Inhibition of airway inflammation, hyperresponsiveness by the fructus schisandrae (wu wei zi) extract in a murine model of allergic asthma
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Abstract: Fructus schisandrae (wu wei zi) extract, a traditional Chinese medicine formula has been used in the treatment of asthma. This study demonstrated the immunoregulatory effect of the fructus schisandrae extract on chronic allergic asthma using the OVA challenged chronic asthmatic murine model. In the current research, we found that the fructus schisandrae extract decreased the airway hyperresponseness (AHR), pulmonary inflammatory cell infiltration, and airway remodeling in OVA mice. The fructus schisandrae extract also decreased Th2 cytokines, IL-4, IL-13 and Th17 (IL-17), but increased IFN-γ in the BALF. Furthermore, fructus schisandrae extract could attenuate TIMP-1 expression in lung, inhibit airway remodeling and AHR. In conclusion, the plant-medicines of fructus schisandrae extract cloud suppressed OVA-induced airway inflammation, remodeling, and hyperresponseness in chronic asthma murine model.

Keywords: Fructus schisandrae (wu wei zi) extract; Allergic asthma; Airway inflammation; Hyperresponsiveness; TIMP-1

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1. Introduction

Asthma is recognized as a common pulmonary disease and it is a serious public health problem worldwide recently. In the last two decades, asthma-related morbidity and mortality have increased[1]. Airway hyperresponsiveness (AHR), chronic pulmonary inflammation with eosinophil infiltration in the lungs, and mucus hypersecretion in the airway are hallmarks of allergic asthma.

Both eosinophils and T-helper (Th) 2 lymphocytes play pathogenic roles in asthma[2]. Eosinophils are commonly associated with allergic inflammation and act as effector cells in the pathogenesis of this disease by releasing cytotoxic granule proteins[3]. Eotaxin is a potent chemoattractant for eosinophils, and its levels are generally elevated after asthma induction. An imbalance between Th1 and Th2 leads to the development of an allergic disease. The levels of Th2 cytokines, including IL-4, IL-5 and IL-13, typically increase during an allergic disease. These cytokines have important roles in airway infiltration such as activating eosinophi, inducing immunoglobulin E (IgE) production, secreting mucus and releasing a variety of inflammatory mediators[4].

Traditional Chinese medicine (TCM) has a long history of human use and is one of the major components of CAM used in the United States. It has a unique (independent) system of theory and diagnosis and treatment tools. The National Center for Complementary and Alternative Medicine at the National Institutes of Health (NIH) defined TCM as a whole medical system[5]. Some publications also provided evidence regarding possible mechanisms underlying the reported clinical efficacy[6]. Fructus schisandrae is a traditional Chinese herbal medicine, can inhibit platelet aggregation, and show antioxidative, calcium antagonism, antitumor-promoting, and anti-HIV (human immunodeficiency virus) effects[7]. It has also been used as an immunomodulating agent in treating immunodeficiency diseases. Our previous study demonstrated the usefulness of fructus schisandrae extract as a component of formula in the treatment of asthma, which can efficiently inhibit airway remodeling, relieve symptoms and reduce the frequency of asthma attacks in a mouse asthma model[8]. However, there have been few reports regarding the role of fructus schisandrae extract on airway inflammation from asthma.

In this study, we report that fructus schisandrae extract inhibits airway inflammation in a mouse asthma model and regulates the TIMP-1 expression in ovalbumin-sensitized mice, providing a novel mechanism for the fructus schisandrae extract inhibitory effect on airway inflammation in animal models of asthma.

2. Methods

2.1. Materials

Fructus schisandrae were obtained from the Anguo Chinese material medica market; Aluminum hydroxide, ovalbumin were purchased from Sigma-Aldrich (St. Louis, MO) (U.S. sigma Chemical company); Interferon gamma (IFN-γ), interleukin IL-4 and IL-13, 17 enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN); TIMP-1 were purchased from Beijing Zhong
Shan Chemical Co.(Beijing, China); BABL/c mice were purchased from the Department of Laboratory Animal Science Center of Qingdao, certificate NO: SCXK(lu)2009007. AniRes 2003 animal lung function analysis system (Beijing Bestlab High-Tech Co., Ltd., China).

Fructus schisandrae was ground into small pieces and extracted twice with five volumes of 80% (v/v) in H2O ethanol under reflux. The extracts were combined and filtered through a paper filter. The pooled extract was concentrated by rota-evaporation under reduced pressure until the removal of all ethanol. Fructus schisandrae extracts was reconstituted in water (with 2g of raw herb in 1ml of extract) and stored at 4°C until use.

2.2. Animal Experimental Protocols

Thirty-six healthy female BABL/c mice, 4 to 6 weeks old, weighing 20 to 26g were randomly divided into 3 groups (n=12): normal control group (A), asthma group (B) and Fructus schisandrae extract group (C). The asthmatic models were established by OVA. The mice were sensitized on 0d, 7d and 14d by intraperitoneal injection of 20mg OVA emulsified in 1mg of aluminum hydroxide in a total volume of 0.2mL in groups B and C. Seven days after the last sensitization, the mice were exposed to 1% OVA aerosol for up to 30 minutes every other day for 7 days. The 1% OVA aerosol was generated by a compressed air atomizer driven by filling a perspex cylinder chamber (diameter 50cm, height 50cm) with a nebulized solution. Saline was used in group A instead of OVA. At the same time, mice in group C were treated with 0.5g/kg Fructus schisandrae extract by gavage every other day for 28 days. Mice were housed under controlled temperature (22±2°C), humidity (50±19%) and lighting (7:30 a.m.—7:30 p.m.) in solid bottom cages with food and water available ad libitum. All experiments were performed under the guidelines of the Experimental Laboratory Animal Committee of Qingdao University. Animal experiments were in strict accordance with the principles and guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Body Weight Changes and Evaluation of Airway Responsiveness

All mice were weighed the night before intragastric administration and on the 5th night of intragastric administration. The body weight changes during the 5d were calculated for comparison and analysis.

Airway responsiveness to methacholine was evaluated using an AniRes 2003 animal lung function analysis system 2h after the last challenge. Mice were anesthetized with pentobarbital sodium (50mg/kg) by intraperitoneal injection. The mice were then intratracheally intubated, placed in a rodent plethysmograph and mechanically ventilated with a constant pressure of 10 cmH2O at a respiratory rate of 90 breaths per min. After reaching a stable tracing, lung resistance (RL) was measured at baseline and following intravenous administration of Mch. Mch was injected via the jugular vein through a micro-infusion pump in progressive doses (0, 100µg/mL×0.1mL, 200µg/mL×0.1mL, 400µg/mL×0.1mL, 800µg/mL×0.1mL, 1.6 mg/mL×0.1mL) about every other 5min. The RL signal was collected continuously from 5s to 1min for each dose, and the mean RL was recorded to reflect the change in airway responsiveness.

2.4. Collection of Bronchoalveolar Lavage (BALF)

At 24 hours after the last challenge, the tracheas of mice were lavaged with two 0.8mL aliquots of cold PBS. The collected fluid was called BALF. Cells from BAL fluid were suspended in phosphate-buffered saline and counted, and cytospins were prepared (2000rpm, 10minutes) and stained with Wright-Giemsma. Differential counts of at least 400 cells were carried out in the high-power field of a microscope, and cells were identified based on their morphologic features.

2.5. Tissue Samples

Lungs were removed from the mice after killing 24 hours after the last challenge. The tissues from the left lung were directly obtained from the surgical suite and immediately fixed in 10% buffered formalin and then embedded in paraffin. Sections (5µm) were prepared and stained with hematoxylin and eosin. Additionally, periodic acid–Schiff staining was performed to identify mucus production in epithelial cells, and the number of positive cells per unit length of basement membrane perimeter was determined. Quantitative analysis was performed blinded as described[9].

2.6. Enzyme-Linked Immunosorbent Assay

The BALF sample was collected and immediately centrifuged at 2000 rpm for 10 minutes at room temperature and stored at -80°C until use. The levels of IFN-γ, IL-4 and IL-13, 17 in BAL were then assayed with ELISA kit according to the manufacturer’s instructions.

2.7. Immunohistochemistry

The expression of TIMP-1 was assessed by semi-quantitative immunohistochemistry. After being deparaffinized, the sections were incubated in 0.01 mol/l citric acid buffer (pH 6.0) for 15min in a microwave for antigen retrieval. After cooling, the sections were incubated in 3g/L H2O2 for 30min, to inactivate endogenous peroxidase. After blocking by 1:10 normal horse serum for 30 min, the supernatant was discarded. Primary anti-mouse TIMP-1 (1:300 dilution with PBS) was added overnight at 4°C. Then,
biotinylated goat anti-rat secondary antibody and streptavidin horseradish peroxidase were added to the slides and incubated for 30 min at room temperature. Staining was completed by incubation with diaminobenzidine chromogen solution at room temperature. The stained cells were mounted and viewed under light microscopy.

2.8. Statistical Analysis
Statistical analysis was performed using SPSS 17.0 software. Data are expressed as mean± standard deviation. Statistical comparisons of the data from the various groups were performed using the Student t test. Differences between groups were considered statistically significant at P<0.05.

3. Results

3.1. Body Weight Changes
As can be seen from Figure 1, the body weight of mice in the Control group increased, but mice in the other groups exposed to OVA increased slowly. The body weight changes between the Model group and the Control group were significant. Compared with the Model group, treatment with astragalus extract group significantly increased compared with the OVA group.

Figure 1. Body Weight Changes. The data were summarized as mean ± standard error of the mean from at least 3 separate experiments. *P<0.05 in comparison with the OVA group.

3.2. Effect on Airway Responsiveness
As shown in Figure 2, mice in the Model group exhibited a significantly elevated RL ratio compared with mice in the Control group. This illustrates that OVA exposure by this method successfully caused AHR. It should be noted that treatment with fructus schisandrae extract could significantly ameliorate AHR symptoms.

Figure 2. RL graphs produced by the AniRes 2003 animal lung function analysis system. RL ratios of mice challenged with gradient doses of Mch. Data are shown as mean±standard error, n=12 per group. *P<0.05, compared with the Control group. #P<0.05, compared with the Model group.

3.3. Effects of fructus schisandrae extract on allergen-induce airway inflammation
To investigate whether fructus schisandrae extract could suppress the OVA-induced infiltration of lungs by inflammatory cells, we performed BAL 24h after the final aerosol challenge and examined each specimen using Wright-giemsa stain. OVA challenge significantly increased the total number of cells in BAL fluid, as well as the numbers of eosinophils, macrophages, lymphocytes (Table 1 and Figure 3).

Histological analysis showed that asthma group mice resulted in the development of significant airway inflammation. Contrast to control group, the asthma
group histological results showed extensive infiltration of inflammatory cells around bronchioles, blood vessels and alveoli, such as eosinophils. After treatment with fructus schisandraceae extract, the extent of inflammation and cellular infiltration in the airway were reduced (Figure 4).

Table 1. Classification and counting of cells in BAL fluid

<table>
<thead>
<tr>
<th>Group</th>
<th>n.</th>
<th>total number</th>
<th>eosinophils</th>
<th>macrophages</th>
<th>lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>12</td>
<td>0.77±0.20</td>
<td>0.02±0.01</td>
<td>0.51±0.10</td>
<td>0.27±0.07</td>
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<tr>
<td>asthma group</td>
<td>12</td>
<td>8.77±1.39</td>
<td>0.75±0.31</td>
<td>3.79±0.57</td>
<td>2.53±0.79</td>
</tr>
<tr>
<td>Fructus schisandraceae extract</td>
<td>12</td>
<td>4.65±1.20</td>
<td>0.23±0.1</td>
<td>2.41±0.68</td>
<td>1.43±0.36</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td>111.36</td>
<td>28.82</td>
<td>39.17</td>
<td>80.14</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 4. Histological examination of peripheral airway tissue. Lung tissue sections obtained from mice 24h after the last OVA challenge were stained with H&E. (A) control group; (B) asthmatic group; (C) fructus schisandraceae extract treated group. All photos were captured at ×200 magnification.

Figure 5. Fructus schisandraceae extract reduced levels of TH2 cytokines and asthma-related chemokines in BAL. BAL was collected from mice subjected to various treatments 24 hours after the final challenge. IL-4, IL-13, 17 and IFN-γ levels were determined by ELISA. The data were summarized as mean ± standard error of the mean from at least 3 separate experiments. #Significant difference versus control group (P>0.05). *Significant difference versus OVA group (P<0.05).
3.4. Fructus schisandrae extract reduced asthma-related chemokines

Inflammation in asthma is considered as a Th2 predominant immune reaction. OVA inhalation in sensitized mice caused a notable increase in IL-4, IL-13 and IL-17 levels into BAL fluid as compared with saline aerosol control. In contrast, BAL fluid level of IFN-γ, a Th1 cytokine, dropped slightly in OVA challenged mice. Noticeably, fructus schisandrae extract markedly upregulated IFN-γ and downregulated IL-4, IL-13 and IL-17 levels in BAL fluid. This finding implies that fructus schisandrae extract is able to modify the Th2-predominant immune activity in our OVA-induced mouse asthma model (Figure 5).

3.5. Influence of fructus schisandrae extract on TIMP-1 expression in mouse lung tissue

To determine whether the therapeutic effect of fructus schisandrae extract on OVA-induced asthma is through TIMP-1 inhibition, TIMP-1 protein was found to be expressed in various cells of the lung including airway epithelial cells, fibroblasts, smooth muscle cells, vascular endothelial cells as well as the infiltrative inflammatory cells in model mice (Figure 5), compared with the control group. Conversely, the TIMP-1 level was significantly lower in the fructus schisandrae extract group. We also assayed TIMP-1 protein levels in the bronchoalveolar lavage fluid and found that TIMP-1 levels were significantly higher in asthmatic mice than those in the control group (Figure 6 and Table 2).

Figure 6. Fructus schisandrae extract suppressed TIMP-1 expression in lung tissues. Expression of TIMP-1 in lung tissue was determined by immunohistochemical staining. A, Control group; B, Asthmatic group; C, fructus schisandrae extract group.

Table 2. ELISA analysis of TIMP-1 protein levels in bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>group</th>
<th>n</th>
<th>TIMP-1(pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>12</td>
<td>231.5±13.5</td>
</tr>
<tr>
<td>Asthmatic group</td>
<td>12</td>
<td>572.7±45.8</td>
</tr>
<tr>
<td>fructus schisandrae extract group</td>
<td>12</td>
<td>324.1±32.8</td>
</tr>
</tbody>
</table>

*Significantly different from control group at P < 0.05.  "Significantly different from OVA-challenged group at P<0.05.

4. Discussion

Asthma is a chronic allergic disease characterized by airway inflammation and remodeling, bronchial hyper responsiveness, variable airflow obstruction, and mucus hypersecrection[10,11]. Using ovalbumin-induced asthma rats model for their reliable, reproducible, and inexpensive are widely used. And the total number of inflammatory cells, eosinophils, neutrophils, lymphocyte percentages in BALF from model group of mice were significantly higher than the control group (P<0.01), illustrate the asthma model was successful.

TCMs have been reported with therapeutic effects on allergic asthma[12,13]. The fructus schisandrae extract, a formula designed following the traditional Chinese medicine theories and clinical experience, has been used to treat asthmatic patients in Asia for decades. Unlike the side effect from using corticosteroids, plant-medicines relieves asthmatic syndrome without total immune suppression. In present study, the immunoregulatory effect and possible mechanism of the fructus schisandrae extract were investigated in OVA-induced chronic allergic asthma murine model. Interleukin 4 (IL-4) and interleukin 13 (IL-13) are two cytokines that are associated with type 2 inflammation[14-18]. A number of studies have implicated IL-4, IL-13, and their receptors in the pathogenesis of asthma and allergy[19-23]. In the current study, we investigated that fructus schisandrae extract significantly reduced the number of infiltrating leukocytes in the airways of OVA-challenged mice, especially eosinophils and lymphocyte. Accordingly, OVA-induced TH2-associated cytokines (IL-4 and IL-13) were significantly suppressed by fructus schisandrae extract treatment. In this study, the fructus schisandrae extract treatment to allergic mice modulated the Th1/Th2 response by increasing IFN-γ, decreasing IL-4 and IL-13.
Furthermore, IL-17 was demonstrated as indispensable to induce granulocyte influx into the lung in allergic asthma model[24-25]. IFN-γ is indicated to limit the IL-17-producing T-cell population[26]. IL-17 is mainly produced by macrophages in allergic inflammation related to asthma[27]. We found that IL-17 production and macrophage infiltration were attenuated while IFN-γ production was increased in the fructus schisandrae extract treated mice. These observations suggest that the fructus schisandrae extract could limit the IL-17 related immune response by increasing IFN-γ production.

Airway inflammation, reactivity, and remodeling in asthma depend upon many interrelated processes. There is increasing recognition that the lung matrix plays an active role in the inflammatory response and resultant remodeling in asthma. Tissue inhibitors of metalloproteinases (TIMPs; specifically TIMP-1 and TIMP-2) modulate the nature and outcome of inflammation, namely healing versus pathologic remodeling[28]. The fructus schisandrae extract exhibited nonspecific anti-inflammatory property with reducing the cell number of all kinds of inflammatory cells in the BALF of OVA mice. Pathological observations also showed that the fructus schisandrae extract reduced inflammatory cell infiltration. Airway remodeling, including lamina thickening and airway structural changes, a central feature of asthma, is closely related to progression of AHR[29-30]. TIMP-1 not only regulates cellular biological processes leading to airway remodeling[31] but also contributes to increased collagen synthesis and AHR[32]. The fructus schisandrae extract treatment decreased the TIMP-1 production in BALF in the lung of OVA mice. Taken together, these results suggest that treatment with the fructus schisandrae extract can suppress AHR by decreasing airway inflammation and mucus hypersecretion associated with TIMP-1 secretion. The properties of fructus schisandrae extract with anti-inflammatory, decreasing airway remodeling, and inhibiting AHR promise this formula an effective therapeutic modality for asthma.

In conclusion, the fructus schisandrae extract suppressed OVA-induced airway inflammation, remodeling, and hyperresponsiveness in chronic asthma murine model. The effect was accompanied by inhibiting Th2 responses and decreasing chemokine expression but elevating IFN-γ and decreasing IL-4 and IL-13 production. In addition, we confirmed that the fructus schisandrae extract could modulate the expression of the TIMP-1, which may involved in modulating airway inflammation, airway remodeling and AHR. Our results support the utility of fructus schisandrae extract as a herbal medicine for asthma treatment and may have application in the development of anti-inflammatory and anti-asthmatic drugs. And provided more clinical advantages over corticosteroids for asthma treatments.

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References
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