



Anti-asthmatic effects of matrine in a mouse model of allergic asthma



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ABSTRACT

The aim of the study was to investigate the anti-asthmatic effects of matrine and the possible mechanisms. Asthma model was established by ovalbumin-induced. A total of 50 mice were randomly assigned to five experimental groups: control, model, dexamethasone (2 mg/kg) and matrine (50 mg/kg, 100 mg/kg). Airway resistance (Raw) was measured, histological studies were evaluated by the hematoxylin and eosin (HE) staining, interleukin-4 (IL-4) and interleukin-13 were evaluated by enzyme-linked immunosorbent assay (ELISA), IL-4 and IL-13 signal protein STAT6 was measured by western blotting. Our study demonstrated that matrine inhibited OVA-induced increases in Raw and eosinophil count; IL-4 and IL-13 were recovered. Histological studies demonstrated that matrine substantially inhibited OVA-induced eosinophilia in lung tissue. Western blotting studies demonstrated that matrine substantially inhibited STAT6 protein level. These findings suggest that matrine may effectively ameliorate the progression of asthma and could be used as a therapy for patients with allergic asthma.

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1. Introduction

Allergic diseases of the airway such as asthma or allergic rhinitis are on the rise throughout the world, especially in western countries [1]. One of the most important diseases is bronchial asthma, that is defined as a chronic inflammation of the bronchial airways and characterized by reversible airway obstruction, increased mucus production and infiltration of the airway with eosinophils, neutrophils, mast cells and T-lymphocytes [2].

Interleukin (IL)-4 and IL-13, expressed by T-helper type 2 (Th2) cells, are key cytokines in the pathogenesis of atopy and atopic asthma [3,4]. Both IL-4 and IL-13 promote acute inflammatory processes and underlying structural changes to the airways, and their receptors are expressed on a number of cell types. Both IL-4 and IL-13, which share the IL-4R α subunit in their cognate receptors, activate signal transducer and activator of transcription factor-6 (STAT-6) [5]. STAT-6 is

required for IL-4 production from antigen-stimulated murine splenocytes [6]. Moreover, activation of STAT-6 is critical for the differentiation of naive T-cells into Th2 effector cells, and STAT-6 regulates IL-4- and IL-13-induced production of Th2 chemokines, including eotaxin, from airway epithelial cells, fibroblasts and smooth muscle cells [7,8]. The IL-4/IL-13/STAT-6 pathway plays a key role in asthma pathogenesis.

Matrine (Mat) and oxymatrine are the major alkaloids of *S. Flavescens*. They have similar molecular structure and also have similar wide spectrum of pharmacological effects, including anti-inflammatory, anti-allergy, anti-virus and antitumor, and are ancillary drugs used clinically in China for protection of liver function and treatment of tumors [9].

Hence, we hypothesized that Mat, which have protective effects in ovalbumin-sensitized asthma *in vivo*, might also be associated with the inhibition of inflammation and immunoregulatory. The present study was undertaken to evaluate the protective effects of Mat and to elucidate the mechanism underlying these protective effects in mice. Our results indicate that Mat pretreatment significantly protected against ovalbumin-sensitized asthma through regulating IL-4/IL-13/STAT-6 pathway.

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2. Materials and methods

2.1. Materials

Mat powder (pure:98.7%) was purchased from National Institutes for Food and Drug Control (Beijing, China) and Mat solution (4 mg/ml) was prepared in distilled water. Dexamethasone was purchased from Xiansheng drug Store (Nanjing, China). Ovalbumin (OVA) was obtained from Sigma Chemical Co., St Louis, MO, aluminium hydroxide from Pierce Biotechnology, Rockford, USA, Wright–Giemsa staining from Nanjing Jiancheng Bioengineering Institute, Nanjing, China, ELISA kits from R&D, Minneapolis, MN, USA, and anti-mouse STAT6 from eBioscience.

2.2. Animals

Specific pathogen-free female BALB/c mice, aged 6–8 weeks, which were routinely screened serologically for relevant respiratory pathogens, were purchased from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Animal experiment was carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University China Pharmaceutical University. A total of 60 female BALB/c mice obtained from Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow ad libitum. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

2.3. Sensitization, airway challenge and treatment

Mice were divided into five groups (each of 10 animals): (1) normal control, (2) asthma-model control, (3) asthma treated with dexamethasone (2 mg/kg, administered by gavage) (4, 5) asthma treated with Mat (50, 100, administered by gavage). Allergic asthma was induced by ovalbumin (Grade V) in five of the groups, using the method described by Oh et al. [10]. Mice were immunized via intraperitoneal (i.p.) injection with 10 µg chicken OVA and 2 mg aluminum hydroxide in 200 µL phosphate buffered saline (PBS) (pH 7.4), on days 0 and 14. Mice were exposed to a 1% (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, and 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to characterize the suppressive effects of GA. A schematic diagram of the treatment schedule is shown in Fig. 1.

2.4. Measurement of airway resistance (Raw)

The forced oscillation technique was used to measure respiratory mechanics. Regular ventilation was interrupted and a computer generated volume signal that consisted of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were measured. Impedance values were obtained before and after the delivery of increasing concentrations of methacholine

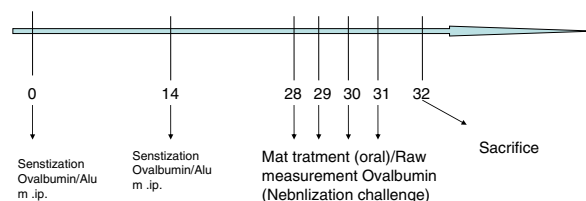


Fig. 1. Sensitization, challenge treatment protocols for the different groups in this study.

aerosols. Prior to the start of the methacholine concentration response curve, two total lung capacity breaths were delivered. Methacholine (Sigma) dissolved in saline was given via an ultrasonic nebuliser (Hudson RCI, Teleflex Medical) in increasing concentrations (0.125 mg ml^{-1} , 0.25 mg ml^{-1} , 0.5 mg ml^{-1} , and 1 mg ml^{-1}). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. In between impedance measurements, regular ventilation was resumed. The peak response for each variable was determined.

2.5. Collection of bronchoalveolar lavage (BAL) fluid

Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 48 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 ml) into a lung, BAL fluid was obtained by three successive aspirations (total volume 1.5 ml) via tracheal cannulation [11]. BAL fluid (BALF) samples were centrifuged at 1500 rpm for 10 min at 4°C , the supernatants were stored in -80°C for analysis of cytokine concentrations and the pellet was resuspended in 100 µL of saline, centrifuged onto slides and 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.

2.6. Histological assessment

Lung tissues and airway tissues were detached from the mice and fixed with Carnoy's solution overnight at 4°C . The fixed tissues were embedded in paraffin and cut into 4-µm sections with amicrotome (Leica, Nussloch, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (Sigma, Korea) in order to examine the cells that had infiltrated into the peribronchial connective tissues and airway tissues. Peribronchial and airway cells count based on a five point scoring system was performed as previously described to estimate the severity of leukocyte infiltration [12]. The scoring system was: 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; and 4, a ring of cells more than 4 cell layers deep.

2.7. Enzyme-linked immunosorbent assay detection of BALF cytokines IL-4 and IL-13

BALF levels of IL-4 and IL-13 were measured by ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). All measurements were performed in duplicate. Briefly, the BALF samples were added in duplicate to 96-well

plates with 100 µl per well. The appropriate biotinconjugated antibodies were added to each well. The samples were incubated at room temperature for 2 h. The wells were then aspirated, and each well was washed 5 times. The substrate solutions were added to each well, and were incubated for 30 min at room temperature in the dark. The optical density (O.D.) of each well was determined using a microplate reader (Bio-Rad Model 680, USA) that was set to 450 nm. A standard curve was created of the average of the O.D. duplicate readings. The results were calculated using Excel 2007 (Microsoft office, USA).

2.8. Measurement of OVA-specific serum and BALF levels of IgE

OVA-specific serum and BALF IgE levels were determined by enzymelinked immunosorbent assay (ELISA) using samples collected 48 h after the last OVA challenge, as described previously (Jain et al., 2002). In brief, a 96-well microtitre plate was coated with OVA (10 mg/ml), and then treated with mouse sera followed by biotin-conjugated rat anti-mouse IgE (Pharmingen, San Diego, CA). Then, avidinehorseradish peroxidase (HRP) solution was added to each well. Units are optical density readings at 405 nm.

2.9. Western blotting of STAT6

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. The samples were loaded to 10% SDS-PAGE gels and were electrotransferred to nitrocellulose. The blots were incubated with the appropriate concentration of specific antibody. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody. The membranes were stripped and reblotted with anti-actin antibody (Sigma) to verify the equal loading of protein in each lane. Quantification of protein expression was normalized to β -actin using a densitometer (Imaging System).

2.10. Statistical analysis

All values were expressed as the mean $\pm$ S.D. and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using SPSS version 13.0 software; a p -value

of less than 0.05 was considered significant and $p < 0.01$ was considered to be statistically very significant.

3. Results

3.1. Effect of Mat on airway resistance (Raw)

Measurements of airway resistance (Raw) during methacholine-induced constriction are shown in Fig. 2. The Raw value of the OVA challenged group was significantly higher than that of the control group ($P < 0.05$). The treatment groups showed a significant decrease with acetylcholine 0.5 mg ml^{-1} and 1 mg ml^{-1} ($p < 0.05$ and 0.01 , compared with model group respectively).

3.2. Effect of Mat on OVA-induced eosinophilia and BAL fluid composition

We examined changes in total cell levels in the BAL fluid to determine the effects of Mat on asthma. Suppression of eosinophilia by Mat in OVA-challenged mice was estimated by counting of cells recruited to the BAL fluid 24 h after the last challenge. As shown in Fig. 3, OVA induced a marked influx of leukocytes into BAL fluid in the control group. Eosinophil levels markedly increased in the BAL fluid of OVA-challenged mice. OVA-sensitized and -challenged mice treated with GA displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction.

3.3. Effect of Mat on OVA-induced eosinophilia in lung tissue and airway tissue

Because Mat inhibits inflammatory cell recruitment into the lung tissue, we next examined the anti-asthmatic effects of the Mat. Lung tissue and were collected 24 h after the final OVA challenge. In OVA-induced asthmatic lung tissue, we observed marked infiltration of inflammatory cells into perivascular, peribronchial connective tissues, compared with the normal tissue. Moreover, most leukocytes were eosinophils. The control group displayed no change in the extent of inflammatory cell infiltration, similar to what was seen in dexamethasone-treated positive control mice. Eosinophilia in OVA-sensitized and challenged mice treated with Mat was

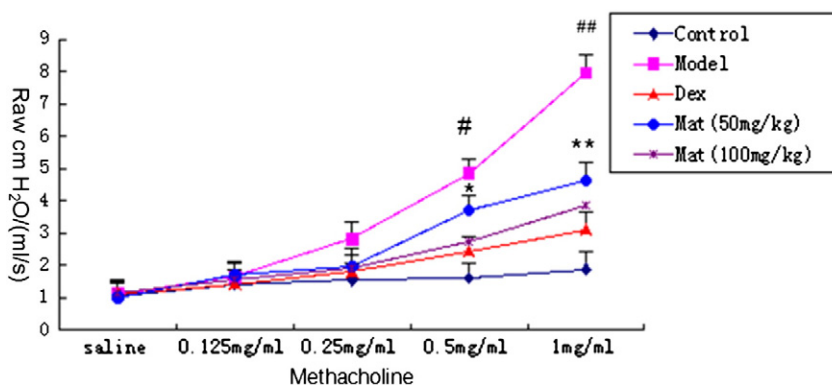


Fig. 2. Effect of Mat on airway Resistance (Raw). Values are expressed as means $\pm$ S.Ds. Compared with control: $^{\#}P < 0.05$, $^{##}P < 0.01$; Compared with model: $^{*}P < 0.05$, $^{**}P < 0.01$.

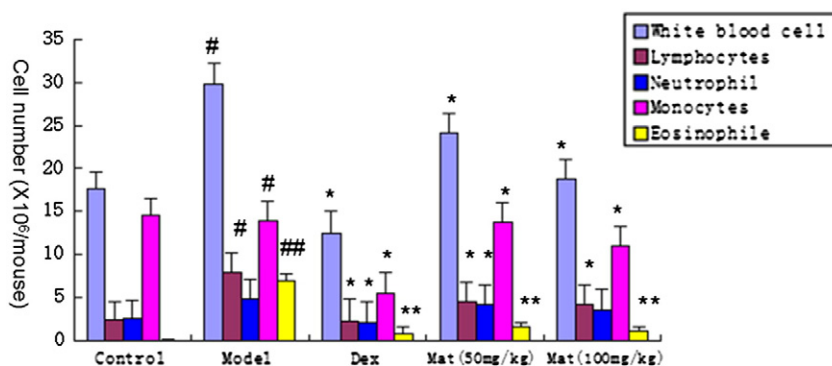


Fig. 3. Effect of Mat on OVA-induced eosinophilia and BAL fluid composition. Values are expressed as means $\pm$ SDs. Compared with control: [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with model: ^{*} $P < 0.05$, ^{**} $P < 0.01$. Columns, mean of six independent experiments; bars, SD.

significantly attenuated compared with the level seen in OVA-challenged mice as shown in Fig. 4.

3.4. Effect of Mat on cytokine levels in BAL fluid

To determine whether Mat influenced cytokine secretion into BAL fluid, IL-4 and IL-13 in the BAL fluid were measured by ELISA 24 h after the final challenge. The results are shown in Fig. 5, from Fig. 5, as for BALF levels of IL-4 and IL-13, compared with control group, levels of IL-4 and IL-13 in model group were markedly increased ($p < 0.05$), while, compared with model group, the treatment groups can markedly decrease levels of IL-4 and IL-13.

3.5. Effect of Mat on OVA-specific serum and BALF levels of IgE

The cross-linking of allergen-specific IgE on the surfaces of mast cells upon allergen challenge is relevant to the initiation of the early asthmatic reaction. The serum and BALF levels of OVA-specific immunoglobulins were measured 24 h after the final airway challenge. We determined that sensitization and challenge with OVA resulted in increased serum and BALF levels of OVA-specific IgE, when compared with the control mice. The treatment of sensitized mice with Mat resulted in reductions in OVA-specific IgE (Fig. 6). These results support the conclusion that Mat suppressed the generation of a Th2-type immune response in this animal asthma model.

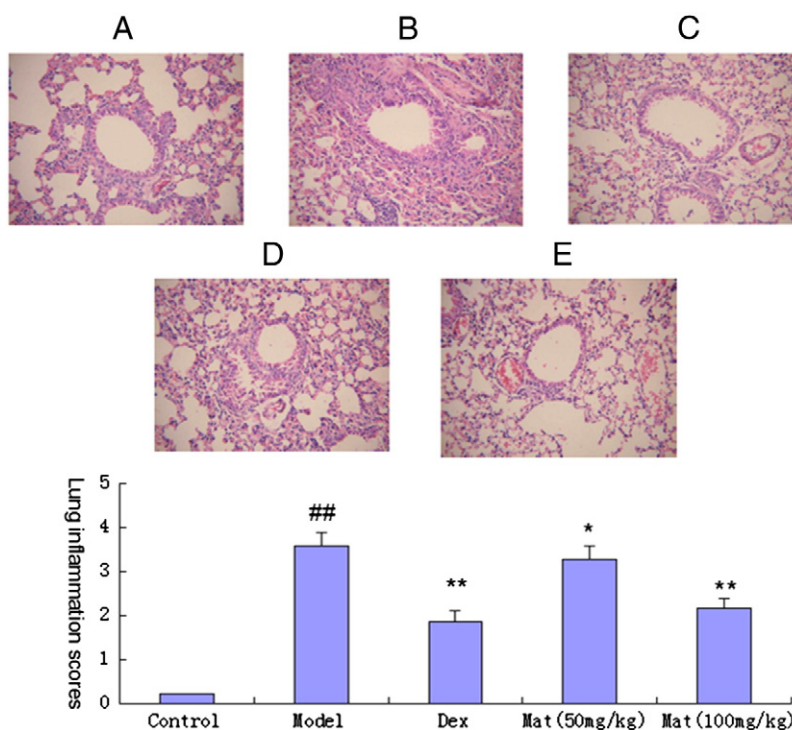


Fig. 4. Effect of Mat on cytokine levels in BAL fluid. Values are expressed as means $\pm$ SDs. Compared with control: [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with model: ^{*} $P < 0.05$, ^{**} $P < 0.01$.

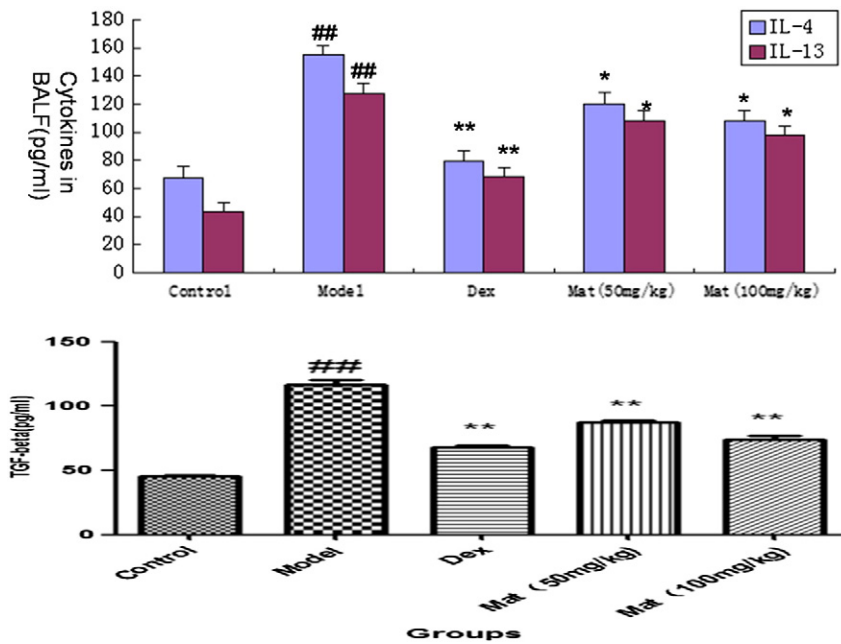


Fig. 5. Pathological changes of lung tissues observed by HE staining (Light microscopy, $\times 200$): A (control); B (model); C (Dexamethasone, 2 mg/kg); D [Mat(50 mg/kg)]; E [Mat(100 mg/kg)]. Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means $\pm$ SDs. Compared with control: $^*P < 0.05$, $^{##}P < 0.01$; Compared with model: $^*P < 0.05$, $^{**}P < 0.01$. Effect of Mat on cytokine levels in BAL fluid. Values are expressed as means $\pm$ SDs. Compared with control: $^*P < 0.05$, $^{##}P < 0.01$; Compared with model: $^*P < 0.05$, $^{**}P < 0.01$.

3.6. Effect of Mat on STAT6

The expression of inflammation-related protein STAT6 was changed by OVA in lung. As shown in Fig. 7 compared with the control group the protein level of STAT6 in model group was significantly increased ($P < 0.05$). In Mat, the protein level of STAT6 was significantly increased in a dose-

dependent manner compared to the model group, respectively ($P < 0.05$).

4. Discussion

Asthma, a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease may cause

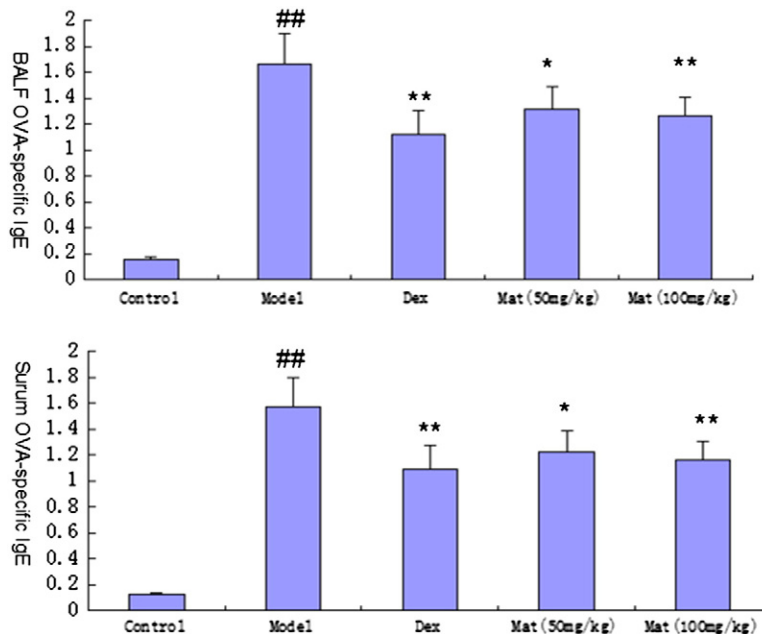


Fig. 6. Effect of Mat on OVA-specific serum and BALF levels of IgE. Values are expressed as means $\pm$ SDs. Compared with control: $^*P < 0.05$, $^{##}P < 0.01$; Compared with model: $^*P < 0.05$, $^{**}P < 0.01$.

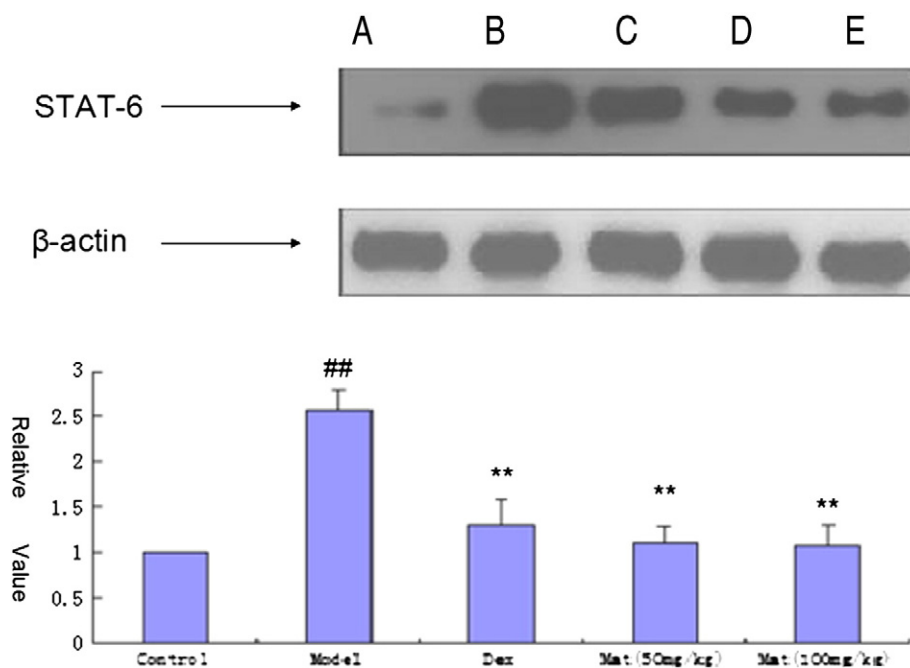


Fig. 7. Effect of Mat on STAT6. A (control); B (model); C (Dexamethasone, 2 mg/kg); D [Mat(50 mg/kg)]; E [Mat(100 mg/kg)]. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$.

severe morbidity, and even mortality if exacerbated. To date, a variety of drugs have been developed to treat asthma; these include steroids, leukotriene inhibitors, mast cell stabilizers, and β_2 adrenergic agonists. Many herbal medicines, including Mat, were commonly used to treat asthma, but the therapeutic efficacies and modes of action of such medicines are currently unclear. Here, using an OVA-induced mouse model of asthma, we show for the first time that Mat can be effective as a therapeutic drug for the treatment of allergic asthma.

Asthma is defined as a variable level of airway obstruction usually accompanied by AHR. Bronchoconstriction attributable to contraction or hypertrophy of airway smooth muscle (ASM), and inflammation within the airway, leads to decreased lung function [13]. AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. We monitored the effects of Mat on AHR by measuring Raw. These experiments disclosed that Mat inhibited the OVA-induced AHR in response to inhaled methacholine.

The mechanisms of recruitment of inflammatory cells associated with and presumably causing AHR have been well-studied. Migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation [14]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that Mat significantly reduces eosinophil numbers in the lung tissue.

Allergic asthma is recognized as a TH2-mediated immune system disease. Asthma and inflammation are associated with enhanced production of TH2 cytokines (IL-4 and IL-13). Our data show that Mat significantly suppresses IL-4 and IL-13 cytokine levels. STAT-6 is required for IL-4 production from antigen-stimulated murine splenocytes. Moreover, activation

of STAT-6 is critical for the differentiation of naïve T-cells into Th2 effector cells, and STAT-6 regulates IL-4- and IL-13-induced production of Th2 chemokines, including eotaxin, from airway epithelial cells, fibroblasts and smooth muscle cells, the IL-4/IL-13/STAT-6 pathway plays a key role in asthma pathogenesis. The present results clearly demonstrate that Mat significantly inhibited STAT-6. Our findings support the possible use of Mat as a therapeutic drug for patients with allergic asthma.

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