



Yan-Hou-Qing formula attenuates allergic airway inflammation via up-regulation of Treg and suppressing Th2 responses in Ovalbumin-induced asthmatic mice

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ABSTRACT

Ethnopharmacological relevance: Yan-Hou-Qing (YHQ), a Chinese medicine formula containing fourteen kinds of materials, has been designed for pharyngitis and cough treatment in Oriental medicine. In the present study, the anti-allergic effects and underlying mechanisms of YHQ in inhibition of airway hyper responsiveness (AHR) was explored in an ovalbumin (OVA)-induced allergic asthma mouse model.

Materials and methods: BALB/c mice were sensitized by OVA and cholera toxin (CT) and challenged with OVA intranasally to induce allergic asthma mouse model. YHQ (200 mg/kg) was orally administered for 3 weeks from week-2 after OVA sensitization. The AHR and histological changes of lung tissues were evaluated by whole-body barometric plethysmography analysis and hematoxylin and eosin (H&E) staining, respectively. The serum concentration of OVA-specific IgE and T helper 2 (Th2) cytokines (IL-4 and IL-13) were determined by enzyme-linked immune sorbent assay (ELISA). Flow cytometry was performed to evaluate the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) in the spleen.

Results: The elevated AHR responses, heavier inflammatory cell infiltration and Th2 cytokines in allergic asthma group indicated Ovalbumin-induced asthmatic mouse models were built successfully. Compared to allergic asthma group, OVA-induced AHR responses and eosinophil infiltration in lung were improved significantly, and the productions of OVA-specific IgE and Th2 cytokines, IL-4 and IL-13, in the serum were also reduced dramatically after the treatment of YHQ. Moreover, YHQ treatment significantly increased the percentage of CD4⁺CD25⁺Foxp3⁺ Treg in OVA-induced allergic asthma mouse model.

Conclusions: YHQ improves the allergic asthma related symptoms via promotion of CD4⁺CD25⁺Foxp3⁺ Treg and suppression of Th2 responses in mouse model, suggesting YHQ can be used as a potent agent to alleviate allergic asthma related symptoms.

1. Introduction

The most recent revised global estimate of asthma suggests that as many as 339 million people have asthma worldwide today, and prevalence is rising (GAN, 2018). Asthma is a Th2 lymphocyte-driven disease that is characterized by chronic airway inflammation, airway hyper-responsiveness (AHR), excessive mucus secretion, and remodeling of airway walls. Besides, accumulation of eosinophils in the lungs, enhanced mucus secretion, and elevation of IgE and a number of T helper type2 (Th2) cytokines including interleukin (IL)-4 and IL-13 are commonly found in asthmatics (Bittar et al., 2015). Specifically, IL-

4 and IL-13 synergistically regulate IgG Isotype switching to IgE in B cells, and IL-13 promotes the development of AHR and increases mucus secretion (Murdoch and Lloyd, 2010). The pathogenesis described above leads to bronchial hyper-responsiveness and causes air flow obstruction. On the other hand, steroidal anti-inflammatory drugs are not universally effective in all patients although they are the recommended medication for asthma in clinical practice, and long-term use of steroidal anti-inflammatory drugs can cause complications such as pneumonia, fracture, hyperglycemia and cataract (Akdis, 2012).

The CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) are known to play important roles in the regulation of Th2-induced allergic responses

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Table 1
Formula of YHQ.

Formula YHQ	Amount	Formula YHQ	Amount
<i>L. japonica</i> Thunb.	125 g	<i>S. tonkinensis</i> Gagnep.	25 g
<i>S. ningpoensis</i> Hemsl.	125 g	<i>B. chinensis</i> (L.) DC.	25 g
<i>C. album</i> (Lour.) DC.	125 g	<i>T. sagittata</i> Gagnep.	25 g
<i>S. lychnophora</i> Hance	125 g	<i>P. alkekengi</i> L.	25 g
<i>T. chebula</i> Retz.	125 g	<i>F. cirrhosa</i> D. Don	25 g
<i>P. grandiflorum</i> (Jacq.) A. DC.	125 g	<i>C. camphora</i> (L.) Presl	2.5 g
<i>S. grosvenorii</i> (Swingle) C. Jeffrey ex A.M. Lu & Zhi Y. Zhang	125 g	<i>M. canadensis</i> L.	1.5 g

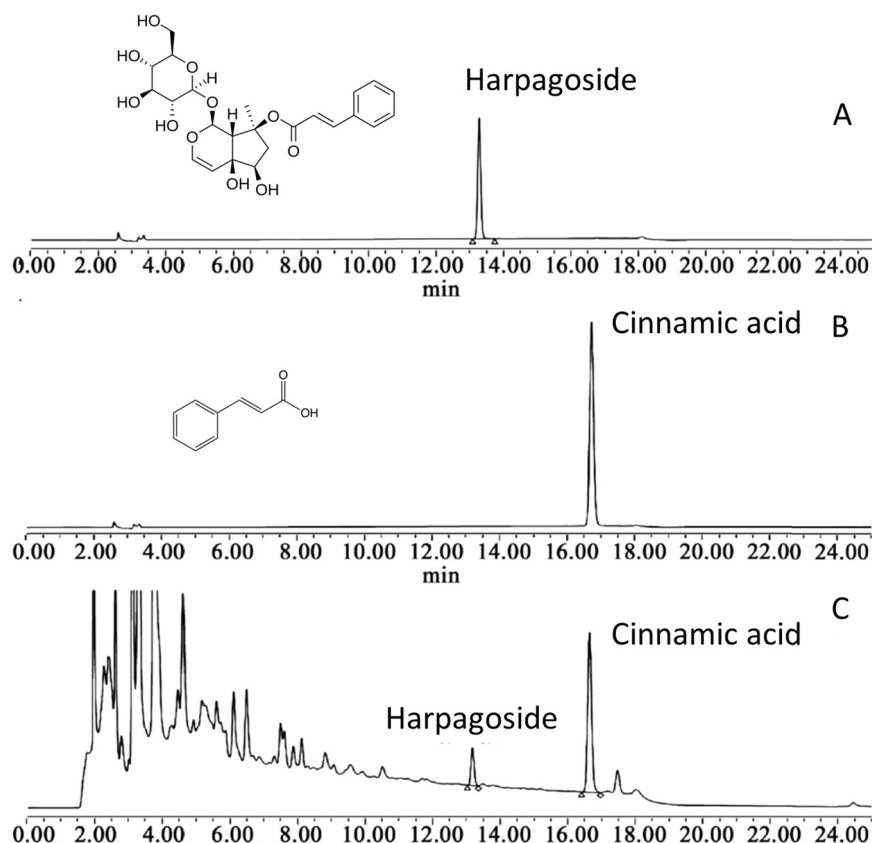


Fig. 1. Representative HPLC-DAD chromatogram (at 279 nm) of the chemical standard of harpagoside (A), cinnamic acid (B) and YHQ formula (C).

(Ray et al., 2010). Treg that express the fork head/winged helix transcription factor (Foxp3) are known to have an anti-inflammatory role and to maintain tolerance to self-components by direct contact with cells or by releasing anti-inflammatory cytokines (Li et al., 2015). It has been reported that the levels of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg were significantly low in pediatric patients bronchial asthma (Lee et al., 2007). Thus, enhancement of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg is an attractive strategy for asthma treatment.

Herbal medicine has a long history of use in humans in Asian countries and is well received by patients (Li and Brown, 2009). Yan-Hou-Qing formula (YHQ) was developed based on the clinical experiences and the principles of traditional Chinese medicine (TCM) by Shenzhen Institute of Ear Nose & Throat (ENT) and Longgang ENT hospital (Qiu, 2007; Qiu et al., 2012). Clinical trials for YHQ has been conducted between 2009 and 2014, and YHQ has been used as a hospital preparations and under the application for approval of China Food and Drug Administration (CFDA) to market a new drug (Li et al., 2016; Yi-Sheng et al., 2017). This formula is composed of 14 species of medicinal herbs (detailed information see Table 1), designed for treating respiratory diseases such as laryngopharyngitis and tonsillitis (Qiu, 2007).

As a type of respiratory diseases, pharyngitis is characterized by a high

inflammatory cell recruitment in airways and other structures of the lung, and the pathogenesis of allergic-related pharyngitis are linked with allergic asthma. However, anti-allergic effect of YHQ still remains unclear, and the therapeutic effects of YHQ pharyngitis and cough inspired us to explore the modulation of YHQ formula on allergic airway hyperresponsiveness. Thus, the effects of YHQ on allergic airway inflammation were investigated via an ovalbumin (OVA)-induced mouse model.

2. Material and methods

2.1. Preparation and quality control of YHQ formula

The botanical origins of these herbal medicines were authenticated by Dr. Yi-Sheng Li, Shenzhen Key Laboratory of ENT, Institute of ENT & Longgang ENT hospital, Shenzhen, China. All voucher specimens (detailed numbers mentioned in following production procedure) were deposited in the specimen room of Shenzhen Key Laboratory of ENT, Institute of ENT, Shenzhen, China.

The production procedures of YHQ formula were performed by Guangzhou Kangyuan Pharmaceutical Co., Ltd (Guangzhou, China) according to the standard operating procedures. Briefly, *L. japonica*

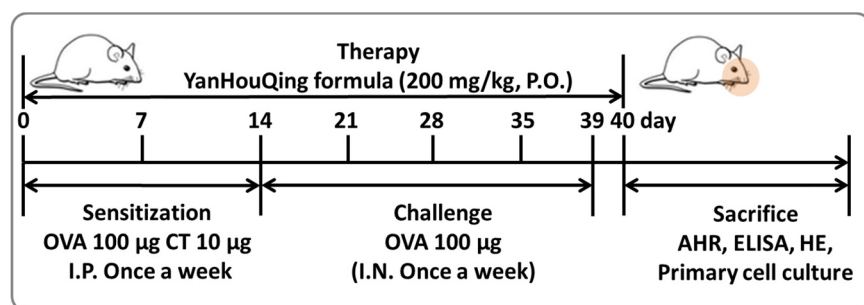


Fig. 2. Schematic diagram of the experimental flow chart. Sensitization: Ovalbumin (OVA) and cholera toxin (CT) via i.p. injection (day 0, 7 and 14). Challenge: OVA via I.N. (day 14, 21, 28, 35 and 39). YHQ and Dexamethasone treatment: Oral administration 2 h prior to OVA challenge. AHR was measured 24 h after the last OVA challenge (day 39). Mice were sacrificed on day 40, and blood, BALF and lung tissues were collected.

Thunb. (No. 2013110201), *S. ningpoensis* Hemsl. Hance (No. 2013110202), *C. album* (Lour.) DC. (No. 2013110203), *S. lychnophora* Hance (No. 2013110204), *T. chebula* Retz. Hance (No. 2013110205) and *S. grosvenorii* (Swingle) C. Jeffrey ex A. M. Lu & Zhi Y. Zhang Hance (No. 2013110206) were extracted with 7.5 L boiling water for 2 h for twice, and then concentrated under reduced pressure at 60 °C. The extract solution of *P. grandiflorum* (Jacq.) A. DC. (No. 2013110207), *B. chinensis* (L.) DC. (No. 2013110208), *S. tonkinensis* Gagnep (No. 2013110209), *T. sagittata* Gagnep. (No. 2013110210) and *P. alkekengi* L. (No. 2013110211) were obtained with aqueous ethanol refluxing using 60% EtOH (1 L × 3, 1 h each time). These two extracts were combined and further spray dried with hot air at 150 °C, and the herbal extract ratio was about 23.3%. Spray dried extract mixed with powder of *F. Cirrhosa* D. Don (No. 2013110212) with addition of powder sweetening agent, and was further granulated spraying with ethanol solution of *M. canadensis* L. (No. 2013110213) and *C. camphora* (L.) Presl. (No. 2013110214). After tablet coating procedures, the Yan Hou Qing formula (YHQ) was finally obtained.

Product quality was monitored by high performance liquid chromatography (HPLC) (Fig. 1). In brief, harpagoside, and cinnamic acid, as the main anti-inflammation components in *S. ningpoensis* Hemsl. was selected as the chemical markers for quality control. Harpagoside and cinnamic acid were prepared in methanol in series of concentrations. 10 tablets of YHQ formula (batch numbers: 140401, 140403, 140403, 140501, 140502, 140503, 140601, 140602, 140603) were ground into powder, extracted with methanol and fixed to 5 ml in volumetric bottle. All the samples were analyzed using the Waters HPLC 2695 system with photodiode array detector (Waters Corporation, MA, USA) with Waters Symmetry C18 column (250 mm × 4.6 mm i.d., 5 µm). The mobile phases consisted of acetonitrile (A) and 0.3% H₃PO₄ (B) in a gradient elution: 20–40% A, 0–15 min; 40–20% A, 15–15.1 min; 20% A, 15.1–25 min. The flow rate was 1.0 ml/min and the column temperature was set at 30 °C. Ten µl of sample was injected and the analytes were monitored with a photodiode array detector (PAD) at the wavelength of 279 nm. The content of harpagoside and cinnamic acid in YHQ formula were 205.5 µg/g (RSD: 0.34%) and 603.6 µg/g (RSD: 0.29%), respectively (Fig. 1).

2.2. Animals

Balb/c mice (female, 6–7 weeks of age weighing 20–25 g) were purchased from Guangdong Medical laboratory Animal Center (Guangzhou, China). All mice were kept under pathogen-free conditions in an air-conditioned environment with a 12 h light/dark cycle. In addition, all mice had free access to food and water during these experiments. This experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) at Shenzhen Institute of ENT (SZENT-2017-007).

2.3. Induction of airway inflammation and animal treatment

The experimental schedule was performed according to the previous study (Bonnegarde-Bernard et al., 2014; Kianmeher et al., 2016).

Briefly, the mice were sensitized by intraperitoneal (i.p.) injections with 100 µg of ovalbumin (OVA) and 10 µg cholera toxin (CT) (Sigma-Aldrich, St. Louis, MO, U.S.A.) on days 0, 7 and 14. Subsequently, mice received an intratracheal (i.t) challenge with OVA (1% OVA in phosphate buffered saline (PBS)) every other day from days 14–39. OVA-sensitized mice were treated with YHQ (200 mg/kg every other day, animal dose is converted according to human equivalent dose based on body surface area) (FDA, 2005) and Dexamethasone (3 mg/kg) by oral administration 2 h before OVA challenge, respectively. The mice in OVA-challenged (Allergic asthma, AA) group were treated only with PBS by oral administration 2 h before OVA challenge. The control (Control) group was treated only with PBS by oral administration without OVA challenge. Mice were analyzed using non-invasive lung function measurements, Buxco Small Animal Whole Body Plethysmography analysis (DSI, MN, U.S.A), to assess airway hyper-responsiveness (AHR) on day 38. Mice were sacrificed and their lung tissues were collected for other analyses on day 39. These experiments were performed three times to clarify that YHQ has significant effects on the attenuation of OVA-induced airway inflammatory responses (Fig. 2).

2.4. Collection of bronchoalveolar lavage fluid (BALF) and quantification of inflammatory cells

BALF was collected from the mice by endotracheal intubation using a catheter followed by lavage with 3 ml of dPBS (GE Healthcare-Hyclone Laboratories, Logan, Utah, U.S.A). After lavage, approximately 2 ml of BALF was recovered, and then BALF was further centrifuged (2000 × g) immediately for 10 min at 4 °C. The cell pellets were re-suspended in PBS, placed on a slide and stained with Giemsa stain (LEAGENE, Beijing, China). Cells with red cytoplasmic granules were counted as eosinophils.

2.5. Measurement of airway hyper-responsiveness (AHR)

Non-invasive measurements of airway responsiveness, Buxco Small Animal Whole Body Plethysmography analysis (DSI, MN, U.S.A), were used in this study. Airway responsiveness was assessed in unrestrained conscious mice 24 h after the last challenge with OVA as described in other studies (Verheijden et al., 2014). Mice were placed in a barometric plethysmography chamber (DSI, MN, U.S.A), and baseline readings were obtained when normal mice challenged with saline. Penh was calculated according to the manufacturers' protocol and is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals. Penh was used as a measure of airway resistance to methacholine. The results were expressed as the percentage increase in Penh after challenge with each concentration of methacholine. Penh values averaged for 3 min after each nebulization were evaluated. The results were expressed as the relative increase in Penh following challenge with each concentration of methacholine (0, 6.25, 12.5, 25, and 50 mg/ml).

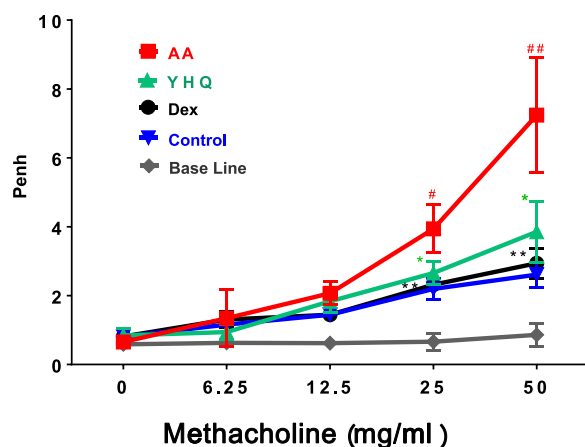


Fig. 3. The effects of YHQ on airway hyper responsiveness in OVA-challenged asthma models. Twenty-four hours after the final intratracheal OVA or PBS administration, whole body barometric plethysmographic analysis was performed to evaluate the inhibitory effects of OVA on airway hyper-responsiveness. The mice were stimulated with increasing doses of aerosolized methacholine (0, 6.25, 12.5, 25 and 50 mg/ml). PBS-challenged mice treated with PBS (Baseline), OVA-challenged mice treated with PBS (Control), OVA-challenged mice (Allergic asthma, AA), OVA-challenged mice treated with YHQ (200 mg/kg) (YHQ), OVA-challenged mice treated with Dexamethasone (3 mg/kg) (Dex). Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted by one-way ANOVA followed by a Tukey's Multiple Comparison test ($\#p < 0.05$ vs. Control, $\##p < 0.01$ vs. Control; $*p < 0.05$ vs. Allergic asthma, $**p < 0.01$ vs. Allergic asthma; $n = 5-6$).

2.6. Measurement of cytokines and OVA-specific IgE in serum

The concentrations of the Th2 cytokines such as IL-4, IL-13 and OVA-specific IgE were measured using a quantitative sandwich enzyme-linked immunoassay kit (R&D, Minneapolis, MN, U.S.A.). All ELISA experiments were strictly performed according to the manufacturer's instruction. Absorbance was measured at 450 nm using SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, CA, U.S.A.).

2.7. Isolation of splenic lymphocyte

Briefly, the whole spleen were dissected out from the mouse, then it was mechanically minced and passed through 70 μ m nylon cell strainer, and layered on Histopaque 1077 density gradient (Sigma, St. Louis, MO, U.S.A) and subjected to density gradient centrifugation for 20 min at 400 $\times$ g. Lymphocyte layers were carefully removed from the buffy layer interface. Cells from this layer were washed twice and grown in RPMI-1640 culture medium (ATCC, U.S.A) supplemented with 10% fetal bovine serum (FBS) (Gibco, U.S.A) in a 5% CO₂ humidified incubator at 37 $^{\circ}$ C.

2.8. Labeling and gating of cells for the flow cytometric analysis

After isolation of lymphocytes, Foxp3 fixation working solution was added, and incubated over night at 4 $^{\circ}$ C in the dark. Fixed cells were spun at 500 $\times$ g for 10 min, and washed twice with 150 μ l permeabilization buffer, then resuspended in 75 μ l of permeabilization buffer and incubated for 30 min at 4 $^{\circ}$ C. Cell staining was performed in round-bottomed 96 well plates via incubating with anti-mouse CD4-APC, anti-mouse CD25-FITC and anti-Foxp3-PE monoclonal antibody (biolegend, San Diego, CA, USA) for 30 min at 4 $^{\circ}$ C. For analysis, desired cell population on dot plot SSC versus CD4 were gated, and further analyzed for CD25 and Foxp3 co-expressions.

2.9. Preparation of lung tissues and histology

Lung tissues were removed from the mice, and the right lower lobes of the lungs were removed for histological analysis. Four percent paraformaldehyde fixing solution was infused into the lungs and the specimens were dehydrated and embedded in paraffin. For histological examination, 4 μ m sections of embedded tissues were cut using a rotary microtome, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (H&E). Images of lung tissue sections stained with H&E were acquired with a Nikon Eclipse Ci-L/S microscope (Nikon, Tokyo, Japan) equipped with a Digital Imaging System (Nikon, Tokyo, Japan).

2.10. Statistical analyses

Data are presented as the mean $\pm$ S.E.M. Analyses of data were performed using the GraphPad Prism software (GraphPad Software 7.01, La Jolla, CA, U.S.A.). The differences between each group were determined via one-way ANOVA followed by Tukey's Multiple Comparison tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Oral administration of YHQ alleviated AHR challenged by methacholine

To determine the inhibitory effects of YHQ on AHR, Buxco Small Animal Whole Body Plethysmography analysis (DSI, MN, U.S.A) was performed. The Penh values increased along with increasing inhaled methacholine. The Penh value (3.94 ± 0.70 , $\#P < 0.05$) of the allergic asthma group was significantly higher than that of control group (2.37 ± 0.02) when inhaled 25 mg/ml methacholine (Fig. 3). The Penh value (6.47 ± 1.45 , $\##P < 0.01$) of the allergic asthma group was significantly higher than that of control group (3.02 ± 0.11) when inhaled 50 mg/ml methacholine. However, there were no significant responses with 0, 6.25, and 12.5 mg/ml of methacholine (Fig. 3). Compared to the allergic asthma group, after challenging with 25 and 50 mg/ml of methacholine, the YHQ treatment reduced Penh values, 2.85 ± 0.06 ($*P < 0.05$) and 3.60 ± 1.08 ($*P < 0.05$) significantly, respectively. While, YHQ group showed Penh values similar to that of the control group and Dexamethasone treated group (Dex) challenged of methacholine at 0, 6.25, 12.5, 25 and 50 mg/ml (Fig. 3). These results indicate that YHQ can relieve AHR in allergic asthma mouse model.

3.2. Oral administration of YHQ abrogated the infiltration of inflammatory cell induced by OVA

Lungs were lavaged at the end of experimentation, and the inflammatory cells in BALF were counted to evaluate the inhibitory effect of YHQ on the influx of inflammatory cells. As shown in Fig. 4a, total inflammatory cell numbers ($618.66 \pm 60.80 \times 10^3$, $\###P < 0.001$) in the OVA group were markedly increased compared with that ($101.67 \pm 27.89 \times 10^3$) of the normal group. Eosinophilic inflammation is a hallmark of asthma and, as expected, treatment with YHQ (200 mg/kg) reduced the number of total cells (from $618.66 \pm 60.80 \times 10^3$ (AA) to $420.00 \pm 44.38 \times 10^3$ (YHQ), $*P < 0.05$) including eosinophils (from $333.33 \pm 22.74 \times 10^3$ (AA) to $195 \pm 27.91 \times 10^3$ (YHQ), $*P < 0.05$) in BALF (Fig. 4b). These data indicated that YHQ treatment inhibited airways cellular infiltration in OVA-induced asthmatic mouse models.

To observe whether YHQ exerted effects on OVA-challenged lung tissue damage and inflammatory cell infiltration into airways, lung tissue sections were stained with H&E. Histological sections of lung tissue from mice that were challenged with OVA exhibited airway inflammation and were found to have heavy infiltration of inflammatory

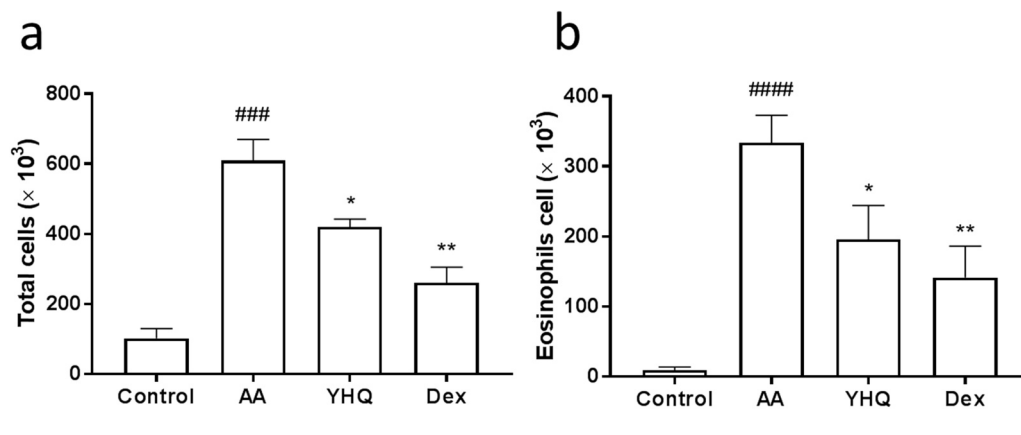


Fig. 4. Effects of YHQ on the numbers of total inflammatory cells and eosinophils in BALF of OVA-induced allergic asthmatic mice. Mice were administered with YHQ (200 mg/kg) and Dex (3 mg/kg) as described in materials and methods. BALF was collected at 24 h after the last OVA challenge to measure the numbers of total cells (a) and eosinophils (b). Data are presented as the means $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Tukey's Multiple Comparison test ($n = 5$ or 6 in each group). ^{###} $P < 0.001$, ^{####} $P < 0.0001$ vs. control group; ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs. AA group.

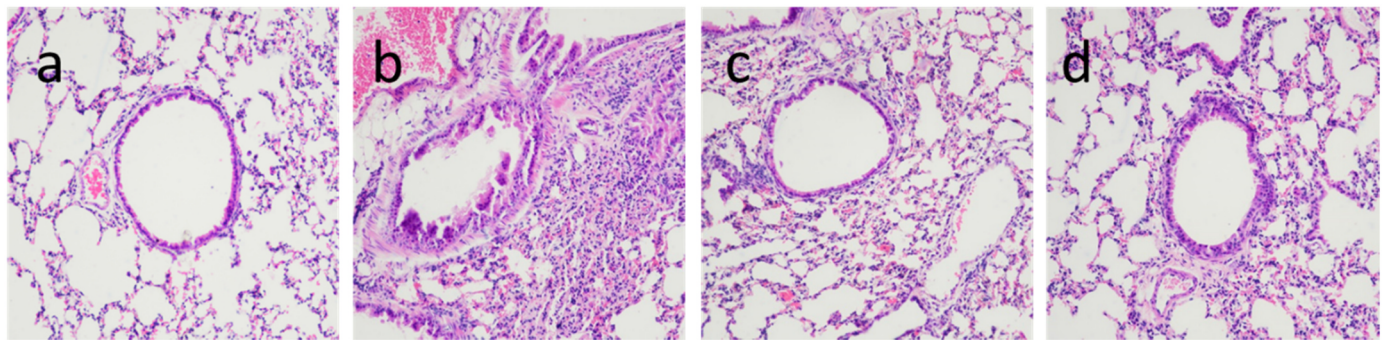


Fig. 5. Effect of YHQ on the recruitment of inflammatory cells to lung tissues in murine model of asthma exacerbation. Lung tissue slices were fixed, embedded, and stained with hematoxylin and eosin (H & E) ($100\times$ magnification). Representative photomicrographs from each group ($n = 6$ per group) were shown as follows: (a) Normal mice (Control group), (b) OVA-induced allergic asthma mice, (c) YHQ treated allergic asthma mice, (d) Dexamethasone treated allergic asthma mice. Arrows indicate areas with peribronchial cell infiltration.

cells in the peribronchial regions of the lung in allergic asthma group. Changes observed in lung tissue sections indicated that the YHQ (200 mg/kg) treated group displayed less inflammatory cells in the peribronchial regions when compared to the allergic asthma group (Fig. 5).

3.3. YHQ decreased Th2 cytokine production in mouse serum

ELISA was used to evaluate differences in the expression of inflammatory molecules such as the Th2 cytokines IL-4 and IL-13 between the control, allergic asthma and YHQ treatment group. IL-4 ($##P < 0.01$) and IL-13 ($##P < 0.01$) were augmented significantly in OVA-induced allergic asthma group when compared to the control group, and these up-regulated IL-4 and IL-13 were decreased after treatment with YHQ and Dexamethasone (detailed data see Fig. 6).

Importantly, the treatment of YHQ (200 mg/kg) significantly suppressed the secretion of IL-4 ($*P < 0.05$) and IL-13 ($*P < 0.05$) in serum when compared to the allergic asthma group. Our data demonstrate that there were strong inhibition effect 200 mg/kg of YHQ on the secretion of IL-4 and IL-13. Most of all, treatment with doses of 200 mg/kg YHQ showed a potent suppressive effect on the secretion of Th2 cytokines that was similar to the control group (IL-4 ($P > 0.05$) and IL-13 ($P > 0.05$)) (Fig. 6).

3.4. The inhibition effect of YHQ on production of OVA-specific IgE in sera

Allergic asthma is characterized by an increase in circulating allergen-specific IgE (sIgE), and OVA-specific IgE is an important component in OVA-induced allergic asthma model. Therefore, serum OVA-specific IgE level was measured in OVA-challenged mice (allergic

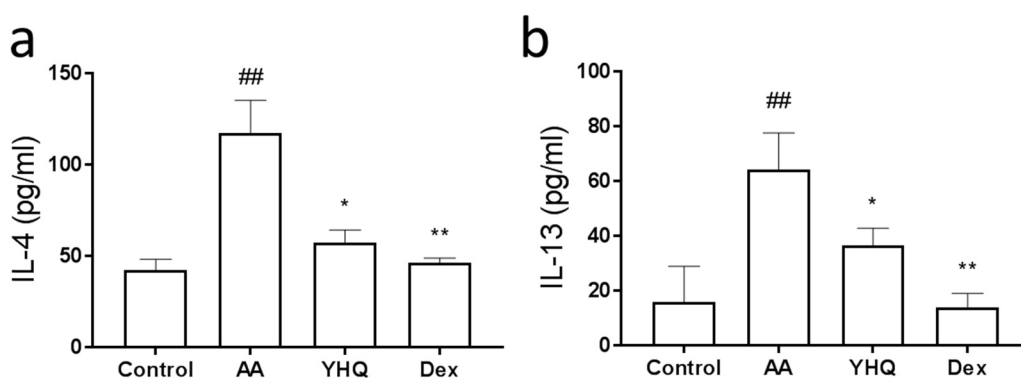


Fig. 6. The effects of YHQ on IL-4 and IL-13 levels in serum. PBS-challenged mice treated with PBS (Control), OVA-challenged mice (Allergic asthma, AA), OVA-challenged mice treated with YHQ (200 mg/kg) (YHQ), OVA-challenged mice treated with Dexamethasone (3 mg/kg) (Dex). (a) Levels of IL-4; (b) Levels of IL-13. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Tukey's Multiple Comparison test ($##p < 0.01$ vs. Control; $*p < 0.05$, $**p < 0.01$ vs. Allergic asthma; $n = 5-6$).

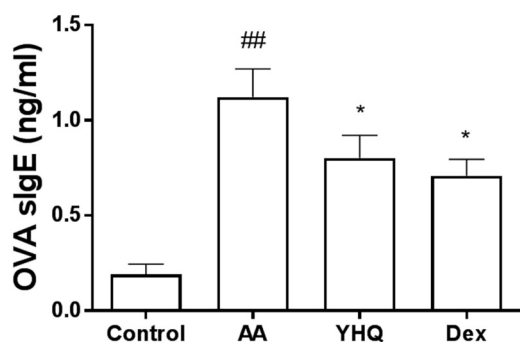


Fig. 7. Serum levels of OVA-specific IgE. PBS-challenged mice treated with PBS (Control), OVA-challenged mice (Allergic asthma, AA), OVA-challenged mice treated with YHQ (200 mg/kg) (YHQ), OVA-challenged mice treated with Dexamethasone (3 mg/kg) (Dex). Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Tukey's Multiple Comparison test (## $p < 0.01$ vs. Control, * $p < 0.05$ vs. AA; $n = 5-6$).

asthma group) and the PBS (control group), YHQ treated group and Dexamethasone treated group (Dex). The OVA-specific IgE level in serum significantly increased in the allergic asthma group (# $P < 0.01$) when compared to the control group, indicating that the induction of allergic airway inflammation was successful, and the concentration of serum IgE levels, similar to that in Dexamethasone treated group, were significantly reduced in the YHQ (200 mg/kg) treated mice (* $P < 0.05$), which suggest that YHQ treatment could reduce the OVA specific reaction in OVA-induced asthma model (Fig. 7).

3.5. YHQ promoted the generation of $CD4^+CD25^+Foxp3^+$ T cells from mouse spleen in vivo

Since $Foxp3^+$ regulatory T cells (Treg) play an important role in regulation of Th2-related inflammatory response, allergen-specific Treg induction has been recently considered as one of attractive strategies for allergic asthma therapy. In the current study, the Treg percentage drop from 6.33% in control group to 3.45% (# $P < 0.05$) in allergic asthma group, and these phenomena were reversed with Treg percentage upregulated to 9.91% and 11.2% (** $P < 0.001$, compared to allergic asthma group) after the treatment of YHQ and Dexamethasone, respectively. In the current study, YHQ treatment significantly increased the number of $CD4^+CD25^+Foxp3^+$ cells in spleen compared with OVA-induced allergic asthma group (Fig. 8). As supporting evidences for our study, allergen-specific $CD4^+CD25^+$ T cells was associated with inhibition of AHR and Th2 inflammation in a murine model of allergic airways disease, and Treg attenuated allergic immune responses in a mouse model of OVA-induced allergic airway disease (Preston et al., 2011). Here we demonstrate that YHQ herbal formula can stimulate $CD4^+CD25^+Foxp3^+$ Treg to alleviate allergic asthma related symptoms.

4. Discussion

Asthma is characterized by airway obstruction attributable to hypertrophy of airway smooth muscle and inflammation within the airway, resulting in decreased lung function (Papi et al., 2018). Although corticosteroids and β_2 -agonists are effective in managing asthma symptoms, there is no curative therapy. The chronic nature of asthma and the lack of preventive and curative therapy are leading patients with asthma in Western societies to seek complementary and Traditional Chinese medicine (TCM). TCM has a long history of human use and it has a unique system of theory and diagnosis and treatment tools. Chinese herbal medicines are one of the major components in TCM practice and are prescribed in hospitals in China as either

monotherapy or complementary therapy to conventional Western therapy. The modulation of Chinese medicine asthma related symptoms have been studied prevalently, and accumulating evidences suggest that Chinese medicine can improve the asthma symptoms and attenuate the inflammation (Zhou et al., 2014; Chen et al., 2016; Kao et al., 2018; Li and Brown, 2009; Wang et al., 2014). In contrast to the long human use history and popularity of TCM herbal medicines for asthma, evidence-based research into their efficacy and mechanisms of efficacy is still in its infancy. Although a number of antiasthma herbal formulas are recorded in TCM textbooks and used in TCM practice only a few have been studied.

YHQ, the crystallization of experience of clinical doctors, contains 14 kinds of herbal medicines (Table 1), and individual herbs involved in YHQ have been documented to have various biological effects (Table 2). In addition, chlorogenic acid and iridoid derivatives from flower buds of *L. japonica* Thunb. exhibit allergy-preventive effects (Oku et al., 2011). In another study, saponins from *P. grandiflorum* (Jacq.) A. DC. inhibit the dinitrophenyl (DNP)-IgE antibody-induced systemic PCA reaction in mice and reduced the β -hexosaminidase and histamine release from anti-DNP-IgE-sensitized RBL-2H3 cells (Han et al., 2009). Irisflorelin from *B. chinensis* (L.) DC. modifies properties of mouse bone marrow-derived dendritic cells and reduces the allergic contact hypersensitivity responses (Fu et al., 2015). The therapeutic potency of YHQ should be attributed to its combined and synergistic effects on multiple targets as a result of its diverse components.

AHR is a measure of the bronchial constriction commonly observed in individuals with asthma, and the effects of YHQ on AHR were evaluated by measuring Penh in this study. Our results demonstrated that YHQ inhibited the OVA-induced AHR in response to inhaled methacholine challenge-induced AHR. The migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation (Papi et al., 2018). The present results clearly demonstrate that YHQ significantly reduces eosinophil infiltration in lung tissue.

Accumulating evidences indicated that the underlying mechanism that drives the asthmatic inflammatory response appears to be attributable to Th2-type immune responses (Fahy, 2015). Recently, regulatory T cells (Treg) cells have been described to play an important role in regulating the immune response, especially on the B cells IgE-Class-switching and production of OVA-specific IgE (Ray et al., 2010). After treatment with YHQ, the ratio of $CD4^+CD25^+Foxp3^+$ T cell in CD4 T cell in OVA + CT induced allergic asthma were elevated significantly, and Th2 cytokines (IL-4 and IL-13) were decreased significantly. Th2 cytokines (IL-4 and IL-13) are involved in IgG Isotype switching to IgE in the pathogenesis of allergy. Binding of IL-4/IL-13 to receptors on B cell surface member further phosphorylate signal transducer and activator of transcription 6 (STAT6), which activates transcription at the IgE isotype-specific S ϵ switch region. Treatment with YHQ lowered the concentrations of IL-4 and IL-13 which are consistent with depressed OVA-sIgE. Treg can reduce inflammation and allergic reactions through their inhibitory functions. Many studies documented that the diminished levels of Treg result in increased airway hyperreactivity, suggesting that Treg is involved in the pathophysiology of asthma (Martín-Orozco et al., 2017). Given the capacity of Treg to regulate or suppress other disease-promoting cells in the immune system, enhancing Treg function in patients represents an attractive therapeutic strategy for allergic asthma treatment. In this study, our data showed that YHQ significantly decreased the levels of Th2 cytokines, IL-4 and IL-13, which further interrupted the process of IgE-Class-switching in B cells and suppressed OVA-specific IgE production. What's more, treatment of YHQ promoted the expression of $CD4^+CD25^+Foxp3^+$ T cells in mouse spleen significantly. Here we demonstrate that YHQ herbal formula can induce Treg differentiation to further alleviate allergic asthma related symptoms. Taken together, our findings support the possible use of YHQ as a therapeutic agent for allergic asthma patients.

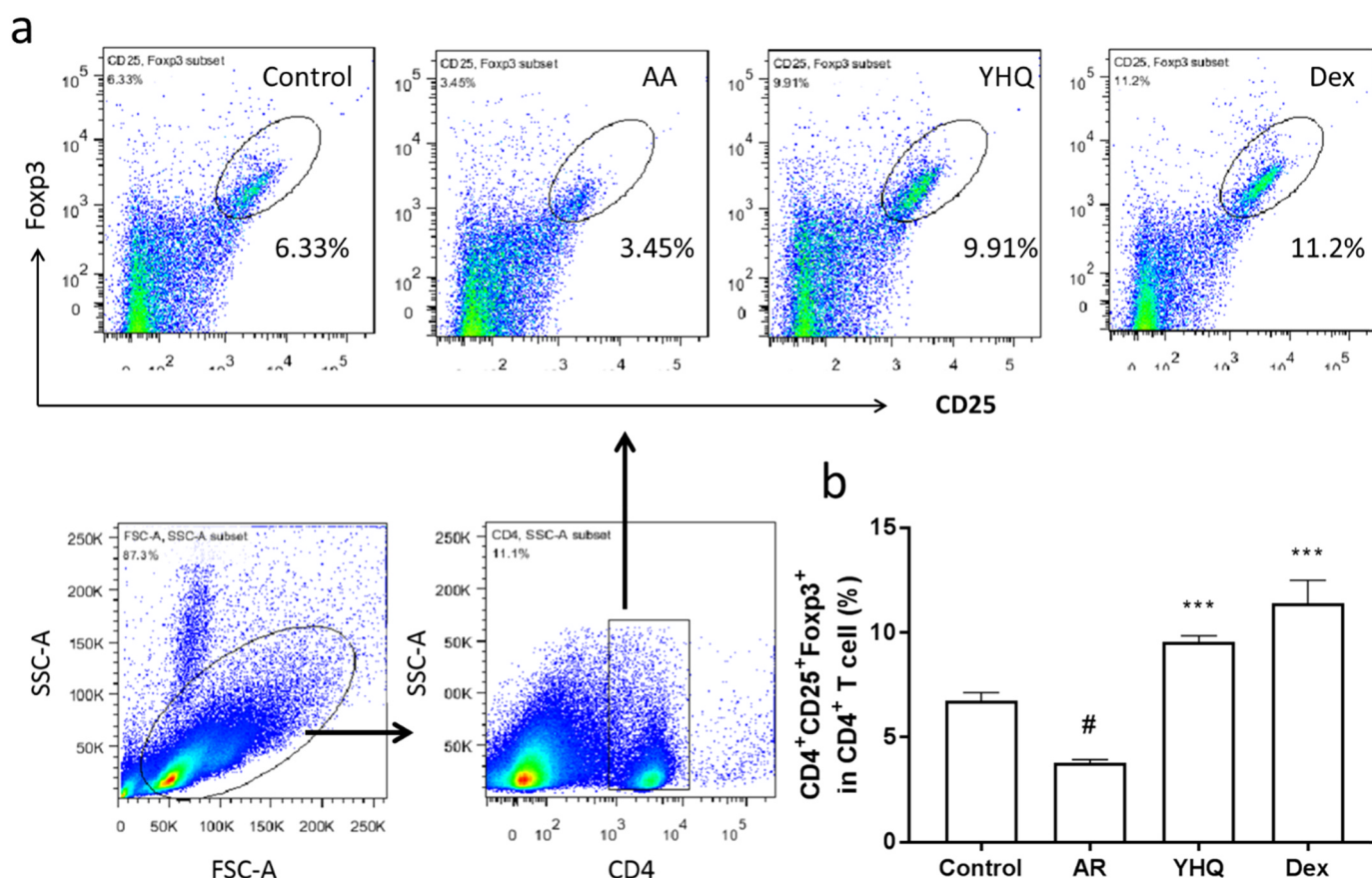


Fig. 8. Flow cytometric analysis of the percentage of CD25⁺Foxp3⁺ T (Treg) cells in the CD4⁺ T cell population in each group. (a) Typical CD25⁺Foxp3⁺ Treg in the CD4⁺ T cell population in Normal mice (Control group), OVA-induced allergic asthma mice (AA), YHQ (200 mg/kg) treated allergic asthma mice (YHQ), and Dexamethasone (3 mg/kg) treated allergic asthma mice (Dex); (b) the bars indicate the percentage of CD25⁺Foxp3⁺ Treg in the CD4⁺ T cell population in each group. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Tukey's Multiple Comparison test (# $p < 0.05$ vs. Control; *** $p < 0.001$ vs. Allergic asthma; $n = 5-6$).

Table 2

Main biological components in YHQ formula.

Species	Main components	Activity
<i>L. japonica</i> Thunb. <i>S. ningpoensis</i> Hemsl.	Chlorogenic acid, Isochlorogenic acid. Harpagoside, Harpagide, Scrophularianine.	Anti-inflammation, antibacterial, anti-oxidant, antiviral (Shang et al., 2011). Anti-inflammation, antimicrobial, anti-asthma, anti-diabetic (Behl and Kotwani, 2017).
<i>C. album</i> (Lour.) DC. <i>S. lychnophora</i> Hance	Gallic acid, Scopoletin, Scopolone Sterculin, Betulinic acid.	Antioxidant and antiglycation (Kuo et al., 2015). Anti-inflammation, antimicrobial, treatment for laryngopharyngitis diseases (El-Sherei et al., 2016).
<i>T. chebula</i> Retz.	Gallic acid, Arjunic acid, Rutin, Quercetin dihydrate, Quercetin-3-O-rhamnoside.	Anti-HIV, antimicrobial, antibacterial, antifungal (Mongalo et al., 2016).
<i>S. grosvenorii</i> (Swingle) C. Jeffrey ex A. M. Lu & Zhi Y. Zhang <i>P. grandiflorum</i> (Jacq.) A. DC.	Mogroside, Neomogroside, Quercetin, Siratic acid, Kaempferol. Platycodin, Favoplatycoside, Lutelin-7-O-glucoside, Quercetin-7-O-rutinoside.	Anti-tussive, phlegm-expelling, and dyspnea-relieving functions, immune-modulation, liver protection, anti-diabetic effects (Li et al., 2014). Apophlegmatic, antitussive, anti-inflammation, anti-tumor, and anti-diabetic (Zhang et al., 2015).
<i>B. chinensis</i> (L.) DC.	Irisflorentin, Dichotomitin, irigenin, tectorigenin	Anti-tussive, anti-asthmatic, antibacterial, anti-inflammation, antioxidant, and antidiabetic activity (Zhang et al., 2016).
<i>S. tonkinensis</i> Gagnep <i>T. sagittata</i> Gagnep. <i>P. alkekengi</i> L.	Senepodine A-G, Formononetin. Palmatine, Jatrorrhizine. Physalin, Luteolin, Phytolaccin.	Antibacterial, anti-oxidant, and anti-inflammation (Yoo et al., 2017). Anti-inflammation, antibacterial, antimicrobial (Yu et al., 2007). Anti-inflammation, antibacterial, anti-tumor, immunomodulation (Li, A.-L. et al., 2018).
<i>F. cirrhosa</i> D. Don <i>M. canadensis</i> L.	Puquienine, Puquedione, Imperialine, Verticine. Menthol, Menthone.	Antitussive, anti-inflammation, antibacterial (Wang et al., 2016). Anti-oxidative, anti-histamine, antimicrobial, anti-parasitical and insecticidal (Farzaei et al., 2017).
<i>C. camphora</i> (L.) Presl	1,8-cineole, Carene, β -isobiotol, Camphor.	Anti-asthmatic, antibacterial, anthelmintic, anti-inflammation, (Li, Y.-R. et al., 2018; Singh and Jawaidd, 2012).

In summary, the treatment of YHQ attenuated airway inflammation and bronchial hyperresponsiveness with suppression of OVA-specific IgE, IL-4 and IL-13 and reduced recruitment of eosinophils in mice with

OVA-induced asthma. Moreover, YHQ effectively recovered percentage of CD4⁺CD25⁺Foxp3⁺ Treg in lymphocytes in mouse spleen, which may play a vital role in the process of ameliorating symptoms of allergic

asthma. Our findings support the possible use of YHQ as a therapeutic drug for patients with allergic asthma.

5. Conclusions

YHQ suppressed airway hyper responsiveness, eosinophil infiltration, secretions of serum Th2 cytokines and OVA-specific IgE production in OVA challenged mouse asthma model. Moreover, YHQ significantly increased the population of CD4⁺CD25⁺Foxp3⁺ Treg expression in mouse spleen of allergic asthma. Thus, our findings suggest that YHQ can be used as a potent immunoregulatory agent for allergic asthma prevention and treatment via enhancement of Treg and inhibition of Th2 responses.

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