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Original Article

Chinese medicine ShenqiBufei attenuates chronic obstructive pulmonary disease

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Abstract: Objective: To evaluate the therapeutic effect of Chinese medicine ShenqiBufei (SBF) onchronic obstructive pulmonary disease (COPD) in a rat COPD model. Methods: SBF was prepared from Astragalus, Codonopsis, Psoraleae, Salviae, Stemonae, Cortex Mori and Radix asteris. The effect of SBF on cigarette smoke extract (CSE)-induced growth of rat airway smooth muscle cells (AMSC), histone deacetylase 2 (HDAC2) and nuclear factor kappa B (NF-κB) p65 protein expression were determined by MTT assay and Western blot. Preclinical COPD model was induced by cigarette-smoke exposure for 28 days plus lipopolysaccharide intratracheal instillation on day 1 and day 14 (CSL) in Sprague-Dawley male rats. The rats either received SBF (35.2 g/kg body weight per day for 28 days) or without SBF treatment. Rats in sham group received only PBS without CSL and SBF treatment. Rat respiratory parameters such as respiratory rate, $FEV_{0.3}$/FVC, resistance inspiration (Ri), resistance expiration (Re) were measured on day 28 before sacrifice. Results: SBF treatment significantly suppressed CSE-induced proliferation of ASMC, inhibited NF-κBp65 but enhanced HDAC2 expression in ASMC *in vitro*. *In vivo*, SBF treatment significantly suppressed the thickening of small pulmonary airway wall, inhibited NF-κBp65 but promoted HDAC2 protein expression in small airway wall, and improved lung function in rat COPD model. Conclusions: The Chinese medicine SBF provides a new approach for the treatment of COPD.

Keywords: Chinese medicine ShenqiBufei, airway smooth muscle cell, cigarette smoke, chronic obstructive pulmonary disease

Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive pulmonary airway inflammatory disease caused by cigarette smoke [1]. COPD is characterized with abnormal lung function [2] due to narrowed airway and impaired relaxation [3]. Patients with COPD often have symptoms such as dyspnea, coughing and susceptible to infections [4], resulting in high morbidity, mortality and economic burden.

Airway smooth muscle cells (ASMC) are a key component in the wall structure of pulmonary small airway and play a central role in the pathogenesis of COPD [5, 6]. In response to pro-inflammatory environmental exposure such as cigarette smoke, ASMC rapidly proliferate leading to the thickness of airway wall, the reduction of airway lumen and the limitation of airflow [7]. It has been shown that histone deacetylase 2 (HDAC2) and nuclear factor kappa B (NF-κB) p65 protein [8, 9] are two essential molecules that regulate smoke-triggered inflammatory response in ASMC [10]. In the last decade, several approaches have been developed to treat COPD, which include corticosteroids, long-acting β2 adrenoceptoragonists, long-acting muscarinic receptor antagonists and theophylline [11, 12]. However, those treatments have very limited therapeutic effect on chronic airway inflammation in COPD, and fail to prevent the progression and mortality of the disease [13]. Thus, the development of novel therapeutics to suppress airway inflammation and wall remodeling in COPD remains attractive.

Here, we report that Chinese medicine ShenqiBufei (SBF) inhibits cigarette smoke-induced

ASMC proliferation, prevents pulmonary small airway wall thickening and improves lung function in a rat preclinical COPD model.

Materials and methods

Preparation of SBF

SBF contains Astragalus (30 g), Codonopsis (15 g), Psoraleae (15 g), Salviae (30 g), Stemonae (15 g), Cortex Mori (30 g) and Radix asteris (15 g) provided by the First Affiliated Hospital, Guiyang Medical College, China. SBF solution was prepared by boiling the mixture of SBF components twice in water, filtered and concentrated to a concentration of 1 g/ml, and kept at 4°C in a refrigerator for *in vitro* and *in vivo* experiments.

Cigarette smoke extract

Lion filter-tipped cigarettes (LiQunTobaccoIndustry Co., Hangzhou, China) containing 13 mg tar, 1.2 mg smoking nicotine (nicotine) and 13 mg carbon monoxide per cigarette were used to prepare cigarette smoke extract (CSE), which was dissolved in DMEM cell culture medium as described [14].

Cell culture

Primary rat AMSC were isolated as described [15], and cultured in DMEM medium with 20% heat-inactivated fetal bovine serum (FBS) (Hyclone, USA) and penicillin/streptomycin antibiotics at 37°C in a 5% CO_2 incubator. After 4-6 passages, AMSC were utilized for *in vitro* experiments.

Cell proliferation assay

ASMC (10^5 cells/ml) were seeded in wells of a 96-well plate and treated with CSE in presence or absence of SBF for 48 hours. ASMC without CSE and SBF served as control. ASMC growth was profiled with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, USA) assay as described [16]. Optical density (OD) at 570 nm was read on a FLUO star OPTIMA machine (BMG Labtech).

Western blot

Rat ASMC were treated with CSE in presence or absence of SBF for 48 hours. Cells without CSE and SBF served as the control. The cells were then washed with cold PBS. Total proteins were extracted from the cells using a RIPA cell lysis buffer (Wolsen, China), separated on 12% SDS polyacrylamide gels, and electrophoretically transferred to apolyvinylidenedifluoride membrane (Millipore, USA). The membrane was treated with mouse anti-rat HDAC2, anti-NF-κBp65 (R&D, USA) or anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibodies (1:1000 dilution) (Sigma, USA). The membrane was then reacted with the horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) before subjected to enhanced chemiluminescent (ECL) detection on an ECL machine (Pierce, USA). The blot was scanned, and the band density was measured on the Quantity One imaging software.

Rat COPD model

Sprague-Dawley rats (Male, 2 months) were purchased from the Experimental Animal Facility in the Third Military University, China, and used to establish a COPD model as described [17, 18]. Briefly, rat COPD model was induced by cigarette-smoke exposure (20 cigarettes, one hour each exposure, twice a day with a 4-hour interval) for 28 days and intratracheal instillation of lipopolysaccharide (LPS) (1 mg/kg body weight) (Sigma, USA) on day 1 and day 14. Rats under the treatment of cigarette-smoke and LPS were alternatively administrated with SBF (35.2 g/kg body weight per day) for 28 days. A sham control group of rats received equal volume of phosphate-buffered saline (PBS) without cigarette-smoke exposure and delivery of LPS. Rat respiratory parameters including respiratory rate, $FEV_{0.3}$/FVC, resistance inspiration (Ri), resistance expiration (Re) were measured on day 28 before sacrifice using a PFT Pulmonary Maneuvers (Buxco, Wilmington, NC) following the instruction from the company as described [19]. Body weight change was monitored on day 14 and day 28 with a digital scale. Ten rats were used per group, with triplication. The use of rats was approved by the Experimental Animal Care and Ethics Committees of Guizhou Provincial People's Hospital.

Rat pulmonary tissue staining

Rat lung tissues were harvested immediately after sacrifice and fixed with 10% formalin for 24 hours. The tissues were then embedded in

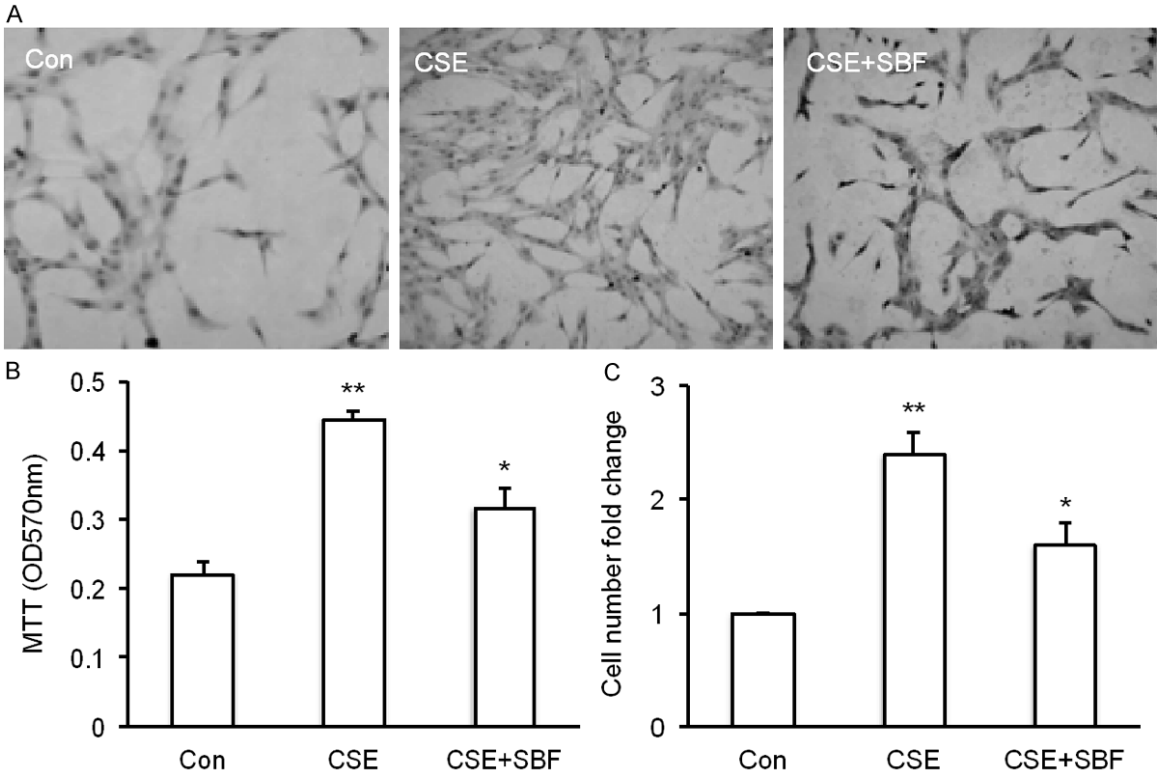

Figure 1. Suppression of CSE-induced growth of ASMC by SBF. A: Primary rat ASMC were cultured in DMEM medium and treated with CSE or CSE plus SBF. ASMC without any treatment served as control. The cells were photographed under a microscope (×20). B: ASMC in the treatments were subjected to MTT assay. OD570 values were measured and presented. C: Alternatively, ASMC in the treated groups or control group were harvested and counted with trypan blue under a microscope. The cell number fold change was calculated based on the control group. **Statistic significant was detected when compared with CSE treatment and control group ($P < 0.01$). *Statistic significant was detected when compared CSE treatment and CSE+BSF treatment ($P < 0.05$).

paraffin. Tissue slides (4-5 mm) were subjected to hematoxylin and eosin (H&E) staining or immunohistochemical staining with anti-rat HDAC2 or NF-κBp65 antibodies. The thickness of rat airway wall or smooth muscle layer was measured under a microscope using Image-Pro Plus 6.0 software. Total airway wall square = out layer wall square (Ao)-inner layer wall square (Ai). Smooth muscle layer square (WAsm) = out layer smooth muscle square (Asmo)-inner layer smooth muscle square (Asmi). The thickness of airway wall or smooth muscle layer was calculated based on the perimeter of the airway wall or the smooth muscle layer (Pi), and presented as WAt/Pi and WAsm/Pi (mm^2/mm) as described [17].

Statistical analysis

All data were presented as mean ± SD (standard deviation). Statistical analyses were performed with one-way analysis of variance (ANOVA) to compare the differences among three groups of treatments using SPSS18.0 software. $P < 0.05$ was considered as statistically significant difference.

Results

SBF suppressed CSE-induced proliferation of ASMC

Cigarette smoke is a key factor to trigger the growth of pulmonary ASMC and the remodeling of small airway wall. To test whether SBF could have inhibitory effect on cigarette smoke-induced ASMC growth, we isolated primary ASMC from rat, and cultured the cells with CSE (Mimicking smoking) in presence or absence of SBF for 48 hours. Analysis of the cells by microscopy showed that CSE stimulation promoted robust proliferation of rat primary ASMC in comparison with control cells without CSE treatment (**Figure 1A**). CSE-induced growth of

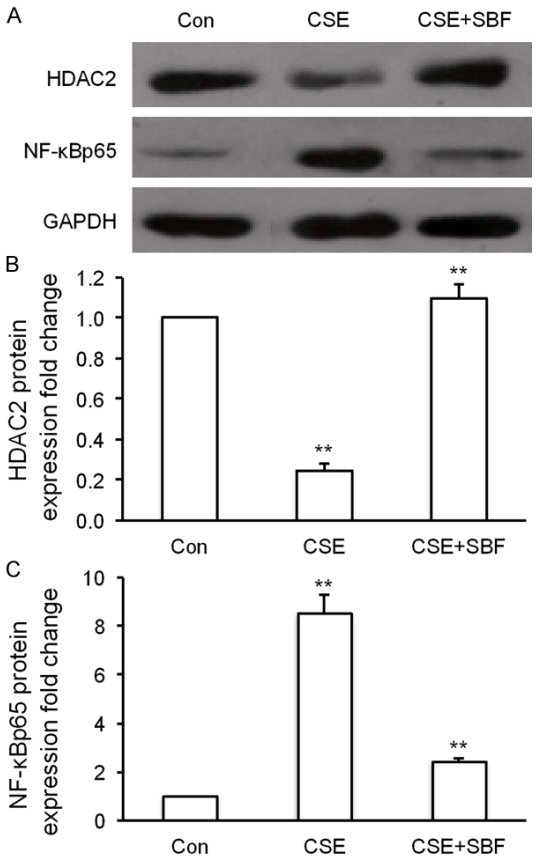

Figure 2. Effect of SBF on HDAC2 and NF-κB protein expression in ASMC *in vitro*. A: Protein lysates from rat pulmonary ASMC treated with CSE, CSE plus SBF or without treatment were subjected to Western blot with anti-rat HDAC2 or NF-κBp65 antibodies, or anti-GAPDH control antibody. B: Protein expression level of HDAC2 in treated groups relative to control group was normalized based on GAPDH protein and presented as fold change. C: NF-κBp65 protein expression level in rat ASMC in treated groups relative to control group was also calculated based on GAPDH protein and presented as fold change. **Statistic significant was detected when compared with CSE treatment and control ($P < 0.01$). **Statistic significant was detected when compared with CSE treatment and CSE+SBF treatment ($P < 0.01$).

ASMC was significantly inhibited by SBF (**Figure 1A**). MTT assay on the treated ASMC confirmed that CSE indeed significantly induced the proliferation of primary ASMC in comparison with control group ($P < 0.01$) and SBF treatment significantly suppressed CSE-induced growth of ASMC in comparison with CSE treatment ($P < 0.05$) (**Figure 1B**). Cell counting further confirmed that CSE treatment significantly increased the absolute cell number of ASMC, 2-fold higher in comparison with the one without CSE treatment ($P < 0.01$) (**Figure 1C**). However, BSF treatment significantly reduced the cell number of primary ASMC in comparison with CSE treatment without BSF ($P < 0.05$) (**Figure 1C**).

SBF enhanced HDAC2 but inhibited NF-κBp65 protein expression in CSE-stimulated ASMC

To examine the mechanistic action of BSF on ASMC, primary ASMC were stimulated with CSE in presence or absence of SBF. Western blot with anti-HDAC2 or anti-NF-κBp65 antibodies demonstrated that CSE treatment significantly reduced HDAC2 protein production and increased NF-κBp65 protein expression in CSE-treated ASMC (**Figure 2A**), 4-fold decrease of HDAC2 expression ($P < 0.01$) (**Figure 2B**) and 8-fold increase of NF-κBp65 production in comparison with the ones in control ASMC without CSE treatment ($P < 0.01$) (**Figure 2C**). However, SBF significantly restored HDAC2 expression in CSE-treated ASMC ($P < 0.01$) (**Figure 2B**), and inhibited NF-κBp65 protein production in ASMC in comparison with CSE treatment ($P < 0.01$) (**Figure 2C**).

SBF suppressed the thickening of small pulmonary airway wall in rat COPD model

Small pulmonary airway wall thickening is a key pathogenic factor leading to pulmonary airflow limitation [20]. To test whether SBF treatment can abrogate cigarette smoke-induced thickening of small pulmonary airway wall *in vivo*, we established a rat COPD model through cigarette smoke exposure and intratracheal instillation of LPS. H&E staining on the tissues of small pulmonary airway wall demonstrated that cigarette smoke and LPS (CSL) treatment promoted the remodeling of small airway wall in comparison with sham treatment (**Figure 3A**). However, SBF treatment markedly abrogated cigarette smoke-induced airway wall remodeling in CSL-treated rats (**Figure 3A**). Measurement of the thickness of the small airway wall or smooth muscle layer demonstrated that CSL-treated rats had thicker airway wall ($P < 0.01$) (**Figure 3B**) and smooth muscle layer ($P < 0.05$) (**Figure 3C**) in comparison with rats in sham group. SBF significantly reduced the thickness of small airway wall ($P < 0.05$) (**Figure 3B**) or smooth muscle layer ($P < 0.05$) (**Figure 3C**) in comparison with CSL-treated rats without SBF.

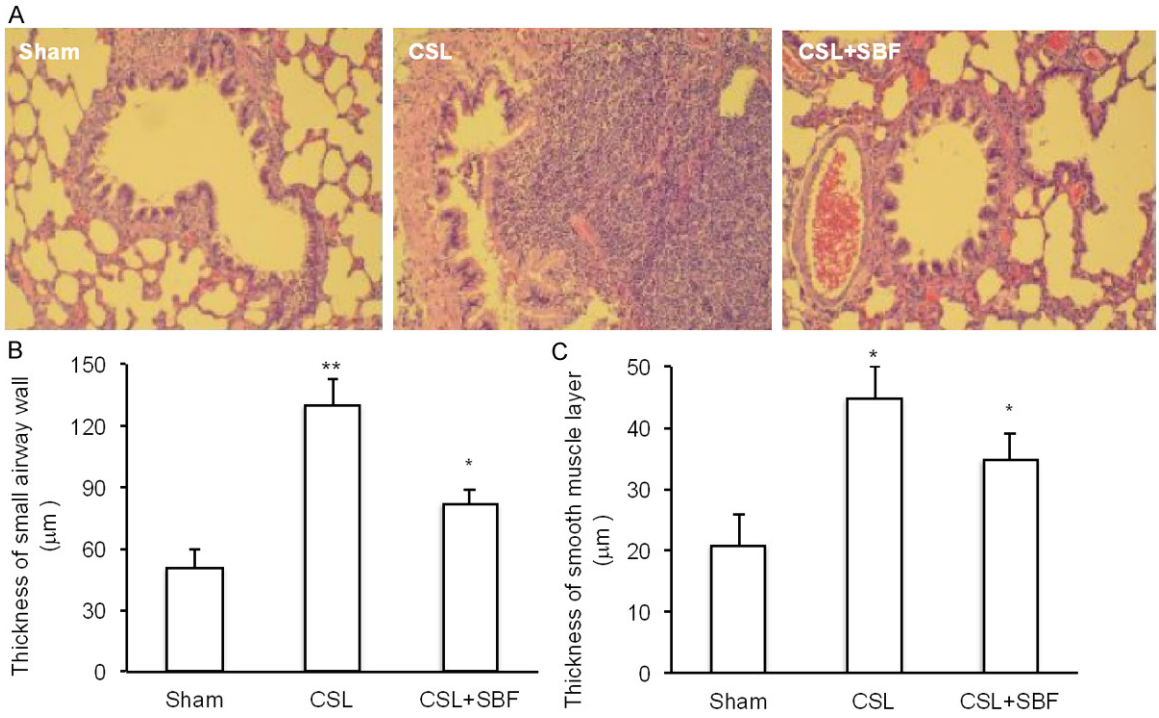

Figure 3. Inhibition of thickness of small airway wall *in vivo* by SBF. (A) Rat COPD model was induced by CSL treatment, and treated with or without SBF. Rats received PBS only without CSL or SBF treatment served as control (Sham) (n=10 per group). Lung tissues harvested from the three groups of rats were immediately subjected to H&E staining and photographed under a microscopy. (B, C) Thickness of airway wall (B) or smooth muscle layer (C) was calculated based total airway wall square or smooth muscle layer square and divided by the perimeter of the airway wall or the smooth muscle layer. Statistic significant was detected when compared with CSL treatment and sham (*P < 0.05, **P < 0.01). *Statistic significant was detected when compared with CSL treatment and CSL+SBF treatment ($P < 0.05$).

SBF promoted HDAC2 but inhibited NF-κBp65 protein expression in small airway wall in vivo

HDAC2 and NF-κB proteins are two crucial molecules involved in the regulation of inflammation in small pulmonary airway wall. HDAC2 functions as a suppressive molecule and NF-κB acts as an inflammation-activator [21]. To examine the effect of SBF on the expression of HDAC2 or NF-κB in small pulmonary airway wall *in vivo*, we performed immunohistochemical assay on the tissues of rat small airway wall isolated from the three groups of rats in the COPD model. Consistent with the results from cell culture *in vitro* (**Figure 2**), immunohistochemical staining with anti-HDAC2 or anti-NF-κBp65 antibodies demonstrated that CSL treatment reduced HDAC2 protein expression in smooth muscle layer of small airway wall in the treated rats (**Figure 4A**) in comparison with sham treatment (**Figure 4A**). However, SBF treatment markedly enhanced HDAC2 production in small airway wall (**Figure 4A**), compared with the one without SBF treatment (**Figure 4A**). Analysis of NF-κBp65 protein expression showed that CSL-treatment induced robust expression of NF-κBp65 in small airway wall of the treated rats (**Figure 4B**) in comparison with sham treatment (**Figure 4B**). However, SBF treatment markedly reduced the expression of NF-κBp65 protein in small airway wall of CSL-treated rats (**Figure 4B**) in comparison with the one without SBF treatment (**Figure 4B**).

SBF improved pulmonary function in rat COPD model

To explore whether SBF treatment could lead to the improvement of lung function *in vivo*, we monitored the respiratory parameters including respiratory rate, $FVE_{0.3}$/FVC, Ri and Re in the three groups of rats in the COPD model. Measurement of those parameters demonstrated that CSL treatment significantly increased respiratory rate ($P < 0.05$) (**Figure 5A**), Ri ($P < 0.01$) (**Figure 5C**) and Re ($P < 0.05$)

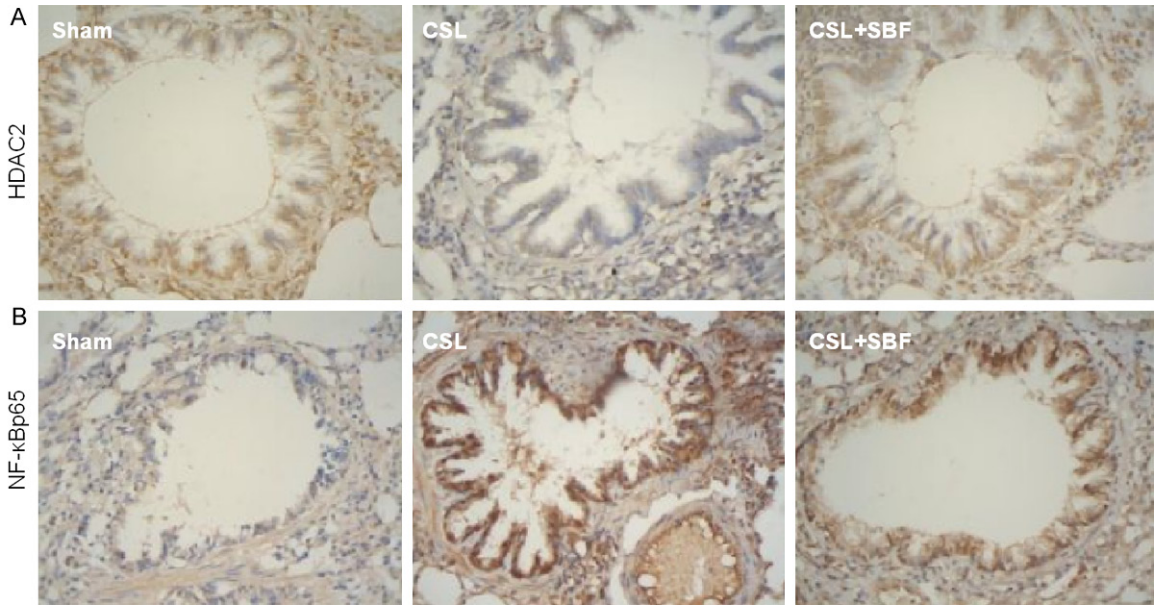

Figure 4. Effect of SBF on HDAC2 and NF-κB protein expression in rat airway wall *in vivo*. Pulmonary airway wall tissues were harvested from rats (n=10 per group) treated with CSL, CSL plus SBF (CSL+SBF), or without treatment (Sham), and subjected to immunohistochemical staining with anti-rat HDAC2 (A) or anti-NF-κBp65 antibodies (B). The images were representatives of 10 rat samples per group, photographed under a microscope (10×).

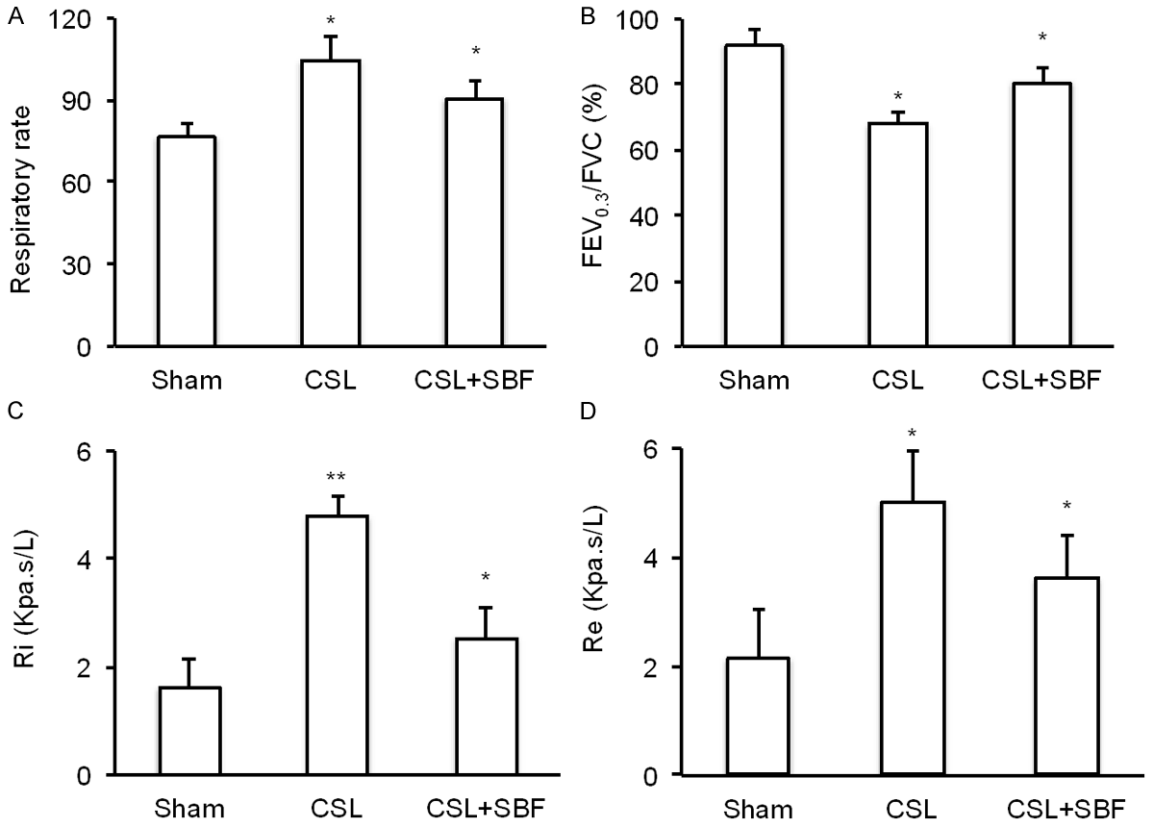

Figure 5. Therapeutic effect of SBF on COPD *in vivo*. Rat in the three groups of rats (n=10 per group) treated with CSL, CSL+SBF, or sham were subjected to respiratory parameter test using a PFT Pulmonary Maneuvers. The parameters include respiratory rate (A), $FEV_{0.3}$/FVC (B), Ri (C), and Re (D). Statistic significant was detected when compared with CSL treatment and sham (*P < 0.05, **P < 0.01). *Statistic significant was detected when compared with CSL treatment and CSL+SBF treatment (*P* < 0.05).

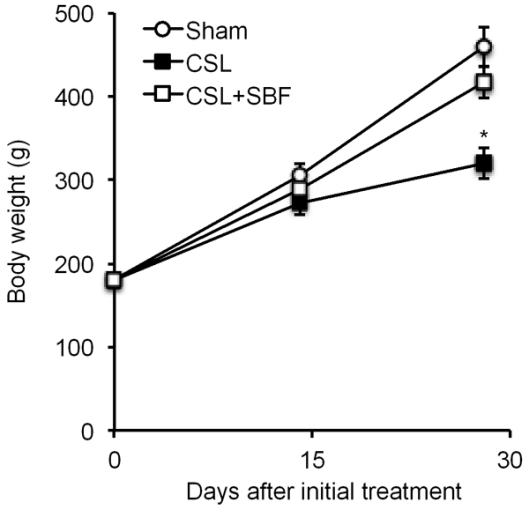

Figure 6. Effect of SBF on rat body weight change. Body weight in the three groups of rats (n=10 per group) treated with CSL, CSL+SBF or sham was measured with a digital scale on day 0, 14 and 28. Data were presented as mean ± SD and analyzed by One-way ANOVA. *$P < 0.05$, comparison between sham and CSL treatment. *$P < 0.05$, comparison between SBF+CSL treatment and and CSL treatment.

(**Figure 5D**), with decreased ratio of $FEV_{0.3}$/FVC ($P < 0.05$) (**Figure 5B**) in the treated ratsin comparison with sham treatment respectively. However, SBF treatment significantly reduced respiratory rate ($P < 0.05$) (**Figure 5A**), Ri ($P < 0.05$) (**Figure 5C**) and Re ($P < 0.05$) (**Figure 5D**) respectively, and increased $FEV_{0.3}$/FVC ratio ($P < 0.05$) (**Figure 5B**) in treated rats in comparison with CSL treatment in absence of SBF. Monitoring total body weight change in the three groups of rats demonstrated that CSL treatment significantly suppressed the growth of rats ($P < 0.05$), with 32% decrease of total body weight on day 28 in comparison with the one in sham group (**Figure 6**). SBF treatment significantly restored the growth of CSL-treated rats in comparison without SBF treatment ($P < 0.05$) (**Figure 6**). There was no significant difference of body weight change between sham group and SBF-treated group (**Figure 6**).

Discussion

In this study, we demonstrate that SBF-a Chinese medicine has a suppressive effect on cigarette smoke-induced proliferation of primary rat ASMC and inflammatory response in the cells. SBF treatment significantly inhibits the thickening of small airway wall and improves lung function in rat COPD model, which suggests that SBF has potent therapeutic effect on COPD and could be utilized to treat the disease.

NF-κB plays a central role in chronic inflammatory airway diseases, contributing to the excessive proliferation of ASMC and pulmonary airway remodeling [21, 22]. Therapeutic targeting NF-κB can suppress cigarette smoke-induced airway inflammatory responses and inhibit the proliferation of ASMC. Inhibitors against NF-κB and its up-stream signaling molecule IκB kinase (IKK) have been shown to reduce airway inflammation [23]. However, some of the inhibitors could not suppresss moke-evoked airway inflammation. Inhibition of NF-κB by SBF significantly reduces cigarette smoke-induced growth of rat pulmonary ASMC indicates that SBF has a potent anti-inflammation activity and could serve as a potential therapeutic for the treatment of COPD. Indeed, SBF treatment markedly inhibited the expression of NF-κB in small airway wall, reduced the thickness of smooth muscle layer and small airway wall, and improved lung function in rat COPD model. Those results further inform the mechanistic action of SBF through the suppression of NF-κB in COPD.

HDAC2, a class I histone deacetylase family member [24], has a property to suppress inflammatory gene expression in patients with COPD [25]. HDAC2 collaborates with histone acetyltransferases to maintain the balance between histone acetylation and deacetylation [26]. It has been shown that HDAC2 is linked to cell-cycle progression and cell proliferation [27], and important for the anti-inflammation effect of glucocorticoid treatment [28]. HDAC2 expression level and activity in lung parenchyma and bronchial biopsies from patients with COPD are significantly lower, which is correlated with the severity of inflammation and the progression of the disease [28]. Corticosteroids are widely used for the treatment of COPD, but have very limited effect to contain the underlying chronic inflammation when utilized as monotherapy due to the decreased expression of HDAC2 [25, 29-31]. In this study, we observed that CSE exposure significantly reduced HDAC2 protein expression in rat pulmonary ASMC. We also discovered that the HDAC2 level can be restored by SBF. CSL-

induced COPD rat model was utilized to evaluate inflammatory response and drug therapeutic efficacy [18, 32]. Chinese medicines have been shown to possess potential therapeutic effect to improve lung function in COPD [33-37]. In this study, we utilized the similar rat COPD model to examine the therapeutic effect of Chinese medicine SBF on COPD in rats. Consistent with *in vitro* finding, we demonstrated that SBF treatment restored HDAC2 level in the small airway wall of the treated rats in the COPD model. Our data also showed that SBF treatment improved lung function without significant side effect on total body weight in rat COPD model, suggesting that SBF has limited toxicity *in vivo*.

In conclusion, we have demonstrated that Chinese medicine SBF has potent capability to inhibit inflammatory response and improve lung function in rat COPD model. We propose that SBF could offer a new approach for the treatment of COPD.

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Disclosure of conflict of interest

None.

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