Three Tiaobu Feishen therapies protect human alveolar epithelial cells against cigarette smoking and tumor necrosis factor-α-induced inflammation by nuclear factor-kappa B pathway

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

OBJECTIVE: To examine the efficacy of BJF, BYF and YZF on the production of inflammatory cytokines, including TNF-α and interleukin (IL)-8, IL-6, matrix metalloproteinases (MMP)-9, and IL-10 in CSE or TNF-α-induced A549 cells. And their related transcription factors and signaling pathway were also analyzed.

RESULTS: The results showed that BJF, BYF and YZF could significantly decrease the expression levels of the pro-inflammatory cytokines induced by CSE or TNF-α. Furthermore, BJF, BYF and YZF could suppress CSE- or TNF-α-induced activation of nuclear factor-kappa B (NF-κB) transcription factors and its corresponding pathways. Taken together, these data implied that BJF, BYF and YZF effectively inhibited CSE- or TNF-α-induced inflammatory response in alveolar epithelial cell, which was due to their inhibition effect on NF-κB pathways.

CONCLUSION: Our findings suggest that the Tiaobu Feishen therapies may protect human alveolar epithelial cells against cigarette smoking and TNF-α-induced inflammation. NF-κB pathway may involve in the actions.

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Keywords: Chinese medical formula; Cigarette smoking; Tumor necrosis factor-alpha; A549 cells

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), characterized by progressive and irreversible airflow limitation, is projected to become the third most prevalent...
cause of mortality in the world by 2020, and mortality rates continue to rise. Cigarette smoking (CS), a complex mixture of thousands of injurious agents and reactive oxidants, is the main risk factor for development of emphysema and lung inflammation in COPD. The small particulate matter component of less than 5 μm in cigarette smoking can reach the small airways, evoke pathological changes in the alveolar walls, and cause oxidative stress and progressive lung inflammation that is associated with the influx of neutrophils, macrophages and lymphocytes, and increases in the levels of inflammatory mediators including chemokines such as IL-8 and pro-inflammatory cytokines such as IL-1β, IL-6 and MMP-9.

In addition, tumor necrosis factor-α (TNF-α) plays a key role in lung inflammation, and exerts its proinflammatory action through recruiting inflammatory cells and increasing the levels of pro-inflammatory mediators. Many studies also demonstrate that TNF-α participates in development of many inflammatory pulmonary diseases such as COPD, pulmonary fibrosis or asthma. For instance, in COPD, TNF-α can mediate neutrophilic inflammation locally in the airways and lung parenchyma.

In previous works, we demonstrated that three Tiaobu Feishen formulae (TBFS), including Bufei Jianpi formula (BJF), Bufei Yishen formula (BYF), and Yiqi Zishen formula (YZF), exerted extensive pharmacological effects on COPD patients, such as alleviating the clinical symptoms of stable COPD patients, reducing the exacerbation frequency, delaying acute exacerbation, and improving pulmonary function and exercise capacity. We also established COPD rat model, and experimentally demonstrated that three formulae had beneficial effect on COPD rats by inhibiting inflammatory cytokines expression, protease-antiprotease imbalance and collagen deposition. However, the anti-inflammatory mechanisms of BJF, BYF and YZF on alveolar epithelial cell remain poorly understood and warrant further investigation.

In this study, we established CS- and TNF-α-induced inflammatory response model in A549 cell, and evaluated the effects of BJF, BYF and YZF on cigarette smoking extract (CSE)- and TNF-α-induced expression of the IL-8, TNF-α, SOD, MMP-9 and TIMP-1 in A549 cells. We also aimed to investigate anti-inflammatory mechanisms underlying the actions of BJF, BYF and YZF.

MATERIALS AND METHODS

Cell culture
The alveolar epithelial cell line (A549) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI 1640 media (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY, USA) and penicillin (100 U/mL), and streptomycin (100 mg/mL). Cells were grown in 25 cm² plastic culture flasks (Costar, Cambridge, MA, USA) at 37 °C with 95% air and 5% CO₂.

Preparation of cigarette smoking extract (CSE)
Fresh CSE was prepared for each experiment. Briefly one commercial filtered cigarette (Hongqi Canal Filter tip cigarette, Henan Tobacco Industry, Zhengzhou, China) was slowly bubbled into 10 mL of preheated DMEM using a peristaltic pump and subsequently filtered through a 0.22 μm filter. The solution was considered 100% CSE and was diluted for each experiment.

Drug preparation
Bufei Jianpi formula (BJF) was prepared as follows: Huangqi (Radix Astragali Mongolici) 15 g, Huangqin (Rhizoma Polygonati Sibirici) 15 g, Danshen (Radix Codonopsis) 15 g, Bai Zhu (Rhizoma Atractylodis Macrocephalae) 12 g, Fulin (Portia) 12 g, Zhebeimu (Balbus Frullariellae Thunbergii) 9 g, Houpu (Cortex Magnoliae Officinalis) 9 g, Chenpi (Pericarpium Citri Reticulatae) 9 g, Zhiwan (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, Shanzhu (Fructus Corni) 12 g, Gouqizi (Fructus Lycii) 12 g, Wuwei (Fructus Schisandrae Chinensis) 9 g, Yinyanghuo (Herba Epimedii Brevicornus) 9 g, Zhebeimu (Balbus Frullariellae Thunbergii) 9 g, Chishao (Radix Paeoniae Rubrae) 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, Huangqi (Rhizoma Polygonati Sibirici) 15 g, Wuwei (Fructus Schisandrae Chinensis) 9 g, Gouqizi (Fructus Lycii) 12 g, Dihuang (Radix Rehmanniae) 15 g, Zhebeimu (Balbus Frullariellae 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, Magon (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 guidelines of the Committee on the Care and Use of Laboratory Animals of the First Affiliated Hospital, Henan University of Traditional Chinese Medicine, China.

Preparation of drug-containing sera
Sprague-Dawley rats (220-250 g) were divided into four groups with 10 rats each: vehicle group (volumematched 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 administrated twice per day. On day 4, after 1 h of the last gastric lavage, the rats were anesthetized and then blood was drawn from the abdominal aorta. Serum was separated from blood and fractionated after sterilization. Sera were then incubated in a 56 °C water bath for 30 min to inactivate complements and antibodies present in the sera. Subsequent to 0.22 μm filter-sterilization, the sera were stored in sterile centrifuge tubes at ~20 °C.

**Cell viability assay**

The cells were cultured in 96 well tissue culture plates and subsequently incubated with various concentrations of BJF-, BYF- and YZF-containing serum for 48 h. The cells were incubated with 0.5 mg/mL MTT in fresh medium for a further 4 h. The formazan was dissolved by adding dimethyl sulfoxide (SigmaAldrich, US) and was spectrophotometrically measured at a wavelength of 570 nm using a Thermo Scientific Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA).

**Cytokine analysis**

A549 (at a concentration of 2 × 10^5 cells/well) were seeded in triplicate, into 96 well tissue culture plates. The cells were stimulated with 5% CSE or 20 ng/mL TNF-α and treated with BJF-, BYF- and YZF-containing serum for 24 h. Supernatants were collected and cytokines were measured with ELISA kits (Boster Biological Engineering, Wuhan, China) according to the manufacturer’s protocols.

**Electrophoretic mobility shift assay (EMSA)**

A549 cell was treated with 20 ng/mL of TNF-α or 10% CSE and BJF-, BYF- and YZF-containing serum for 24 h. Nuclear protein was extracted from using 4% non-fat-milk and then incubated with primary antibodies at 4 °C 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 were presented as mean ± standard error of mean. The data for each condition were subject to one-way analysis of variance with the SPSS 17.0 package (SPSS Inc., Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago, IL, USA). A P < 0.05 was the significant level.

**RESULTS**

**Cell viability of A549 cell**

As shown in Figure 1, the MTT assay results showed that 10%, 20%, 40% of TBFS containing serum exerted no significant suppression on A549 cell viability at 24 h. Thus, in the subsequent experiments, TBFS containing serum was used at concentration of 20% and 40% for 24 h.

**Cytokines expression in CSE or TNF-α-stimulated A549 cells**

As shown in Figure 2A-C, treatment with 20% of
TBFS containing serum significantly inhibited IL-8 and IL-6 production induced by CSE. And 20% of BJF and YZF containing serum obviously increased IL-10 expression in A549 cells exposed to CSE. Similarly, 40% of TBFS containing serum markedly suppressed TNF-α-induced the expression of IL-8, MMP-9 and TNF-α in supernatant of A549 cells (Figure 2D-F).

NF-κB binding activity induced by CSE and TNF-α in A549 cells
As shown in Figure 3A and B, 20% of BJF, BYF, and YZF containing serum obviously inhibited NF-κB activity in CSE-induced A549 cells. Similarly, 40% of BJF, BYF, and YZF containing serum obviously inhibited NF-κB activity in TNF-α induced A549 cells.

NF-κB signaling pathway activated by CSE and TNF-α in A549 cells
As shown in Figure 4A, 20% of BJF, BYF, and YZF containing serum could suppress myeloid differentiation primary response 88 (Myd88) and p65 expression in CSE-induced A549 cells. Similar responses were also observed in TNF-α-induced A549 cells, 20% of BJF, BYF, and YZF containing serum inhibited p65 expression, but had no effect on inhibitor of NF-κB (IκB) and p-p65 levels (Figure 4B). Taken together, TNFSF-mediated anti-inflammatory effect on CSE- and TNF-α-treated A549 cells were likely due to at least in part to suppression of NF-κB signaling activation.

DISCUSSION
Exposure to cigarette smoking has been identified as the strongest risk condition associated with the development of COPD. Cigarette smoking induces oxidative stress and then drives an inflammatory response in lung epithelial cells. Generally, in lung tissues, TNF-α exerts proinflammatory action directly by recruiting inflammatory cells and upregulating the other pro-inflammatory cytokines production.1 In present work, we showed that TBFs (BYF, BJF and YZF) exerted anti-inflammatory effect in CSE- or TNF-α-induced inflammatory response in A549 cells, such as suppressing the production of TNF-α, IL-8 and MMP-9, by inhibiting NF-κB activation, which might contribute to their therapeutic effect on COPD patients.

In conclusion, the findings suggest that the Tiaobu Feishen therapies may protect human alveolar epithelial cells against cigarette smoking and TNF-α-induced inflammation. NF-κB pathway may involve in the therapies actions.

REFERENCES
Figure 3 Effect of BJF (Bufei Jianpi formula)-, BYF (Bufei Yishen formula)- and YZF (Yiqi Zishen formula)-containing serum on the NF-κB binding activity
A549 cells were exposed to 10% CSE or 20 ng/mL of TNF-α, or treated with BJF-, BYF- and YZF- containing serum. A: the DNA binding activities of NF-κB in CSE-induced A549 cells were evaluated using the EMSA kit; B: the DNA binding activities of NF-κB in TNF-α-induced A549 cells were evaluated using the EMSA kit. NF-κB: nuclear factor-kappa B; CSE: cigarette smoking extract; TNF-α: tumor necrosis factor-α; EMSA: electrophoretic mobility shift assay.

Figure 4 Effect of BJF (Bufei Jianpi formula)-, BYF (Bufei Yishen formula)- and YZF (Yiqi Zishen formula)-containing serum on the NF-κB signaling pathway
A: the Myd88, p65 and IκB levels in CSE-induced A549 cells were measured by Western blot analysis. B: the IκB, p-p65 and p65 in TNF-α-induced A549 cells were measured by Western blot analysis. A549 cells were exposed to 10% CSE or 20 ng/mL of TNF-α, or treated with BJF-, BYF- and YZF- containing serum. CSE: cigarette smoking extract; TNF-α: tumor necrosis factor-α; Myd88: myeloid differentiation primary response 88; IκB: inhibitor of NF-κB.


