



Pulmonary, gastrointestinal and urogenital pharmacology

Anti-allergic activities of 5,7-dimethoxy-3,4'-dihydroxyflavone via inhalation in rat allergic models

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ARTICLE INFO

Keywords:

5,7-dimethoxy-3,4'-dihydroxyflavone

Anti-allergic activity

Airway hyperresponsiveness

Airway inflammation

Immunoglobulin E

ABSTRACT

Various studies have shown that flavones have several pharmacological activities including anti-allergy activities. However, the bioavailability of oral flavones is very low, and whether inhaled administration can improve efficacy in respiratory disease models is unclear. In the present study, the anti-allergic activities of inhaling 5,7-dimethoxy-3,4'-dihydroxyflavone (MHF), a synthetic flavonoid, was investigated by comparison with disodium cromoglycate (DSCG) and nedocromil sodium (NS) in rat allergic models. In an anti-DNP-IgE-induced asthmatic model, inhaled MHF dose-dependently inhibited the increase in airway resistance after antigen challenge. In an ovalbumin (OVA)-induced asthmatic model, inhaled MHF showed significant suppression of airway hyperresponsiveness; a decrease in eosinophil and neutrophil counts, IL-4, IL-5 and leukotriene D₄ in bronchoalveolar lavage fluid; a reduction in total IgE and OVA-specific IgE levels in serum; and suppression of eosinophil infiltration in lung tissue after antigen challenge. The efficacy of inhaled MHF was comparable to that of NS and DSCG. In conclusion, based on these findings, the report for the first time that that inhaled MHF may be a potential drug for the treatment of allergic asthma.

1. Introduction

Bronchial asthma (BA) is a heterogeneous disease, usually characterized by airway inflammation along with variable expiratory limitation (Global Initiative for Asthma GINA, 2017). In asthma, airflow obstruction is thought to primarily result from inflammation-triggered airway smooth muscle (ASM) contraction. BA is often sustained by allergic sensitization, which leads to acute bronchoconstriction and airway hyperresponsiveness (AHR) in response to specific and non-specific triggers. For many years, from a pathogenic perspective, the focus of research has been on the role of T cells in the initiation and perpetuation of inflammation (Georas et al., 2005; Kay, 2001; Busse and Lemanske, 2001). In particular, T helper 2 (Th2) cells have been identified to be involved in controlling immunoglobulin E (IgE) production because of their ability to produce interleukin 4 (IL-4) and IL-13, and influence eosinophil function through the action of IL-5 (Romagnani, 2004). The presence of Th2 cells in the bronchoalveolar lavage from atopic asthmatics was clearly demonstrated (Robinson et al., 1992). There is strong evidence that mast cells play an important

role in the early asthmatic reaction following allergen exposure. Mast cell-derived mediators induce the classical features of an early asthmatic reaction in vivo, inducing bronchoconstriction, mucus secretion and mucosal edema (Theoharides et al., 2015; Cruse and Bradding, 2016). Both disodium cromoglycate (DSCG) and nedocromil sodium (NS) are known as the “mast cell stabilizers” because they inhibit histamine release from peritoneal mast cells (Zhang et al., 2016; Theoharides et al., 1980; Eady, 1986) and passive cutaneous anaphylaxis (PCA) in rats (Altounyan, 1967; Eady, 1986; Riley et al., 1987). Recently, there was evidence that DSCG attenuates both vascular leakage in the lung and bronchoconstriction in an IgE-mediated passive pulmonary anaphylaxis model in rats (Wex et al., 2014). Even though DSCG and NS are not therapeutically effective in all patients and have mainly an adjunctive role in asthma, their excellent safety profiles have made them especially useful, especially in children. They also have a therapeutic role in diseases such as mastocytosis and allergic conjunctivitis (Zhang et al., 2016). In the present study, DSCG and NS are used as positive control drugs to compare with the efficacy of MHF in the models.

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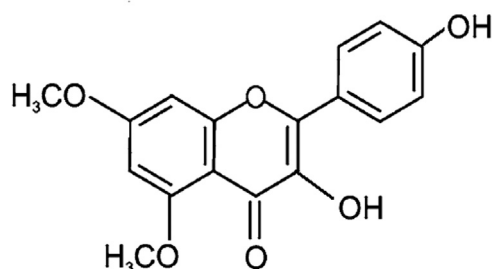


Fig. 1. Chemical structure of 5,7-dimethoxy-3,4'-dihydroxyflavone (MHF).

Flavones are a class of naturally occurring polyphenolic compounds. Various studies have shown that flavones have several pharmacological activities, such as antioxidant, anti-inflammatory, antimicrobial, cytotoxic, anti-tumor and anti-allergic (Kawai et al., 2007). The natural flavonoid 3,5,7,3',4'-pentahydroxyflavone (quercetin) was shown to be more potent than cromolyn in inhibiting photosensitivity in humans (Weng et al., 2012). Quercetin (Mlcek et al., 2016; Kim et al., 2014) and 3',4',5,7-tetrahydroxyflavone (luteolin) are natural anti-allergic and antioxidant flavonoids (Middleton et al., 2000; Theoharides et al., 2007), which also inhibit the release of histamine, tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF) (Kempuraj et al., 2005), leukotrienes (LTs) and prostaglandin D₂ from mast cells (Kimata et al., 2000). Our recent study showed that MHF (5,7-dimethoxy-3,4'-dihydroxyflavone) (Fig. 1) is a synthetic flavonoid based on the structure of luteolin, in which the hydroxyls at the 5- and 7- positions are replaced with methoxy groups and the 3'-hydroxyl is removed and exchanged for a 3-hydroxyl. Further, our study showed that MHF is a more potent mast cell inhibitor, more metabolically stable, as well as more orally and inhalation efficacious than luteolin (unpublished data). The vast majority of these investigated anti-allergic effects of flavonoids in vivo are administered orally, and whether or not inhaled delivery provides better efficacy remains to be studied. There are many advantages to aerosols, including noninvasive targeted delivery, a large drug absorption area, the potential for fewer systemic side effects, decreased drug metabolism, and patient convenience (Rubin and Williams, 2014). In the present study, our aim is to clarify whether MHF is active, and second, if inhaled MHF has anti-allergic activities when compared to NS and DSCG in rat allergic models.

2. Material and methods

2.1. Materials

MHF (5,7-dimethoxy-3,4'-dihydroxyflavone); (C₁₇O₆H₁₄; MW: 314; purity > 98%) was synthesized and provided by Prof. Yuhua Lu from the Department of Organic Synthesis, Institute of Material Medica, Chinese Academy of Medical Sciences, Beijing. The structure of MHF is shown in Fig. 1.

2.2. Animals

Female Sprague-Dawley (SD) rats weighing 150–175 g were purchased from the Laboratory Animal Center of Zhejiang University and were used after one week of quarantine and acclimation. The animals (4–5 mice/cage) were housed in an SPF environment under a 12 h light/dark cycle and received food and water ad libitum. All of the animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Zhejiang University. Animals were terminally euthanized by inhalation of CO₂ or anesthetized with urethane.

OVA-induced asthmatic model: Fifty-six rats were divided into seven groups of eight rats in each group. To sensitize the rats, a solution of 1 mg OVA (Grade V, Sigma Chemical Co., St. Louis, MO) absorbed in

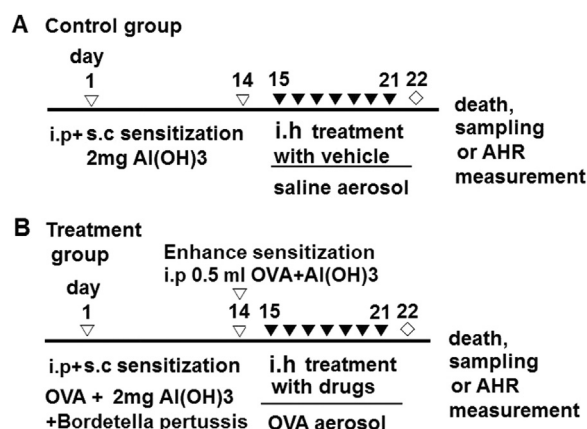


Fig. 2. The OVA sensitization, challenge, and drug treatment procedure in rats.

100 mg aluminum hydroxide adjuvant in 1.0 ml saline was used. Each rat was intraperitoneally injected (i.p.) with 0.5 ml, and subcutaneously injected (s.c.) with 0.05 ml/site on four footpads (4 sites), neck (one site), back (3 sites), and groin (2 sites). Then, every rat was injected (i.p.) with 1×10^{10} Bordetella pertussis adjuvant (Shanghai Institute of Biological Products, China) on experiment day 0. Control rats were injected with only aluminum hydroxide adjuvant in saline following the same protocol. To enhance sensitization, rats were treated with 0.5 ml antigen (OVA 0.5 mg + aluminum hydroxide 50 mg) via repeated i.p. injection on day 14. Starting on day 15, rats from the vehicle (model), MHF, DSCG and NS treatment groups were placed in a 45 cm × 45 cm × 15 cm plastic box and challenged by exposure to an aerosol of OVA (20 mg/ml in saline), which was generated in a jet nebulizer (particle size 1–5 μm; BARI, MASTER, Germany) for 20 min, and repeated daily until day 21. In a similar process, control rats were exposed to an aerosol of saline. The OVA sensitization, challenge, and drug treatment procedure is shown in Fig. 2.

Anti-DNP-IgE-induced asthmatic model: Forty-eight rats were divided into six groups of eight rats in each group, and every rat was passively sensitized by intravenous injection (i.v.) of 10 μg of anti-dinitrophenol (DNP) monoclonal mouse IgE (SPE-7, Sigma-Aldrich, St. Louis, MO). One day later, rats in the vehicle (model), MHF, DSCG and NS treatment groups were challenged by i.v. administration of 1.5 μg of the antigen DNP-BSA (Sigma-Aldrich, St. Louis, MO) in saline.

2.3. Drug administration

MHF and reference drugs nedocromil sodium (Fisons Pharmaceutical Corporation) and disodium cromoglycate (Shanghai Xinyi Pharmaceutical Factory Co. LTD) were prepared directly before each experiment. In the OVA-induced rat model, MHF and the reference drugs were inhaled for 15 min with a jet nebulizer (particle size 1–5 μm; BARI, MASTER) once daily from days 15–21 (Fig. 2). In the anti-DNP-IgE-induced asthmatic model, MHF and the reference drugs were given by i.h. administration once for 15 min, and 10 min before administration of 1.5 μg of DNP-BSA antigen by i.v.

2.4. The preparation of bronchoalveolar lavage fluids

Seven days after the OVA challenge, the rats were anesthetized with urethane (2 g/kg, i.p.). The BAL fluid was obtained by through cannulation of the trachea and lavaging with phosphate buffer solution (PBS) containing 1% bovine serum albumin (BSA) and 5000 IU/L heparin. The BAL fluid was centrifuged at $500 \times g$ for 10 min at 4 °C, and the supernatant was stored at –80 °C. The pelleted BAL fluid cells were resuspended in PBS, and the total number of leukocytes were counted in a Neubauer chamber. Two hundred cells from the BAL fluid stained with Wright-Giemsa were identified under a light microscope. The total

number of each cell type was determined by multiplying the percentage of each cell type by the total number of cells. The results are expressed as the number of each cell type in 1 ml of BAL fluid.

2.5. The measurement of AHR and bronchoconstriction

The changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were used to evaluate the changes in airway function after intravenous administration of methacholine (MCh, Sigma, St. Louis, USA). The anesthetized and tracheostomized rats were assessed as previously described (Guan et al., 2018; Cao et al., 2011). Briefly, each anesthetized animal was placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fleisch tube connected to the airway in a pressure transducer. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. To measure the pleural pressure, a needle (No. 12) with a multiholed tip was directly inserted into the pleural cavity (between the fourth and fifth rib at the left thorax) through a port in the connecting tube with a differential pressure transducer. The transpulmonary pressure was calculated as the difference between the mouth and pleural pressures. The signals of all pressure transducers were continuously computed (MedLab, Nanjing Biotech Instruments, Nanjing, China) by fitting flow, volume, and pressure to an equation of motion. Cumulative dose-effect curves were thus established, and the provocation dose (PD) of methacholine which cumulatively produced a 100% increase in R_L (PD_{100}) or a 25% decrease in C_{dyn} (PD_{25}) was determined for each animal. These values were used as indices of airway responsiveness. Cumulative doses of 0.025, 0.05, 0.1, and 0.2 mg/kg (i.v. via a cannula placed in the jugular vein) were administered for a period of 5 min per dose with lung function measurements taken between each dose.

2.6. Histopathological examination

The left lung was fixed in 10% neutral formalin for 7 days. The tissue was sectioned within paraffin-embedded blocks and stained with hematoxylin and eosin (H&E) to evaluate inflammatory cell infiltration using light microscopy, based on the peribronchial cell counts and the severity of the infiltration of inflammatory cells. A 5-point scoring system was used as described previously (Cao et al., 2011; Duan et al., 2004). Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section. Briefly, the scoring system was: 5 - marked, 4 - moderate, 3 - medium, 2 - mild, 1 - minimal and 0 - no eosinophil cells. Mean scores were obtained from eight animals.

2.7. Determination of serum total IgE and OVA-specific IgE

Total serum IgE was measured by a sandwich ELISA (Bethyl Laboratories, Inc. Montgomery, TX) according to the manufacturer's instructions. Briefly, blood was collected 24 h after the last antigen challenge. Rats were anaesthetized with urethane (2 g/kg, i.p.) and bled by femoral artery, and serum was prepared and kept at -80°C until needed for measurement. Briefly, plates were coated with purified anti-rat IgE (Bethyl Laboratories, Inc.), washed and blocked, and then diluted serum (1:25) was added. Biotinylated anti-rat IgE (Bethyl Laboratories, Inc.) was used for antibody detection. Purified rat IgE was used as the standard. After incubation with substrate (OPD), plates were read using a Bio-Rad microplate reader.

Serum OVA-specific IgE levels were measured by ELISA. Briefly, to determine the OVA-specific IgE, 96-well plates were coated with 100 μl /well of mouse anti-rat IgE heavy chain antibody (Serotec Ltd., Oxford, UK) at a concentration of 2.5 $\mu\text{g}/\text{ml}$. The next day, after the plates were blocked and washed, 100 μl of each serum sample (1:5 in blocking buffer) were applied. After incubation overnight, the plates

were treated with 100 μl /well of biotinylated OVA allergen (5 $\mu\text{g}/\text{ml}$) prepared using the Sulfo-NHS-LC-Biotinylation kit (Pierce Biotechnology, Rockford, IL) and horseradish peroxidase-streptavidin (1:1500, Sigma, St. Louis, MO) for 1 h each at room temperature. For the allergen-specific serum IgE assay, 96-well plates were coated with 100 μl of OVA at a concentration of 1 mg/ml in coating buffer and incubated overnight at 4°C . The next day, after the plates were blocked and washed, 200 μl of blocking buffer were added to each well followed by a 2 h incubation at 37°C . Diluted serum (1:50) was applied (100 μl /well) and followed by incubation overnight at 4°C . Wells were then washed and treated with 100 μl of mouse anti-rat biotinylated IgE (1:5000, AbDSerotec, OX, UK) and horseradish peroxidase-streptavidin (diluted 1:1500) for 1.5 h each at 37°C . Finally, 100 μl /well TM Blue (Sigma, St. Louis, MO) was applied as a substrate for horseradish peroxidase, and the reaction was allowed to develop at room temperature for at least 10 min. The plate was read at 650 nm by a Bio-Rad microplate reader.

2.8. Determination of IL-4, IL-5 and leukotriene D_4 levels in BAL fluid

To investigate the effects of MHF on IL-4, IL-5 and leukotriene D_4 levels in rat lungs in the OVA-induced allergic model, rat BAL fluid was collected after treatment and the levels were determined using a commercially available ELISA kit (rat leukotriene D_4 was purchased from Cayman Chemical, Ann Arbor, MI, USA; rat IL-4 and IL-5 were purchased from BOSTER, Wuhan, China). The BAL fluid was centrifuged at $2000 \times g$ for 60 min at 4°C . The concentrations of IL-4, IL-5 and leukotriene D_4 in BAL fluid were measured using a commercially available ELISA kit.

2.9. Statistical analysis

The data are expressed as the mean $\pm$ S.E.M. Statistical tests were performed using SPSS software (version 16.0; SPSS, Chicago, IL, USA). To avoid inflation of the type I error because of multiple comparisons in Fig. 3, the differences in the dose-response curves to methacholine stimulation were assessed with the variance analysis of repeated-measures method (with Greenhouse–Geisser epsilon correction and Tukey test), and the PD_{50} and PD_{20} values were calculated by the weighted probit analysis of Bliss method. For the other figures, one-way analysis of variance followed by Dunnett's test was used to determine significant

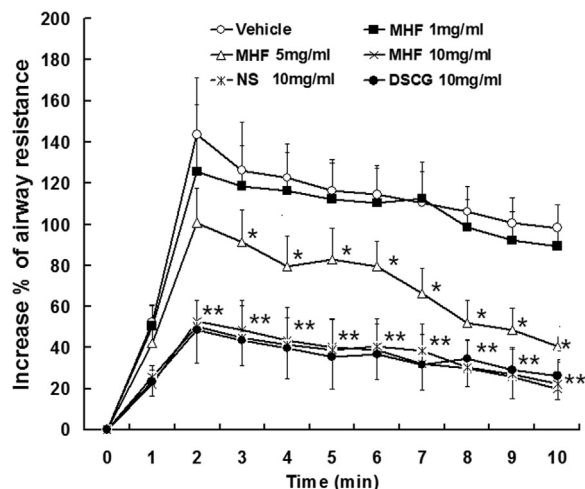


Fig. 3. Effect of MHF on anti-DNP-IgE-induced bronchoconstriction in rats. Rats were passively sensitized by i.v. administration of 10 μg of anti-DNP-IgE one day before the experiment. MHF, nedocromil sodium (NS) and disodium cromoglycate (DSCG) were administered i.h. 15 min before i.v. administration of antigen (1.5 μg of DNP-BSA in saline). Data is shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. * $P < 0.05$, ** $P < 0.01$ vs vehicle group.

differences between treatment groups. The critical level for significance was set at $P < 0.05$.

3. Results

3.1. MHF inhibits anti-DNP-IgE-induced bronchoconstriction

The effects of MHF on anti-DNP-IgE-induced asthmatic models were investigated. In a bronchoconstriction model, MHF dose-dependently inhibited the anti-DNP-IgE-induced increase in airway resistance, and the 5 mg/ml inhalation dose for 15 min showed statistically significant suppression. MHF at 10 mg/ml significantly reduced airway resistance (all $P < 0.01$), which is comparable to both NS and DSCG treatment at 10 mg/ml (Fig. 3).

3.2. MHF suppresses OVA allergen-induced airway responsiveness to methacholine

The OVA-sensitized rats were repeatedly challenged with aerosolized OVA for 7 consecutive days. The R_L and C_{dyn} were measured before and after intravenous injection of each dose of MCh. In the vehicle + OVA group, AHR was demonstrated by a significant decrease in R_L PD₁₀₀ value in addition to a significant increase in maximum R_L . Similarly, the cumulative dose-effect curves for methacholine had significantly lower C_{dyn} PD₂₅ values with a decreased minimum C_{dyn}

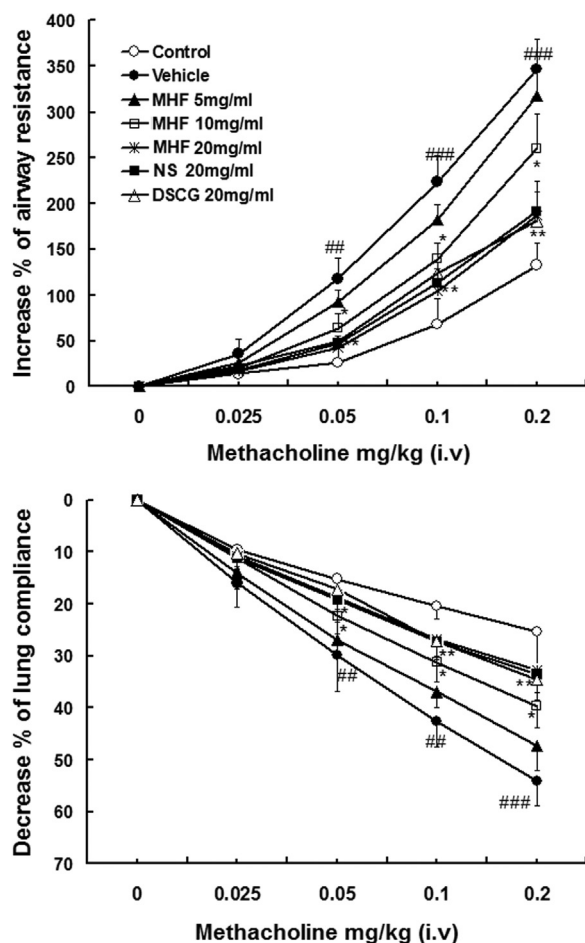


Fig. 4. Effect of MHF on airway responsiveness to methacholine. Relative changes in percentage of baseline control before methacholine provocation are given in the legend. Intravenous administration of methacholine- (MCh) induced airway hyperresponsiveness 24 h after the last OVA challenge. Data are shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. ## $P < 0.01$, ### $P < 0.001$ vs control group; * $P < 0.05$, ** $P < 0.01$ vs vehicle group.

Table 1

Effect of MHF and reference drugs on methacholine PD₁₀₀ (airway resistance, R_L) and PD₂₅ (lung dynamic compliance, C_{dyn}) in OVA-induced asthmatic rats.

Groups	R_L PC ₁₀₀ (95% CI) mg/kg, i.v.	C_{dyn} PC ₂₅ (95% CI) mg/kg, i.v.
Control	0.158(0.118–0.196)	0.202(0.126–0.256)
Vehicle	0.045(0.027–0.062)	0.043(0.026–0.056)
MHF 5 mg/ml	0.055(0.031–0.078)	0.046 (0.030–0.61)
MHF 10 mg/ml	0.061 (0.035–0.81)	0.062 (0.046–0.081)
MHF 20 mg/ml	0.105 (0.085–0.139)	0.088(0.054–0.121)
NS 20 mg/ml	0.081(0.058–0.124)	0.085(0.062–0.119)
DSCG 20 mg/ml	0.091(0.064–0.135)	0.083(0.064–0.125)

CI: confidence interval.

(Fig. 4). These changes in AHR due to MCh were quantitatively observed as a 3.51-fold decrease in R_L PD₁₀₀ and a 4.7-fold decrease in C_{dyn} PD₂₅ (Table 1). MHF at doses of 10 and 20 mg/ml i.h. inhibited AHR to MCh compared to the vehicle + OVA group. The provocative doses of MCh in the 5, 10 and 20 mg/ml MHF i.h. groups saw R_L elevated by 2.87-, 2.59- and 1.50-fold, and 4.39-, 3.26- and 2.30-fold elevated C_{dyn} . The reference drugs NS and DSCG at 20 mg/ml delivered similar potent suppressant effects when compared to the 20 mg/ml MHF i.h. group. The results of the statistical significance tests for the R_L and C_{dyn} parameters to MCh stimulation within the repeated measurements with variance analysis using the Greenhouse–Geisser correction model for the relative changes in the dose-response curves compared with baseline data are given in Fig. 4. The curve changes in R_L with the Greenhouse–Geisser correction: between-subject effects ($F = 8.36$, $P < 0.01$); within-subject effects ($F = 88.30$, $P < 0.01$); and subject-by-dose interaction ($F = 5.25$, $P < 0.01$). The curve changes in C_{dyn} with Greenhouse–Geisser correction: between-subject effects ($F = 4.58$, $P < 0.01$); within-subject effects ($F = 96.19$, $P < 0.01$); and subject-by-dose interaction ($F = 2.78$, $P < 0.05$).

3.3. MHF decreases inflammatory cells in BAL fluid and reduces eosinophil infiltration in lung tissue

To further study the effects of MHF (i.h.) on antigen-induced airway inflammation, the inflammatory cell infiltration in OVA-sensitized rats was evaluated. Twenty-four hours after the final OVA challenge, inflammatory cells, including total white blood cells, eosinophils, neutrophils, lymphocytes and macrophages in BAL fluid were counted. The total number of inflammatory cells in BALF of vehicle-OVA rats was 4-fold greater than in control rats. MHF (i.h.) dose-dependently decreased the total number of inflammatory cells in BALF. Classification of these inflammatory cells showed that in vehicle-OVA rats, the number of eosinophils and neutrophils in BALF increased approximately 700- and 17-fold, respectively, compared to the numbers observed in control rats (Fig. 5). MHF (i.h.) significantly decreased eosinophil and neutrophil counts in BAL fluid. MHF at a dose of 20 mg/ml is comparable to both NS and DSCG treatment at 20 mg/ml (Fig. 5).

The effect of MHF (i.h.) on antigen-induced inflammatory response was also evaluated by lung histopathology. Lung tissues were harvested 24 h after the final OVA challenge. The vehicle-OVA rats exhibited an obvious eosinophil cell infiltration into the peribronchiolar and perivascular connective tissues compared with control rats. MHF (i.h.) markedly inhibited the OVA-induced eosinophil infiltration compared with vehicle-OVA rats ($P < 0.01$), and MHF at 20 mg/ml is comparable to both NS and DSCG treatment at 20 mg/ml (Fig. 6).

3.4. MHF reduces serum IgE levels

In the present study, the effects of MHF on serum total IgE levels as well as allergen-specific IgE levels in the specific OVA allergen-induced allergic rat model were investigated. Total serum IgE was measured by ELISA and the results are shown in Fig. 7. The vehicle group exhibited

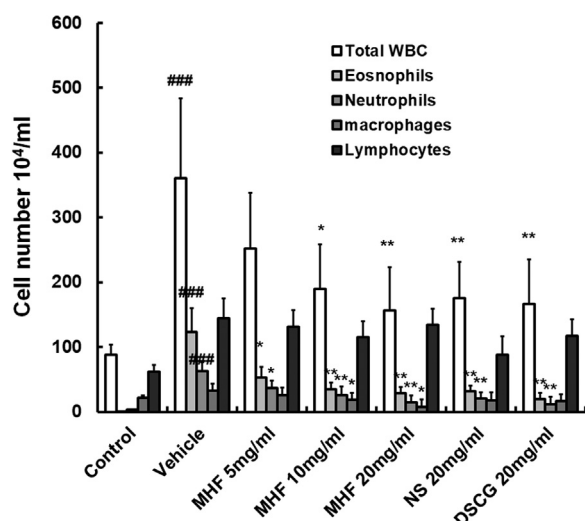


Fig. 5. Effect of MHF on inflammatory cells in BAL fluid. The total number of inflammatory cells in BAL fluid were counted, and cell classification was performed on a minimum of 200 cells to classify eosinophils, neutrophils, lymphocytes and macrophages 24 h after the final antigen challenge. Data are shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. $###P < 0.01$ vs control group; $*P < 0.05$, $**P < 0.01$ vs vehicle group.

significantly higher IgE levels than the control group ($P < 0.01$). MHF at either 10 mg/ml or 20 mg/ml i.h. produced a marked decline in total serum IgE levels ($P < 0.05$ and $P < 0.01$, respectively). In comparison, NS and DSCG at 20 mg/ml i.h. significantly inhibited total serum IgE levels ($P < 0.01$). Furthermore, the experiment also evaluated the potential effects of MHF on the OVA-specific IgE levels in the rat OVA-induced allergic model. OVA-specific serum IgE was measured by ELISA, and the data are shown in Fig. 7. A significant increase in OVA-specific serum IgE levels was observed in the vehicle group compared with the control group, and MHF i.h. treatment at either 10 mg/ml or 20 mg/ml significantly inhibited OVA-specific IgE production ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, NS and DSCG at 20 mg/ml also significantly inhibited the elevation of OVA-specific serum IgE levels (both $P < 0.01$).

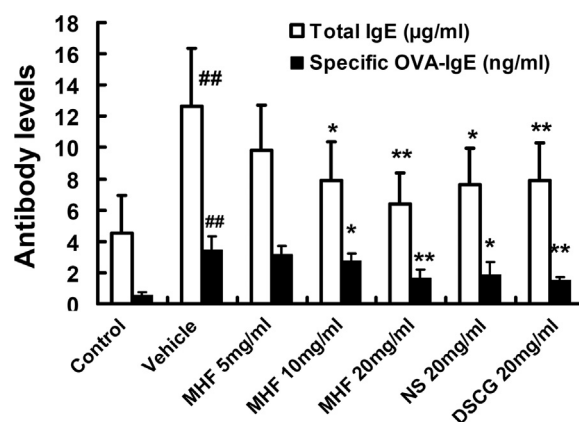


Fig. 7. Effect of MHF on serum total IgE and OVA-specific IgE levels. Sera were harvested 24 h after the last antigen challenge. Total IgE and OVA-specific IgE were measured by sandwich ELISA as described in Methods. Data is shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. $###P < 0.01$ vs control group; $*P < 0.05$, $**P < 0.01$ vs vehicle group.

3.5. MHF alleviates IL-4, IL-5 and leukotriene D_4 levels

To assess the role of MHF on the inhibition of inflammatory mediators, the effects of an antigen challenge on IL-4, IL-5 and leukotriene D_4 production in BAL fluid were examined. As shown in Fig. 8, MHF at 10 and 20 mg/ml i.h. and NS and DSCG at 20 mg/ml i.h. significantly inhibited IL-4, IL-5 and leukotriene D_4 ($P < 0.05$ and $P < 0.01$, respectively).

4. Discussion

In the present study, the effects of the synthetic flavonoid MHF on changes in bronchoconstriction in an anti-DNP-IgE asthmatic model, and on changes in AHR and airway inflammation were investigated in an OVA-induced asthmatic model. MHF exhibited similar activity after inhalation compared with two inhalable reference compounds, DSCG and NS.

Flavonoids have multiple and specific effects on inflammatory and allergic processes. *In vitro*, flavonoids and related compounds were

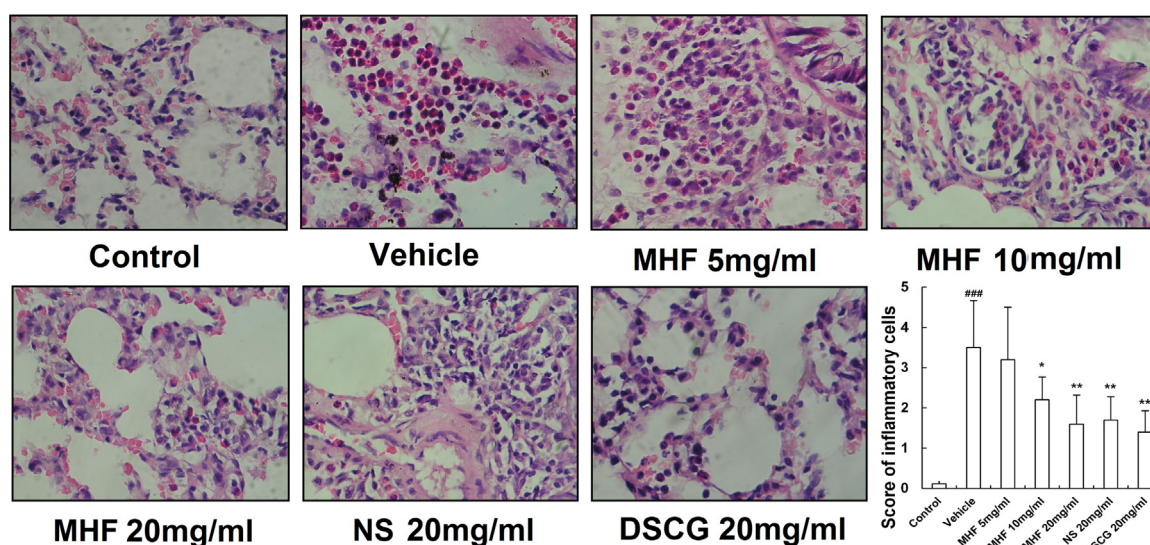


Fig. 6. Effect of MHF on changes in lung histopathology. H&E-stained lung tissues were evaluated and scored for inflammatory cell infiltration. Compared with the control group, a marked infiltration of eosinophil cells in the peribronchiolar space and perivascular space were observed in the vehicle group. There was also less eosinophil cell infiltration present in the MHF (10 mg/ml and 20 mg/ml doses), NS and DSCG groups (photos). Inflammatory cell infiltration was graded based on the severity of inflammation (histogram). Data are shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. $###P < 0.01$ vs control group; $*P < 0.05$, $**P < 0.01$ vs vehicle group.

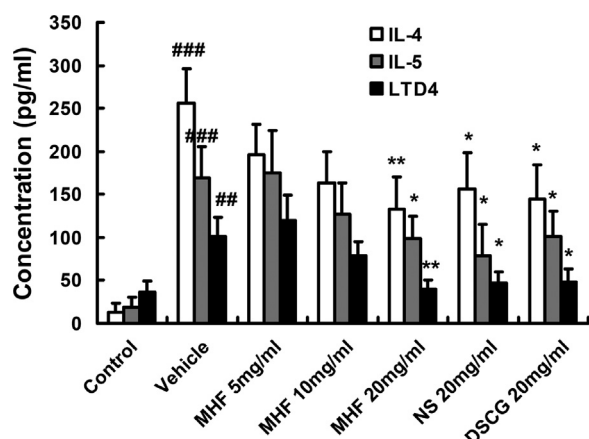


Fig. 8. Effect of MHF on inflammatory mediators. BAL fluid was harvested 24 h after the final OVA challenge. BAL fluids were measured by sandwich ELISA as described in Methods. Data is shown as the mean $\pm$ S.E.M.; $n = 8$ for each group. ## $P < 0.01$, ### $P < 0.01$ vs control group; * $P < 0.05$, ** $P < 0.01$ vs vehicle group.

tested for inhibition of IL-4 and IL-13 production in basophils stimulated with anti-IgE antibody, and quercetin, luteolin and apigenin were found to be the strongest inhibitors, with IC_{50} values of 1–2 μM (Hirano et al., 2004; Kawai et al., 2007). Luteolin also inhibited IL-5 activity with an IC_{50} value of 18.7 μM . The most effective compound, however, was luteolin 4'-O-glucopyranoside with an IC_{50} of 3.7 μM and 95% inhibition at 30 μM (Park et al., 1999). Luteolin and quercetin were also found to inhibit hexosaminidase release (degranulation marker) from mast cells (RBL-2H3 cells) as an estimate for anti-allergic actions, with IC_{50} values below 10 μM . Similar to luteolin and quercetin, MHF was found to be potent with an IC_{50} value of 6.5 μM for mast cell degranulation (unpublished data). *In vivo*, flavonoids including luteolin and quercetin (Kawai et al., 2007; Mlcek et al., 2016; Seelinger et al., 2008; Cheong et al., 1998) showed potent anti-allergic and anti-inflammatory activities in the mouse allergic asthma model. The anti-allergic mechanism of action of luteolin is via induction of foxp3 in $CD4^+CD25^+$ Treg cells (Kim et al., 2018). Quercetin reduced airway allergic inflammation by inhibiting the activation of nuclear transcription factor kappa B (NF- κB), P-selectin expression and mucus production in the lung (Rogerio et al., 2010). In the rat allergic asthma model, intraperitoneal injection of luteolin inhibits inflammatory cell number and IL-4 expression in BAL fluid via decreasing the activities of peroxisome proliferator-activated receptor- γ (PPAR γ) and p38 mitogen-activated protein kinase (p38MAPK) in pulmonary tissue (Zeng et al., 2014). However, until now, it was unknown whether flavonoids, including luteolin and quercetin, can inhibit bronchoconstriction and AHR in anti-DNP-IgE- or OVA-induced rat asthmatic models. In this study, the anti-DNP-IgE- and OVA-induced rat asthmatic models to assess MHF inhibitory activity were established. Antigen-induced bronchoconstriction is a common feature in allergic asthmatics. It is therefore important that animal models of asthma show the similar features of mast cell inflammation and reactivity to inhaled allergens (Li et al., 2014). Allergic asthmatics frequently suffer from acute bronchoconstriction upon antigen inhalation, and this acute phase is then often followed by an extended period of airway hyperresponsiveness (AHR), for which an MCh challenge is used. The physiological phenotype of asthma can thus be divided into two phases. Mouse models, however, typically only exhibit the second (AHR) phase. Indeed, published reports show that an often-used OVA mouse model of asthma does not exhibit any measurable bronchoconstriction upon inhalation of OVA (Zosky et al., 2008). Therefore, in this study, an anti-DNP-IgE-induced rat asthmatic model to assay the activity of MHF on antigen-induced acute bronchoconstriction was used. Our results demonstrated that inhaled MHF dose-dependently inhibited

bronchoconstriction, which depends on mast cell and eosinophil release by inflammatory factors, including histamine, LTs, cytokines and chemokines. Then, the OVA-induced rat asthmatic model to evaluate MHF activity on MCh-induced AHR and airway inflammation were used. Our results demonstrated that inhaled MHF dose-dependently alleviated AHR and airway infiltration in inflammatory cells, and reduced Th2, cytokines, and IL-4 and IL-5 production, and decreased the LTD₄ level in BAL fluid. The Th1/Th2 imbalance correlates with the changes in the total serum IgE and allergen-specific serum IgE levels, airway responsiveness and eosinophilia. Thus, in the present study, our results suggest that inhalation of MHF decreases asthmatic acute phase bronchoconstriction in addition to improving AHR associated with the regulation of the Th1/Th2 balance.

Despite anti-allergic properties of oral MHF, similar to other flavonoid derivatives (Scalia et al., 2013; Pápay et al., 2017), the use of MHF in the pharmaceutical field may be limited due to its poor aqueous solubility, instability in physiological media and rapid photodegradation. The clinical application of MHF may be limited by its poor bioavailability and low stability in aqueous medium. The achievement of effective concentrations of MHF in the lung tissue is difficult; therefore, higher doses and administration frequency are required, which leads to higher treatment costs as well as increased unpredictable side effects. Thus, it is necessary to develop new dosage forms and administration routes for MHF with increased solubility and improved bioavailability. The inhalation route is frequently used to administer drugs for the management of respiratory diseases such as asthma or chronic obstructive pulmonary disease. Compared with other routes of administration, inhalation offers a number of advantages in the treatment of these diseases. For example, via inhalation, a drug is directly delivered to the target organ, conferring high pulmonary drug concentrations and low systemic drug concentrations. Therefore, drug inhalation is typically associated with high pulmonary efficacy and minimal systemic side effects (Borghardt et al., 2018).

5. Conclusions

In this study, two hypersensitive rat models and inhalation delivery to evaluate the anti-allergic effects of MHF were used. Our results suggest that inhaled MHF has significant inhibitory effects in the two allergic models, while inhaled drug delivery has a better effect directly on the target organs, is more conducive to the treatment of allergic asthma, and may reduce possible adverse reactions to MHF systemic administration. It is anticipated that MHF will undergo clinical evaluation as a therapeutic anti-allergic agent.

Acknowledgements

This work was supported by grants obtained from the National Natural Science Foundation of China (No. 81872876 and No. 81573498).

Conflicts of Interest

The authors declare no conflict of interest.

References

- Altounyan, R.E.C., 1967. Inhibition of experimental asthma by a new compound, cromolyn sodium "Intal". *Acta Allergol.* 22, 487–495.
- Borghardt, J.M., Kloft, C., Sharma, A., 2018. Inhaled therapy in respiratory disease: the complex interplay of pulmonary kinetic processes. *Can. Respir. J.* 2018, 2732017.
- Busse, W.W., Lemanske Jr., R.F., 2001. Asthma. *N. Engl. J. Med.* 344, 350–362.
- Cao, R., Dong, X.W., Jiang, J.X., Yan, X.F., He, J.S., Deng, Y.M., et al., 2011. M₃ muscarinic receptor antagonist bencycloquidum bromide attenuates allergic airway inflammation, hyperresponsiveness and remodeling in mice. *Eur. J. Pharmacol.* 655, 83–90.
- Cheong, H., Ryu, S.Y., Oak, M.H., Cheon, S.H., Yoo, G.S., Kim, K.M., 1998. Studies of structure activity relationship of flavonoids for the anti-allergic actions. *Arch. Pharm.*

- Res. 21, 78–80.
- Cruse, G., Bradding, P., 2016. Mast cells in airway diseases and interstitial lung disease. *Eur. J. Pharmacol.* 778, 125–138.
- Duan, W., Chan, J.H., Wong, C.H., Leung, B.P., Wong, W.S., 2004. Anti-inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *J. Immunol.* 172, 7053–7059.
- Eady, R.P., 1986. The pharmacology of nedocromil sodium. *Eur. J. Respir. Dis. Suppl.* 147, 112–119.
- Georas, S.N., Guo, J., De Fanis, U., Casolaro, V., 2005. T-helper cell type-2 regulation in allergic disease. *Eur. Respir. J.* 26, 1119–1137.
- Global Initiative for Asthma (GINA), 2017. *Global strategy for asthma management and prevention, 2017*. GINA website. <<http://ginasthma.org>>.
- Guan, Y., Zhu, J.P., Shen, J., Jia, Y.L., Jin, Y.C., Dong, X.W., Xie, Q.M., 2018. Salvianolic acid B improves airway hyperresponsiveness by inhibiting MUC5AC overproduction associated with Erk1/2/P38 signaling. *Eur. J. Pharmacol.* 824, 30–39.
- Hirano, T., Higa, S., Arimitsu, J., Naka, T., Shima, Y., Ohshima, S., et al., 2004. Flavonoids such as luteolin, fisetin and apigenin are inhibitors of interleukin-4 and interleukin-13 production by activated human basophils. *Arch. Allergy Immunol.* 134, 135–140.
- Kawai, M., Hirano, T., Higa, S., Arimitsu, J., Maruta, M., Kuwahara, Y., et al., 2007. Flavonoids and related compounds as anti-allergic substances. *Allergol. Int.* 56, 113–123.
- Kay, A.B., 2001. Allergy and allergic diseases. Second of two parts. *N. Engl. J. Med.* 344, 109–113.
- Kempuraj, D., Madhappan, B., Christodoulou, S., Boucher, W., Cao, J., Papadopoulos, N., et al., 2005. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. *Br. J. Pharmacol.* 145, 934–944.
- Kim, M., Lim, S.J., Kang, S.W., Um, B.H., Nho, C.W., 2014. Aceriphyllumrossii extract and its active compounds, quercetin and kaempferol inhibit IgE-mediated mast cell activation and passive cutaneous anaphylaxis. *J. Agric. Food Chem.* 62, 3750–3758.
- Kim, S.H., Saba, E., Kim, B.K., Yang, W.K., Park, Y.C., Shin, H.J., et al., 2018. Luteolin attenuates airway inflammation by inducing the transition of CD4(+)CD25(-) to CD4(+)CD25(+) regulatory T cells. *Eur. J. Pharmacol.* 820, 53–64.
- Kimata, M., Inagaki, N., Nagai, H., 2000. Effects of luteolin and other flavonoids on IgE-mediated allergic reactions. *Planta Med.* 66, 25–29.
- Li, S., Aliyeva, M., Daphtary, N., Martin, R.A., Poynter, M.E., Kostin, S.F., et al., 2014. Antigen-induced mast cell expansion and bronchoconstriction in a mouse model of asthma. *Am. J. Physiol. Lung Cell Mol. Physiol.* 306, L196–L206.
- Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 52, 673–751.
- Mlcek, J., Jurikova, T., Skrovankova, S., Sochor, J., 2016. Quercetin and its anti-allergic immune response. *Molecules* 21, E623.
- Pápay, Z.E., Kósa, A., Böddi, B., Merchant, Z., Saleem, I.Y., Zariwala, M.G., et al., 2017. Study on the Pulmonary Delivery System of Apigenin-Loaded Albumin Nanocarriers with Antioxidant Activity. *J. Aerosol Med. Pulm. Drug Deliv.* 30, 274–288.
- Park, K.Y., Lee, S.H., Min, B.K., Lee, K.S., Choi, J.S., Chung, S.R., et al., 1999. Inhibitory effect of luteolin 4'-O-glucoside from *Kummerowia striata* and other flavonoids on interleukin-5 bioactivity. *Planta Med.* 65, 457–459.
- Riley, P.A., Mather, M.E., Keogh, R.W., Eady, R.P., 1987. Activity of nedocromil sodium in mast-cell-dependent reactions in the rat. *Int. Arch. Allergy Appl. Immunol.* 82, 108–110.
- Robinson, D.S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A.M., et al., 1992. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326, 298–330.
- Rogério, A.P., Dora, C.L., Andrade, E.L., Chaves, J.S., Silva, L.F., Lemos-Senna, E., Calixto, J.B., 2010. Anti-inflammatory effect of quercetin-loaded microemulsion in the airways allergic inflammatory model in mice. *Pharmacol. Res.* 61, 288–297.
- Romagnani, S., 2004. Immunologic influences on allergy and the TH1/TH2 balance. *J. Allergy Clin. Immunol.* 113, 395–400.
- Rubin, B.K., Williams, R.W., 2014. Emerging aerosol drug delivery strategies: from bench to clinic. *Adv. Drug Deliv. Rev.* 75, 141–148.
- Scalia, S., Haghi, M., Losi, V., Trotta, V., Young, P.M., Traini, D., 2013. Quercetin solid lipid microparticles: a flavonoid for inhalation lung delivery. *Eur. J. Pharm. Sci.* 49, 278–285.
- Seelinger, G., Merfort, I., Schempp, C.M., 2008. Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. *Planta Med.* 74, 1667–1677.
- Theoharides, T.C., Sieghart, W., Greengard, P., Douglas, W.W., 1980. Antiallergic drug cromolyn may inhibit histamine secretion by regulating phosphorylation of a mast cell protein. *Science* 207, 80–82.
- Theoharides, T.C., Kempuraj, D., Iliopoulou, B.P., 2007. Mast cells, T cells, and inhibition by luteolin: implications for the pathogenesis and treatment of multiple sclerosis. *Adv. Exp. Med. Biol.* 601, 423–430.
- Theoharides, T.C., Valent, P., Akin, C., 2015. Mast cells, mastocytosis, and related disorders. *N. Engl. J. Med.* 373, 163–172.
- Weng, Z., Zhang, B., Asadi, S., Sismanopoulos, N., Butcher, A., Fu, X., et al., 2012. Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. *PLoS One* 7, e33805.
- Wex, E., Thaler, E., Blum, S., Lamb, D., 2014. A novel model of IgE-mediated passive pulmonary anaphylaxis in rats. *PLoS One* 9, e116166.
- Zhang, T., Finn, D.F., Barlow, J.W., Walsh, J.J., 2016. Mast cell stabilisers. *Eur. J. Pharmacol.* 778, 158–168.
- Zeng, W., Wu, C., Dai, Y., 2014. Regulatory effects of luteolin on airway inflammation in asthmatic rats. *Zhonghua Yi Xue Za Zhi.* 94, 2535–2539.
- Zosky, G.R., Larcombe, A.N., White, O.J., Burchell, J.T., Janosi, T.Z., Hantos, Z., 2008. Ovalbumin-sensitized mice are good models for airway hyperresponsiveness but not acute physiological responses to allergen inhalation. *Clin. Exp. Allergy* 38, 829–838.