

Oral administration of honokiol attenuates airway inflammation in asthmatic mouse model

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Abstract: Allergic asthma is a disease that pathologically characterized by eosinophilia infiltration, airway inflammation and hyper responsiveness. In this study, we evaluated the anti-inflammatory and anti-allergy possibilities of honokiol, a bi-phenolic compound obtained from species of the genus *Magnolia*, which has long been involved in traditional Chinese prescriptions for asthma-related lung diseases, in an ovalbumin-induced mouse model of allergic asthma. We found honokiol significantly inhibited the eosinophilia infiltration, reduced the airway inflammation and suppressed the production of inflammatory cytokines as well as the IgE in serum. Moreover, MMP-9 and? (IL-4 and IFN- NF-κB were found to be involved in the honokiol induced biological process. These results suggested that honokiol may be a possible candidate in the treatment of lung asthma related diseases.

Keywords: Honokiol, asthma, anti-allergy, anti-inflammation.

INTRODUCTION

Asthma, whose prevalence and morbidity have been increasing worldwide over decades, is a chronic inflammation of the airways which pathologically characterized by hyper-responsiveness of airway, inflammatory infiltration of eosinophils in the bronchial walls, as well as the elevated serum IgE levels. The processes related to allergic inflammation are manipulated by the Th2 lymphocytes, which secrete IL-5 and IL-4 leading to production improvement of IgE by B cells as well as the generation and recruitment of eosinophils, respectively (Finiasz *et al.*, 2011; John *et al.*, 2001; Paul *et al.*, 2006). Current therapies are effective in reducing airway allergic inflammation and hyper-responsiveness; however, some asthmatic patients still exhibit airway hyper-responsiveness after a prolonged treatment with steroids. Moreover, the chronic asthmatic patients in the long-term glucocorticoids treatment may develop serious side effects. Thus there is a need for new or alternative medications to deal with asthma, especially for those who respond poorly to conventional ones (Stephen *et al.*, 2006; Nicola, 2008; Paolo *et al.*, 2009).

Honokiol is a bi-phenolic compound with the formula of C₁₈H₁₈O₂ and a relatively small molecular weight of 266 grams/mole. The molecule is found in several species of the genus *Magnolia* including *Magnolia officinalis*, *Magnolia obovata*, and *Magnolia grandifolia*. In the mean time, it is an isomer of another compound also found in *Magnolia* materials called magnolol, which has 1000 times the antioxidant potentiality of vitamin E. The dried bark of *M. officinalis* usually has about 2-10% magnolol and 1-5% honokiol. Honokiol has been demonstrated to possess effects of anti-oxidation, anti-

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inflammation, anti-angiogenesis, anti-cancer and anti-lipid peroxidation *in vitro* and *in vivo* (Byung *et al.*, 2008; Traci *et al.*, 2005; Park *et al.*, 2006; Lioua *et al.*, 2003; Chen *et al.*, 2007; Cuia *et al.*, 2007). Recently, anti-allergy, anti-autoimmunity as well as anti-contraction of the trachea smooth muscle were added to the item of honokiol's biological functions by several research teams (Chien *et al.*, 2003; Han *et al.*, 2007; Melissa *et al.*, 2007). As a natural chemical with multiple biological functions, therefore it is reasonable to believe that honokiol could be supposed to be potentially effective in lung allergic diseases, such as asthma which is featured by contraction of the trachea smooth muscle activity resulting from allergic responses and inflammatory infiltrations in the bronchial walls.

In this study we have shown that honokiol has potential therapeutic effects in murine model of bronchial asthma, based on the supportive observations in the behavioral, pathological and serological areas. This experiment showed us the possibility that honokiol itself, or as combination with other drugs, could be helpful in administration and attenuation of asthmatic symptoms, especially the chronic types.

MATERIALS AND METHODS

Experimental therapy of allergic asthma in mice model
Female SPF BALB/c mice (8–10 weeks old) were obtained from Sichuan University and raised in the SPF Laboratory Animal Center of Sichuan people's hospital (Ethical approval reference number:伦审(研)2011年第2号). This study was performed under the NIH guidelines for care and use of laboratory animals. Twenty-one BALB/c mice were randomly assigned to a control

group, an ovalbumin (OVA) group, and a honokiol group. The protocol for sensitization and inhalational challenge has previously been described in detail. Briefly, mice in the OVA group and honokiol group were sensitized on days 0, and 14 by intraperitoneal injection of 20 μ g chicken egg ovalbumin (Sigma) emulsified in 2.25 mg of aluminium hydroxide in a total volume of 100 μ l. Two weeks after the last sensitization, the mice were exposed to 1% aerosolized OVA for 30 min/d, 2 days per week for 5 weeks. For aerosolized challenges, the mice were placed in a Plexiglas box (50cm \times 30cm \times 20cm) and underwent aerosolized OVA using an ultrasonic nebulizer (NE-U11B, Omuron). During the last 5 weeks, the mice in the honokiol group were administered orally with *Magnolia officinalis* originated and endotoxin-free honokiol (CenterBio) at a dosage of 50 mg/kg. The control group was injected and nebulized with normal saline instead of OVA.

Analysis of bronchoalveolar lavage fluid (BALF) and serum

Mice were bled 12 hours after the last OVA exposure by retroorbital puncture using heparinized capillary tubes. After centrifugation (10 minutes, 4°C, 1000 \times g), serum was stored at -70°C until use. Lungs were washed three times with 0.5 ml saline to collect BALF which was then centrifuged (10 minutes, 4°C, 1000 \times g), and the total number of inflammatory cells and eosinophilias was counted with a hemocytometer. Differential cell counts were conducted using cytopspin techniques and Wright's staining by counting all the cells in 0.1ml BALF. The levels of IL-4, IFN- γ and IgE in the serum (Jingmei Biotech) were determined by ELISA according to the manufacturer's protocol.

Histological and immunohistochemical analysis of lung tissue

Twelve hours after last challenge, the left lungs taken from sacrificed mice were immersed in 10% formaldehyde overnight, and then embedded in paraffin and sections (5 μ m thickness) were stained with either hematoxylin/eosin to assess the inflammatory cell infiltrates, and Alcian blue/periodic acid-Schiff (AB/PAS) to quantify airway goblet cells. Photomicrographs showed the mucus-producing cells with the distinctive colors for PAS positivity (pink) and AB/PAS positivity (purple). For quantitative analysis, the percentages of AB/PAS-positive cells were calculated from the number of AB/PAS-positive epithelial cells per bronchus divided by the total number of epithelial cells in each bronchiole. Immunohistochemical analysis was performed using an avidin-biotin complex (ABC) kit and manufacturer's protocol (Vector Laboratories). Sections were incubated with purified polyclonal antibodies. As a negative control, the primary antibody was replaced with PBS.

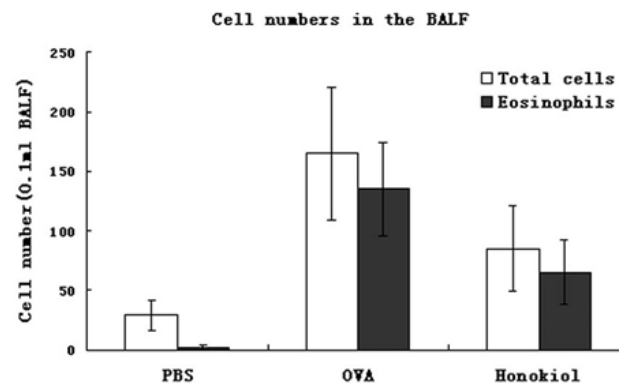
STATISTICAL ANALYSIS

All of the data were calculated as the mean \pm SEM. Statistical analyses were performed using a two-tailed Student *t* test. Significant differences were defined by $P < 0.05$.

RESULTS

Honokiol inhibited chronic airway inflammation

In the mouse asthma model, sensitization and challenge of OVA resulted in a substantial increase of total leukocytes in the BALF. Total eosinophils counts in the BALF were significantly decreased in the honokiol-treated mice with comparative to the control group (fig. 1). The results of histological examination of lung tissues resembled the cell numbers in the BALF. Marked infiltration of inflammatory cells into the airway and around the blood vessels were observed in the OVA-sensitized/challenged mice, but not in the control mice. As to the honokiol-treated mice, the attenuated inflammatory cell infiltration into the peribronchiolar and perivascular regions was noted. Furthermore, we find less swelling of the bronchiolar wall and shrinkage of the bronchiolar cavity in the control and honokiol team, compared with non-treatment group (fig. 2). Besides, we also observed less coughing, wheezing and upsetting behaviours in the honokiol team when challenging with OVA aerosol, which suggested attenuation of bronchiolar airway inflammation.



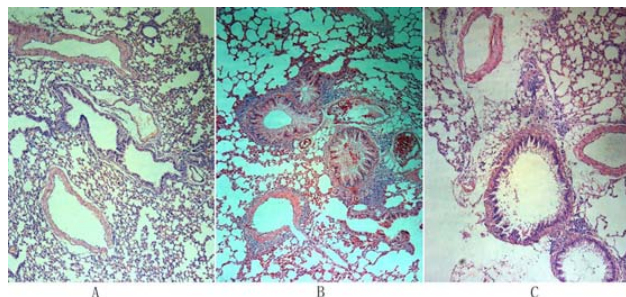
Honokiol treatment reduced the number of inflammatory cells in the BALF after OVA challenge. Cell counts were measured as in the Methods section. PBS, PBS-challenged mice; OVA, OVA-challenged mice; Honokiol, asthmatic mice treated with honokiol (50mg/kg). Values are expressed as mean \pm SEM, n=5/group in four separate experiments. *Significant difference from OVA, $P < 0.05$.

Fig. 1: Inflammatory cells and Eosinophils counts in the BALF

The influence of honokiol on the levels of IgE and Th1/Th2 cytokines in serum

The marked increase of IgE and Th2 cytokine IL-4 in the total serum were recorded in the airway challenged group

when compared to those of the control and honokiol group, which means the administration of honokiol could reduce the concentrations of Th2 cytokines and IgE in the serum to some extent. At the same time, the IFN- γ which secreted by Th1 cells, was found to be elevated in the honokiol treated group. The change of cytokines detected by ELISA suggested the influence of honokiol in the function of T helper cell lines and the possible application in the management of asthmatic diseases. (fig. 3).



The lung tissues were fixed, embedded, cut into slices, and stained with H&E solution, which enables us to distinguish the infiltration of inflammatory cells, swelling of the bronchiolar wall and shrinkage of the bronchiolar cavity: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) asthmatic mice treated with honokiol (50mg/kg). Original magnifications: $\times 200$.

Fig. 2: Pathological observation of mouse lung tissue

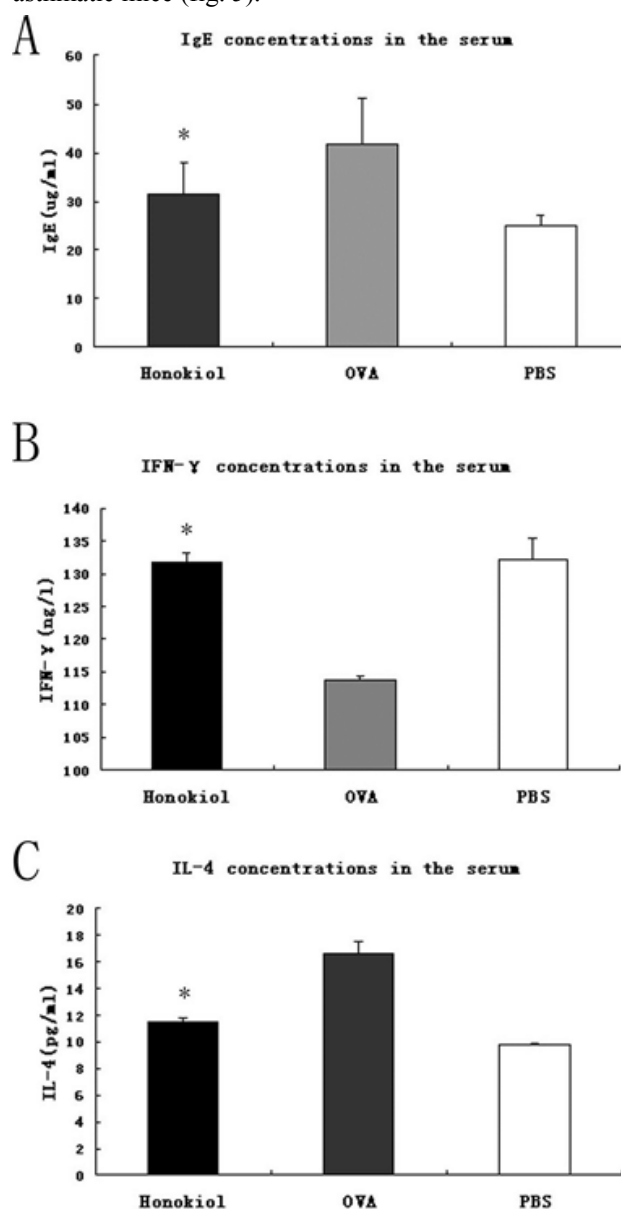
Honokiol suppressed the goblet cell secretion

Sequential alcian blue (AB) and periodic acid-Schiff (PAS) staining of airway tissue sections allows clear visualization of mucin which is produced by secretory cells. In the untreated group of mice, less AB-PAS positive epithelial cells were observed. However, in contrast, honokiol administration induced a significant decrease in the number of goblet cells and mucus-stained areas (fig.4). Therefore, it is reasonable to think that the marked decrease of mucus production in the lungs of honokiol-treated mice may be attributable to a significant variance of some cytokine levels, considering the involvements of MMP-9 and NF- κ B in the biological process of asthmatic airway epithelium reported by different teams, we made a further pathological test on these two proteins.

Molecules involved in the honokiol induced biological process

MMP-9 is a matrix metalloproteinase which is reported to be present in low quantities in the healthy lung, but much more abundant in some lung diseases, including idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), acute and chronic asthma (Jeffrey *et al.*, 2001). NF- κ B manipulates the expression of multiple chemokines, cytokines, and cell adhesion molecules that are deeply involved in the pathogenesis and progress of asthma. Selective attenuation of NF- κ B function in airway epithelium has been shown to be effective in reducing the OVA-induced mucus production

in mice model (Poynter *et al.*, 2004; Broide *et al.*, 2005). These two molecules in the honokiol team were shown to be degraded significantly compared to the untreated asthmatic mice (fig. 5).

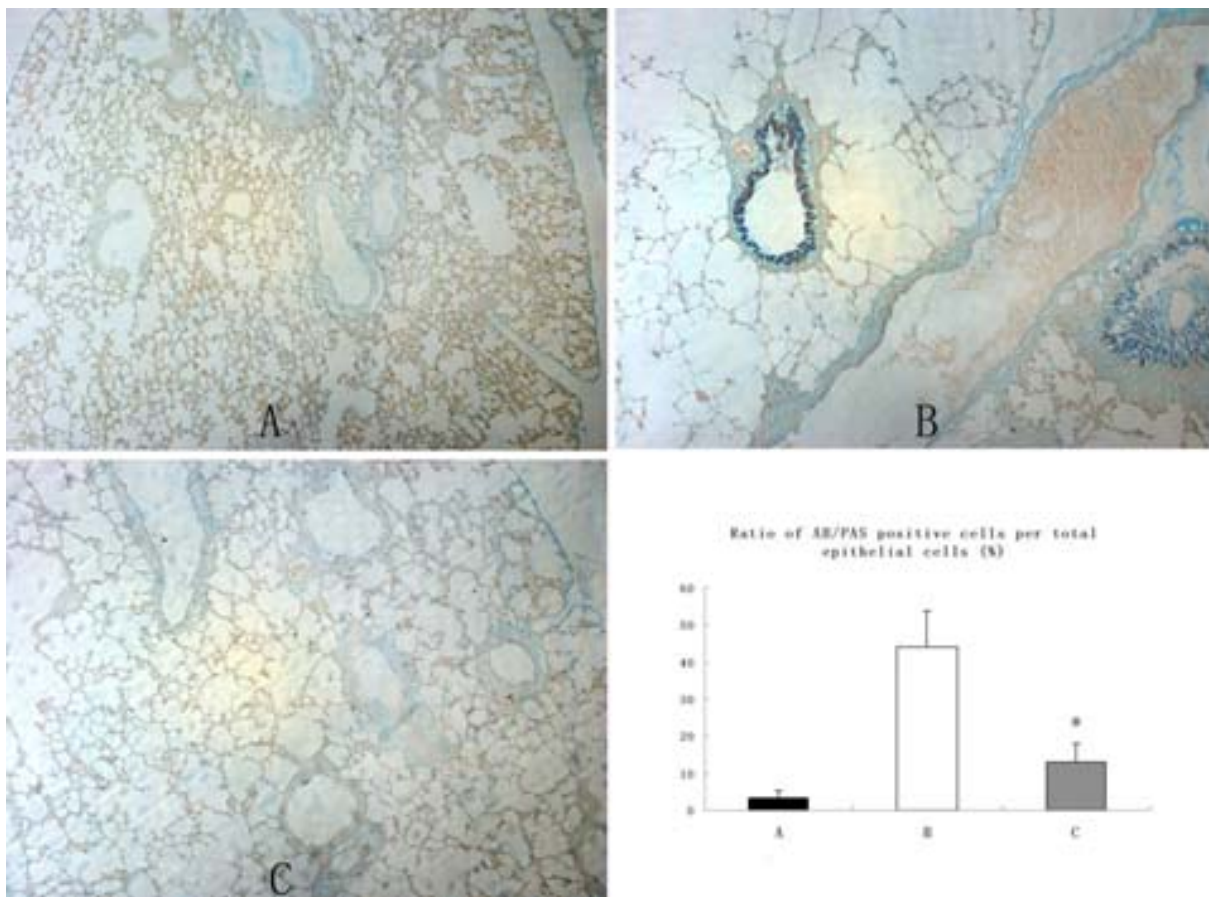


Effect of honokiol on IgE, IL-4 and IFN-g level in serum. Mice serums were collected 12 h after the last OVA challenge. Levels of these molecules were analyzed using ELISA (n=6). PBS, PBS-challenged mice; OVA, OVA-challenged mice; Honokiol, asthmatic mice treated with honokiol (50mg/kg). Values shown are the mean \pm SEM. * Significant difference from OVA, $P < 0.05$.

Fig. 3: Serum IgE, IL-4 and IFN-g quantification

DISCUSSION

Honokiol, an bio-active component isolated and purified from the *Magnolia officinalis*, has been demonstrated by several teams to possess the multi-functions of anti-oxidation, anti-inflammation and anti-lipid peroxidation

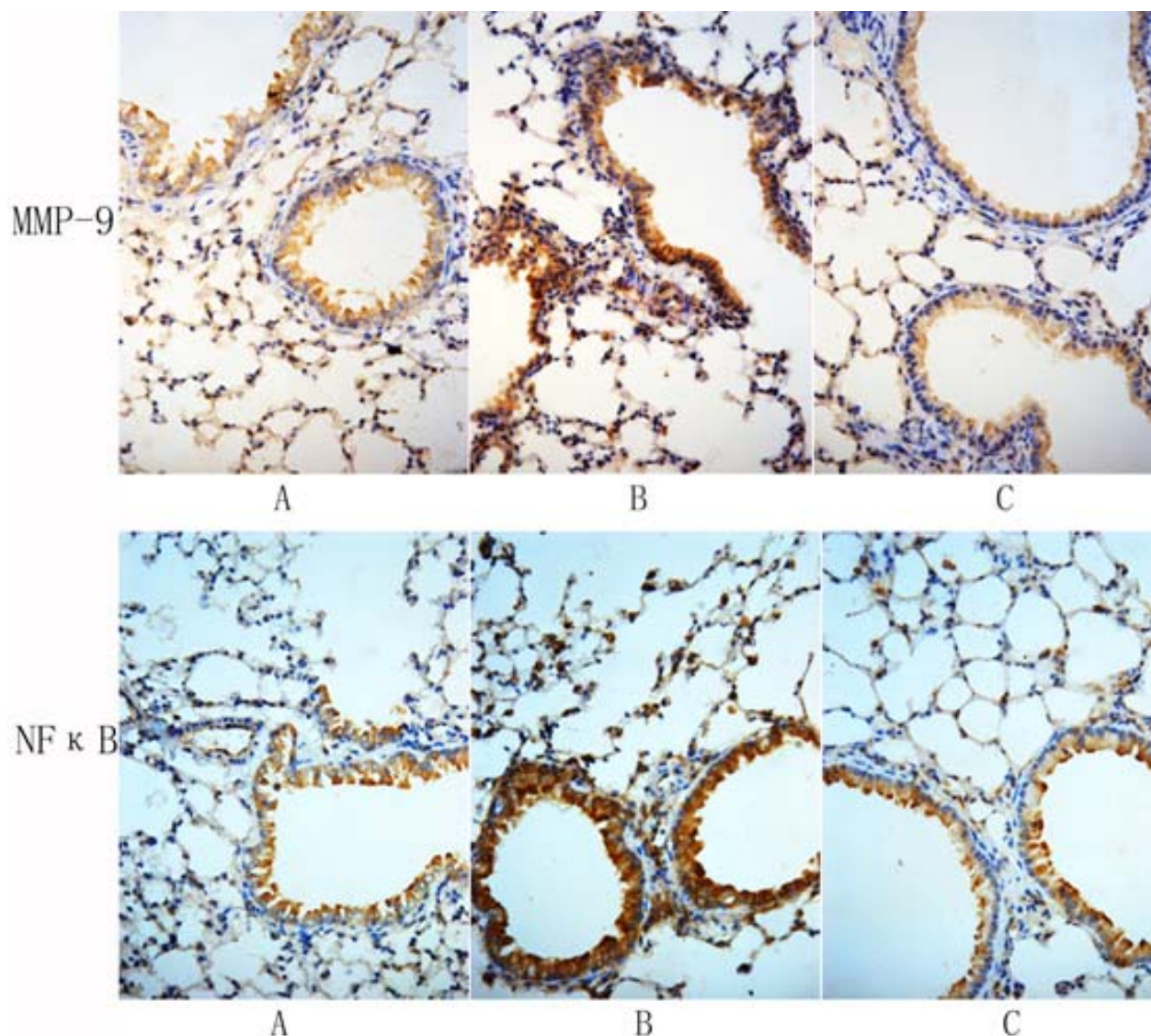


The lung sections were stained with AB/PAS solution, which enables us to see the hyperplasia of goblet cells, the mucus-producing cells, and mucus within airway or bronchi: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) asthmatic mice treated with honokiol (50mg/kg). The percentages of AB/PAS positive cells were calculated from the number of AB/PAS positive epithelial cells per bronchus divided by the total number of epithelial cells in each bronchiole. * Significant difference from OVA, $P < 0.05$. Original magnifications: $\times 200$.

Fig. 4: AB/PAS staining of lung tissue

in vitro and *in vivo*. Benefited from these biological effects, honokiol was found to show protection effect in liver cells, neutrophils and neural cells, by inhibition of several enzymes, such as NAPDH oxidase, myeloperoxidase, cyclooxygenase, GSH peroxidase, ATPase. Especially, oral administration of honokiol in advance could decrease the experimental cerebral ischemia and reperfusion injury in mice model. Furthermore, the fibrosis process in the chronic inflammation of liver was reported to be suppressed by the honokiol through the induction of apoptosis of hepatic stellate cells (Park *et al.*, 2006; Lioua *et al.*, 2003; Chen *et al.*, 2007; Cuia *et al.*, 2007). Recently, honokiol has also been shown to be a systemically available and non-toxic inhibitor of angiogenesis and tumorigenesis. Besides, suppressive effect, such as induction of cellular apoptosis, has been revealed by *in vitro* studies in the murine endothelial cells, human colorectal carcinoma cells, human leukemia cell line HL-60 and human squamous lung cancer cells (Byung *et al.*, 2008; Traci *et al.*, 2005).

More interestingly, anti-allergy and anti-autoimmunity activity were added to the item of honokiol's biological functions by several research teams. In experiments *in vitro* and *in vivo*, honokiol's suppression of the degranulation of RBL-2H3 cell and the expression of cytokines (IL-4 and TNF- α), as well as the relief of the itch caused scratching in the mice skin allergy test, induced by antigen complex, were observed (Han *et al.*, 2007). Besides, the mice model of the collagen induced arthritis and the genetic related psoriasis, diseases highly related with over activation of autoimmunity, were obviously weakened by honokiol administration (Melissa *et al.*, 2007). Therefore, the anti-allergy and anti-inflammation function of honokiol made it a candidate drug for diseases with aberrant immunity and inflammation involvement, such as allergic asthma which is featured by the airway inflammation and allergy induced airway shrink. Interestingly, researchers in Taiwan reported the honokiol could inhibit the shrinkage of the trachea smooth muscle, which is resulted from the



Effect of honokiol on MMP-9 and NF- κ B production. The lung tissues were fixed, embedded, cut into slices, and stained with MMP-9 and NF- κ B monoclonal antibodies: (A) PBS-challenged mice; (B) OVA-challenged mice; (C) asthmatic mice treated with honokiol (50mg/kg). Original magnifications: $\times 200$.

Fig. 5: MMP-9 and NF- κ B staining of mouse lung tissue

carbachol and potassium, through the decrease of cell calcium in flow (Chien *et al.*, 2003). As a natural chemical with multiple biological functions, therefore it is reasonable to believe that honokiol could be supposed to be potentially effective in several diseases.

In this experiment, the allergic asthma in mice model was somehow attenuated by honokiol administration orally in the dosage of 50mg/kg. First of all, the allergy related behaviours, such as shortness of breath, cough and irritability; as well as the quantification of eosinophil cells were less observed in honokiol treated mice. Pathological analysis revealed, compared with the control group, the decrease of smooth muscle thickness in the bronchial wall and reduction of the lymphocyte infiltration. AB/PAS staining of mucopolysaccharide also

showed the function suppression of goblet cells in the bronchial wall. Furthermore, the ELISA assay of asthma associated cytokines and antibody (IFN- γ , IL-4 and IgE) revealed the relief of the asthmatic physiology process, which suggested a beneficial bias in the balance between Th1 and Th2 cell lines. Immunohistochemistry analysis of some asthma related proteins (MMP-9 and NF- κ B) in paraffin embedded lung tissues also suggested suppression of some pathophysiological processes involved in asthmatic progression.

CONCLUSIONS

It has been hundreds of years in China since *Magnolia officinalis*, the botanical which honokiol was isolated

from, was commonly used with other botanicals in the treatment of asthma related lung diseases. As a disease featured with acute or chronic inflammation caused by unbalanced state between Th1 and Th2 cell functions, as well as the airway hyper-responsiveness, it is proper to take advantage of those chemicals with multiple functions involving anti-inflammation, anti-allergy, and dilatation of trachea smooth muscle. This experiment showed us the possibility that honokiol itself, or as combination with some drugs, could be helpful in administration and attenuation of asthmatic symptoms, especially the chronic types.

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