

The Effect of Synthetic Salidroside on Cytokines and Airway Inflammation of Asthma Induced by Diisocyanate (TDI) in Mice by Regulating GATA3/T-bet

Jing Wang,¹ Lu Xiao,¹ Lingpeng Zhu,¹ Mei Hu,¹ Qiujuan Wang,¹ and Tianhua Yan^{1,2}

Abstract—This study was conducted to explore the anti-asthma effects of synthetic salidroside on cytokines and airway inflammation of asthma induced by diisocyanate (TDI) in mice. The experiment was carried out 60 female BALB/C mice which were randomly assigned to six experimental groups: control, vehicle, model, dexamethasone (2 mg/kg), and salidroside (24 and 48 mg/kg). After the experiment, histological studies were evaluated by the hematoxylin and eosin staining, the bronchoalveolar lavage fluid (BALF) and blood were collected from the animals, and the composition of the induced inflammatory cells, and the concentrations of certain cytokines (IL-4, INF- γ) were evaluated. GATA3 and T-bet mRNAs were evaluated by QPCR. Our study demonstrated that salidroside inhibited TDI-induced increases in eosinophil count; IL-4 and INF- γ were recovered. Histological studies demonstrated that salidroside substantially inhibited TDI-induced eosinophilia in lung tissue. Salidroside can improve T-bet mRNA and reduce GATA3 mRNA in lung. These findings suggest that salidroside may effectively ameliorate the progression of asthma and could be used as a therapy for patients with allergic asthma by regulating GATA3/T-bet balance.

KEY WORDS: salidroside; diisocyanate (TDI); asthma model; cytokines.

INTRODUCTION

Occupational asthma (OA) is the most common occupational lung disease in many industrialized countries. Diisocyanates, highly reactive low molecular weight compounds used in the production of polyurethanes, are the most commonly identified cause of OA, a disease that accounts for nearly 10 % of all adult-onset asthma [1]. It is estimated that as many as 5 % of workers exposed to diisocyanates develop asthma, which may continue indefinitely even in the absence of continued exposure [2]. Clinically, diisocyanate asthma shows similar manifestations to allergic asthma induced by high molecular weight allergens, illustrating common pathogenic processes, although clinical studies have highlighted a few important differences, such as a low association with atopy, a low prevalence of specific immunoglobulin E (IgE) antibodies

[3], a mixed T helper 1/T helper 2 response and the presence of high numbers of antigen-specific CD8 T cells [4]. Salidroside has the anti-hypoxia, anti-tumor and anti-aging effect, what's more, animal experiments and clinical data show salidroside have obvious therapeutic effect on rats' lung injury and pulmonary dysfunction which accept burn excitation. But the protective mechanism of salidroside is unclear. We conducted a asthma model induced by TDI to observe the therapeutic effect of salidroside and further researched the possible mechanism.

MATERIALS AND METHODS

Animals

Female BALB/c mice (approximately 20 g and 8 weeks old) were kept in the animal center of China Pharmaceutical University with 12-h dark/light cycles. They received tap water and pelleted pathogen-free food. All experimental protocols were approved by the Animal Studies Committee of China Pharmaceutical University.

¹Department of Physiology and Pharmacology, China Pharmaceutical University, Tongjiexiang 24, Nanjing, China

²To whom correspondence should be addressed at Department of Physiology and Pharmacology, China Pharmaceutical University, Tongjiexiang 24, Nanjing, China. E-mail: machunhuabest@126.com

Main Reagents and Kits

We used synthetic salidroside (Sal, provided by the Second Military Medical University, Shanghai, China; purity $\geq 99\%$) and Dexamethasone (Dex; Xiansheng Drug Store, Nanjing, China). The vehicle (AOO) used to dissolve TDI consisted of a mixture of 2 vol. of acetone and 3 vol. of olive oil for sensitization, and 1 vol. of acetone and 4 vol. of olive oil for the challenge. Concentrations of TDI are provided as percent (v/v) in AOO [5]. Toluene diisocyanate was obtained from Merck-Schuchardt (Hohenbrunn, Germany). ELISA kits (IFN- γ , IL-4; R&D Systems, Inc., Minneapolis, MN, USA); TRIzol (Gibco BCR Inc., Shanghai, China).

Experimental Protocol

Referring to the protocol as previously described [5]. A total of 68-week-old female BALB/c mice were randomized to the following six groups (10 mice/group): (1) sham-sensitization plus challenge with PBS; (2) vehicle (AOO)-sensitized, AOO-challenged and PBS-treated mice (AOO group); (3) TDI-sensitized (0.3 % TDI, AOO: 2 vol. of acetone and 3 vol. of olive oil), TDI-challenged (0.01 % TDI, AOO: 1 vol. of acetone and 4 vol. of olive oil), and PBS-treated mice (TDI group); (4) TDI-sensitized, TDI-challenged, and dexamethasone (2 mg/kg)-treated mice (DEX group); (5) TDI-sensitized, TDI-challenged, and salidroside 24 mg/kg treated mice; (5) TDI-sensitized, TDI-challenged, and salidroside (48 mg/kg)-treated mice [6]. Mice were immunized intraperitoneally (i.p.) and subcutaneous injection of 40 μ l 0.3 % TDI on days 1 and 8. Mice were challenged via the nasal inhalation with 0.01 % TDI on days 15, 18 and 21. All the drugs were given via lavage administration each day from days 15 to 21 on consecutive days.

Blood Cytology Examination

Whole blood was collected 24 h after the last challenge from the orbit. Blood (20 μ l) was mixed with 0.38 ml eosinophils (EOS) counting fluid, and then we counted eosinophils under the optical microscope. Furthermore, we smeared and fixed blood in the slide and stained with Wright-Giemsa staining. The slides were quantified for differential cell count by counting a total of 200 cells/slide at 40 magnification and then we calculated the various kinds of white blood cells percentage. The remaining blood was centrifuged at 3,000 rpm/min for 10 min. Samples of the supernatant were secured and stored at -80°C for further study.

Inflammatory Cell Counts in Bronchoalveolar Lavage Fluid (BALF)

Mice were sacrificed with an overdose of 50 mg/kg of pentobarbital 24 h after the last challenge, and tracheotomies were performed [7]. After ice-cold PBS (0.5 ml) was instilled into the lungs, BALF was obtained by three successive aspirations (total volume, 1.5 ml) via tracheal cannulation [8]. The BALF was centrifuged at 1,200 rpm/min for 10 min at 4°C . Samples of the supernatant were secured and stored at -80°C for further study. Cell pellets were resuspended in 1 ml of PBS, then stained for 8 min with Wright-Giemsa staining. The slides were quantified for differential cell count by counting a total of 200 cells/slide at 40 magnification [9].

Histological Assessment

Airway tissues and lung tissues were isolated from the mice and fixed with Carnoy's solution overnight at 4°C . The fixed tissues were embedded in paraffin and cut into 4-mm sections with a microtome. The sections were placed on slide glasses, deparaffinized, stained with hematoxylin and eosin (Sigma, Korea) in order to check the cells which had infiltrated into airway tissues and the peribronchial connective tissues. Airway and peribronchial cells count based on a 5-point scoring system was performed as previously described to estimate the severity of leukocyte infiltration [10]. The scoring system was as follows: 0, no cells; 1, a few cells; 2, a ring of cells, 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep; 4, a ring of cells, more than 4 cell layers deep.

ELISA Detection of Serum and BALF Cytokines IL-4, IL-6 and IFN- γ RNA Extraction and Real-Time PCR Analysis

The lung tissue was homogenized in 1 ml Trizol reagent (Invitrogen) using a glass homogenizer. The total RNA was isolated according to the manufacturer's protocol and stored at -70°C . cDNA was synthesized from total RNA by reverse transcriptase (Fermentas) with the primer oligo(dT) 18 and was stored at -20°C . The primers for PCR are listed in Table 1. The SYBR green PCR Master Mix was used according to the manufacturer's instructions. The thermo cycling programmers consisted of 35 cycles at 94°C , 30 s; 60°C , 30 s; and 72°C , 45 s for all. The amplified products were electrophoresed with 1.5 % agarose gel and visualized by GoldViewTM (SBS Genetech, Beijing,

Table 1. The Primers for T-bet, GATA3, and GAPDH

Genes	Sequence (5'–3')	Sequence (3'–5')
T-bet	5-AGCAAGGACGGCGAATGTT-3	5-GGGTGGACATATAAGCGGTTC-3
GATA3	5-CTCGGCCATTCGTACATGGAA-3	5-GGATACCTCTGCACCGTAGC-3
GAPDH	5-TATGTCGTGGAGTCTACTGGT-3	5-GAGTTGTCATATTCTCGTGG-3

China) and UV irradiation. T-bet: F: 5-AGCAAGGACG GCGAATGTT-3, R: 5-GGGTGGACATATAAGCGGTTC-3; GATA3: 5-CTCGGCCATTCGTACATGGAA-3, R: 5-GGATACCTCTGCACCGTAGC-3; GAPDH: F: 5-TATGTCGTGGAGTCTACTGGT-3, R: 5-GAGTTGTC ATATTCTCGTGG-3.

Statistical Analysis

The results were expressed as mean±SD and analyzed by one-way ANOVA, with the Statistical Product and Service Solutions (SPSS 17.0, USA). A *P* value <0.05 was taken as statistically significant.

RESULTS

Effect of Salidroside on TDI-Induced Eosinophilia in Blood

There was no significant difference between the normal group and the vehicle group. There was significant difference between the TDI group and the normal group

(*P*<0.01). Compared with the TDI group, the content of eosinophils of DEX group, salidroside (24 mg/kg) and salidroside (48 mg/kg) significantly decreased (*P*<0.01), as shown in Fig. 1.

Effect of Salidroside on Different Cells BALF

The percentage of eosinophil, lymphocyte, and neutrophil granulocyte BALF was calculated. The results showed that compared with the normal control group, eosinophil percentage of TDI group increased markedly and lymphocyte percentage decreased significantly (*P*<0.01) in both blood and BALF. There was no difference between the normal group and TDI group in neutrophil granulocyte percentage (*P*>0.05) in blood. But neutrophil granulocyte percentage of TDI group increased markedly compared with the normal group in BALF (*P*<0.01). Treatment with Dex (2 mg/kg) or salidroside (24 and 48 mg/kg) could dramatically decrease eosinophilic percentage and increase lymphocyte (*P*<0.01) percentage in both blood and BALF, and dramatically decrease neutrophil granulocyte percentage in BALF, as shown in Fig. 2.

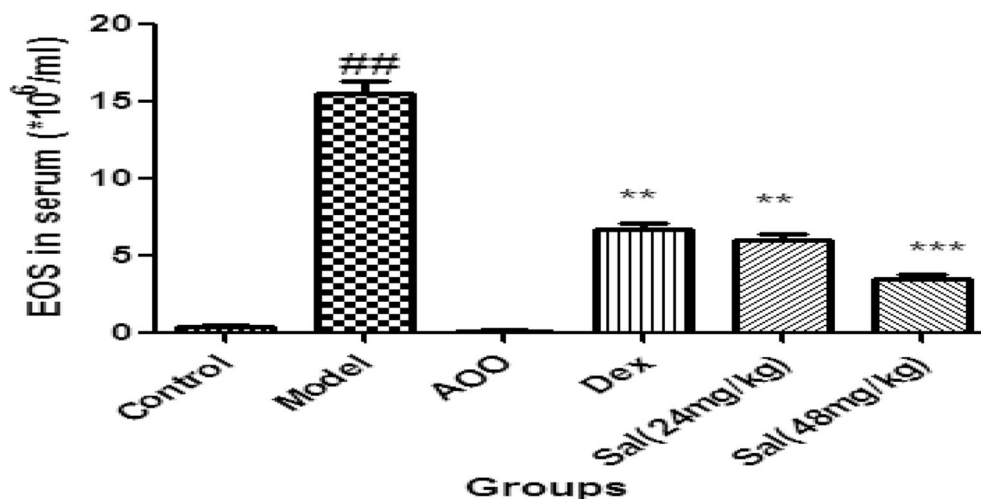


Fig. 1. Effect of salidroside on TDI-induced eosinophilia in blood. Values are expressed as mean±SD. Compared with control: **P*<0.05, ***P*<0.01; compared with model: **P*<0.05, ***P*<0.01.

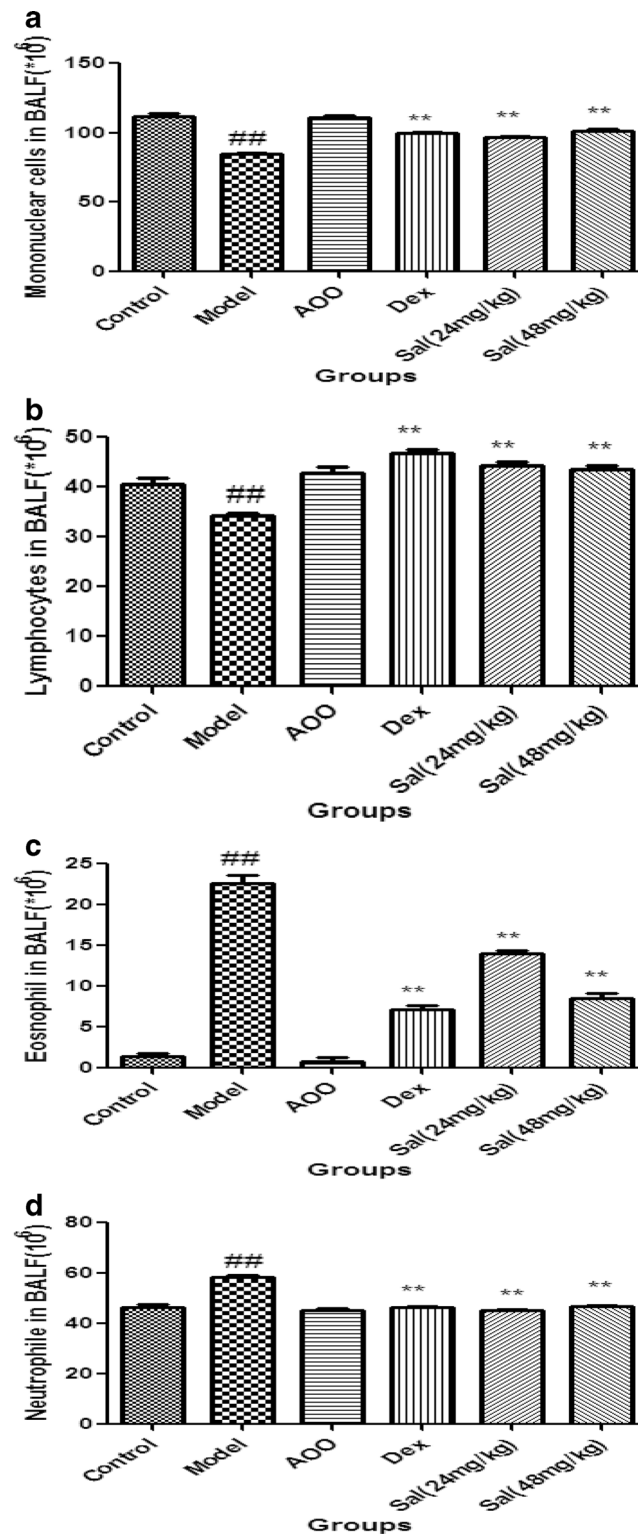


Fig. 2. Effect of salidroside on different cells BALF. Values are expressed as mean $\pm$ SD. Compared with control: $^{\#}P<0.05$, $^{##}P<0.01$; compared with model: $^{*}P<0.05$, $^{**}P<0.01$.

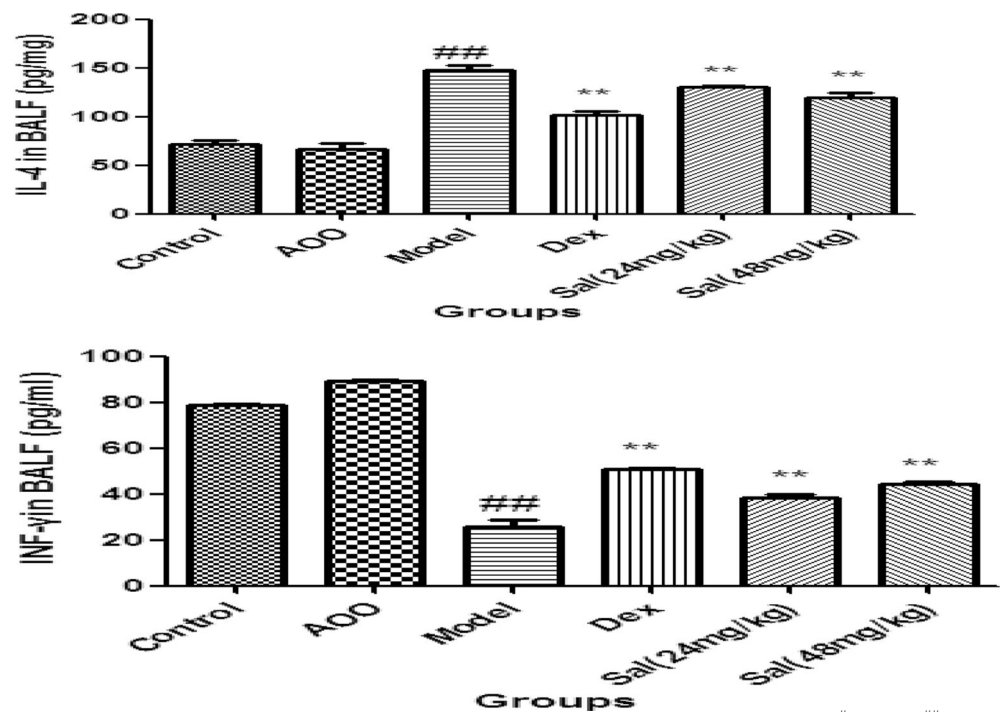


Fig. 3. Effects of salidroside on cytokine in BALF. Values are expressed as mean±SD. Compared with control: [#] $P<0.05$, ^{##} $P<0.01$; compared with model: ^{*} $P<0.05$, ^{**} $P<0.01$.

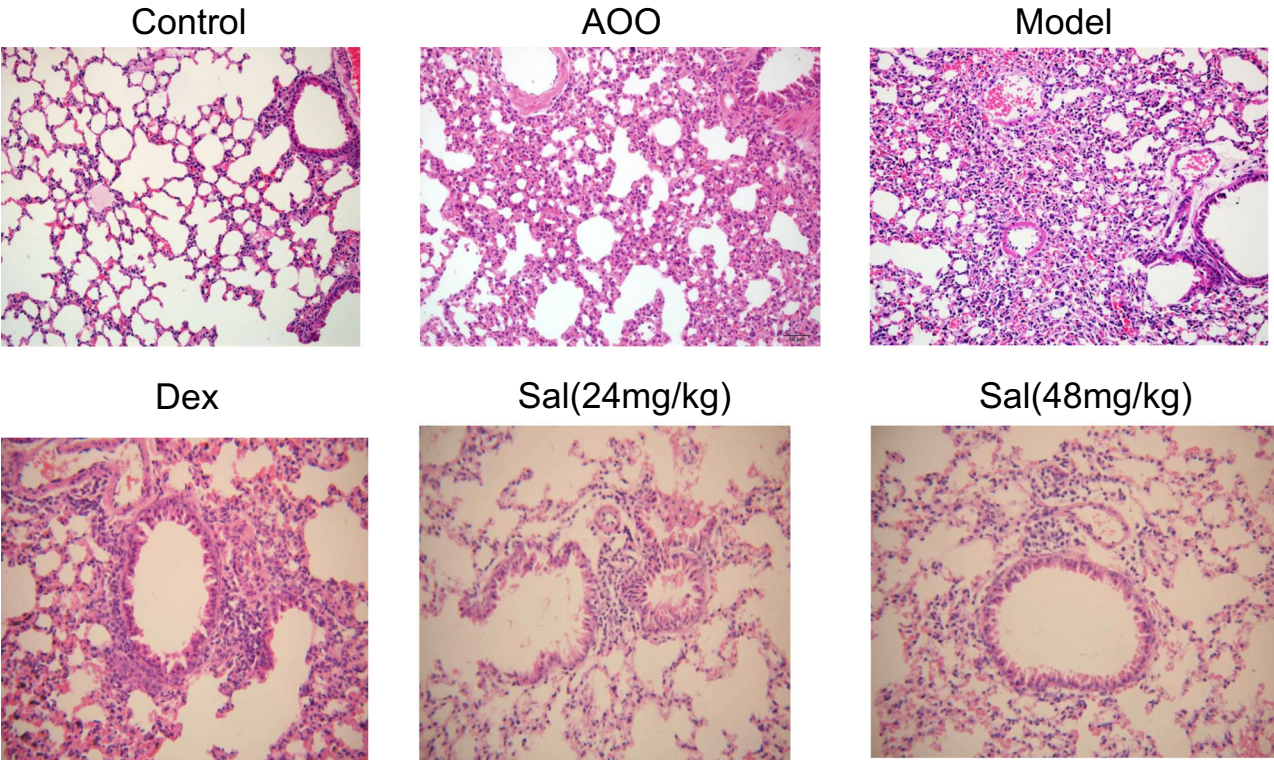


Fig. 4. Pathological changes of lung tissues observed by HE staining (light microscopy, ×200).

Effects of Salidroside on Cytokine in BALF

Cytokines levels in the BALF were measured by enzyme immune assays according to the manufacturer's introduction. As shown in Fig. 3, the concentration of IL-4 and IL-6 produced by Th2 increased and IFN- γ originating from Th1 decreased in TDI group compared to normal group ($P<0.01$) both in BALF and serum. Treatment with Dex (2 mg/kg) or salidroside (24 and 48 mg/kg) caused a reduction on the level of IL-4 and increase on IFN- γ ($P<0.01$) both in BALF and serum.

Histological Examination

Compared with the control mice (Fig. 4), histological analyses of TDI-induced mice showed typical pathologic features of asthma. The histological observation of TDI-induced mice indicated large numbers of inflammatory cells including goblet cells and eosinophils infiltrated around the bronchioles and vessels. There were also wall thickening, heals wall necrosis and removal. Mice treated

with Dex (2 mg/kg) and salidroside (24 and 48 mg/kg) significantly alleviated the pathological changes.

Effects of Salidroside on GATA3 and T-bet

The effect of salidroside on the mRNA levels of T-bet and GATA3 genes in Fig. 5, GATA3 mRNA was increased in model group compared to control group, GATA3 mRNA of the groups treated with salidroside was decreased compared to the model group; T-bet mRNA was decreased in the model group compared to the control group. Furthermore, T-bet mRNA of the groups treated with salidroside was increased compared to the model group.

DISCUSSION

Asthma is an inflammatory disease of the lungs characterized by increased leukocytes infiltration, especially eosinophils, into the airways, and reduced respiratory

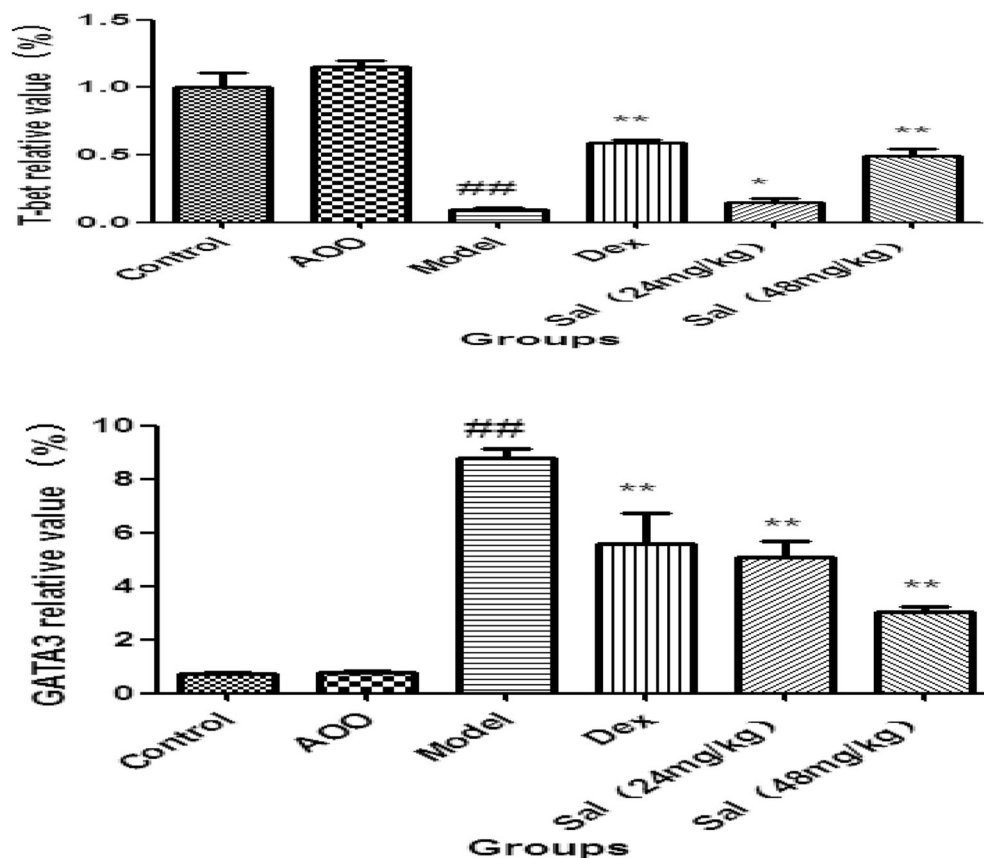


Fig. 5. Effects of salidroside on GATA3 and T-bet. Values are expressed as mean $\pm$ SD. Compared with control: $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared with model: $^{*}P<0.05$, $^{**}P<0.01$.

function. The inflammation causes bronchoconstriction, increased AHR, and mucus production [8]. Animal models of disease are invaluable to investigate therapeutic targets and mechanisms. Murine models have been extensively used to study allergic asthma. Mouse ovalbumin models have successfully duplicated many features of allergic asthma such as eosinophilic airway inflammation, Th2 T cell responses and airway hyperresponsiveness (AHR) [11]. The first animal studies of diisocyanate induced OA were investigated in guinea pigs, and they demonstrated the acute inhalational toxicity and immunogenicity of diisocyanates. Guinea pig models show many of the clinical features of human diisocyanate-induced OA, including AHR, epithelial damage, neutrophilic inflammation, airway eosinophilia, and a late-phase asthmatic reaction. These studies have also revealed that exposure through skin or airways, common routes of exposure in the workplace, can lead to dermal or respiratory sensitization [12]. In this study, we use a murine model of TDI induced asthma. We use Subcutaneous and intraperitoneal injection with 0.3 % TDI to sensitize the mouse and use Intranasal administration with 0.01 % TDI to motivate. we found increased numbers of total cells, eosinophils, and neutrophils in serum and BALF raised levels of IL-4, IL-6 and IFN- γ and more infiltration of inflammatory cells in pulmonary alveoli of TDI-treated mice. Taken together, these findings suggest that a successful TDI-induced asthma model was developed in our study. Many nature products were widely used in the treatment of asthma, but the therapeutic efficacy and modes of action of these medicines are still unclear. Here, using a TDI-induced mouse model of asthma, we indicate that salidroside can be effective as a therapeutic drug for the treatment of OA the first time. Migration of inflammatory cells, especially eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation [13]. A rise in the number of eosinophils in the BALF is a characteristic of asthma [14]. The present results clearly demonstrate that salidroside significantly reduces eosinophil numbers in the lung tissue. Allergic asthma is recognized as a TH2-mediated immune system disease. The induction of allergen-specific TH1 cells can attenuate allergic asthma. Several studies have shown that administration of TH1 cytokines, such as IFN- γ , at the time of sensitization can inhibit the induction of TH2-driven inflammation [15]. Asthma and inflammation are associated with enhanced production of IL-4 and decreased production of IFN- γ [16]. Our data indicate that salidroside significantly decreases

IL-4 cytokine levels and significantly increases IFN- γ cytokine levels. In summary, salidroside effectively decreased IL-4, improved IFN- γ , and inhibited the recruitment of eosinophils in mice with TDI-induced asthma. Airway inflammation was attenuated in the presence of salidroside. Our findings support the possible use of salidroside as a therapeutic drug for patients with OA. Thus, the results support the notion that use of salidroside is beneficial in the treatment of TDI-induced asthma, and its exact mechanism and clinical application need further investigation.

ACKNOWLEDGMENTS

This work was supported by National twelve five major drug discovery project (2011ZX09102-002-01).

REFERENCES

1. Gautrin, D., A.J. Newman-Taylor, H. Nordman, and J.L. Malo. 2003. Controversies in epidemiology of occupational asthma. *European Respiratory Journal* 22: 551–559.
2. Johnson, V.J., J.M. Matheson, and M.I. Luster. 2004. Animal models for diisocyanate asthma: Answers for lingering questions. *Current Opinion in Allergy and Clinical Immunology* 4: 105–110.
3. Scheerens, H., T.L. Buckley, T.L. Muis, et al. 1999. Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. *American Journal of Respiratory and Critical Care Medicine* 159: 1074–1080.
4. Matheson, J.M., V.J. Johnson, and M.I. Luster. 2005. Immune mediators in a murine model for occupational asthma: Studies with toluene diisocyanate. *Toxicological Sciences* 84: 99–109.
5. Song, Jiafu, Haijin Zhao, et al. 2013. Mechanism of E-cadherin redistribution in bronchial airway epithelial cells in a TDI-induced asthma model. *Toxicology Letters* 220: 8–14.
6. Bu-Chun Zhang et al. 2012. Salidroside decreases atherosclerotic plaque formation in low-density lipoprotein receptor-deficient mice. *Evidence-Based Complementary and Alternative Medicine* Article ID 607508, 5 pages.
7. Lee, Meeyoung, et al. 2009. Anti-inflammatory and anti-asthmatic effects of resveratrol, a polyphenolic stilbene, in a mouse model of allergic asthma. *International Immunopharmacology* 9: 418–424.
8. Djukanovic, R., W.R. Roche, J.W. Wilson, C.R. Beasley, O.P. Twentyman, R.H. Howarth, et al. 1990. Mucosal inflammation in asthma. *American Review of Respiratory Disease* 142: 434–457.
9. Świerczyńska-Machura, D., et al. 2012. Immunological determinants in a murine model of toluene diisocyanate-induced asthma. *International Journal of Occupational Medicine and Environmental Health* 25(4): 492–498.
10. DuanW, Chan J.H., C.H. Wong, et al. 2004. Anti inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *Journal of Immunology* 172: 7053–7059.
11. Gelfand, E.W. 2002. Pro: Mice are good model of human airway disease. *American Journal of Respiratory and Critical Care Medicine* 166(1): 5–6.

12. Ebino, K., H. Ueda, H. Kawakatsu, Y. Shutoh, T. Kosaka, E. Nagayoshi, et al. 2001. Isolated airway exposure to toluene diisocyanate results in skin sensitization. *Toxicology Letters* 121: 79–85.
13. Elsner, J., and A. Kapp. 1999. Regulation and modulation of eosinophil effector functions. *Allergy* 54: 15–26.
14. Chunhua Ma, et al. 2013. Anti-asthmatic effects of baicalin in a mouse model of allergic asthma. *Phytotherapy Research*.
15. Gavett, S.H., D.J. O’Heam, X. Li, S.K. Huang, F.D. Finkelman, et al. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *Journal of Experimental Medicine* 182: 1527–1536.
16. Wong, C.K., C.Y. Ho, et al. 2001. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and the cytokines (IFN- γ , IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clinical and Experimental Immunology* 125: 177–183.