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Original Article

A chinese herbal formula ameliorates COPD by inhibiting the inflammatory response via downregulation of p65, JNK, and p38

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ABSTRACT

Background: Bufei Yishen formula (BYF), a traditional Chinese medicine (TCM), is an effective therapeutic strategy for patients with chronic obstructive pulmonary disease (COPD).
Purpose: To evaluate the efficacy of BYF and investigate its therapeutic mechanisms.
Methods: A total of 134 patients completed the study: 68 patients treated by BYF combined with conventional Western medicine in the trial group; and 66 patients treated using conventional Western medicine in the control group. The efficacy of BYF was evaluated by a subgroup analysis of data obtained from a four-center, open-label, randomized controlled trial of comprehensive TCM interventions. A rat model of COPD was treated with the key active molecules (KAM) of BYF for 8 weeks. An in vitro model of COPD was also treated with KAM.
Results: Patients treated with BYF had reduced frequency of acute exacerbation of COPD ($p < 0.001$) and duration ($p = 0.028$), dyspnea scale ($p = 0.007$), 6-min walking distance ($p = 0.048$). There were no differences observed in forced vital capacity in one second (FVC), forced expiratory volume in one second (FEV1), and FEV1 percentage of the predicted value (FEV1%). The five KAM of BYF (KAM-BYF) improved lung function, including tidal volume, minute ventilation, peak expiratory flow, FVC, FEV0.1, and FEV0.3, and pathological changes in COPD rats. Treatment with KAM-BYF markedly decreased the levels of interleukin 6 (IL6), tumor necrosis factor-α (TNF-α), matrix metalloproteinase 9 (MMP9), and MMP12 in serum and bronchial alveolar lavage fluid. In airway epithelial cells, KAM-BYF decreased the levels of TNF-α-induced IL8 and IL6. Finally, we discovered that the anti-inflammatory effects of KAM-BYF in COPD rats and BEAS-2Bs were mediated through inhibition of nuclear factor-kappaB (NF-κB) p65, c-Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase signaling.
Conclusions: BYF exerts beneficial effects in patients with COPD via inhibition of inflammation.

Introduction

Chronic obstructive pulmonary disease (COPD), which is characterized by a progressive and partially reversible airflow limitation, is a leading cause of morbidity and mortality (GOLD, 2020). Abnormal inflammatory responses and proteolytic activity contribute to poor lung function (Maigeng et al., 2019). Therefore, the control of inflammatory processes and proteolytic activity is a potential approach to suppressing the progression of COPD. However, present strategies for the prevention of progression or reversal of COPD are limited.

Abbreviations: AECOPD, acute exacerbation of COPD; BYF, Bufei Yishen formula; COPD, chronic obstructive pulmonary disease; CTCMI, comprehensive TCM interventions; FEV1, forced expiratory volume in 1 s; FEV1%, FEV1 percentage of the predicted value; FVC, forced vital capacity; KAM, key active molecules; TCM, traditional Chinese medicine; MV, minute ventilation; PEF, peak expiratory flow; TV, tidal volume; mMRC, modified Medical Research Council; 6MWD, 6-min walking distance.

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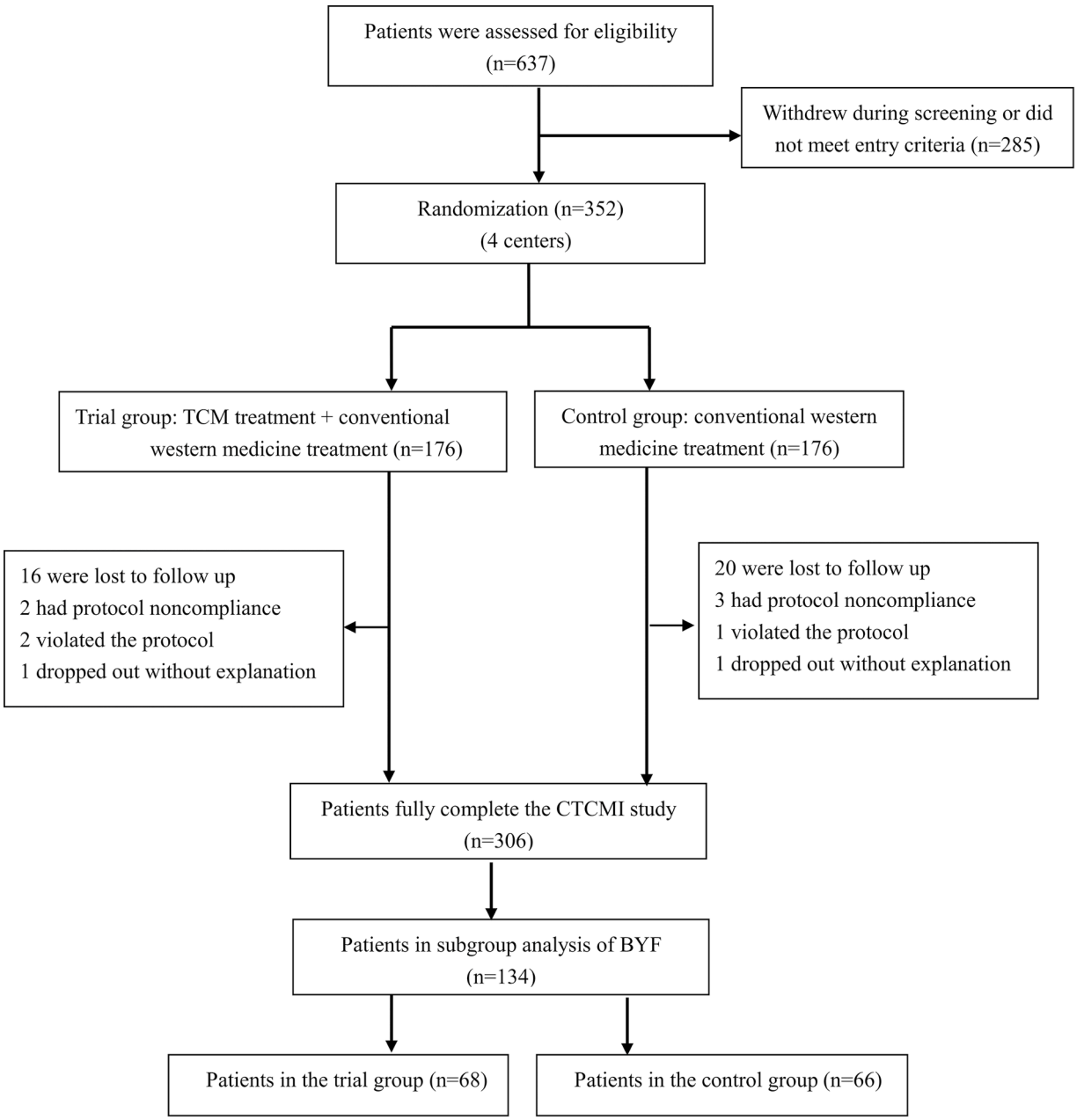

Fig. 1. The flow diagram of the clinical trial approach.

At present, the treatment of COPD is mainly based on the classes of medications recommended by the global initiative for chronic obstructive lung disease (GOLD) (GOLD, 2020). Although more alternative approaches and appropriate treatment plans are promoted in patients with COPD, definite evidence on the effect of treatment with traditional Chinese medicine (TCM) remains limited. Use of the TCM pattern, which reflects the concepts of individualized therapy, has potential advantages for patients with COPD (Li et al., 2012). This pattern is a specific stratification of a disease according to a group of symptoms, which can be regarded as a summary of the body's condition at a certain stage in a disease process. The Bufei Yishen formula (BYF) is prescribed for COPD patients with the typical TCM patterns, including panting, shortness of breath, lassitude, spontaneous sweating, weakness in the lower back and knees, tinnitus, vertigo, frequent nocturia, soreness, and weakness of the waist and knees. Previous studies have shown the efficacy of BYF in patients with COPD, improving their quality of life and reducing the frequency of exacerbation (Li et al., 2012, 2013). However, limited evidence has been found concerning one specific herbal intervention based on a single TCM pattern for patients with COPD. Therefore, we performed a subgroup analysis of data obtained from the comprehensive TCM interventions (CTCMI) study to evaluate the efficacy of BYF corresponding to the lung-kidney qi deficiency TCM pattern.

BYF contains thousands of compounds, and its efficacy is achieved by a combination of effective components rather than a single compound (Li et al., 2015). However, the complexity of these effective components complicates the elucidation of the therapeutic mechanisms involved in this process. Thus, we previously applied the method of effective-component compatibility in an in vivo model to identify the key active molecules in BYF (KAM-BYF), which could achieve bioactive equivalence compared with the original BYF (Li et al., 2020). In addition, airway epithelial cells are activated by cigarette smoke and other irritants, and release proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin 1B (IL1B), IL6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and C-X-C motif chemokine

ligand 8 (CXCL8), IL6 and IL8, via autocrine and paracrine mechanisms (Barnes, 2016). Cytokines can recruit inflammatory cells, leading to airway inflammation and airspace enlargement. Moreover, mitogen-activated protein kinases (MAPKs) and the nuclear factor-kappaB (NF-κB) signaling pathway are activated to upregulate inflammatory genes in airway epithelial cells (Gaffey et al., 2013). Inhibition of MAPKs and NF-κB signals is an effective approach to suppressing airway inflammation in COPD (Banerjee et al., 2012). Therefore, we investigated the therapeutic effect of the KAM-BYF on COPD rats, and the anti-inflammatory mechanisms in airway epithelial cells induced by TNF-α.

In this study, we performed a subgroup analysis of data obtained from the CTCMI study to evaluate the efficacy of BYF. We also comprehensively investigated the mechanism of action of the KAM-BYF in vivo and in vitro.

Materials and methods

Clinical study

The CTCMI study, a four-center, open-label, randomized controlled trial was conducted following the Good Clinical Practice guidelines. The study was approved by the Ethical Research Committees of the First Affiliated Hospital of Henan University of Chinese Medicine (batch number: YFYKTLL2007–1), and was registered in the Chinese Clinical Trial Register Center (ChiCTR-TRC-11,001,406). All patients received the treatment voluntarily and signed the written informed consent before inclusion. A total of 352 patients who met the inclusion criteria were randomly divided into the trial and control groups of the CTCMI study; two patients who violated the protocol were excluded; and 44 patients who did not fully complete the study were withdrawn from the analysis. Therefore, 306 patients fully completed the study. The stratified and block randomization design was adopted, with a ratio of 1-to-1 and length of block four. The design was provided by the DME department of Guangzhou University of TCM. Regarding the subgroup analysis on the efficacy of BYF, 134 patients from four research centers were included, with 68 and 66 patients allocated to the trial and control groups, respectively (Fig. 1).

Patients

Patients met the inclusion criteria: met the COPD diagnostic criteria, met the TCM pattern of lung-kidney qi deficiency criteria of COPD, patients in the stable status and met the diagnosis of mild to severe COPD (GOLD 1, 2, 3), age between 40 and 80 years, no experience in other interventional trials in the previous one month, in which were enrolled from the out-patient department. If patients had confusion, dementia or any type of mental illness, and serious diseases such as tumor, heart failure, liver and kidney diseases, and allergic to treatment drugs were excluded.

Interventions

In the control group, conventional Western medicine was applied to patients with COPD recommended by GOLD (2007). Albuterol sulfate (Ventolin, 100 μg/dose; GlaxoSmithKline) was used for GOLD1 patients (100 μg). Formoterol fumarate dehydrate (Oxis Turbuhaler, dose: 4.5 μg twice daily; AstraZeneca) was used for GOLD2 patients. Salmeterol/-fluticasone propionate (Seretide; GlaxoSmithKline) was used for GOLD3 patients (dose: 50/250 μg twice daily).

In the trial group, in addition to treatment with conventional Western medicine, patients received BYF granules for lung-kidney qi deficiency (batch number: 080,102) (4.25 g per bag, three bags each time, twice daily for 6 months). The granules were compound preparations of TCM and produced by Jiang Yin Tian Jiang Pharmaceutical Co. Ltd. The quality of the granules was consistent with the required quality standards.

Outcomes

The frequency and duration of acute exacerbation of COPD (AECOPD) during treatment were recorded over 12 months. AECOPD refers to acute exacerbation of the original conditions of patients (e.g., dyspnea, cough, and/or expectoration) in the process of disease, which exceeds the daily routine variation and requires a change in treatment. Usually, in the course of AECOPD, aggravation of shortness of breath is often accompanied by dyspnea, chest tightness, worsening of cough, increased sputum volume, changes in color and/or viscosity of sputum, and fever (2007; Li JS, 2012). If the interval between the onset of AECOPD was $\leq$ 1 week, it was counted as one exacerbation. Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and FEV1 percentage of the predicted value (FEV1%) were tested. The modified Medical Research Council Dyspnea Scale questionnaire was utilized, and the 6-min walking distance (6MWD) was evaluated. The timepoints of evaluation were before treatment, at months 3 and 6 during the treatment period, and at 12 months of the follow-up period (18 months).

Chemicals and animals

Klebsiella pneumoniae (strain ID: 46,114) was purchased from the National Center for Medical Culture Collection (Beijing, China). Cigarettes were obtained from Henan Tobacco Industry (Hongqi Canal® Filter tip cigarette; tobacco type, tar: 10 mg; nicotine content: 1.0 mg; carbon monoxide: 12 mg; Zhengzhou, China). Aminophylline (APL) was obtained from Shandong Xinhua Pharmaceutical Co., Ltd. (Shandong, China). TNF-α, IL6, matrix metalloproteinase 9 (MMP9), and MMP12 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster Biological Engineering (Wuhan, China). Antibodies against NF-κB p65, p38 MAPK, or stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) were purchased from Cell Signaling Technology (Danvers, MA, USA). KAM-BYF (35.0625 mg/ml) is composed of 20-S-ginsenoside Rh1 (6.25 mg/ml), astragaloside IV (1.25 mg/ml), icariin (25 mg/ml), nobiletin (1 mg/ml), and paeonol (1.5625 mg/ml). The purity of these compounds was > 99%.

Fifty Sprague–Dawley rats (25 males and 25 females, age: 6–8 weeks, weight: 200 ± 20 g) were purchased from the Experimental Animal Center of Henan Province (Zhengzhou, China). All animals were acclimatized to laboratory conditions for seven days before proceeding to the in vivo studies. The animal experiments were conducted with the approval of Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Chinese Medicine (DWLL201903210). Animals were housed in filter-top cages under standard conditions of humidity (50% ± 10%), temperature (25 ± 2 °C), and light (12-h light/dark cycle), and fed with a lab rat maintenance diet (SPF (Beijing) Biotechnology Co.,Ltd.) and purified water (Millipore, Milli-Q).

COPD model and treatment

The COPD model in rats was established as previously described (Li et al., 2015, 2012a, 2012b). Briefly, rats were placed in a chamber and exposed to smoke of eight cigarettes for 30 min twice daily, with 3 h of smoke-free intervals during the first 2 weeks. Subsequently, they were exposed to the smoke of 15 cigarettes for 30 min thrice daily, with 3 h of smoke-free intervals for the next 10 weeks. *Klebsiella pneumonia* suspension (6×10^8 CFU/ml, 100 μl) was inoculated every 5 days for the first 8 weeks. Control rats received the same treatment, but were exposed to air rather than cigarette smoke.

On week 9, rats were divided into four subgroups: (1) normal group ($n = 12$): rats that intragastrically received normal saline (2 ml); (2) COPD model group ($n = 12$): COPD rats that intragastrically received normal saline (2 ml); (3) COPD with KAM-BYF treatment group ($n = 12$):

COPD rats intragastrically treated with KAM-BYF (5.5 mg/kg) once daily for 8 weeks; and (4) COPD with APL treatment group ($n = 12$): COPD rats that received oral gavage with APL (54 mg/kg) once daily for 8 weeks.

Pulmonary function analysis

Pulmonary function was detected through computer-controlled unrestrained pulmonary function-testing plethysmography (Buxco Inc., Wilmington, NC, USA). Rats were anesthetized with pentobarbital sodium (35 mg/kg, ip) and placed in the chamber with a reference chamber, which was connected to the Buxco air flow transducers. Tidal volume (TV), peak expiratory flow (PEF), and minute ventilation (MV) were evaluated every 4 weeks. At week 16, rats were anesthetized, and a tracheotomy was performed. FVC and FEV0.1 were determined.

Histopathology

Tissue samples collected from the left lower lobe were fixed in 10% formalin, embedded in paraffin, and sliced into 4-μm sections. For histological examination, paraffin sections were stained with Mayer's hematoxylin and 1% eosin. Sections were observed and evaluated by optical microscopy and a photographic system (Olympus, Tokyo, Japan) (Li et al., 2015). The alveolar mean linear intercept (μm) and the mean alveolar number (mm^2) were counted using the counting tool of the Adobe Photoshop CC software.

ELISA assay

The levels of IL6, TNF-α, MMP9, and MMP12 in the serum and bronchial alveolar lavage fluid (BALF) supernatants, as well as those of IL6, TNF-α, and IL8 in cell culture supernatant were detected using ELISA kits (Boster Biological Engineering) according to the instructions provided by the manufacturer.

Cell culture and treatment

The human bronchial cell line BEAS-2B (ATCC) was cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) at 5% CO_2 and 37 °C. The cells were treated with KAM-BYF (35.0625 μg/ml) or vehicle (cell culture/0.1% dimethyl sulfoxide) 3 h prior to stimulation with TNF-α (10 ng/ml). Samples for analysis by ELISA and western blotting were collected at 24 h and 3 days, respectively.

Western blotting

Total proteins from lung tissues or cells were obtained using radioimmunoprecipitation assay lysate buffer according to the instructions provided by the manufacturer. Proteins were stored at −80 °C for western blotting assay; lung tissue and cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred through electrophoresis to polyvinylidene difluoride membranes. The membranes were blocked with 10% fat-free milk for 1 h, and incubated with these primary antibodies overnight at 4 °C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, signals were visualized using enhanced chemiluminescence (Pierce, Holmdel, NJ, USA)

Statistical analysis

For the clinical study, differences between two groups were analyzed by independent-samples t-tests or Mann–Whitney *U*-tests based on data distribution. The differences of time continuous observations were measured by analysis of variance (ANOVA) of repeated measures. The absolute frequency was described for numerical data. *P*-values were two-tailed and the α level of significance was set at 0.05. For the animal and cell study, comparisons of the data were performed using one-way ANOVA and Tukey's post-hoc test. *P*-values < 0.05 denoted statistically significant differences. All statistical analyses were performed using SAS9.2 (SAS Institute Inc., Cary, NC, USA)

Table 1
Baseline Characteristics of the Patients.

Characteristics	Trial group ($n = 68$)	Control group ($n = 66$)	*p*
Age (Years)	63.35±9.28	65.88±8.45	0.102
Gender			
Male	52	47	0.557
Female	16	19	
Course of disease (Month, $x \pm s$) □	139.81±117.01	165.26±131.23	0.239
BMI ($x \pm s$)	23.24±2.07	23.92±3.05	0.127
Exacerbation ■			
Frequency (times, $x \pm s$)	3.45±2.23	3.25±2.16	0.593
Duration (days, $x \pm s$)	9.70±5.42	10.14±6.01	0.663
Level of education (Case)			
Illiterate / semi illiterate	4	7	0.862
Primary school	18	17	
Middle school	18	16	
Senior high school	14	11	
College degree or above	14	15	
Smoking status (Case) ●			
None-smoking	21	27	0.351
Used to smoke and quit	6	4	
Less smoking	3	6	
Heavy smoking	38	29	
GOLD classification (Case)			
GOLD 1	6	11	0.334
GOLD 2	25	25	
GOLD 3	37	30	

Notes: □The course of disease was calculated in months. ■Exacerbations during the 12 months before screening were self-reported. ●The Fisher's exact test.

Results

General information

Patient demographics are described in Table 1. There was no significant difference in age, sex, course of disease, body mass index, exacerbations, level of education, smoking status, and GOLD classification of lung function between the two groups ($p > 0.05$).

Comparison of the frequency and duration of AECOPD

At the time points of 6 and 18 months, there was a significant reduction in the frequency of AECOPD in the treatment group ($p = 0.002$ and $p = 0.016$, respectively). The average frequency and the constituent ratio were significantly reduced in the treatment group ($p = 0.000$ for both). The average duration of AECOPD was significantly different between the two groups ($p = 0.028$). The results are shown in Table 2.

Comparison of lung function

At 3, 6, and 18 months, a downward trend was observed for FVC, FEV1, and FEV1%. However, there were no significant differences in these measures between the two groups ($p = 0.890$, $p = 0.477$, and $p = 0.814$, respectively). The results are shown in Table 3.

Comparison of the dyspnea scale

The mean scores of the dyspnea scale and 6MWD of the trial group were significantly lower over time compared with those of the control group ($p = 0.007$ and $p = 0.048$, respectively). At 3, 6, and 18 months, there were significant differences in the mean scores of the dyspnea scale

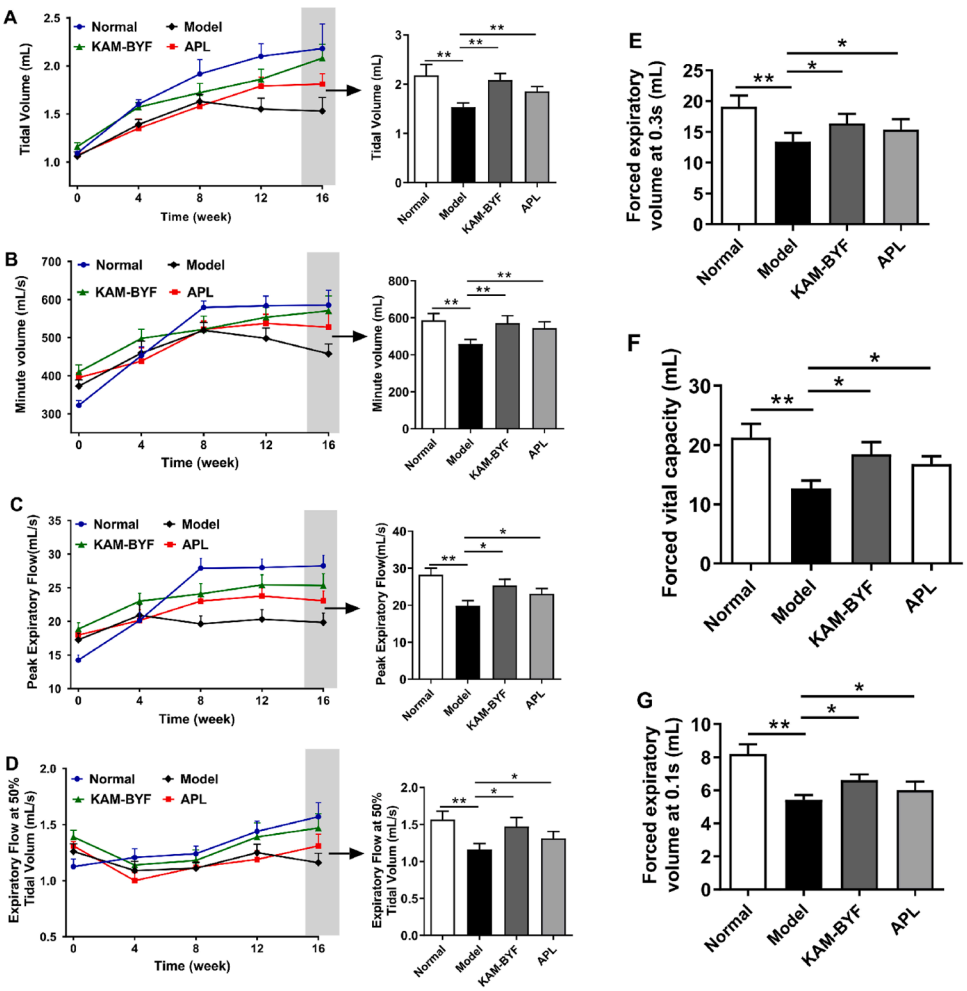

Fig. 2. Effect of KAM-BYF on the lung functions of COPD rats. COPD rats were treated with KAM-BYF (5.5 mg/kg) and aminophylline (APL; 54 mg/kg) for 8 weeks (weeks 9–16). Changes in (A) tidal volume, (B) maximum minute ventilation, (C) peak expiratory flow, and expiratory flow at 50% tidal volume were examined every 4 weeks. Forced expiratory volume at 0.3 s, forced vital capacity, and forced expiratory were detected at week 16. All data are presented as the mean ± SEM. $n = 6$ for each group. * $p < 0.05$, ** $p < 0.01$.
BYF, Bufei Yishen; COPD, chronic obstructive pulmonary disease; KAM, key active molecules; SEM, standard error of the mean.

Table 2
Comparison of the frequency and duration of AECOPD.

Variable	Trial group ($n = 68$)	Control group ($n = 66$)	t/Z	p
Frequency (times)				
Month 0	3.45±2.23	3.25±2.16	0.535	0.593
Month 6	0.37±0.52	0.77±0.87	−3.257	0.002
Month 18	0.59±0.80	0.95±0.94	−2.438	0.016
Average frequency	0.64±0.70	1.15±0.93	−3.612	0.000
Frequency (constituent ratio)				
Have exacerbation	15	34	12.529	0.000
No exacerbations	53	32		
Duration(days)				
Average duration	4.62±5.85	6.77±5.32	−2.227	0.028

and 6MWD in the treatment group compared with the control group (dyspnea scale, $p = 0.013$, $p = 0.001$, and $p = 0.003$, respectively; 6MWD, $p = 0.037$, $p = 0.012$, and $p = 0.006$, respectively). The results are shown in Table 4.

Effect of kam-byf on the pulmonary function of copd rats

Next, we used a rat model of COPD to investigate the potential mechanisms of action. Histopathological changes and airflow limitation are the hallmark features of COPD. Therefore, we assessed the effects of KAM-BYF on lung tissue morphology and lung mechanics. From week 4, exposure to smoke decreased the TV, MV, and PEF (Figs. 2A–D). Treatment with KAM-BYF and APL could restore these parameters toward values in the control group. In addition, we found that FVC, FEV0.1, and FEV0.3 were decreased in COPD rats. Also, KAM-BYF and the positive control APL improved lung function (Figs. 2E–G).

Histopathological analysis of lung tissue revealed that exposure to smoke induced severe pathological changes in COPD rats, such as alveolar destruction and alveolar cavity expansion. Administration of KAM-BYF and APL suppressed lung damage. Image quantification also showed that the mean alveolar number in the KAM-BYF and APL groups was significantly increased compared with that recorded in the model group. In contrast, the mean linear intercept was significantly decreased (Fig. 3).

Effect of KAM-BYF on the levels of pro-inflammatory cytokines and proteases in the COPD rat model

We next examined the pulmonary inflammatory responses by measuring the levels of pro-inflammatory cytokines in the serum and BALF. As shown in Fig. 4, the levels of IL6 and TNF-α increased in COPD rats, and this increase was inhibited by the administration of KAM-BYF and APL.

Increased production of proteases is an important mechanism for the development of COPD. We measured the levels of MMP9 and MMP12 in the serum and BALF. As shown in Fig. 5, the levels of MMP9 and MMP12 were significantly increased in the COPD rat model, and KAM-BYF and APL could suppress the overproduction of proteases.

Subsequently, we focused on the mechanisms underlying the effect

Table 3
Comparison of the FVC, FEV1, FEV 1% of lung function.

Variable	N	Month 0	Month 3	Month 6	Month 18	F	*p*
FVC							
Trial group	68	2.65±0.91	2.67±0.82	2.62±0.75	2.58±0.78	0.019	0.890 △
Control group	66	2.63±0.91	2.62±0.75	2.68±0.85	2.62±0.81	0.799	0.477 ▲
t/Z		0.085	0.064	−0.408	−0.287	0.278	0.814 ●
p		0.933	0.949	0.684	0.774		
FEV1							
Trial group	68	1.36±0.50	1.40±0.49	1.40±0.46	1.33±0.48	0.989	0.322 △
Control group	66	1.28±0.41	1.34±0.40	1.34±0.41	1.24±0.41	6.837	0.000 ▲
t/Z		0.966	0.775	0.770	1.205	0.27	0.833 ●
p		0.336	0.439	0.442	0.231		
FEV 1%							
Trial group	68	52.36±10.40	53.15±11.71	53.67±9.89	51.96±9.69	1.427	0.234 △
Control group	66	50.76±12.08	52.44±12.67	51.76±11.93	48.57±10.73	3.565	0.017 ▲
t/Z		0.826	0.333	1.011	1.921	0.786	0.492 ●
p		0.411	0.739	0.314	0.057		

Notes: △The main effect of grouping factor. ▲The main effect of time. ●The crossover effect of time and grouping factor.

Table 4
Comparison of the mMRC and 6MWD.

Variable	N	Month 0	Month 3	Month 6	Month 18	*F*	*p*
mMRC							
Trial group	68	1.93±0.72	1.50±0.72	1.31±0.76	1.26±0.68	7.629	0.007 △
Control group	66	1.97±0.66	1.80±0.66	1.70±0.61	1.61±0.63	35.365	0.000 ▲
t/Z		−0.363	−2.53	−3.266	−3.006	4.066	0.014 ◆
p		0.717	0.013	0.001	0.003		
6MWD							
Trial group	68	363.50±75.19	394.13±65.63	412.18±68.13	409.49±66.21	3.969	0.048 △
Control group	66	360.64±82.19	368.68±74.31	381.11±73.17	376.44±71.50	34.586	0.000 ▲
t/Z		0.211	2.103	2.545	2.777	6.881	0.001 ◆
p		0.834	0.037	0.012	0.006		

Notes: △The main effect of grouping factor. ▲The main effect of time. ●The crossover effect of time and grouping factor.

of treatment with KAM-BYF on the inflammatory response in COPD rats. We investigated the activation of three main signaling pathways (p38, NF-κB, and SAPK/JNK), which have been shown to play an important role in inflammatory responses in COPD. The phosphorylated levels of p38, NF-κB, and SAPK/JNK were increased in lung tissues of COPD rats, and significantly decreased in KAM-BYF-treated COPD rats. Total p38 MAPK, NF-κB, and SAPK/JNK did not change regardless of treatment (Fig. 6).

Effect of KAM-BYF on the inflammatory response in beas-2b cells induced by TNF-α

Bronchial epithelial cells play an important role as immune effector cells and may generate and release mediators of inflammation. To investigate whether treatment with KAM-BYF suppresses the inflammatory response in bronchial epithelial cells, BEAS-2B cells were treated with 10 ng/ml TNF-α and KAM-BYF; the release of IL8, IL6 was detected. As shown in Fig. 7, IL8 and IL6 were induced following exposure of BEAS-2B to TNF-α. Interestingly, treatment with the KAM-BYF clearly inhibiting the production of these cytokines.

To further investigate the mechanisms underlying the effects of KAM-BYF on the release of IL8, IL6, and TNF-α, we assessed the activation of p38 MAPK, JNK, and NF-κB, which play a role in inflammatory responses. As shown in Fig. 8, phosphorylation of SAPK/JNK, NF-κB, and p38 MAPK was increased in BEAS-2B cells after exposure to TNF-α. Treatment with KAM-BYF significantly decreased the phosphorylation of SAPK/JNK, NF-κB, and p38 MAPK; however, it had no effect on their protein levels.

Discussion

In this study, we demonstrated that treatment with BYF reduced the frequency and duration of AECOPD (based on the dyspnea scale and 6MWD of patients with COPD). KAM-BYF could suppress the decrease of pulmonary function, pathological changes, and inflammation in COPD rats. Furthermore, KAM-BYF protected against TNF-α-induced inflammatory response in epithelial cells. This influence was partially mediated through the inhibition of JNK, p38, and NF-κB p65.

The major goal of COPD clinical therapy is to reduce the frequency and duration of exacerbations, delay the decline of lung function, ameliorate symptoms, and improve exercise endurance. Over the 6-month treatment and 12-month follow-up periods, BYF had beneficial effects against COPD. The frequency and duration of AECOPD in the trial group were 0.64 times per year and 4.62 days, respectively, while those of the control group were 1.15 times per year and 6.77 days, respectively. The rate of decline in FVE1 in the trial and control groups was approximately 30 ml and 40 ml per year, respectively. These rates are consistent with the results of other large trials. The improvement in modified Medical Research Council Dyspnea Scale was 34.71% and 18.27% in the trial and control groups, respectively. The improvement in the 6MWD was 45.99 m and 15.80 m, respectively. Treatment with BYF reduced the symptoms and improved the exercise tolerance of COPD patients. This may be due to the combined effect of conventional Western medicine and Chinese medicines. Systemic inflammation was evaluated in the CTCMI study (Li et al., 2012). The levels of serum C-reactive protein, IL8, TNF-α were measured at 6 months. After treatment, the levels of C-reactive protein and TNF-α in the two groups were lower than prior to treatment, and the levels in the trial group were significantly lower than those recorded in the control group.

BYF contains thousands of molecules; only a fraction of those are the KAM of BYF for the treatment of COPD (Li et al., 2015). Thus, we identified the KAM (i.e., 20-S-ginsenoside Rh1, astragaloside IV, icariin, nobiletin, and paeonol) in the herbal medicine BYF, which could achieve bioactive equivalence compared with the original BYF (Zhang et al.,

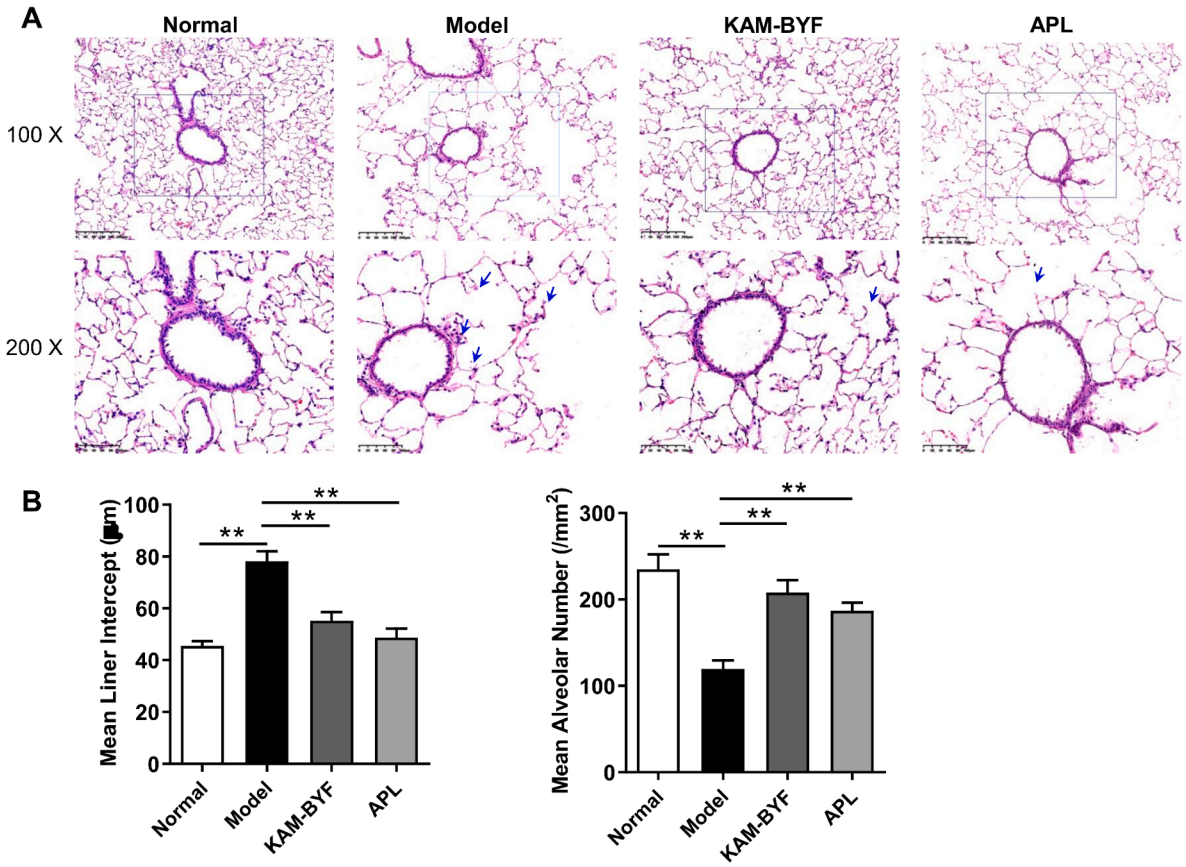

Fig. 3. Effect of KAM-BYF on the pathological changes in COPD rats. (A) HE staining (magnification, × 100). (B) Alveolar number. (C) Alveolar diameter. All data are presented as the mean ± SEM. $n = 6$ for each group. ** $p < 0.01$.
BYF, Bufei Yishen; COPD, chronic obstructive pulmonary disease; HE, hematoxylin and eosin; KAM, key active molecules; SEM, standard error of the mean.

2015; Li et al., 2020). Previous studies have shown that these KAM possess significant anti-inflammatory properties. For instance, 20-S-ginsenoside Rh1 exerts an anti-inflammatory effect on a variety of cell lines in vitro by different mechanisms, such as attenuating the phosphorylation and activation of NF-κB, p38 MAPK, and JNK (Choi et al., 2011; He et al., 2014). In addition, icariin can suppress lipopolysaccharide-induced acute inflammatory responses by inhibiting NF-κB (Xu et al., 2010). In this study, COPD rats were treated with KAM-BYF to investigate its therapeutic effect. We demonstrated that KAM-BYF prevented a reduction in lung function (i.e., TV, MV, PEF, FVC, FEV0.1, and FEV0.3) induced by exposure to smoke. We also observed that KAM-BYF suppressed the pathological changes, namely increasing the mean alveolar number and decreasing the mean liner intercept. KAM-BYF suppressed the levels of inflammatory cytokines and proteases. In addition, MMPs degrade extracellular matrix components in COPD processes. For instance, tobacco smoke induced overexpression of MMP9 and MMP12 in the lung of patients with COPD may cause the degradation of lung tissue, leading to progressive airway destruction and remodeling(Barnes, 2014). In this investigation, we found that KAM-BYF could decrease the protein levels of MMP9 and MMP12 in the serum and BALF. These data demonstrated that the KAM derived from BYF could be effective compounds for the treatment of COPD in rats.

Airway epithelial cells form an essential barrier for the defense against airborne stimulants and pathogens. Moreover, they produce cytokines (e.g., TNF-α, IL6, and IL8) that recruit various inflammatory cells, thus driving airway inflammation (Barnes, 2016). Furthermore, TNF-α usually precedes and generally promotes the production of numerous other inflammatory mediators, thereby further aggravating the inflammatory response in airway epithelial cells by activation of JNK, p65, and p38 MAPK (Di Stefano et al., 2014). To verify its anti-inflammation effect and underlying mechanisms, we investigated whether treatment with KAM-BYF could inhibit TNF-α-induced inflammatory responses in airway epithelial cells. The data showed that KAM-BYF suppressed the inflammatory response by decreasing the levels of IL8, IL6, and TNF-α. In previous research, we demonstrated that BYF could suppress inflammation via the JNK/p38 and NF-κB signaling pathways (Li et al., 2014). In the present study, we also detected the effect of KAM-BYF on the critical pathways, and demonstrated that it could inhibit the active forms of p65, p38, and JNK in BEAS-2B cells induced by TNFα and in lung tissues of rats with COPD. These results suggest that KAM-BYF exerts its ameliorative effect on COPD via inhibition of the inflammatory response by decreasing the activity of p65, p38, or NF-κB.

There are some limitations in this study. Firstly, patients with COPD were treated with conventional Western medicine and BYF.

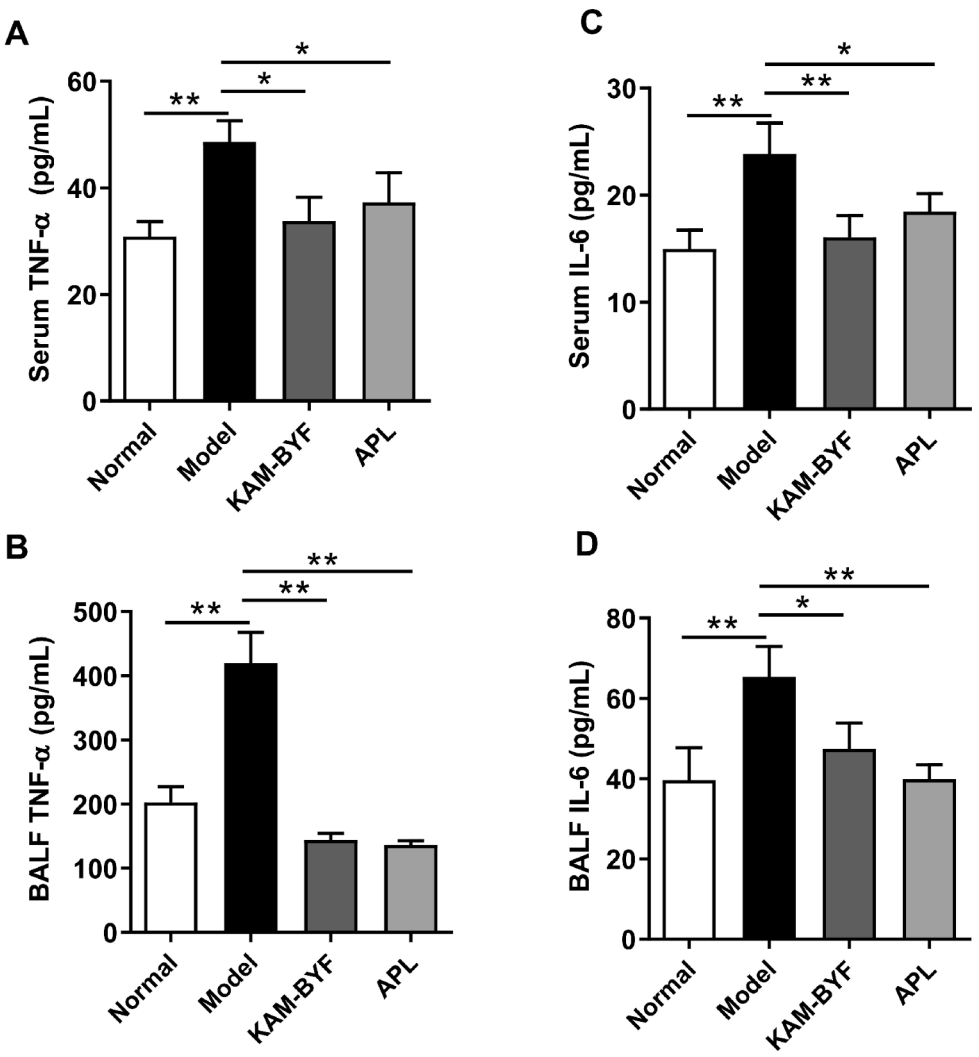

Fig. 4. Effect of the Bufei Yishen formula (KAM-BYF) on the TNF-α and IL6 in the serum and BALF of COPD rats. The levels of TNF-α and IL6 cytokines in the serum and BALF of rats were detected by ELISA. Data are presented as the mean ± SEM ($n = 6$ mice per group). * $p < 0.05$, ** $p < 0.01$.
BALF, bronchial alveolar lavage fluid; BYF, Bufei Yishen; COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; IL6, interleukin 6; KAM, key active molecules; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

Investigation of the effect of BYF without involvement of Western medicine was not performed in this work. According to the special statement of the retrospective registration, the Chinese Clinical Trial Registry (ChiCTR) extended the deadline for retrospective registration to January 1, 2013. Therefore, with the retrospective registration status, the CTCMI study was registered in ChiCTR in May 2011 after the completion of the study based on the ChiCTR requirement. In addition, we did not identify the therapeutic target of these key compounds in this work. Thus, further study on BYF without involvement of Western medicine should be performed to examine whether this effect is due to the combined treatment; such investigations should be prospectively registered prior to the initiation of the study. This approach would provide more details on the anti-inflammatory mechanisms and target of KAM-BYF.

Conclusion

BYF and KAM-BYF were effective in the treatment of patients with COPD and rat models, respectively. These effects were due to inhibition of the inflammatory response and the corresponding signals.

Author contributions

Jiansheng Li and Brian G. Oliver designed the outline of the study. Yang Xie, Suyun Li, and Minghang Wang performed the clinical study. Peng Zhao, Yange Tian, and Yanqin Qin performed the animal and cell experiments, conceived the study, and drafted and revised the manuscript. All authors contributed to the data analysis, as well as the drafting and critical review of the manuscript. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work with regard to the integrity and accuracy of the data.

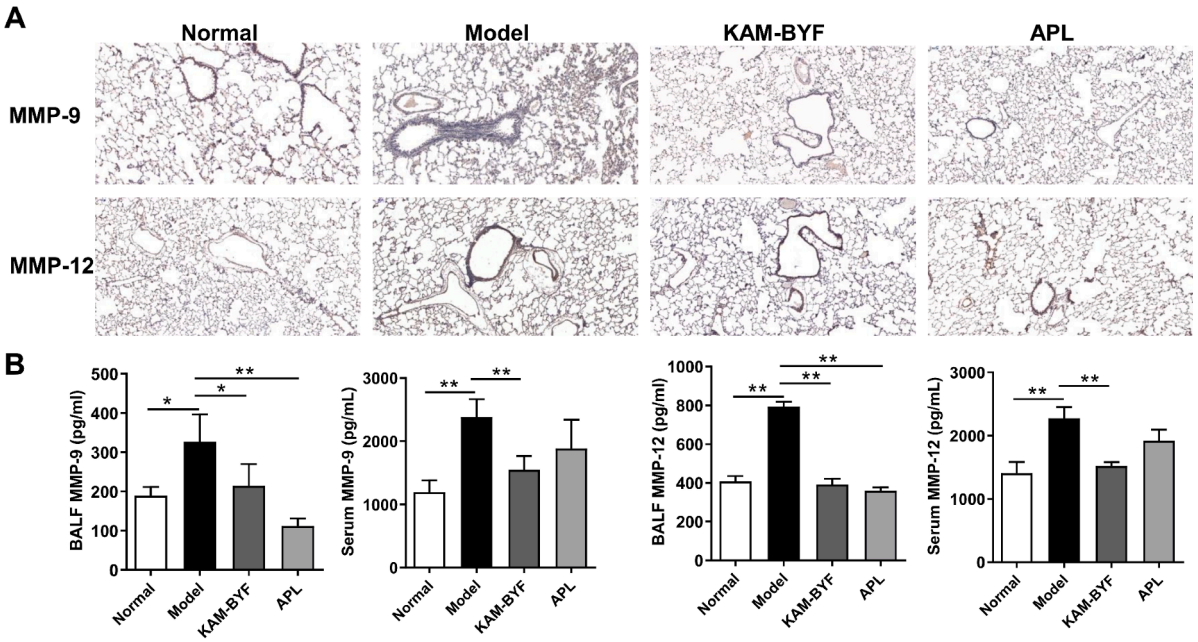

Fig. 5. Effect of the Bufei Yishen formula (KAM-BYF) on the MMP9 and MMP12 in the lung, serum and BALF of COPD rats. The levels of MMP9 and MMP12 expressed in lung tissues were detected by immunohistochemistry (A). The levels of MMP9 and MMP12 in the serum and BALF of rats were detected by ELISA (B). Data are presented as the mean ± SEM ($n = 6$ mice per group). * $p < 0.05$, ** $p < 0.01$.
BALF, bronchial alveolar lavage fluid; BYF, Bufei Yishen; COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; KAM, key active molecules; MMP9, matrix metalloproteinase 9; SEM, standard error of the mean.

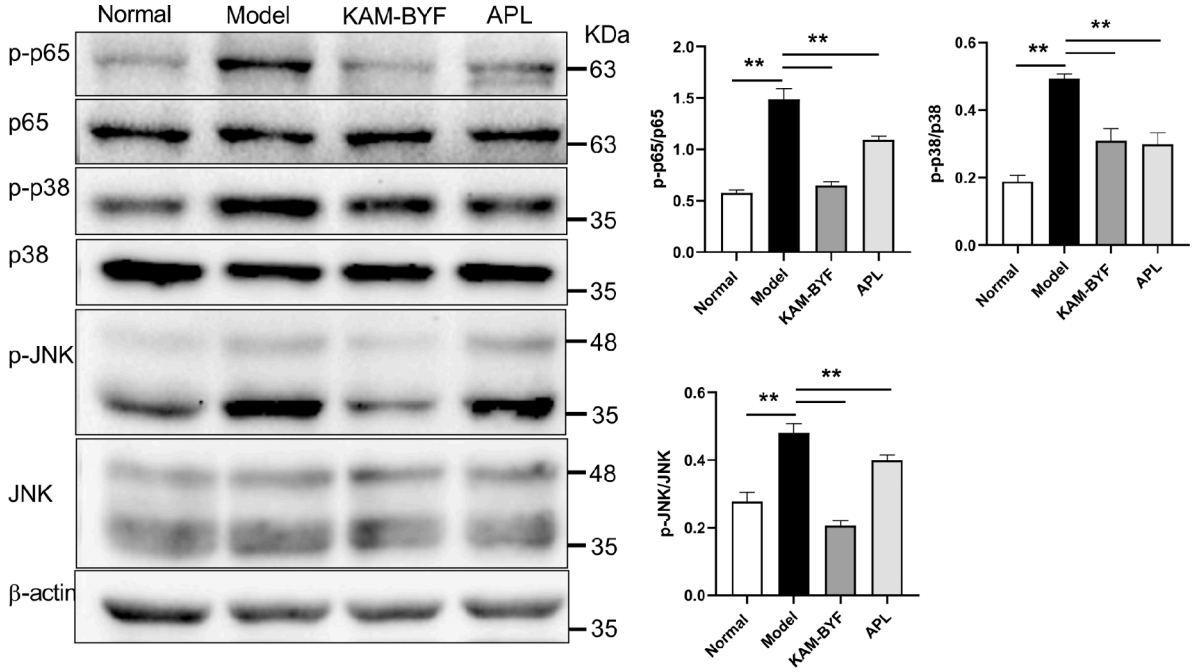

Fig. 6. KAM-BYF suppressed the activation of p38 mitogen-activated protein kinase (MAPK), NF-κB, and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) in the lung tissue of COPD rats. We detected the levels of (A) NF-κB p65, (B) p38 MAPK, and (C) JNK phosphorylation; the levels of their total proteins were also detected by western blotting. Images are representative of $n = 6$ per group.
BYF, Bufei Yishen; COPD, chronic obstructive pulmonary disease; KAM, key active molecules; NF-κB, nuclear factor-kappaB.

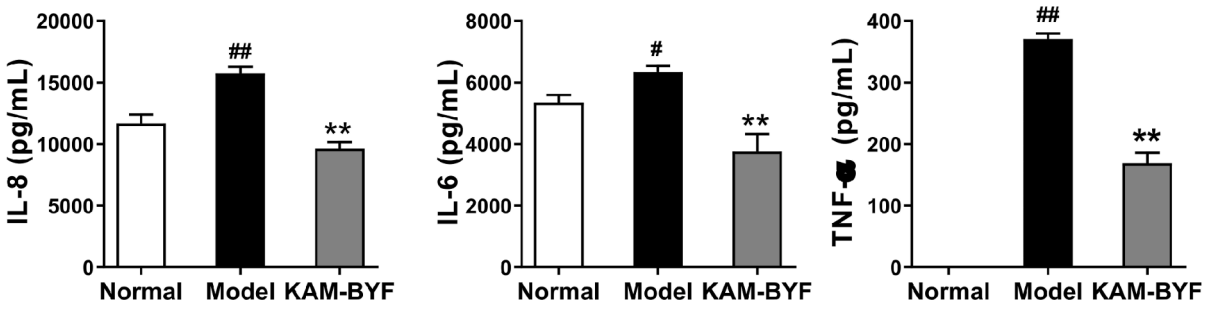

Fig. 7. KAM-BYF decreased the levels of cytokines in BEAS-2B cells induced by TNF-α. BEAS-2B cells were treated with TNF-α and/or KAM-BYF. The levels of IL8, IL6, and TNF-α were detected by ELISA. Data are presented as the mean ± SEM ($n = 3$). Significant differences were denoted by $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ compared with control and ** $p < 0.01$ compared with model.
BYF, Bufei Yishen; ELISA, enzyme-linked immunosorbent assay; IL6, interleukin 6; KAM, key active molecules; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

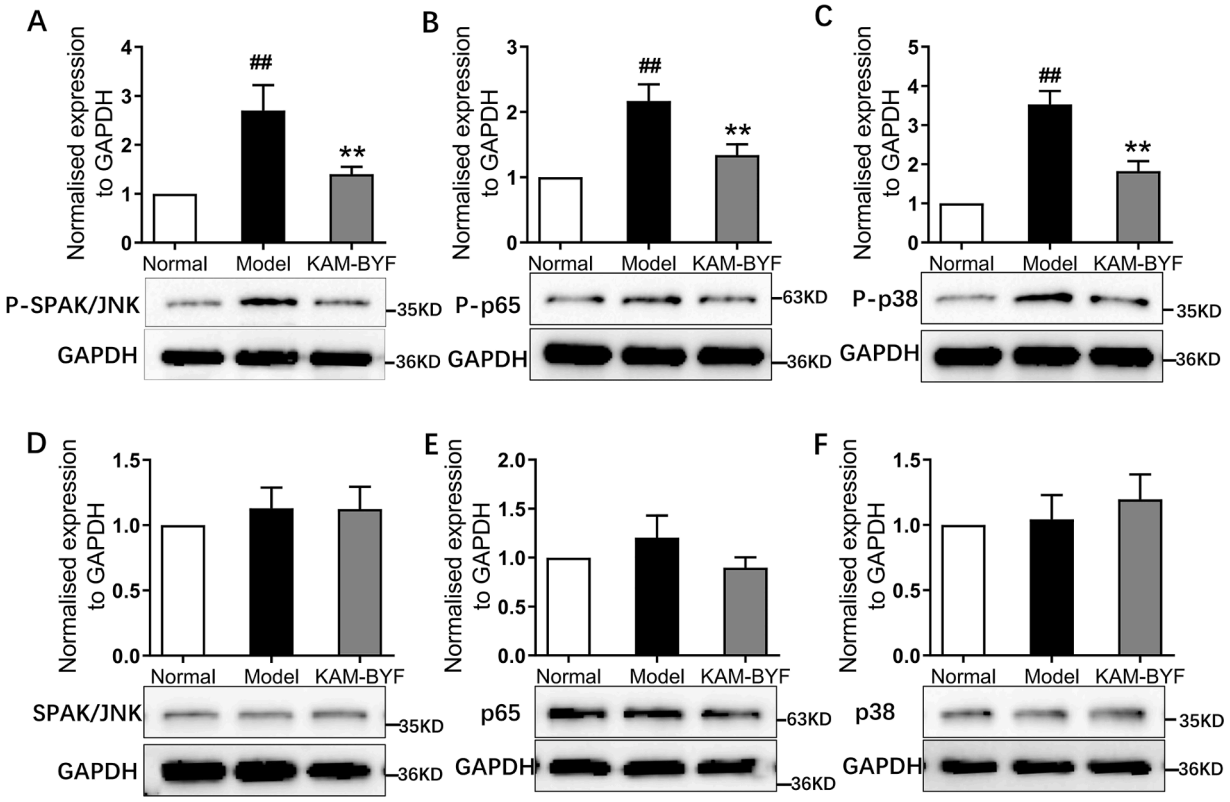

Fig. 8. KAM-BYF suppressed the activation of p38 mitogen-activated protein kinase (MAPK), NF-κB, and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK). BEAS-2B cells were challenged with TNF-α and/or treated with KAM-BYF. The levels of JNK (A), NF-κB p65 (B), and p38 MAPK (C) phosphorylation were detected by western blotting. The levels of total JNK (D), NF-κB p65 (E), or p38 MAPK (F) was also detected. Data are presented as the mean ± SEM. $n = 3$. Significant differences were denoted by $^{\#\#}p < 0.01$ compared with control and ** $p < 0.01$ compared with model.
BYF, Bufei Yishen; KAM, key active molecules; NF-κB, nuclear factor-kappaB; TNF-α, tumor necrosis factor-α; SEM, standard error of the mean.

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Declaration of Competing Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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