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Original Research Article

Trifolirhizin mitigates ovalbumin-induced lung inflammation and tissue damage in neonatal rats via inhibition of the NF-κB signaling pathway

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Abstract

Purpose: *To investigate the effect of trifolirhizin on neonatal rat model of asthma, and the mechanism of action involved.*
Methods: *Neonatal rats (n = 50) were randomly assigned to 5 groups (10 pups/group): sham, asthma and three treatment groups. With the exception of sham group, the rat pups were sensitized intraperitoneally with ovalbumin (OVA) at a dose of 20 μg/kg on days 7 and 21 postpartum. Rats in the treatment groups received trifolirhizin intragastrically at doses of 2, 4 and 5 mg/kg on day 7 postpartum. Eosinophils in bronchoalveolar lavage fluid (BALF) were counted using hematological analyzer. Serum immunoglobulin (Ig)E and interleukin (IL)-4, IL-5 and IL-13 levels in BALF were determined using their respective enzyme-linked immunosorbent assay (ELISA) kits. Messenger RNA (mRNA) expressions of mucin 5AC (Muc5AC), mucin 5B (Muc5B), tumor necrosis factor α (TNF-α) and intercellular adhesion molecule-1 (ICAM-1) were determined using immunohistochemical staining, while the protein expression of inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells alpha (IκBα) was assayed by Western blotting.*
Results: *Serum IgE level was significantly higher in asthma group than in sham group, but was significantly and dose-dependently reduced after treatment with trifolirhizin ($p < 0.05$). Lung tissue damage was also significantly mitigated in the treatment groups, relative to asthma group ($p < 0.05$). Trifolirhizin treatment significantly and dose-dependently downregulated the mRNA expressions of Muc5AC, Muc5B, TNF-α and ICAM-1, but upregulated IκBα protein expression significantly and dose-dependently ($p < 0.05$). Bronchoalveolar lavage fluid (BALF) levels of IL-4, IL-5 and IL-13 were significantly higher in asthma group, but were significantly and dose-dependently reduced after treatment with trifolirhizin ($p < 0.05$).*
Conclusion: *These results indicate that trifolirhizin mitigates OVA-induced lung inflammation and tissue damage in neonatal rats via inhibition of NF-κB signaling pathway, thus affording a potential therapeutic strategy for the management of asthma*

Keywords: *Asthma, Bronchoalveolar lavage fluid, Inflammation, Interleukins, Ovalbumin*

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INTRODUCTION

Asthma is a chronic disease of the respiratory system. Statistics show that it affects more than 335 million people annually [1,2]. Asthma is characterized by hyper-responsiveness of the airway, inflammation, increased mucus secretion and remodeling of pulmonary airways [1,2]. Some symptoms of the disease are wheezing, difficulty breathing, cough and chest tightness [2]. The pathogenesis of asthma is complex, and is yet to be fully elucidated. Helper T (Th) cells, the most important cells in adaptive immunity, are required for almost all adaptive immune responses. They not only help activate B cells to secrete antibodies and macrophages that destroy ingested microbes, they also help activate cytotoxic T cells to kill infected target cells. Increased activation of T helper 2 (Th2) cells has been demonstrated to contribute to the pathogenesis of asthma [3].

Studies have revealed an imbalance in the Th1/Th2 ratio in asthmatic patients. Inhalation of exogenous allergens leads to the differentiation of Th0 cells into CD4+ Th2 cells, thereby stimulating the production of inflammatory cytokines [3]. The resultant increase in cytokine level induces IgE production, and also elevates eosinophil level and mucus secretion, thereby resulting in asthma [4]. The drugs presently used for the treatment of asthma do not completely eradicate the symptoms: they only relieve inflammation of lung airways [5]. This has necessitated the search for novel compounds that can effectively alleviate the symptoms of asthma. Hyper-responsiveness, the hallmark of asthma is caused by chronic inflammation of lung airways [4]. Clinically, asthma is managed mainly by daily inhalation of corticosteroids (ICS) in combination with long-acting β2 agonists (LABAs) [1,6]. However, administrations of monoclonal antibodies which effectively target IL-5 pathway have been shown to significantly reduce eosinophilic inflammation, while reducing the symptoms in asthmatic patients [7].

Sophora flavescens is a herb used in Traditional Chinese Medicine (TCM) for treating various ailments. Its root is used for treating inflammation, microbial infections, asthma and mental disorders [8]. Trifolirhizin, a pterocarpan flavonoid isolated from the roots of *Sophora flavescens* possesses varied biological and pharmacological properties, such as hepatoprotective and anti-inflammatory effects [9,10]. The present study investigated the effect of trifolirhizin on neonatal rat model of asthma, and the mechanism of action involved.

EXPERIMENTAL

Materials

Bicinchoninic acid (BCA) protein kit was purchased from Beijing Gede Biological Co. Ltd. Polyclonal antibodies of IκBα and β-actin, and IL-4, IL-5, IL-13 and IgE ELISA kits were obtained from Abcam (UK). Horseradish peroxidase (HRP)-labeled antibody was obtained from Shanghai Biyuntian Biotechnology Co. Ltd. 3**,** 3′-Diaminobenzidine (DAB) was purchased from Santa Cruz Biotechnology (USA), while enhanced chemiluminescence (ECL) kit was a product of Boster Biological Technology (USA).

Rats

The rat pups (n = 50) used for this study were littered by pregnant female Sprague Dawley rats (n = 15) obtained from the Animal Centre of Shandong University (Jinan). The pups were housed in metal cages under standard conditions and allowed free access to standard feed and water. Prior to commencement of study, the pups were acclimatized to the laboratory environment for 3 days. They were exposed to 12 h light/12 h dark cycle and maintained at 24 ± 1 °C and 60 % humidity. The study protocol was approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology (approval no. 007/HU/17), and the study procedures were carried out according to the National Institute of Health (NIH) guidelines for Laboratory Animal Use [9].

Establishment of neonatal rat model of asthma

A total of fifty pups were randomly assigned to 5 groups (10 pups/group) viz: sham, asthma and three treatment groups. With the exception of sham group, the pups were sensitized by intraperitoneal injection of OVA at a dose of 20 µg/kg bwt on days 7 and 21 postpartum. Rats in the treatment groups received trifolirhizin intragastrically at doses of 2, 4 and 5 mg/kg bwt, respectively, on day 7 postpartum. The pups were thereafter challenged with 1 % OVA for 40 min using TurboBOY N air nebulizer (PARI GmbH) on day 45 postpartum. On day 46 postpartum, they were anesthetized with sodium pentobarbital (60 mg/kg bwt) prior to collection of blood samples for biochemical analysis. Moreover, BALF and lung tissues were obtained

on day 47 under sodium pentobarbital (200 mg/kg bwt) anesthesia.

Preparation of serum and BALF, and biochemical analysis

Blood samples obtained from orbital vascular plexuses of rat pups were centrifuged at 15,000 rpm for 15 min to obtain sera which were used for assay of IgE using commercial ELISA kits. Bronchoalveolar lavage fluid (BALF) was obtained via infusion of phosphate-buffered saline (PBS) (200 µL) thrice into the left lung using cannula. The BALF samples were then centrifuged at 120 rpm for 6 min at 4˚C, and the supernatants obtained were stored at -80 ˚C. Eosinophils in BALF counted with hematology analyzer which uses Wright-Giemsa dye.

Histopathological examination of lung tissues

The right lung tissues were subjected to histological examination using hematoxylin and eosin (H & E) staining, and the pathological changes were scored. The score ranged from 0 to 4, with higher histologic scores indicating more serious impairment of the lung.

Determination of airway hyper-responsiveness

The hyper-responsiveness of lung airways was determined via measurement of airway resistance after exposure of rat pups to graded concentrations of methacholine (Mch). The measurement was done using FlexiVent system (SCIREQ). The pups were anesthetized with 1 % sodium pentobarbital and subjected to tracheostomy, followed by mechanical ventilation. The ventilation was maintained at 200 breaths per min, while the tidal volume was kept at 10 mL/kg. Each rat pup was challenged with Mch aerosol for 10 sec prior to recording of airway resistance.

Immunohistochemistry

Lung tissue sections (3µm thick) were subjected to immunohistochemistry (IHC). The sections were deparaffinized, rehydrated, and incubated with 3 % H_2O_2 for 10 min to reduce non-specific background staining. Then, the tissue samples were agitated for 15 min in 10 mM citrate buffer (pH 6.0) in a microwave oven. In order to further reduce background staining, the sections were incubated for another 10 min at room temperature in Ultra V Block solution, and further blocked with 1 % bovine serum albumin (BSA) for 45 min. They were thereafter incubated at 4 ˚C with rabbit anti-TNF-α, ICAM-1, Muc5AC and Muc5B monoclonal antibodies overnight, each at 1: 1000 dilution. Then, incubation with secondary antibody was carried out at 37 ˚C for 1 h. Antibody binding was determined using Ultra-vision LP System according to the manufacturer's instructions. The sections were developed using DAB, and counterstained with hematoxylin. Tumor necrosis factor α (TNF-α), ICAM-1, Muc5AC and Muc5B mRNA expressions were determined randomly in 10 fields of the tissues. Staining was scored as percentage of cells with positive cytoplasm. Zero (0) score was considered negative expression, while 1+ to 4+ were taken for overexpression.

Western blotting

Cell suspensions resulting from trypsinization of lung tissues were washed twice with PBS and lysed with ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (1:5 volume ratio). The resultant lysates were centrifuged at 13500 rpm for 25 min at 4 °C, and the protein concentrations of the supernatants were determined using bicinchoninic (BCA) assay kit. A portion of total cell protein (30 µg) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added to the membrane, with gentle shaking at 37 ºC and incubated to block non-specific binding of the blot. Thereafter, the membranes were incubated overnight at 4 ◦C with the primary antibodies: rabbit polyclonal anti- IκBα and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blots were developed using an X-ray film. Grayscale analysis of the bands was performed using ECL. The various protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using Graphpad Prism (7.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Effect of trifolirhizin on serum IgE level

Serum IgE level was significantly higher in asthma group than in sham group, but was significantly and dose-dependently reduced after treatment with trifolirhizin ($p < 0.05$; Figure 1).

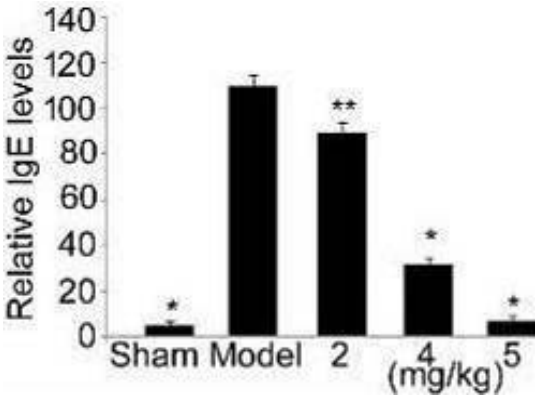

Figure 1: Effect of trifolirhizin on total IgE levels. $^{*}P < 0.05$; $^{**}p < 0.01$, compared with asthma group

Effect of trifolirhizin on lung tissue histology

The alveolar septa of the asthmatic rats were characterized by aggregation of inflammatory cells and edema in pulmonary tissues. However, lung tissue damage was significantly mitigated in the treatment groups, when compared to the untreated asthma group (histological scores were significantly and dose-dependently reduced by trifolirhizin treatment) ($p < 0.05$). These results are shown in Figure 2.

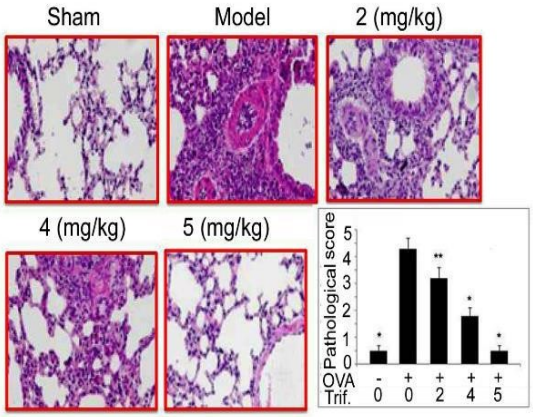

Figure 2: Effect of trifolirhizin on lung injury. **(A):** Histopathological changes in the lungs of rat pups as revealed by H & E staining (x 200); and **(B):** Histologic scores as determined by evaluating the infiltration of inflammatory cells. $^{*}P < 0.05$; $^{**}p < 0.01$, compared with asthma group

mRNA expression levels of Muc5AC and Muc5B

As shown in Figure 3, trifolirhizin treatment significantly and dose-dependently downregulated the mRNA expressions of Muc5AC and Muc5B in asthmatic rat lungs ($p < 0.05$).

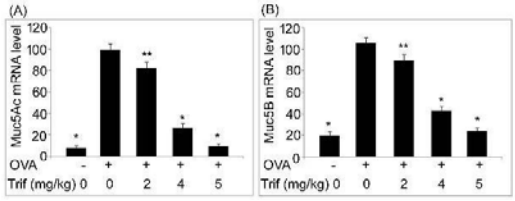

Figure 3: Levels of expression of Muc5AC and Muc5B mRNAs. **(A):** Level of expression of Muc5AC mRNA; and **(B):** mRNA expression of Muc5B; $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with asthma group

Effect of trifolirhizin on IL-4, IL-5 and IL-13 levels

The levels of IL-4, IL-5 and IL-13 in BALF were significantly high in asthma group, but they were significantly and dose-dependently reduced after treatment with trifolirhizin ($p < 0.05$). These results are shown in Figure 4.

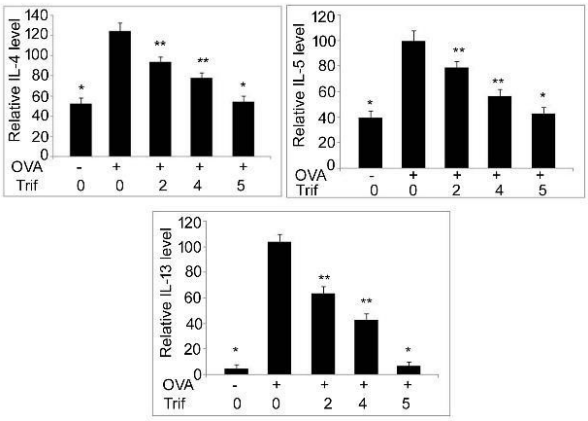

Figure 4: Effect of trifolirhizin on IL-4, IL-5 and IL-13 levels in neonatal rat BALF. $^{*}P < 0.05$; $^{**}p < 0.01$, compared with asthma group

Expression levels of TNF-α and ICAM-1 in rat BALF

As shown in Figure 5, trifolirhizin treatment significantly and dose-dependently downregulated the mRNA expressions of TNF-α and ICAM-1 in BALF of the asthmatic rats ($p < 0.05$).

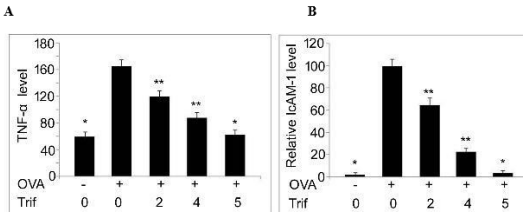

Figure 5: Levels of expression of TNF-α and ICAM-1 in rat BALF. (A): mRNA expression of TNF-α; (B): mRNA expression of ICAM-1; $^{*}p < 0.05$; $^{**}p < 0.01$, compared with asthma group

Effect of trifolirhizin on NF-κB signaling pathway

Treatment of asthmatic rat pups with trifolirhizin led to significant and dose-dependent upregulation of IκBα protein expression ($p < 0.05$; Figure 6).

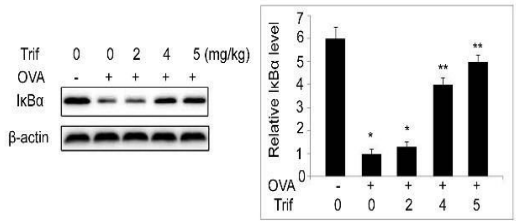

Figure 6: Effect of trifolirhizin on NF-κB signaling pathway. $^{*}P < 0.05$ and $^{**}p < 0.01$, compared with asthma group

DISCUSSION

Asthma is a heterogeneous disorder regulated by multiple molecular mechanisms. Recent studies show that overactivation of Th2 cells contributes to the pathogenesis of asthma [11]. High eosinophil count and Th2 cytokines such as IL-4, IL-5 and IL-13 have been reported in asthmatic patients [12]. Interleukin 4 (IL-4) regulates B-lymphocyte function and also promotes IgE production during inflammation [13]. The production, activation, differentiation and migration of eosinophils to inflammatory sites are regulated by IL-5 [14]. The function of IL-13 is similar to that of IL-4: it regulates type-II immune response [15].

The present study investigated the effect of trifolirhizin in neonatal rat model of asthma, and the mechanism involved. The results revealed high levels of inflammatory cells, upregulated IL-4, IL-5 and IL-13, and accumulation of eosinophils in BALF of asthmatic rat pups. However, trifolirhizin treatment significantly and dose-dependently mitigated the effect of asthma on the levels of these inflammatory cytokines. These results indicate that trifolirhizin may alleviate the symptoms of asthma via regulation of the inflammatory response (inhibition of inflammatory cytokine aggregation).

Asthma is characterized by airways remodeling which seriously affects lungs function [16]. Airway remodeling may be due to Th2 cell-mediated allergic inflammation or some other non-inflammatory processes [17]. In this study, alveolar septa of asthmatic rats were thickened with aggregation of inflammatory cells and edema. However, lung tissue damage was significantly mitigated in the treatment groups, relative to the untreated asthma group.

The inflammatory cytokine TNF-α has been shown to contribute to asthma in children and in refractory asthmatic patients [18]. It causes bronchial hyper-responsiveness [19]. The levels of vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 are coordinately regulated by TNF-α [19].

The results of this study showed that trifolirhizin treatment significantly downregulated the expressions of Muc5AC, Muc5B, TNF-α and ICAM-1 mRNA in asthmatic rat pups.

The NF-κB pathway has been shown to play a central role in the release of inflammatory cytokines. As a regulatory molecule, it participates in innate and adaptive immune responses [20]. In the inactive form, the dimeric molecule NF-κB comprises P65 and P50 cytoplasmic subunits linked to IκB (inhibitory protein) [21]. During asthmatic attacks, various stimuli induce inflammation via activation of NF-κB which in turn stimulates pro-inflammatory cytokine release [22]. In this study, treatment of asthmatic rat pups with trifolirhizin led to significant and dose-dependent upregulation of IκBα protein expression, an indication that the anti-asthmatic effect of trifolirhizin may be exerted via the regulation of NF-κB signaling pathway.

CONCLUSION

Trifolirhizin mitigates OVA-induced lung inflammation and tissue damage in neonatal rats via inhibition of NF-κB signaling pathway. Therefore, this compound has the potential to be developed for the management of asthma.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Li Rong and Pan Lei contributed to this work equally. Huang Qing - conceived and designed the study; Li Rong, Pan Lei, Li Yi, Wu Yupei, Gong Rui, Liu Xin, Xie Huimin - collected and analyzed the data; Li Rong, Pan Lei, Li Yi, Wu Yupei -wrote the manuscript. All authors read and approved the manuscript for publication.

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