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RESEARCH ARTICLE

Three Tiaobu Feishen formulae reduces cigarette smoke-induced inflammation in human airway epithelial cells

Dong Haoran, Liu Xuefang, Zheng Wanchun, Feng Suxiang, Li Jiansheng, Qin Yanqin, Wu Yaosong, Chen Yulong, Yin Sugai, Zhao Peng

Dong Haoran, Liu Xuefang, Zheng Wanchun, Feng Suxiang, Li Jiansheng, Qin Yanqin, Wu Yaosong, Chen Yulong, Yin Sugai, Zhao Peng, Henan Key Laboratory of Chinese Medicine for Respiratory Disease, Henan University of Chinese Medicine, Zhengzhou 450046, China; Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment & Chinese Medicine Development of Henan Province, Henan University of Chinese Medicine, Zhengzhou 450046, China

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Correspondence to: Prof. Li Jiansheng, Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment & Chinese Medicine Development of Henan Province, Henan University of Chinese Medicine, Zhengzhou 450046, China. li_js8@163.com

Telephone: +86-371-65676568

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Abstract

OBJECTIVE: To investigate the therapeutic efficacy of Tiaobu Feishen formulae (TBFS) on cigarette smoke-induced inflammation *in vitro* using lipopolysaccharide (LPS)-induced and cigarette smoke extract (CSE)-induced NCI-H292 cells.

METHODS: We evaluated the inhibitory effects of Bufei Jianpi formula (BJF), Bufei Yishen formula (BYF), and Yiqi Zishen formula (YZF) on the expressions of inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-8, matrix metalloproteinase (MMP)-9, tissue inhibitor of matrix metalloprotease (TIMP)-1, and superoxide dismutase (SOD) in H292 cells stimulated with LPS or CSE. Their related transcription factors and signaling pathways were also analyzed.

RESULTS: BJF, BYF, and YZF significantly inhibited the LPS- or CSE-induced expressions of TNF-α, IL-8, MMP-9, TIMP-1, and SOD in H292 cells, and suppressed the activation of transcription factors including nuclear transcription factor (NF)-κB, activator protein (AP)-1, and signal transducers and activators of transcription (STAT) 3 and their corresponding pathways, including NF-κB, mitogen-activated protein kinase (MAPK), STAT3, and peroxisome proliferator-activated receptor (PPAR).

CONCLUSION: BJF, BYF, and YZF effectively suppressed inflammatory responses, protease-antiprotease imbalance, and oxidative stress induced by LPS and CSE, an effect that was closely associated with the inhibition of the NF-κB, MAPK, STAT3, and PPAR pathways.

Keywords: Pulmonary disease, chronic obstructive; Lipopolysaccharides; Cigarette smoking; Inflammation; Epithelial cells; Tiaobu Feishen formulae

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), a major cause of morbidity and mortality, is characterized by airflow obstruction, chronic bronchitis, and systemic inflammation.[1] Airway epithelial cells release various proinflammatory cytokines induced by lung inflamma-

tory stimuli, which might have an important role in the development and progression of COPD.[2,3]

Bacterial infection as well as cigarette smoking and air pollutants, are thought to be common causes of lung inflammation in COPD.[4] Lipopolysaccharide (LPS), a component of Gram-negative bacteria, and cigarette smoke induce innate immune responses by Toll-like receptor 4 (TLR4) expressed on airway epithelial cells.[5,6] Furthermore, mitogen-activated protein kinase (MAPK) and janus kinase (JAK)-signal transducers and activators of transcription (STAT) 3 activation subsequently activates the downstream transcriptional factors nuclear transcription factor (NF)-κB, activator protein (AP)-1, STAT 3 and peroxisome proliferator-activated receptor (PPAR), which mediate the transcription and translation of proinflammatory mediators.[7] The expressions of these factors in airway epithelial cells leads to the release of cytokines, which aggravate airway inflammation and promote the migration of inflammatory cells.[6,8]

In previous studies, we found that Tiaobu Feishen formulae (TBFS), consisting of Bufei Jianpi formula (BJF), Bufei Yishen formula (BYF), and Yiqi Zishen formula (YZF), possessed extensive pharmacological effects on COPD, including alleviating the clinical symptoms of stable COPD patients, reducing the exacerbation frequency, delaying acute exacerbation, and improving pulmonary function and exercise capacity.[9] Moreover, we experimentally demonstrated that three formulae consisting of BJF, BYF, and YZF, which had inhibitory effects on inflammatory cytokine expression, protease-antiprotease imbalance and collagen deposition, were effective for the treatment of COPD in rats.[10-13] However, the anti-inflammatory mechanisms of BJF, BYF, and YZF on airway epithelial cells remain poorly understood and warrant further investigation.

In the present study, we investigated the efficacy of BJF, BYF, and YZF on the LPS- and CSE-induced expressions of tumor necrosis factor (TNF)-α and interleukin (IL)-8, matrix metalloproteinase (MMP)-9, tissue inhibitor of matrix metalloprotease (TIMP)-1, and superoxide dismutase (SOD) in human airway epithelial H292 cells to examine the mechanism underlying their beneficial actions.

MATERIALS AND METHODS

Cell culture

H292 cells (ATCC, Manassas, VA, USA) were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL), and incubated at 37 ℃ with 5% CO_2. All experiments were performed with exponentially growing cells.

Preparation of cigarette smoke extract (CSE)

CSE was prepared as previously described.[14] Briefly, one cigarette (Hongqi Canal® Filter tip cigarette, Henan Tobacco Industry, Zhengzhou, China) was combusted and cigarette smoke was slowly bubbled into 10 mL culture medium (100% CSE).

Drug preparation

The Bufei Jianpi formula (BJF) was as follows: Huangqi (*Radix Astragali Mongolici*) 15 g, Huangjing (*Rhizoma Polygonati Sibirici*) 15 g, Dangshen (*Radix Codonopsis*) 15 g, Baizhu (*Rhizoma Atractylodis Macrocephalae*) 12 g, Fuling (*Poria*) 12 g, Zhebeimu (*Bulbus Fritillariae Thunbergii*) 9 g, Houpu (*Cortex Magnoliae Officinalis*) 9 g, Chenpi (*Pericarpium Citri Reticulatae*) 9 g, Ziwan (*Radix Asteris Tatarici*) 9 g, Dilong (*Pheretima Aspergillum*) 12 g, Aidicha (*Radix Ardisiae Japonicae*) 15 g, Yinyanghuo (*Herba Epimedii Brevicornus*) 6 g. Bufei Yishen formula (BYF) was prepared as follows: Renshen (*Radix Ginseng*) 9 g, Huangqi (*Radix Astragali Mongolici*) 15 g, Shanzhuyu (*Fructus Macrocarpii*) 12 g, Gouqizi (*Fructus Lycii*) 12 g, Wuweizi (*Fructus Schisandrae Chinensis*) 9 g, Yinyanghuo (*Herba Epimedii Brevicornus*) 9 g, Zhebeimu (*Bulbus Fritillariae Thunbergii*) 9 g, Chishao (*Radix Paeoniae Rubra*) 9 g, Dilong (*Pheretima Aspergillum*) 12 g, Zisuzi (*Fructus Perillae Argutae*) 9 g, Aidicha (*Radix Ardisiae Japonicae*) 15 g, Chenpi (*Pericarpium Citri Reticulatae*) 9 g. Yiqi Zishen formula (YZF) was prepared as follows: Renshen (*Radix Ginseng*) 9 g, Huangjing (*Rhizoma Polygonati Sibirici*) 15 g, Wuweizi (*Fructus Schisandrae Chinensis*) 9 g, Gouqizi (*Fructus Lycii*) 12 g, Dihuang (*Radix Rehmanniae*) 15 g, Zhebeimu (*Bulbus Fritillariae Thunbergii*) 9 g, Mudanpi (*Cortex Moutan Radicis*) 12 g, Zisuzi (*Fructus Perillae Argutae*) 9 g, Baibu (Radix Stemonae) 9 g, Chenpi (*Pericarpium Citri Reticulatae*) 9 g, Maidong (*Radix Ophiopogonis Japonici*) 15 g, Rougui (*Cortex Cinnamomi Cassiae*) 3 g, Dilong (*Pheretima Aspergillum*) 12 g. The herbal drugs were identified and prepared in fluid extract. The experiments were conducted in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the First Affiliated Hospital, Henan University of Chinese Medicine, China.

Preparation of drug-containing sera

Sprague-Dawley rats (body weight 220-250 g) were divided into four groups with 10 rats in each as follows: vehicle group (volume-matched normal saline); BJF group (14 g/kg); BYF group (18 g/kg), and YZF group (20 g/kg). Rats in the different groups were intragastrically administered treatments twice every day. On day 4, 1 h after the last gastric lavage, rats were anesthetized and blood was drawn from the abdominal aorta. Serum was separated from blood and fractionated after sterilization. Sera were then incubated in a 56 ℃ water bath for 30 min to inactivate complement and antibodies present in the sera. After 0.22-μm filter sterilization, the sera were stored in sterile centrifuge tubes at − 20 ℃.

Cell viability assay

For cell viability assays, H292 cells (at a concentration of 2 × 10^4 cells/well) were plated in sextuplicate 96-well plates for 24 h. These cells were then treated with various concentrations of BJF-, BYF- and YZF-containing serum for 24 and 48 h. Subsequently, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) solution was added. Plates were incubated for 4 h, the supernatants were removed and 150 μL/well dimethyl sulfoxide was added to dissolve formazan crystals. The absorbance was recorded at 570 nm.

Cytokine analysis

H292 cells (at a concentration of 2 × 10^4 cells/well) were seeded in triplicate, into 96-well tissue culture plates. The cells were stimulated with 1 μg/mL of LPS or 10% CSE and treated with BJF-, BYF- and YZF-containing serum for 48 h. Supernatants were collected and human cytokines were measured with ELISA kits (Boster Biological Engineering, Wuhan, China) according to the manufacturer's protocols.

Electrophoretic mobility shift assay (EMSA)

H292 cells were treated with 1 μg/mL of LPS or 10% CSE and BJF-, BYF- and YZF-containing serum. Nuclear proteins from the H292 cells were extracted using a total nuclear protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Protein concentration was measured using the bicinchoninic acid method. The DNA binding activities of AP-1, PPAR, NF-κB, and STAT3 in nuclear extracts were evaluated by EMSA using the EMSA kit (Beyotime, Shanghai, China).

Western blotting assay

H292 cells were treated with 1 μg/mL of LPS or 10% of CSE and 20% of BJF-, BYF- and YZF-containing serum. Cells were washed with ice-cold PBS and then lysed RIPA lysis buffer (Solarbio Life Sciences, Beijing, China). Protein concentration of cell lysates was tested by BCA protein assay. Then, proteins were mixed with SDS sample buffer, boiled, and separated by SDS polyacrylamide gel electrophoresis before being transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked for 1 h at room temperature with 5% nonfat-milk and then incubated with primary antibodies at 4 ℃ overnight followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. The bands were visualized by film exposure with ECL reagent.

Statistical analysis

Values are presented as the mean ± standard error of mean (SEM). The data for each condition were subject to one-way analysis of variance using SPSS 17.0 software package (SPSS Inc., Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA). $P < 0.05$ was determined to be statistically significant.

RESULTS

Effect of TBFS on the viability of H292 cells

First, we examined the effects of TBFS at different concentrations on cell viability. H292 cells were treated with different concentrations of TBFS containing serum for 24 and 48 h. As shown in Figure 1, the MTT assay results showed that 10% and 20% TBFS containing serum exerted no significant suppression on H292 cell viability at 24 and 48 h. However, 40% TBFS containing serum markedly inhibited H292 cell viability at 48 h. Thus, 20% TBFS containing serum was considered an appropriate dose for the subsequent experiments.

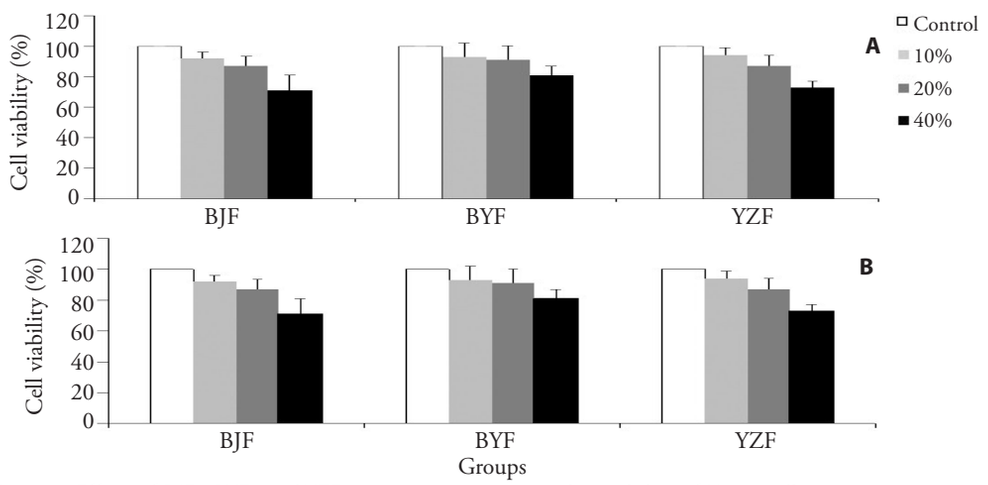

Figure 1 Effect of BJF-, BYF- and YZF-containing serum on the viability of H292 cells

A: 24 h; B: 48 h. H292 cells were plated in 96-well plates, then treated with 10, 20, and 40% BJF-, BYF- and YZF-containing serum for 24 or 48 h. The cell viability was analyzed by the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide method. BJF: Bufei Jianpi formula; BYF: Bufei Yishen formula; YZF: Yiqi Zishen formula. Data are expressed as the mean ± standard error of mean, $n = 3$.

Effect of TBFS on the expressions of cytokines in H292 cells induced by LPS or CSE

TBFS suppressed the levels of inflammatory cytokines, and imbalance of protease-antiprotease in cigarette smoke- and bacterial infection-induced COPD rats.[10-12] Thus, we proposed the hypothesis that TBFS treatment decreases inflammatory cytokines, matrix metalloproteinases, and increases antioxidant enzymes in LPS- and CSE-treated H292 cells.[15-18] To test this hypothesis, we evaluated the effect of TBFS on the expressions of IL-8, TNF-α, MMP-9, TIMP-1, and SOD in H292 cells. In LPS-induced H292 cells, BJF significantly suppressed TNF-α and increased SOD. BYF markedly decreased IL-8, TNF-α, and MMP-9, and increased SOD, and TIMP-1/MMP-9. YZF inhibited TNF-α and MMP-9, and increased SOD, and TIMP-1/MMP-9 (Figure 2). As shown in Figure 3, BJF and YZF suppressed TNF-α and MMP-9, and increased SOD, TIMP-1, and TIMP-1/MMP-9. BYF suppressed MMP-9 and increased SOD and TIMP-1/MMP9 in CS-induced H292 cells. Based on the above data, we found that TNFS, consisting of BJF, BYF, and YZF suppressed inflammatory responses and protease-antiprotease imbalance, and oxidative stress in LPS- or CSE-treated H292 cells.

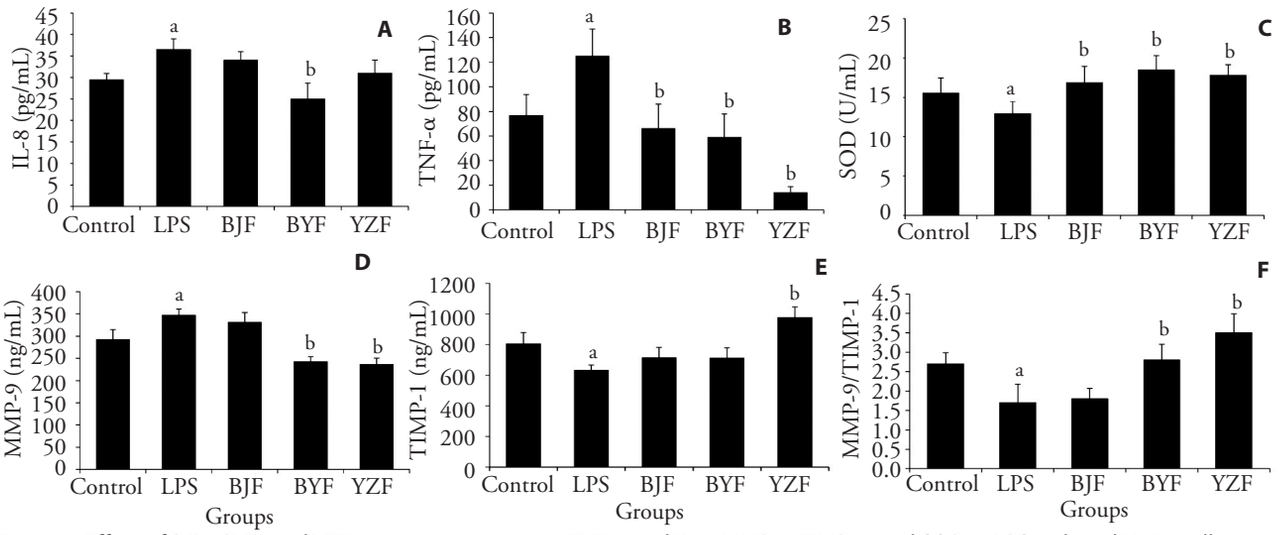

Figure 2 Effect of BJF-, BYF- and YZF-containing serum on TNF-α and IL-8, MMP-9, TIMP-1, and SOD in LPS-induced H292 cells
A: IL-8; B: TNF-α; C: SOD; D: MMP-9; E: TIMP-1; F: TIMP-1/MMP-9. H292 cells were plated in 96-well plates, stimulated with 1 μg/mL of LPS and treated with 20% BJF-, BYF- and YZF-containing serum for 48 h. $^{a}P < 0.01$ *vs* the control; $^{b}P < 0.01$ *vs* the LPS. BJF: Bufei Jianpi formula; BYF: Bufei Yishen formula; YZF: Yiqi Zishen formula; TNF-α: tumor necrosis factor-α; IL-8: interleukin-8; MMP-9: matrix metalloproteinase-9; TIMP: tissue inhibitor of matrix metalloprotease-1; SOD: superoxide dismutase. Data are expressed as the mean ± standard error of mean, $n = 3$.

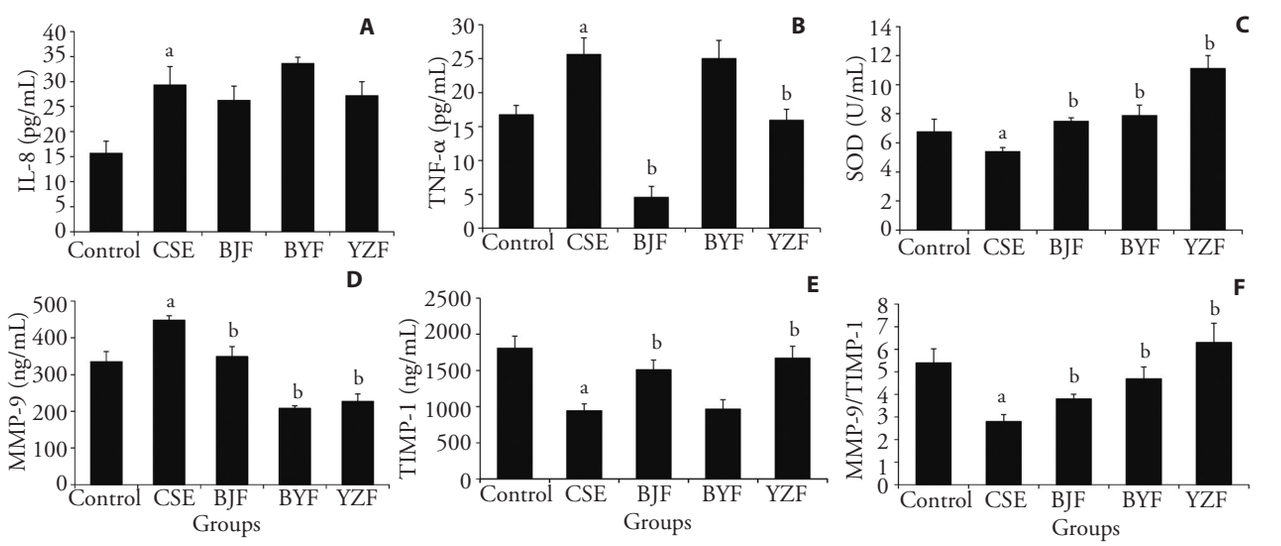

Figure 3 Effect of BJF-, BYF- and YZF-containing serum on TNF-α and IL-8, MMP-9, TIMP-1, and SOD in CSE-induced H292 cells
A: IL-8; B: TNF-α; C: SOD; D: MMP-9; E: TIMP-1; F: the ratio of TIMP-1/MMP-9. H292 cells were plated in 96-well plates, stimulated with 10% CSE and treated with 20% BJF-, BYF- and YZF- containing serum for 48 h. BJF: Bufei Jianpi formula; BYF: Bufei Yishen formula; YZF: Yiqi Zishen formula; TNF-α: tumor necrosis factor-α; IL-8: interleukin-8; MMP-9: matrix metalloproteinase-9; TIMP: tissue inhibitor of matrix metalloprotease-1; SOD: superoxide dismutase; CSE: cigarette smoke extract. $^{a}P < 0.01$ *vs* the control; $^{b}P < 0.01$ *vs* the LPS. Data are expressed as the mean ± standard error of mean, $n = 3$.

Effect of TBFS treatment on NF-κB, AP-1, PPAR, and STAT3 binding activity in H292 cells induced by LPS and CSE

According to our previous studies, LPS-induced cytokine expression resulted from the activation of NF-κB and AP-1 binding, whereas CSE-mediated cytokine expression was associated with the activation of STAT3 and PPAR in H292 cells. Thus, we investigated the effects of TBFS, consisting of BJF, BYF, and YZF, on NF-κB and AP-1 binding activity induced by LPS, and the activation of STAT3 and PPAR caused by CSE in H292 cells. The results showed that BJF treatment significantly suppressed NF-κB and AP-1 binding activity, and that BYF and YZF inhibited AP-1 and NF-κB activity, respectively, in LPS-induced H292 cells (Figure 4A, 4B). In addition, TBFS markedly inhibited the activation of STAT3 and increased the activation PPAR in CSE-induced H292 cells (Figure 4C, 4D).

Effect of TBFS exposure on NF-κB, MAPK, STAT3, and PPAR signaling pathways in H292 cells activated by LPS and CSE

TLR4 activation activates NF-κB signaling, including IκB phosphorylation or degradation, and NF-κB transactivation and promoter binding.[19-21] In addition, AP-1 and STAT3 binding activity are regulated by MAPK and JAK/STAT pathway signaling.[22, 23] Based on the above results, we evaluated the effect of TBFS on the NF-κB and MAPK signaling pathways activated by LPS, and JAK/STAT and PPAR pathways induced by CSE in H292 cells. As shown in Figure 5A, TBFS significantly decreased the expression levels of TLR4, p-P65, P65, IκB, p-MAPK14, and JUN induced by LPS. In addition, BYF and YZF suppressed the activation of JAK1/2, but had no effect on STAT3 and PPAR induced by CSE. Furthermore, BJF had no effect on pathways induced by CSE (Figure 5B).

DISCUSSION

Airway inflammation and remodeling are major characteristics of COPD that are usually related to increased expression levels of inflammatory cytokines, MMP-9, and oxygen-free radicals.[24, 25] Bacterial infection and cigarette smoking are important risk factors and are highly implicated in airway inflammation and remodeling, and the development of COPD.[20] Studies reported that the inhibition of NF-κB, MAPK, and STAT3 activity are a focus for COPD treatment.[26] These pathways are essential for the regulation of inflammatory responses and many other biological processes, and are activated

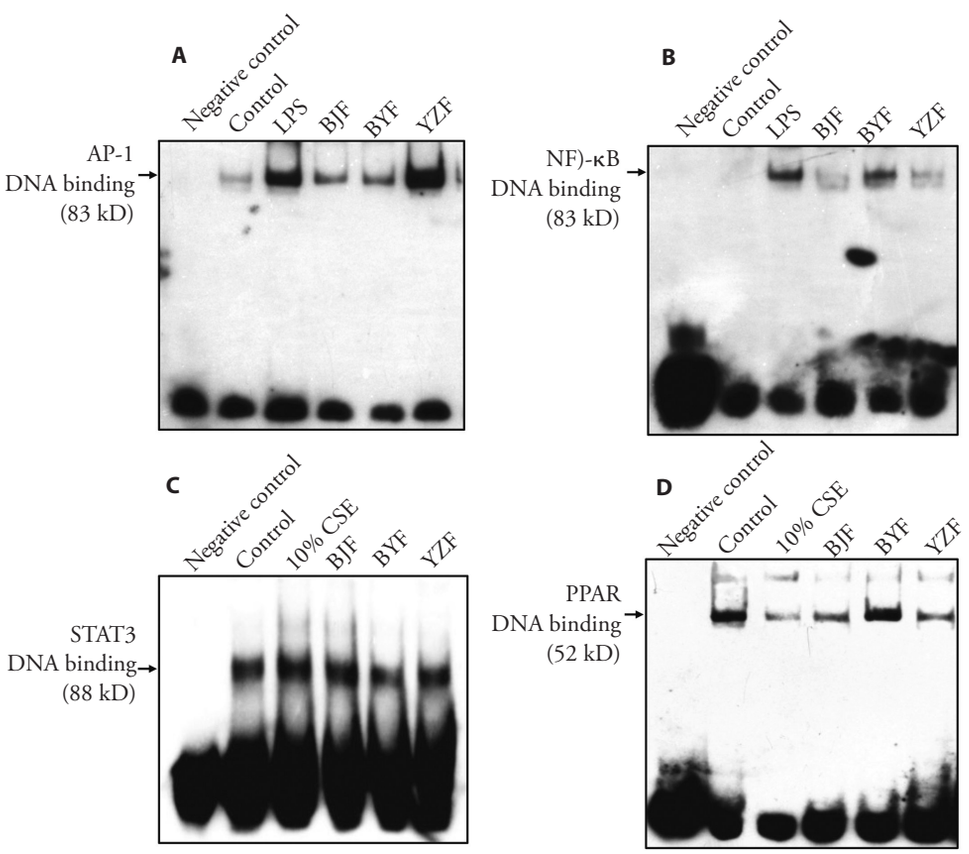

Figure 4 Effect of BJF-, BYF- and YZF-containing serum on transcription factor binding activity

A: the DNA binding activities of activator protein (AP)-1; B: nuclear transcription factor (NF)-κB in LPS-induced H292 cells; C: DNA binding activities of signal transducers and activators of transcription (STAT) 3; D: peroxisome proliferator-activated receptor (PPAR) in CSE-induced H292 cells were evaluated using the electrophoretic mobility shift assay kit. H292 cells were treated with 2.5 μg/mL of LPS or 10% CSE and BJF-, BYF- and YZF-containing serum for 24 h. BJF: Bufei Jianpi formula; BYF: Bufei Yishen formula; YZF: Yiqi Zishen formula; CSE: cigarette smoke extract.

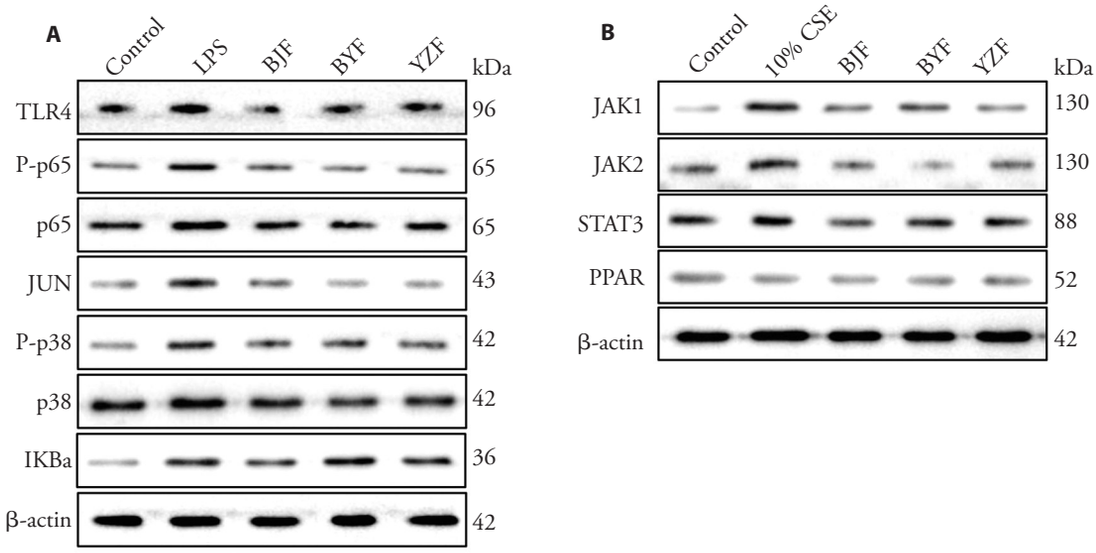

Figure 5 Effect of BJF-, BYF- and YZF-containing serum on the NF-κB, MAPK, STAT 3, and PPAR signaling pathways
A: TLR4, p65, IκB, Jun, and phosphorylated p-65 and pMAPK14 in LPS-induced H292 cells were measured by Western blot analysis; B: JAK1/2, STAT3, and PPAR in CSE-induced H292 cells were measured by Western blot analysis. H292 cells were treated with 2.5 μg/mL of LPS or 10% CSE and BJF-, BYF- and YZF-containing serum for 24 h. BJF: Bufei Jianpi formula; BYF: Bufei Yishen formula; YZF: Yiqi Zishen formula; NF-κB: nuclear transcription factor; MAPK: mitogen-activated protein kinase; STAT: signal transducers and activators of transcription; PPAR: peroxisome proliferator-activated receptor; CSE: cigarette smoke extract.

by various inflammatory cytokines and environmental factors, such as bacterial infection and cigarette smoking,[27-29] which are believed to play a central role in regulating the expressions of MMP-9 and proinflammatory cytokines such as TNF-α in airway epithelial cells of COPD patients.[30, 31] Here, we found that TBFS significantly decreased IL-8, TNF-α, and MMP-9, and increased SOD and TIMP-1 levels in LPS- or CSE-induced H292 cells, which might be associated with the regulation of NF-κB, MAPK, STAT3, or PPAR signaling pathways and their transcription factors such as NF-κB, AP-1 or STAT3.

In conclusion, the present study demonstrated that TBFS attenuated inflammatory responses, protease-antiprotease imbalance, and oxidative stress in LPS- and CSE-stimulated H292 cells, clarifying the anti-COPD mechanism of TBFS.

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