALF)

The right lung was lavaged three times with 0.4 mL of saline, with a recovery rate of 70%–80%. The BALF samples were centrifuged at 1200 g for 5 min, and the supernatants were removed to a EP tube and stored at –20°C for measurements of inflammatory cytokines. The pelleted cells were resuspended in 0.2 mL PBS, and the total cell number was counted by a hemocytometer. Differential cell count was performed by cytocentrifugation at 1200 g for 5 min and stained with Wright's stain (200 cells were counted for each mouse).

1.4 Levels of Inflammatory Cytokines IL-6 and TNF-α Detected by Enzyme-linked Immunosorbent Assay

Levels of IL-6 and TNF-α in the BALF were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits for mouse cytokines (Elabscience Biotechnology Co., Ltd., China) according to the manufacturer's instructions.

1.5 Lung Histopathology Examination

The left lung tissues were cut into 3 mm-thick slices and immersed in a 4% paraformaldehyde solution for 72 h to allow complete fixation. Thereafter, the slices were embedded in paraffin, and 4-μm thick sections were cut and stained with hematoxylin and eosin (HE). Then light microscopy was used to observe the morphological changes.

1.6 qRT-PCR for mRNA Expression of TGF-β1, Smad2 and Smad3

Total RNA was extracted from the lung tissues using TRIzol reagent (Aidlab, China). The content and purity of RNA were detected by ultraviolet light spectrophotometer (Junyi Electrophoresis Equipment Company, China). The obtained total RNA was reversely transcribed into complementary DNA (cDNA) using oligo (dT) primer and MuLV reverse transcriptase. Further, PCR was performed with the gene-specific primers using Taq DNA polymerase according to the manufacturer's instruction. The reaction systems were prepared following the instructions of the kits. The initial activation was at 95°C for 15 s, 60°C for 15 s and 72°C for 30 s on an ABI 7900 real time instrument (ABI, USA). The primers of TGF-β1, Smad2 and Smad3 were designed and synthesized by Biofavor Biotech Co. Ltd. (China) and sequences were as follows: TGF-β1 (forward: 5′-TTGCTTCAGCTCCACAGAGA-3′, reverse: 5′-TGGTTGTAGAGGGCAAGGAC-3′); Smad2 (forward: 5′-GACTACACCC ACTCCATTCC-3′, reverse: 5′-GCAGGTTCCGAGTAAGTAA-3′); Smad3 (forward: 5′-TGGAACTTACAAGGCGACAC-3′, reverse: 5′-TGGGAGACTGGACGAA AA-3′); GAPDH (forward: 5′-ATGGGTGTGAACCACGAGA-3′, reverse: 5′-CAGGGATGATGTTCTGGGCA-3′).

1.7 Western Blot Analysis for Expression of TGF-β1, Smad2 and Smad3

Lung tissues were homogenized in the RIPA buffer containing 100 mmol/L Tris, 150 mmol/L NaCl, 0.1% SDS, 1% Triton-X, 1 mmol/L EDTA, EGTA and PMSF, protease and phosphatase inhibitors. Then the RIPA was centrifuged at 4°C at 12 000 g for 5 min. The concentrations of total protein in the supernatant were detected, then the protein denaturalized at 95°C for 5 min. Fifty mg of isolated soluble protein was separated by SDS-PAGE, transferred to PVDF membranes (Millipore, USA), then incubated with primary antibody, anti-TGF-β1, anti-Smad2, and anti-Smad3 monoclonal antibodies according to the instructions and HRP-conjugated secondary antibodies (Wuhan Boster Biological Engineering Co., Ltd., China).

1.8 Statistical Analysis

Statistical analysis was carried out using SPSS software 22.0, and all values were expressed as mean±standard deviations (SD). Multi groups comparison was made using variance analysis, followed by the LSD-*t* between two groups. A significant difference was defined as $P<0.05$.

2 RESULTS

2.1 Histopathological Changes of Bronchial Lung Tissue in COPD Mice

Berberine prevented airway histopathological changes in CSE-induced mice. In our study, the pathological changes of bronchial lung tissue in COPD model group were consistent with features of COPD. As compared with control group, the bronchial lung tissue in COPD model group showed varying degrees of damage, such as airway epithelium thickening, enlargement of alveolus, and inflammatory cell infiltration (fig. 1). Such changes were significantly attenuated by berberine pretreatment, especially with high dose berberine.

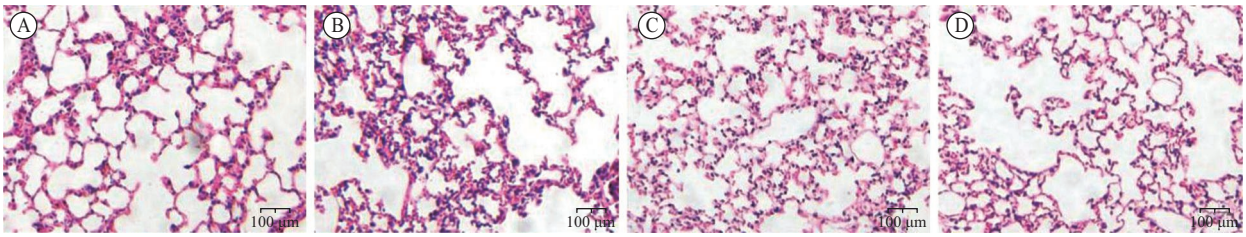

Fig. 1 Preventive effect of berberine on airway histopathological changes in CSE-induced mice
Lung tissues were analyzed by hematoxylin and eosin staining. A: control group; B: CSE group; C: CSE+Ber (L) group; D: CSE+Ber (H) group. Scale bars=100 μm

2.2 Number of Inflammatory Cells in BALF

As compared with control group, the total cell counts and differential cells counts in BALF of COPD model group were significantly increased ($P<0.05$). High-dose berberine pretreatment significantly reduced the counts of total cells and differential cells in BALF of CSE-induced mice ($P<0.05$), whereas the difference was not statistically significant in the counts of total cells and differential cells in BALF of low-dose berberine group ($P>0.05$) (fig. 2).

2.3 Levels of Inflammatory Cytokines TNF-α and IL-6 in BALF

The levels of inflammatory cytokines TNF-α and IL-6 in BALF were significantly higher in the COPD model group than in the control group ($P<0.05$). Pretreatment with high-dose berberine significantly reduced the levels of inflammatory cytokines TNF-α and IL-6 in BALF as compared with the COPD model group ($P<0.05$), but these findings were not seen in the low-dose berberine group ($P>0.05$) (fig. 3 and 4).

2.4 mRNA Expression of TGF-β1, Smad2 and Smad3 by qRT-PCR

The mRNA expression of TGF-β1, Smad2 and

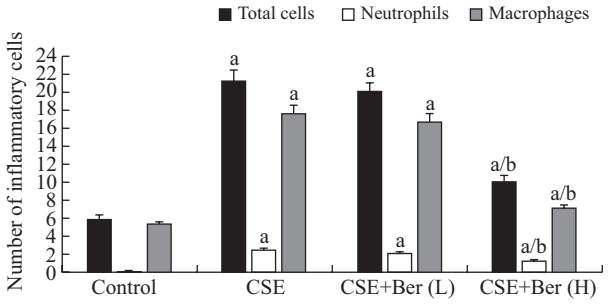

Fig. 2 The number of inflammatory cells in BALF
[a]$P<0.05$ *vs.* control group, [b]$P<0.05$ *vs.* CSE group

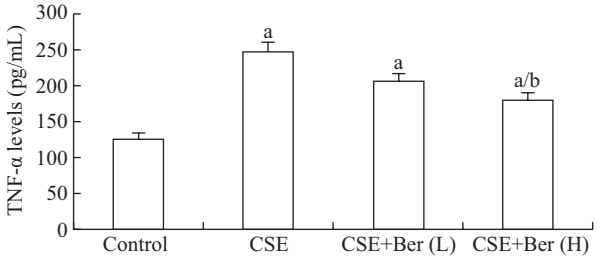

Fig. 3 Levels of TNF-α in BALF
[a]$P<0.05$ *vs.* the control group, [b]$P<0.05$ *vs.* the CSE group

Smad3 in the COPD model group was significantly up-regulated as compared with the control group (P<0.05). High-dose berberine down-regulated the mRNA expression of Smad3 significantly (P<0.05), whereas this change in the low-dose berberine group was not significant (P>0.05). Berberine down-regulated the mRNA expression of TGF-β1 and Smad2 in a dose-dependent manner as compared with the COPD model group (P<0.05) (fig. 5).

2.5 Protein Expression Levels of TGF-β1, Smad2 and Smad3 Detected by Western Blotting

The expression of TGF-β1, Smad2 and Smad3 proteins was markedly up-regulated in the COPD model group as compared with the control group (P<0.05). High-dose berberine down-regulated the protein expression of TGF-β1 compared to the COPD model group (P<0.05), but this kind of effect was not seen in the low-dose berberine group (P>0.05). Berberine down-regulated the protein expression of Smad2 and Smad3 in a dose-dependent manner as compared with the COPD model group (P<0.05) (fig. 6).

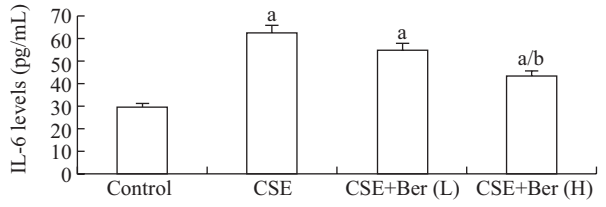

Fig. 4 Levels of IL-6 in BALF
[a]P<0.05 *vs.* the control group, [b]P<0.05 *vs.* the CSE group

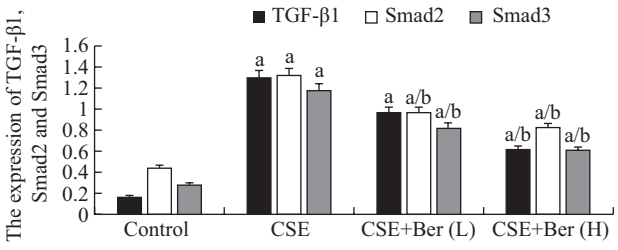

Fig. 5 The mRNA expression levels of TGF-β1, Smad2 and Smad3
[a]P<0.05 *vs.* the control group, [b]P<0.05 *vs.* the CSE group

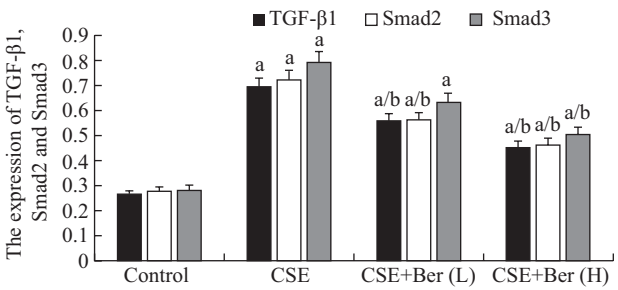

Fig. 6 The protein expression levels of TGF-β1, Smad2 and Smad3
[a]P<0.05 *vs.* the control group, [b]P<0.05 *vs.* the CSE group

3 DISCUSSION

COPD is a chronic inflammatory airway disease, and anti-inflammatory therapy serves as one of the fundamental treatments[17]. The anti-inflammatory activity of berberine had been reported abundantly. Our study demonstrated that high-dose berberine can relieve the airway inflammation induced by CSE via regulating the TGF-β1/Smads pathway.

The pathogenesis of COPD is complex and neutrophils, macrophages and lymphocytes are the main inflammatory cells[18]. These cells release a large number of inflammatory mediators involved in COPD, such as TNF-α, IL-6, IL-8, LTB4, IL-1, TGF-β[19, 20]. IL-6 was reported to have various biological activity, including promoting necrophilia oxidation reaction, delaying necrophilia apotheosis and causing persistent inflammatory reaction[21]. Liang *et al* found that the COPD patients had a higher IL-6 level and greater WBC counts in serum than normal people[22]. Ferrari reported that IL-6 increased significantly after 3 years compared to baseline measurements in COPD patients and was associated with worse 6MWD performance[23].

TNF-α is a cytokine released primarily from macrophages, and is thought to play a vital role in the progression of COPD[24]. TNF-α can prompt granulocytes adhesion, increase the activity of cellular proteolytic enzymes and stimulate endothlin-1 production from airway smooth muscle cells. Chiang *et al* reported that TNF-α is associated with clinical severity and airflow limitation of COPD in an additive manner[25]. All of these inflammatory cells and cytokines are involved in the pathogenesis and clinical course of COPD, thus, to some extent, inhibiting the inflammatory cells and cytokines may offer viable choices for the therapy of COPD.

Our study observed that high-dose berberine could attenuate the airway inflammation of COPD by inhibiting the release of inflammatory cells and cytokines TNF-α and IL-6 into BALF. The results were consistent with the study that reported that berberine attenuates cigarette smoke-induced airway inflammation and mucus hypersecretion in mice[14]. However, this change did not happen to the low-dose berberine. The difference may be related to the experimental animals and the sensitivity to berberine in C57/BL6 mice and Wistar rats. The application of berberine in COPD is not yet mature and the causes remain to be further discussed.

TGF-β1 is thought to be the strongest ECM deposition agent[26]. Verhamme *et al* reported that TGF-β1 involved in the pathogenesis of obstructive lung diseases[27]. Genetic studies had confirmed an association of gene polymorphisms of the TGF-β1 with COPD[28]. TGF-β1 is correlated with the increase of ECM and submucosal collagen in the patients

with COPD[29]. A previous study suggested that pharmacologic inhibition of TGF-β signaling can prevent the murine lung from altered lung histology, impaired lung function and a panel of injury measures that accompany cigarette smoke-induced COPD[30].

Smads are thought to be the sole active substrate of TGF-β1 and the key intermediary molecule transmitting TGF-β1 signal from extracellular areas to nucleus as well. Moreover, many subtypes of Smads are involved in the progress of airway remolding in COPD, such as Smad2, Smad3 and Smad4, which are the most important cytokines accelerating fibrosis and active substrates secreted by ECM from TGF-β1[31].

The previous studies on TGF-β1/Smads are mainly concentrated on the pathogenesis and therapeutic effects of pulmonary fibrosis, asthma and pulmonary hypertension[32–35]. Whereas, the present studies found that TGF-β1/Smad2 signaling pathway is also involved in the progress of inflammation and remodeling in COPD[36]. Chitra *et al* reported that berberine can effectively inhibit TGF-β1/Smads signaling pathway of bleomyci-induced pulmonary fibrosis[34]. However, it was not confirmed whether the suppressive effect of berberine on TGF-β1/Smads signaling pathway is viable in COPD model mice. In order to confirm the possible mechanism of berberine in COPD, the expression levels (mRNA and protein) of TGF-β1, Smad2 and Smad3 were detected in our study. We observed that high-dose berberine decreased TGF-β1, Smad2 and Smad3 expression in C57BL/6 mice, suggesting the activation of TGF-β1/Smads signaling pathway might be inhibited by high-dose berberine. To some extent, it also implies high-dose berberine may exert a potential role in the treatment of COPD.

In summary, our study demonstrated that pretreatment with berberine can attenuate CSE-induced airway inflammation, and TGF-β1/Smads signaling might be involved in the mechanism. However, our present study only involved the anti-inflammatory effects of berberine in COPD mice. It is well known that TGF-β1/Smads signaling is also related to airway remodeling of COPD[4]. Whether berberine could inhibit airway remodeling activity through restraining the over-activation of TGF-β1/Smads signaling in COPD remains further studies.

Conflict of Interest Statement

There are no conflicts of interest.

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