

ORIGINAL ARTICLE

Tripterygium Polyglycosid Attenuates the Established Airway Inflammation in Asthmatic Mice*

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ABSTRACT **Objective:** To investigate the effect of Tripterygium polyglycosid on establishing airway eosinophil infiltration and related airway hyperresponsiveness of asthmatic mice. **Methods:** A mature murine asthmatic model was made with ovalbumin sensitized and challenged C57BL/6 mice. Forty mice were divided into four groups with 10 mice in each group: mice sensitized and challenged with saline (WS group), mice sensitized and challenged with ovalbumin (WO group), mice sensitized and challenged with ovalbumin and treated with Tripterygium polyglycosid (TP group) and Dexamethasone (DXM group). The mice were intraperitoneally injected with 20 μ g chicken ovalbumin emulsified in injected alum on days 0 and 14, then were challenged with an aerosol generated from 1% ovalbumin on days 24, 25 and 26. Tripterygium polyglycosid was injected intraperitoneally at 50 mg/kg on days 25, 26 and 27 after ovalbumin challenge. Dexamethasone was administrated to mice at 2 mg/kg on day 21, 23 before ovalbumin challenge. The airway hyperresponsiveness, mucus production, eosinophils in parabronchial area and bronchoalveolar lavage fluid and the level of interleukin-5, granulo-macrophage clone stimulating factor in bronchoalveolar lavage fluid were measured as indexes of inflammation. **Results:** Tripterygium polyglycosid treatment after ovalbumin challenge completely inhibited eosinophil infiltration in bronchoalveolar lavage fluid $[(0.63 \pm 0.34) \times 10^4$ vs. $(75.0 \pm 14.8) \times 10^4$, $P < 0.05$] and the peribronchial area (12.60 ± 3.48 mm² vs. 379.0 ± 119.3 mm², $P < 0.05$), mucus overproduction in airway (2.8 ± 1.7 vs. 7.1 ± 5.6 , $P < 0.05$), and increased interleukin-5 levels in bronchoalveolar lavage fluid (28.8 ± 2.8 pg/mL vs. 7.5 ± 3.5 pg/mL, $P < 0.05$). Meanwhile, Tripterygium polyglycosid treatment after ovalbumin challenge also partially inhibited airway hyperresponsiveness. The level of granulo-macrophage clone stimulating factor in bronchoalveolar lavage fluid didn't change with drugs intervention. **Conclusions:** The administration of Tripterygium polyglycosid could inhibit the established airway inflammation and reduce the airway hyperresponsiveness of allergic asthmatic mice. It provides a possible alternative therapeutic for asthma.

KEYWORD Tripterygium polyglycosid, asthma, mouse, airway hyperresponsiveness, eosinophils

Tripterygium wilfordii hook f (TwHF), a vinelike member of the Celastraceae plant family, has been used in Chinese medicine for 2,000 years. It has attracted more attention recently for its clinical application in the therapy of autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus etc. and renal diseases.⁽¹⁻³⁾ Its anti-inflammatory effects were also shown in a recent study.⁽⁴⁾ Apart from more delicate molecular analysis, the common regimen is the extract with variable efficacies and human safety profiles produced by various TwHF processing methods, which reduces its toxicity and improves efficiency significantly.^(5,6) These characteristics extend their clinical application widely. Among these extracts, Tripterygium polyglycosid (TP) is the one that keeps most of its activity.⁽¹⁾

inflammation characterized by the infiltration of inflammatory cells such as T cells, eosinophils etc., mucus production and airway hyper-responsiveness (AHR).⁽⁷⁾ The inflammation process is dominated by T help2 (Th2) cells and relevant cytokines such as

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Asthma is a disease of chronic airway

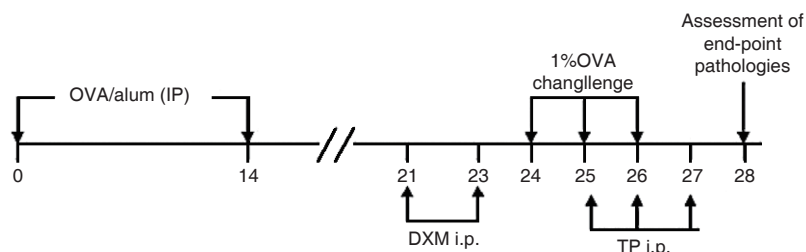


Figure 1. Time Line Presentation of the OVA Protocol Used and the Administration of DXM and TP

Notes: i.p.: intraperitoneal injection; OVA: ovalbumin; DXM: Dexamethasone; TP: Tripterygium polyglycosid

interleukin (IL)-4 and IL-5, etc. Eosinophils are the most important effector cells to cause the inflammation. These cells release relative cytokines and inflammatory mediators, which cause the spasm of smooth muscle and mucus overproduction and lead to the acute exacerbation of asthma. Targeting on eosinophils and AHR is crucial for asthma treatment. TP has been utilized to treat asthmatic patients in some small size studies.⁽⁸⁾ However, little is known about its effect on airway inflammation and hyperresponsiveness.

In the present study we investigated the effect of TP on airway eosinophil infiltration and related inflammation in asthmatic mice. We also measured the cytokine concentration in bronchoalveolar lavage fluid (BALF) to investigate the possible mechanism.

METHODS

Animals

Forty C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang University and housed in a specific pathogen-free facility in our campus. All the mice were maintained in micro-isolator cages housed in specific pathogen-free animal facilities, and the male mice of 8–12 weeks were used for experiments. Experimental protocols were approved by the Ethical Committee for Animal Studies of Zhejiang University.

Ovalbumin Sensitization/ Aerosol Challenge and Administration of TP and Dexamethasone

Mice were divided into 4 groups with 10 mice in each group: mice sensitized and challenged with saline (WS group), mice sensitized and challenged with ovalbumin (OVA, WO group), mice sensitized and challenged with ovalbumin and treated with TP (TP group) or Dexamethasone (DXM group). The mice were sensitized by an intraperitoneal injection (100 μ L) of 20 μ g chicken OVA emulsified in injected alum [2.25 mg Al (OH)₃, 2 mg Mg (OH)₂, Pierce, USA] on days 0

and 14. Then, they were subsequently challenged with an aerosol generated from 1% OVA in saline, or saline alone, for 20 min by ultrasonic nebulization (DeVilbiss, USA) on days 24, 25 and 26.

DXM was injected intraperitoneally at 2 mg/kg on day 21, 23 before aerosol challenge. TP in tween 80 was injected intraperitoneally at 50 mg/kg on day 25, 26 and 27. Mice were assessed for pulmonary cellular infiltrates, histopathologies, bronchoalveolar lavage (BAL) cytokine levels, and lung function on day 28. A time line of these experimental manipulations is presented in Figure 1.

Measurement of AHR in Response to Methacholine Challenge

AHR was assessed by whole-body plethysmography, inducing airflow obstruction with a methacholine aerosol.⁽⁹⁾ This methodology (Buxco Electronics, Troy, USA) relies on pressure differences between a chamber containing conscious/unrestrained mice and a reference chamber to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (Penh). Penh is a dimensionless parameter that is a function of total pulmonary airflow in mice (i.e., the sum of the airflows in the upper and lower respiratory tracts) during the respiratory cycle of the animal and closely correlates with airway resistance as measured by traditional invasive techniques using ventilated animals.⁽¹⁰⁾ Dose-response data were plotted either as percent baseline Penh vs the log₁₀ of the methacholine solution (milligrams per milliliter) used to generate the aerosol.

Assessments of Allergic Airway Inflammation: Pulmonary Cellular Infiltrates and OVA-Induced Histopathologies

The cellularity of the airspaces was determined by BAL as previously described.⁽¹¹⁾ Calculation of eosinophils in lung tissue sections (4 μ m) was

performed by hematoxylin/eosin (HE) stain. Quantity of eosinophils was achieved by using an image analysis software program (Image ProPlus, Silver Spring, USA). The density of eosinophils in lung tissue was calculated by sum of eosinophils infiltrated in peribronchial area per bronchiole dividing the number of bronchioles in slides.

Histopathologic changes of the airways, including mucus cell content of the epithelium, were assessed in paraffin-embedded lungs following formalin fixation [lungs were inflated with a fixed volume (0.5 mL) of fixative]. Parasagittal sections (4 μ m) were stained with periodic acid-Schiff's (PAS) reagent and counterstained with hematoxylin/methyl green to assess mucus production and goblet cell metaplasia. The mucus content of airways (both proximal and distal) were assessed by brightfield microscopy ($n=5$ mice per group) using the same image analysis software program to derive an airway mucus index that is reflective of both the amount of mucus per airway and the number of airways affected.⁽¹¹⁾ All pathologic samples were read by a blinded reviewer.

Cytokine Assays

Cytokine levels in BAL fluid were determined by enzyme-linked immunosorbent assay (ELISA). Mouse interleukin (IL)-5 and granulo-macrophage clone stimulating factor (GM-CSF) levels were assessed using immunoassay kits (R&D Systems, Minneapolis, USA) under the manufacturer's instructions. The limits of detection for GM-CSF and IL-5 assay were 7 pg/mL.

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation. Differences between groups were tested using One-way analysis of variance (ANOVA) or the Mann-Whitney test using Sigma-stat 3.5 software. P -values of less than 0.05 were considered significant.

RESULTS

Administration of TP Inhibited the Infiltration of Eosinophils in Airway

Firstly, we sought to examine eosinophil cell infiltration in BALF. The BALF data in Figure 2A showed that OVA sensitization and challenge induced the accumulation of eosinophils in BALF $[(75.0 \pm 14.8) \times 10^3]$. The administration of TP or DXM inhibited the infiltration of eosinophils in airway lumen

completely $[(0.63 \pm 0.34) \times 10^4$ and $(0.23 \pm 0.10) \times 10^4$ respectively, $P<0.05$]

The distribution of tissue eosinophils in the lungs of mice was determined by HE staining. Normally few eosinophils could be seen in the lung while OVA sensitization and challenge lead to significantly augmented recruitment of eosinophils into airway ($379.0 \pm 119.3/\text{mm}^2$). Both TP and DXM diminished this tissue infiltration of eosinophils effectively (Figure 3). Further quantitative assessments showed that peribronchial eosinophils surrounding individual airways in mice treated with TP were significantly decreased ($12.60 \pm 3.48 \text{ mm}^2$, $P<0.05$, Figure 2B).

TP-Inhibited OVA-Induced Airway Epithelial Mucus Production

Airway pathologic changes including hyperplasia of epithelial cells, mucus overproduction, discontinuous epithelium, thickened basal layer and hypertrophy of smooth muscle were evident in either

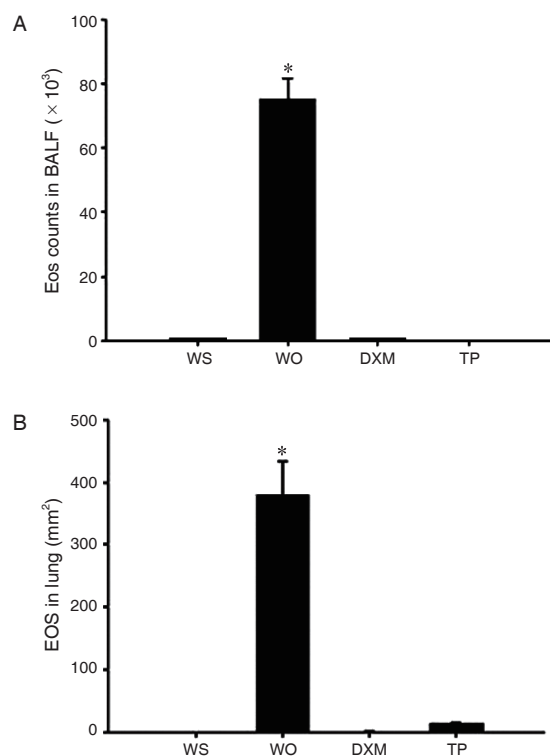


Figure 2. Administration of TP Eliminated the Infiltration of Eosinophils in Airway

Notes: A: showed that the mice in the WS group do not have eosinophils in BALF, but OVA sensitization and challenge induced the infiltration of eosinophils. The administration of TP and DXM inhibited the infiltration of eosinophils in BALF significantly. B: accumulation of eosinophils in the lungs of the WO group was inhibited completely by TP or DXM. $P<0.05$, compared with the WS group

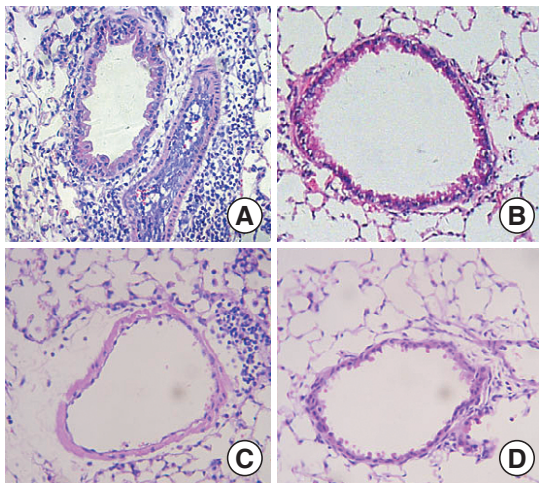


Figure 3. Administration of TP or DXM Resulted in A Reduction of Eosinophils Distribution in OVA-Treated Mice (HE Staining, $\times 200$)

Notes: A: WO group; B: WS group; C: TP group; D: DXM group

OVA-treated mice. It didn't occur in the lungs of DXM-treated mice. In the lungs of TP-treated mice, the mucus production was clearly reduced (2.8 ± 1.7 vs. 7.1 ± 5.6 , $P < 0.05$, Figure 4). The quantitative assessment of mucus production via mucus index in Figure 5 further confirmed this finding.

Influence of TP on BALF Cytokines

Cytokines in BALF are related to inflammation in airway. OVA sensitization and challenge did

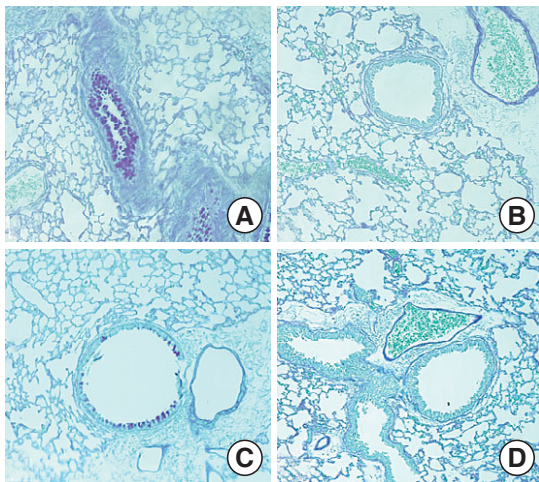


Figure 4. Administration of TP or DXM Inhibited OVA Induced Mucus Over- Production (PAS Staining, $\times 400$)

Notes: Lung sections from formalin-fixed/paraffin-embedded tissue were stained for the presence of mucin with periodic acid-Schiff's reagent (Counter-stain: hematoxylin). The photographs presented show representative examples of the staining that occurs in bronchioles. A: WO group; B: WS group; C: TP group; D: DXM group

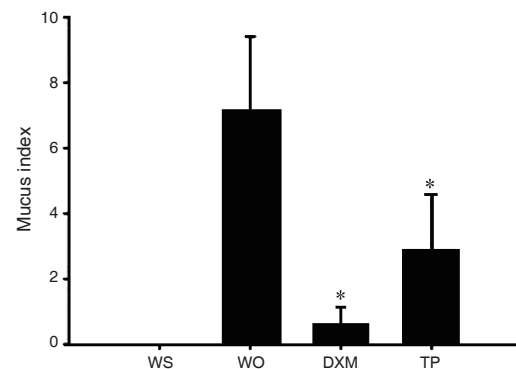


Figure 5. Measurement of Mucus Accumulation in Airway

Notes: The mucus index of the WS group was close to zero. OVA sensitization and challenge increased it significantly. TP or DXM treatment both decreased this overproduction. $n=5-10$ mice/group; * $P < 0.05$, compared with the WO group

not influence the low expression level of GM-CSF in bronchial lumen, nor did TP or DXM. But OVA sensitization and challenge induced a significant increase of BALF IL-5 levels (28.8 ± 2.8 pg/mL vs. 7.5 ± 3.5 pg/mL, $P < 0.05$), which was absent in normal mice. Both TP and DXM significantly diminished BALF IL-5 levels (7.5 ± 3.5 pg/mL, $P < 0.05$ and 5.4 ± 2.1 pg/mL, $P < 0.001$, respectively; Figure 6).

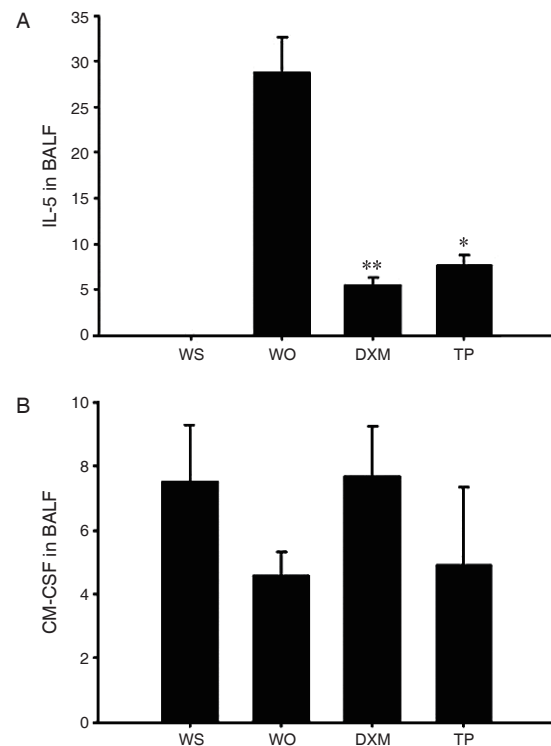


Figure 6. Cytokines Assessment in BALF

Note: * $P < 0.05$, ** $P < 0.01$, compared with the WO group

Allergen-Induced AHR Was Inhibited by TP

Induction of airway hyperresponsiveness is

partly mediated by infiltrated eosinophil cells. Because TP treatment diminishes eosinophil cell infiltration, we hypothesized that TP treatment might also be able to reduce airway hyperresponsiveness. Two days after the final challenge with OVA, the Penh values of different groups were assessed by whole-body plethysmography. The results of a methacholine dose-response are presented in Figure 7. The dose response curve confirmed earlier studies and demonstrated the measurable effect of TP and DXM. Both DXM and TP inhibited mouse response to methacholine. These data together suggest that TP treatment could alleviate airway hyperresponsiveness.

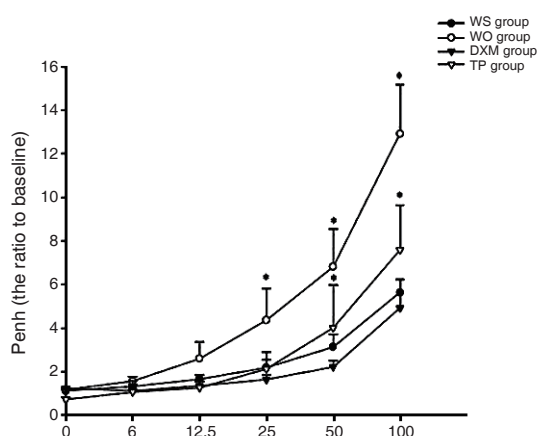


Figure 7. Airway Responsiveness Measurement

Note: * $P < 0.05$, compared with the WS group

DISCUSSION

TP is a powerful immuno-suppressive and anti-inflammatory medicine. Its main active component, triptolide, is involved in different aspects of inflammation such as lymphocyte regulation and cytokine secretion, which are crucial in the mechanism of asthma.⁽¹²⁾ Our study demonstrated that TP was able to inhibit the airway eosinophil infiltration and airway hyperresponsiveness in a murine model of asthma. What distinguishes our study from others is that TP was administrated after OVA challenge, suggesting it was able to reverse the already established airway inflammation.

Our study demonstrated that TP significantly suppressed the IL-5 levels but not the GM-CSF levels in BALF. IL-5 is involved in the development and maturation of eosinophils.^(7,13) It also mediates the interaction of eosinophils and inter-cellular adhesion molecule-1, and modulates eosinophil activation and survival. IL-5 has also been shown to play pivotal

role in the regulation of Th2 immunity and allergic inflammation.⁽¹⁴⁾ Both deactivation of T lymphocytes and apoptosis of eosinophils reduce the secretion of IL-5. GM-CSF is mainly produced by Th2 lymphocytes in the early stage during development of asthma, it can also be produced by activated eosinophils.⁽¹⁵⁾ It was shown that small dose of TP inhibits T cell activation and proliferation *in vitro*.⁽¹⁶⁾ Jenn-Huang, et al⁽¹⁷⁾ reported that TP could induce the spontaneous apoptosis of T cells via activation of caspase-3 without the involvement of Fas/Fas ligand interaction. Its function of downregulating NF-kappaB activation during inflammatory conditions might also contribute to this.⁽¹⁸⁾ In the present study, the concentration of GM-CSF was not affected by OVA-sensitization and challenge, which is in concert with Leonie S' study earlier.⁽¹⁹⁾ These results also suggested that GM-CSF might play an important role in the early stage of airway inflammation.⁽²⁰⁾ Thus, administration of TP after OVA challenge is not able to alter the GM-CSF level.

TP significantly inhibited the infiltration of eosinophils in airway of asthmatic mice. Eosinophils are the most important effector cells in the airway inflammation of asthma.⁽⁷⁾ The infiltration and activation of eosinophils in airway are correlated with the severity of asthma exacerbation and progress of AHR.⁽²¹⁾ The pathologic and physiologic changes disappeared with elimination of airway eosinophils.⁽²²⁻²⁴⁾ Therefore, the inhibition of eosinophils is the key point to treat asthma. There might be two possible mechanisms for TP's role on elimination of eosinophils. The first is that TP directly induced apoptosis of eosinophils. Xu, et al⁽²⁵⁾ demonstrated that TP caused the apoptosis of eosinophils in sputum of asthmatic patients. The other mechanism is that TP could upregulate the number of CD8⁺ T cells, while downregulate the number of CD4⁺ T cells, which results in changing the ratio of CD8⁺ T cells to CD4⁺ T cells.⁽²⁶⁾ Therefore, TP may inhibit the immune response through enhancing T cell activity. Besides, the decrease of IL-5 in airway lumen also shortens the life span of local eosinophils.

The anti-inflammation therapy of TP also reduced the mucus production and AHR of asthmatic mice. The main contributors of mucus production are CD4⁺ T cells and eosinophils.⁽²⁷⁾ The relationship between the number of inflammatory cells, particularly eosinophils, and the level of AHR was also confirmed by previous

studies.⁽²⁸⁾ Therefore, reduction of infiltration of CD4⁺ T cells and eosinophils accounts for the improvement. Moreover, TP could inhibit the differentiation of Th17 cells and peroxynitrite precursor synthesis, attenuate the increased activity of PP2A and consequently protect endothelial barrier function.⁽²⁹⁻³¹⁾ The inhibition of the IL-5 and TNF- α also improved AHR.^(32,33) The beneficial effects of TP treatment may result from the combination of all these effects.

In summary, the administration of TP could inhibit the airway inflammation and reduce AHR of allergic asthmatic mice. Although it is usually used to treat autoimmune diseases and its potential side effects have limited its broad application. This study demonstrated that TP might be used as a novel therapeutics in asthma. Though glucocorticoid is the first choice for the long-term management of asthma, for the small portion of patients who are steroid-resistant or unable to tolerate steroids,⁽²³⁾ TP may provide a possible alternative medicine.

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