Effect of Daiqin phlegm-expelling pill on development of inflammation in rats with chronic obstructive pulmonary disease induced by lipopolysaccharide and smoke

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Supported by: Natural Science Foundation of Jiangsu Province (No. BK20171098); Jiangsu Province 12th Five-Year Plan for the construction of Key Traditional Chinese Medicine Discipline (No. J51302); Project of Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (No. Y17002)

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Accepted: May 6, 2018

Abstract

OBJECTIVE: To investigate the effect of Daiqin phlegm-expelling pill, prepared with Traditional Chinese Medicine (TCM), on the development of inflammation in Sprague-Dawley (SD) rats with chronic obstructive pulmonary disease (COPD) induced by lipopolysaccharide (LPS) and smoke, and to identify the possible underlying mechanism.

METHODS: Sixty male rats were divided into 6 groups (healthy control group, untreated group, Daiqin phlegm-expelling pill low, middle and high dose, and ambroxol hydrochloride tablet group). COPD was established in SD rats by sootiness and tracheal instillation with LPS. The rats were treated with Daiqin phlegm-expelling pill at the indicated doses for 28 d, the inflammatory cells in bronchoalveolar lavage fluid (BALF), the concentration of tumor necrosis factor-α (TNF-α), interleukin (IL)-8 and IL-6 in blood and the inflammation in lung were evaluated.

RESULTS: The number of inflammatory cells in the BALF and TNF-α, IL-8 and IL-6 level in plasma were significantly reduced in rats with COPD when treated with Daiqin phlegm-expelling pill or ambroxol hydrochloride tablet, compared with those without any treatment. The Daiqin phlegm-expelling pill treated rats with COPD had a attenuated inflammation in lung tissue, compared with the untreated group.

CONCLUSION: Daiqin phlegm-expelling pill can significantly restrain the airway inflammation in rats with LPS-smoke induced COPD.

Keywords: Pulmonary disease, chronic obstructive; Daiqin phlegm-expelling pill; Tumor necrosis factor-alpha; Inflammation

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), a pulmonary disease with irreversible air inflow limitation, is a leading cause of morbidity and mortality worldwide. The prevalence of COPD has increased over the decades and will increase worldwide according to the World Health Organization (WHO) expectation. The damage to the airway and lung parenchyma...
caused by chronic inflammation can induce emphysema and small airway fibrosis, which resulting in a COPD. The inflammatory response in COPD involved both innate and adaptive immune responses, with increased number of lymphocytes, monocytes/macrophages and neutrophils in the lumen. Smoking was an evidenced inducer for the occurrence of COPD. Resident macrophages and airway epithelial cells were stimulated by cigarette smoke and other irritants inhaled into the respiratory tract, releasing multiple chemotactic mediators, particularly chemokines, which recruit circulating neutrophils, monocytes, and lymphocytes into the lung.

Neutrophils exerted an important role in the pathogenesis of COPD. There was increased number of activated neutrophils in the sputum and bronchoalveolar lavage fluid (BALF) of patients with COPD. It has been found that neutrophil numbers in induced sputum were positively correlated with COPD disease severity.

Activated lung macrophages secreted granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor, which promote the granulocyte production and release from the bone marrow as well as their survival in the respiratory tract. The up-regulated expression of E-selectin on endothelial cells in the airways of patients with COPD can initiate the neutrophil adhesion, hence facilitating the neutrophil accumulation in the airways and parenchyma. Infiltrated neutrophils in the airways of patients with COPD were activated by the elevated concentrations of granule proteins, such as myeloperoxidase (MPO), and human neutrophil lipocalin. Activated neutrophils mediated the alveolar destruction and mucus hypersecretion by secreting serine proteases, such as neutrophil elastase, proteinase-3, and cathepsin G, as well as matrix metalloproteinase (MMP)-8 and MMP-9. Patients with acute exacerbations of COPD were likely to have a significant increase in neutrophil numbers in the airways. A potential reason may lie on the increased purulence of sputum, which usually contained a increased concentration of neutrophil chemotactic factors, including LTB4 and IL-8.

Cytokines were characteristic in chronic inflammation and had been implicated in COPD. Plasma tumor necrosis factor-α (TNF-α) and its soluble receptor (sTNF-R75) were up-regulated in patients with COPD. The activated TNF-α system in vivo is related to the systemic hypoxemia noted in patients with COPD, which may account for the weight loss. Increased systemic TNF-α, interleukin (IL)-8 and IL-6 had been involved in cachexia and skeletal muscle weakness in patients with COPD. Exposure of the normal host to TNF-α resulted in cachexia, anemia, leukocytosis, and infiltration of neutrophils into organs such as omentum, liver and spleen.

Daiqin phlegm-expelling pill, prepared with Traditional Chinese Medicine (TCM), consists of Qingdai (Indigo Naturalis), Huangqin (Radix Scutellariae Baicalensis), Haifushi (Pumes), Juhong (Exocarpium Citri Reticulatae), Rhi zincola (Rhi nchinesis), Mangxiao (Nardii Salsae) and Xiangfu (Rhizoma Cyperi). The effective compounds in this formula were indirubin and baicalin. Daiqin phlegm-expelling pill is being widely used in the clinical treatment to patients with chronic bronchitis in the hospitals of TCM in China. Clinical survey revealed it was effective to ameliorate the symptoms of cough or pant in patients with COPD. However, the underlying mechanisms in which the Daiqin phlegm-expelling pill functioning as a therapeutic agent is still unknown. In the study, we aimed to investigate the regulatory role of Daiqin phlegm-expelling pill in the development of airway inflammation in rats with COPD induced by LPS and smoke.

METHODS

Establishment of COPD rat model

Adult male Sprague-Dawley rats (SD, 250-280 g) were purchased from Vital River Laboratories Company (Beijing, China), and bred under specific pathogen-free conditions at our Laboratory Animal Services Center. COPD was induced as previously described with slight modification. Briefly, the rats were administrated intratracheally with 200 μL PBS containing 200 μg of LPS (Sigma-Aldrich Corp, St. Louis, MO, USA) on days 1 and 14. On days 2-13 and 15-28, the rats were treated with one cigarette (Shanghai Tobacco Group Co., Shanghai, China) for 0.5 h in a 72 liters airtight box for each day. The experimental protocol was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine and all experiments were performed in accordance with relevant guidelines and regulations.

Treatments

Sixty rats were randomly divided into 6 groups (10 rats for each group): (a) group A: healthy control group, healthy rats were treated with physiological saline (10 mL/kg); (b) group B: COPD suffering rats, rats with induced COPD were treated with physiological saline (10 mL/kg); (c) group C: COPD suffering rats + Daiqin phlegm-expelling pill (low dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (1 g for each kg) for each day; (d) group D: COPD suffering rats + Daiqin phlegm-expelling pill (middle dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (2 g/kg) for each day; (e) group E: COPD suffering rats + Daiqin phlegm-expelling pill (high dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (4 g/kg) for each day; (f) group F: COPD suffering rats + ambroxol hydroxyl hy-
drochloride tablet, rats with induced COPD were orally treated with ambroxol hydrochloride tablet suspension (30 mg/kg) for each day. Rats were treated once for each day from days 1 to 28, and sacrificed on day 30. Daiqin phlegm-expelling pill was provided by Jiangsu Province Hospital of TCM. The ambroxol hydrochloride tablet was purchased from Changzhou Siyao Pharmaceutical Company and served as positive control.

**Collection of BALF and cell counting**

Rats were sacrificed and trachea was exposed. Make a small incision in the trachea, to allow passage of Closed IV Cather System (20 G, BD Intima II, Jiangsu, China). Slowly inject 5 mL cold PBS with 0.1 mM EDTA into lungs. Collect 3-4 mL BALF from lungs using 5 mL syringe into microtubes. Centrifuge microtubes containing 1 mL BALF at 350 g for 5 min, and supernatant was discarded. Performed the “input” and “output” twice, and collected all the cells for differential counting. The number of cells collected from BALF was counted, and 5 × 10² cells in 100 μL PBS were cytospun onto glass slides at 350 rpm for 5 min. After Wright’s staining, differential cell counts was performed under microscope, and constitutively counted 200 cells for each sample.

**Assay of TNF-α, IL-8 and IL-6 in plasma**

The EDTA anti-coagulated blood was from the sacrificed rats, and serum was obtained after 10 min centrifugation at 1000 × g. The concentrations of inflammation-related cytokines TNF-α, IL-8 and IL-6 were measured by ELISA (BioLegend, San Diego, CA, USA).

**Hematoxylin and eosin (HE) staining**

Lung tissues were obtained from sacrificed rats, fixed with 4% PFA and embedded in paraffin. Then 5 μm sections were prepared for HE and periodic acid-Schiff (PAS) staining. Lung sections were deparaffinized in xylene, and stained with HE, respectively.

**Statistical analysis**

All data were expressed as mean ± standard deviation (±). Differences between groups were assessed by one-way analysis of variance (ANOVA) analysis, student t test or Mann-Whitney rank sum test. P < 0.05 was considered significant. All analyses were performed using the SPSS 20.0 (IBM Corp. Released 2011, IBM SPSS Statistics for Windows, Version 20.0, Armonk, NY, USA).

**RESULTS**

**Number of inflammatory cells in BALF and blood**

The total number of white cells, lymphocytes and neutrophils in BALF and blood were significantly increased in the rats with induced COPD, when compared with the healthy subjects (P < 0.05). Treatment with Daiqin phlegm-expelling pill, ambroxol hydrochloride tablet effectively reduced the number of these cells compared with COPD suffering rats without any treatment (P < 0.05). There is no difference in the number of inflammatory cells among the low, middle and high dose of Daiqin phlegm-expelling pill treated groups (Tables 1, 2).

**Production of TNF-α, IL-8 and IL-6 in plasma**

Rats with COPD displayed a significantly elevated level of TNF-α, IL-8 and IL-6 in the plasma than the negative control group (group A) (P < 0.05). Interestingly, the concentrations of TNF-α (group A, 0.158±0.016; group B, 0.185±0.022; group C, 0.162±0.014; group D, 0.151±0.007; group E, 0.154±0.008; group F, 0.150±0.011), IL-8 and IL-6 in plasma were remarkably down-regulated in the rats with COPD in the presence of Daiqin phlegm-expelling pill, or ambroxol hydrochloride tablet (P < 0.05). Again, no statistic difference was found in the TNF-α, IL-8 or IL-6 level in plasma among the rats treated with different

### Table 1: The number of nucleated cells in BALF (10³/mL, ± ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total white cells</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11.72±0.93</td>
<td>0.39±0.18</td>
<td>6.98±1.27</td>
</tr>
<tr>
<td>B</td>
<td>17.86±0.98</td>
<td>0.60±0.13</td>
<td>12.93±1.36</td>
</tr>
<tr>
<td>C</td>
<td>15.72±0.87</td>
<td>0.41±0.08</td>
<td>10.42±1.25</td>
</tr>
<tr>
<td>D</td>
<td>15.46±1.22</td>
<td>0.39±0.06</td>
<td>10.83±1.02</td>
</tr>
<tr>
<td>E</td>
<td>15.69±1.07</td>
<td>0.39±0.08</td>
<td>10.58±1.37</td>
</tr>
<tr>
<td>F</td>
<td>14.54±1.11</td>
<td>0.31±0.05</td>
<td>10.02±1.21</td>
</tr>
</tbody>
</table>

Notes: group A: healthy control group, healthy rats were treated with physiological saline (10 mL/kg); group B: COPD suffering rats, rats with induced COPD were treated with physiological saline (10 mL/kg); group C: COPD suffering rats + Daiqin phlegm-expelling pill (low dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (1 g for each kg) for each day; group D: COPD suffering rats + Daiqin phlegm-expelling pill (middle dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (2 g/kg) for each day; group E: COPD suffering rats + Daiqin phlegm-expelling pill (high dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (4 g/kg) for each day; group F: COPD suffering rats + ambroxol hydrochloride tablet, rats with induced COPD were orally treated with ambroxol hydrochloride tablet suspension (30 mg/kg) for each day. Rats were treated once for each day from days 1 to 28, and sacrificed on day 30. BALF: bronchoalveolar lavage fluid; COPD: chronic obstructive pulmonary disease. *P < 0.01, †P < 0.05, compared with group A; #P < 0.01, compared with group B.
thenumber of nucleated cells in blood, compared with group A; \( < 0.01 \), compared with group B.

Notes: group A: healthy control group, healthy rats were treated with physiological saline (10 mL/kg); group B: COPD suffering rats, rats with induced COPD were treated with physiological saline (10 mL/kg); group C: COPD suffering rats + Daiqin phlegm-expelling pill (low dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (2 g/kg) for each day; group D: COPD suffering rats + Daiqin phlegm-expelling pill (middle dose), rats with induced COPD were orally treated with Daiqin phlegm-expelling pill suspension (4 g/kg) for each day; group F: COPD suffering rats + ambroxol hydrochloride tablet, rats with induced COPD were orally treated with ambroxol hydrochloride tablet suspension (30 mg/kg) for each day. Rats were treated once for each day from days 1 to 28, and sacrificed on day 30. COPD: chronic obstructive pulmonary disease. \( P < 0.01 \), compared with group A; \( P < 0.01 \), compared with group B.

doses of Daiqin phlegm-expelling pill (Figure 1). The COPD suffering rats showed an obvious focal pneumomorrhagia and edema formation around the alveolar, severe dilation and congestion in the pulmonary interstitial capillary, alveolar epithelium necrosis and falling, inflammatory cells infiltration around the bronchus and serious structural damage. On the contrary, the rats treated with Daiqin phlegm-expelling pill, or ambroxol hydrochloride tablet displayed a slight capillary dilation and congestion, little edema and focal pneumomorrhagia, mild inflammatory cell infiltration and relatively intact pulmonary structure.

DISCUSSION

Daiqin phlegm-expelling pill was developed in Jiangsu Province Hospital of TCM, based on the TCM theory and clinical experience. It was designed for clearing heat and transforming sputum, diffusing the lung and suppressing liver, and especially functioned against the phlegm in COPD. Clinical observation revealed Daiqin phlegm-expelling pill possessed therapeutic effect-improving the symptom of coughing, phlegm and pant and facilitating to sustain lung function. Here we found the Daiqin phlegm-expelling pill could suppress the pulmonary inflammation using a rat model of LPS-smoke induced COPD.

The characteristic of airway inflammation in COPD was inflammatory infiltration, including neutrophils, macrophages and lymphocytes, which mediated and sustained the chronic inflammation doing harm to the respiratory system. One of the hallmarks in COPD was the increased number of neutrophils. Sputum neutrophil count increased with the Global Initiative for COPD stage, and higher levels of human neutrophil peptides and neutrophil elastase in spontaneous sputum from COPD patients were associated with greater decline in lung function over 2 years. In our investigation, the LPS-smoke induced COPD rats had a sig-
significantly increased number of total white cells and neutrophils in the BALF and blood, suggesting the successful establishment of a COPD like disease. Daiqin phlegm-expelling pill could effectively reduce the number of total white cells and neutrophils, although there was no difference among the three dosages, indicating a regulatory role of the agent in the activation of inflammatory cells in COPD.

TNF-α, IL-8 and IL-6 are potent activators of NF-κB pathway, which may promote the inflammatory response. 1 TNF-α is produced by peripheral blood monocytes and has been implicated in the cachexia and skeletal muscle apoptosis in some patients with COPD. Gunella and his colleges found smoking induced a higher level of TNF-α secretion from alveolar macrophages. 2-4 IL-8 and TNF-α are capable of recruiting neutrophils, inducing neutrophil degranulation and stimulating the respiratory burst. 5,20 In addition to its pro-inflammatory actions, the direct effects of TNF-α on epithelial cells may be another explanation for the pathogenesis of COPD. TNF-α mediated the airway mucous cell metaplasia and hypersecretion in vitro and in vivo. The elevated TNF-α was associated with the goblet cell metaplasia observed in chronic bronchitis, 6 as well as the reduced interepithelial binding, increased cell death, alveolar collagen deposition in murine alveolar walls, emphysematous lesions and induced production of IL-1, IL-8, and MCP-4. 7-10 von Haehling et al. 10 had reported that increasing airflow obstruction and hypercapnia were associated with an enhanced TNF-α response in COPD.

In our study, the Daiqin phlegm-expelling pill displayed an inflammation suppressive role reflected by the decreased concentration of TNF-α, IL-8 and IL-6 in plasma from rats with COPD, which may be partially contributed to its anti-inflammatory property. Through the intratracheal administration of LPS and smoking, we established the COPD disease model in rats. The HE staining of lung tissue slices showed typical pathological characteristics, such as mucous gland enlargement, increased mucus secretion and inflammatory infiltration in central bronchus. Besides, the airway smooth muscle become thickening, mucociliary disfunction, anomalous appearance of goblet cells in tracheal epithelium, alveolar expansion, thinner alveolar walls and some parts of the fractured alveolar fusion were observed in the lung of COPD suffering rats. To be noted, the Daiqin phlegm-expelling pill treatment could ameliorate the chronic inflammation associated symptoms in COPD, including the extenuation of hemorrhage, decrease in inflammatory cell infiltration and slighter alveolar structure damage, when compared with the untreated rats with COPD. Therefore, the Daiqin phlegm-expelling pill would be an effective agent in the clinical treatment of COPD. Interestingly, no significant difference was found among the groups of rats fed with low, middle or high doses of Daiqin phlegm-expelling pill. This observation may be contributed by the drug overdose, as even in the low dose group, it was four times more dosage than the average of adult. And the middle and high dose were 8 and 16 times more respectively.

In conclusion, our study provided evidence for the Daiqin phlegm-expelling pill in COPD therapy. In the rats with LPS-smoke induced COPD, Daiqin phlegm-expelling pill was capable of decreasing the number of total white cells, neutrophils and lymphocytes in the BALF and blood, reducing the concentration of TNF-α in plasma and extenuating the inflammation related damage in lung tissues. It would be beneficial for the elucidation of molecular mechanism by which the Daiqin phlegm-expelling pill suppressed the airway inflammation and alleviates the pathological symptom of COPD.

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks to all those who have helped me make this study possible and better.

REFERENCES