Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 79 (2016) 185−194

www.jcma-online.com

Original Article

Antiallergic effect of Gami-hyunggyeyeongyotang on ovalbumin-induced allergic rhinitis in mouse and human mast cells

Yong-Seok Im, Bina Lee, Eun-Young Kim, Ju-Hee Min, Dea-Uk Song, Jeong-Min Lim, Ji Whan Eom, Hyun Joo Cho, Youngjoo Sohn, Hyuk-Sang Jung*

College of Korean Medicine, Kyung Hee University, Seoul, South Korea

Received September 22, 2014; accepted August 23, 2015

Abstract

Background: Gami-hyunggyeyeongyotang (GMHGYGT) is a polyherbal medicine derived from an oriental prescription traditionally used in the treatment of allergic diseases such as allergic rhinitis (AR). This study aimed to evaluate the effects of GMHGYGT on ovalbumin (OVA) sensitization/challenge-induced AR in BALB/C mice, through examination of allergic inflammatory response regulation, as well as examination of human mast cells (HMC-1).

Methods: Nasal symptoms were evaluated in the OVA-induced allergic rhinitis mouse model, and total immunoglobulin (Ig)E and OVA-specific IgE levels in serum were investigated. Eosinophil infiltration and thickness of the nasal mucosa, and levels of interleukin (IL)-1β and caspase-1 were also measured by immunohistochemistry. Additionally, the effect of GMHGYGT on the phorbol-12-myristate-13-acetate plus calcium ionophore A23187-induced phosphorylation of extracellular signal-regulated kinase, C-Jun N-terminal kinase and p38 in HMC-1 cells was investigated.

Results: GMHGYGT was demonstrated to have antiallergic effects on the nasal symptoms of the OVA-induced mouse model, decreasing serum levels of OVA-specific IgE and levels of the cytokines IL-5, IL-6, IL-1β, monocyte chemotactic protein-1, and macrophage inflammatory protein-2. GMHGYGT reduced the number of eosinophils in the nasal mucosa and thickness of the nasal septum, and inhibited the expression of IL-1β and caspase-1. Moreover, it inhibited the phosphorylation of extracellular signal-regulated kinase and C-Jun N-terminal kinase, as well as the activation of nuclear factor-κB on protein level in HMC-1 cells.

Conclusion: These results suggest that GMHGYGT has therapeutic potential for the treatment of allergic rhinitis.

Keywords: allergic rhinitis; cytokines; Gami-hyunggyeyeongyotang; immunoglobulin E; nasal symptom; nuclear factor κB

1. Introduction

Allergic rhinitis (AR) is an inflammatory disease of the nasal mucosa.[1,2] It induces an immunoglobulin (Ig)E-mediated reaction resulting from inflammation of the airway mucosa with hypersensitivity caused by seasonal or perennial responses to specific allergens.[3,4] Approximately 500 million people are affected by AR around the world, and it presents with symptoms of sneezing, itching, and respiratory obstruction causing pain.[1,5] Besides these symptoms, AR can also lead to other inflammatory diseases such as asthma, rhinosinusitis, allergic conjunctivitis, otitis media with effusion, nasal polyp, tubal dysfunction, and adenoid hypertrophy.[6]

AR is caused by allergic mediators such as mast cells, inflammatory cytokines, eosinophils, and histamine.[7] They have been recognized to play a key role in the inflammatory reaction, as the mediators interact with each other to cause acute inflammatory reactions against an allergen upon exposure.[8] At the

Conflict of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Hyuk-Sang Jung, College of Korean Medicine, Kyung Hee University, 26, Kyungheedae-ro, Dongdaemun-gu, Seoul, 130-701, South Korea.

E-mail address: jhs@khu.ac.kr (H.-S. Jung).

http://dx.doi.org/10.1016/j.jcma.2015.08.012

beginning of the AR reaction, mast cells combine with immunoglobulin (Ig)E receptors on the basophil surface, which can trigger the release of inflammatory cytokines and histamine.[9] These steps contribute to increasing the recruitment of inflammatory cells and eosinophil migration to affected tissue.[10]

Even though there are multiple treatments available for AR such as medical treatment, immunotherapy, or surgery, the rates of side effects and recurrence are high after stopping medication.[11] Therefore, alternative treatments with improved drug stability are needed.[12] Herbal medicines have been used for the traditional treatment of various allergic diseases to maintain immune balance. Hence, many studies aiming to find an appropriate treatment for AR have focused on traditional medicines, which have fewer side effects and are more stable than the currently used drugs.[13]

Gami-hyunggyeyeongyotang (GMHGYGT) is a polyherbal medicine derived from the Chinese traditional prescription hyunggyeyeongyotang (HGYGT) for the treatment of runny nose, sneezing, and congestion.[14] Some experimental studies have reported that HGYGT and GMHGYGT had effects on fever, pain, and edema.[15] Previous reports also indicated that HGYGT has anti-allergy and anti-inflammatory effects, as well as experimental effects on histamines and serotonin.[16] However, the anti-allergy effect and mechanism of GMHGYGT remain unknown. The present study aimed to evaluate the effect of GMHGYGT on ovalbumin (OVA) sensitization/challenge-induced AR in BALB/C mice through regulation of the allergic inflammatory response. Nasal symptoms were evaluated in an OVA-induced allergic rhinitis mouse model. Cytokine, total IgE, and OVA-specific IgE levels in the serum were investigated to verify the effects of GMHYGYT. Eosinophil infiltration and thickness of the nasal mucosa, and levels of interleukin (IL)-1β and caspase-1 were also measured via immunohistochemistry. To provide more evidence for verification of the anti-AR mechanism, mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and inhibitor of NF-κB (IκB-α) were examined through western blotting in HMC-1 cells *in vitro*.

2. Methods

2.1. Reagents

Phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 were purchased from Sigma−Aldrich (St. Louis, MO, USA). Aqueous nonradioactive cell proliferation assay from Promega Corporation (Madison, WI, USA), Iscove's Modified Dulbecco's Medium and fetal bovine serum from Gibco BRL (Grand Island, NY, USA), and streptomycin from Invitrogen (Carlsbad, CA, USA). Antibodies to phosphorylated extracellular signal-regulated kinase (ERK), ERK, phosphorylated p38, p38, phosphorylated C-Jun N-terminal kinase (JNK) and JNK were purchased from Cell Signaling Technology (Danvers, MA, USA), to NF-κB, IκB-α, Lamin B, and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA), peroxidase IgG from Jackson ImmunoResearch (West Grove, PA, USA), and to caspase-1 and IL-1β from Santa Cruz Biotechnology. Chemical reagents diaminobenzidine tetrachloride, $NiCl_2 \cdot H_2O$, Triton™ X-100, and methyl Green were obtained from Sigma−Aldrich.

2.2. Preparation of GMHGYGT

Herbal components of GMHGYGT were purchased from Omni Herb Inc. (Andong-si, Gyeongbuk, Korea). The substances and composition of GMHGYGT are presented in Table 1. All herbs were authenticated by Professor Youngmin Bu, a medical botanist in the Department of Herbology, College of Korean Medicine, Kyung Hee University, Seoul, Korea; additionally, voucher specimens were preserved at Kyung Hee University. Herbs used in GMHGYGT were mixed according to the ratios listed in Table 1. Dried GMHGYGT was extracted as follows. Mixed GMHGYGT (208 g) was boiled in 2.1 L distilled water (95−100°C) for 2 hours, and filtered using Whatman filter paper No. 3 (Maidstone, Kent, UK). The filtered extract was concentrated in a rotary vacuum evaporator then lyophilized to yield 34 g of dried powder (yield ratio

Table 1
Contents of Gami-hyunggaeyeongyotang (GMHGYGT).

Herbal medicine	Scientific name	Voucher specimen number	Amount Used (g)
Schizonepetae Spica	*Schizonepeta tenuifolia* Briquet	A051	3
Forsythia Fructus	*Forsythia viridissima* Lindley	KHY01	3
Saposhnikovia Radix	*Saposhnikovia divaricata* Schiskin	KHY02	3
Angelicae Radix	*Angelica a cutiloba* kitagawa	KA015	3
Cnidii Rhizoma	*Cnidium officinale* Makino	A061	3
Paeoniae Radix alba	*paeonia lactiflora* pallas	A028	3
Angelicae dahuricae Radix	*Angelica dahurica Bentham* et Hooker	KHY03	3
Bupleuri Radix	*Bupleurum falcatum* Linne	A011	3
Scutellariae Radix	*Scutellaria baicalensis* Georgi	A027	3
Aurantii Fructus Immaturus	*Citurus aurantium* Linne	KHY05	2
Gardeniae Frutus	*Gardenia jasminoides* Ellis	KHY06	3
Platycodi Radix	*Platycodon grandiflorum* A. De candole	KHY09	3
Glycyrrhizae Radix	*Glycyrrhiza uralensis* Fischer	KHY010	3
Ulmi Cortex	*Ulmus macrocarpa* Hance	KHY04	6
Xanthii Fructus	*Xanthium strumarium* Linne	A057	4
Magnoliae Flos	*Magnolia kobus* De Candolle	KHY07	2
Rubiae Radix	*Rubia akane* Nakai	KHY08	2

15.4%). The product was maintained at a temperature of −20°C until use. The chromatogram patterns of high-performance liquid chromatography (HPLC) was shown in Fig. S1.

2.3. Animals

Six-week-old female BALB/c mice were purchased from Nara Biotech (Gangnam-gu, Seoul, Korea). All animal experiments were approved according to guidelines of the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-13-006). The mice were housed under controlled temperature (23 ± 3°C) with a relative humidity of 40−60% and 12 hour light/dark cycles. Food and water were provided *ad libitum*.

2.4. Elicitation and group classification of AR model

The 6-week-old female BALB/C mice were divided into four groups ($n = 8$ per group): (1) control group; (2) OVA sensitized (OVA) group; (3) GMHGYGT group; and (4) cetirizine-treated (Cet) group. After the mice were stabilized for 1 week, they were OVA-sensitized by intraperitoneal injection of 50 μg OVA (chicken egg albumin; Sigma) in 200 μL of phosphate-buffered saline (PBS) containing 2 mg aluminum hydroxide (Alum; Sigma) on Day 0, Day 7, and Day 14. One week after the last injection on Day 21, the mice were challenged with 20 μL PBS containing 50 μg/mL OVA into the bilateral nasal cavities. From Day 21 to Day 31, mice in the Control and OVA groups were given saline by peroral administration, and those in the Cet group received 10 mg/kg cetirizine hydrochloride (Allertec Tab; Korean Drug, Seoul, Korea), and the GMHGYGT group was orally administered 134 mg/kg GMHGYGT 1 hour before intranasal challenge of OVA under the same conditions. Cetirizine, a second-generation antihistamine, is a major metabolite of hydroxyzine and a racemic selective H1 receptor inverse agonist used in the treatment of allergies, angioedema, and urticaria.[17] In this study, cetirizine was used as a positive control.

2.5. Nasal symptom evaluation

The mice (6 weeks old, female) were administrated OVA intranasally, and the nasal symptoms were evaluated 2 minutes later by counting the time of nasal rubbing and number of sneezing events for 10 minutes. This procedure was carried out for 10 days starting from Day 21 (Fig. S2). Three hours after the last observation, the mice were anesthetized with sodium pentobarbital. The blood was then collected via cardiac puncture, and the nasal mucosa and tissue were taken and stored at −20°C until use.

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

Blood of the mice from each experimental group was collected via cardiac puncture. The blood was centrifuged to obtain serum, from which the total IgE and OVA-specific IgE were quantified using an enzyme-linked immunosorbent assay (ELISA).

2.7. Measurement of cytokines in the serum

To demonstrate the effect of GMHGYGT on allergic responses in the AR model, the levels of IL-5, IL-6, IL-1β, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 were measured using a mouse cytokine/chemokine magnetic bead panel kit (EMD Millipore, Billerica, MA, USA). The plate was washed with 200 μL wash buffer for 10 minutes at room temperature, and then 25 μL standard solution and assay buffer were added to the appropriate wells. The standard serum matrix solution (25 μL) was added to the background well. Diluted serum was mixed with assay buffer, and 25 μL of beads was added to all wells. The plate was then covered by foil, and placed at room temperature for 20 minutes. Next, 25 μL of detection antibody was added to the wells and reacted for 1 hour at room temperature, after which streptavidin−phycoerythrin were added to each well and mixed for 30 minutes. After washing twice, 150 μL sheath fluid was added to the wells and mixed for 5 minutes. The plate was measured with the Luminex 200 System (Luminex, Austin, TX, USA).

2.8. Histological evaluation

The heads of the mice were fixed using 10% formalin, then the nasal tissue was separated from the muscle and skin of the head. Nasal tissue was decalcified in 10% EDTA buffer for 14 days. The tissue was embedded in paraffin and sectioned to 5 μm, and the slides were stained with hematoxylin and eosin. The infiltration of eosinophils and thickness of the nasal mucosa were observed.

2.9. Immunohistochemical staining

AR tissue was tested by immunohistochemical staining. AR tissue sections were washed three times with PBS for 5 minutes, then reacted with 0.3% H_2O_2 for 10 minutes. Tissue was reacted for 1 hour in blocking solution, which was a mixture of PBS with 10% normal serum (Jackson Immuno Research). The tissue was then washed three times, and treated with primary antibody overnight at 4°C. Caspase-1 and IL-1β (Santa Cruz Biotechnology) were used as the primary antibodies in a solution with a mixture of bovine serum albumin (GenDepot, Katy, TX, USA) and Triton X-100 with PBS. After reaction, the tissue was incubated with peroxidase IgG for 1 hour. It was then reacted with diaminobenzidine tetrachloride (Sigma) mixed with $NiCl_2 \cdot H_2O$ (Sigma). Finally, the tissue was stained with Methyl Green as a counter stain.

2.10. HMC-1 cell culture

HMC-1 cells were provided by Prof. H.M. Kim (Department of Pharmacology, Kyung Hee University). The HMC-

1 cell line is useful for studying cytokine activation pathways in immediate allergic reactions.[18,19] HMC-1 cells were incubated in Iscove's Modified Dulbecco's Medium with 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum at 37°C, 5% CO_2, and 95% humidity.

2.11. MTS assay for cell viability

Cell viability was tested by the MTS colorimetric assay. MTS assay is using conversion of the tetrazolium salt into a colored aqueous soluble formazan, produced by mitochondrial activity of viable cells. HMC-1 cells (1×10^5) were plated in 96-well plates with various GMHGYGT concentrations (0.1 μg/mL, 1 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL), and the cells were incubated for 24 hours in a 37°C incubator. MTS solution (2 mg/mL) was added to each well, and the cells were placed in an incubator for an additional 2 hours at 37°C. The optical density of the 96-well culture plates was measured at 490 nm with an ELISA reader. The formazan optical density of the untreated control cells was deemed to represent 100% viability.

2.12. Western blot analysis

HMC-1 cells (5×10^6) were treated with various GMHGYGT concentrations (1 μg/mL, 10 μg/mL, and 100 μg/mL) for 1 hour, stimulated with PMA and A23187, and incubated for an additional 2 hours for nuclear proteins, and 30 minutes for MAPKs. Western blot analysis was performed on the cell extracts. Harvested cells were treated by a detergent lysis procedure using lysis buffer to obtain the cell extract [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), KCl, $MgCl_2$, dithiothreitol, phenylmethanesulfonyl fluoride, NaCl glycerol, EDTA]. Samples were heated at 95°C for 5 minutes and centrifuged. Proteins were then resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, then electrotransferred to nitrocellulose membranes. The membrane were blocked for 1 hour with 1 L phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) containing 5% skimmed milk, and then incubated with primary antibodies overnight at 4°C. Blots were incubated with peroxidase-conjugated secondary antibodies at room temperature, and the antibody-specific proteins were visualized by an enhanced chemiluminescence procedure, using an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.13. Statistical analysis

The data of symptom scores, eosinophil infiltration in nasal mucosa, expression of caspase-1 and IL-1β were analyzed by use of nonparametric Kruskal–Wallis analysis of variance and Mann–Whitney *U* test. The data of IgE level, proinflammatory cytokines and *in vitro* assay were analyzed for statistical significance by one-way analysis of variance, followed by Dunnett's multiple comparison test. Statistical analysis was presented using the GraphPad PRISM Software (Graphpad Software Inc., La Jolla, CA, USA). Data were presented as mean ± standard error of the mean, and $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of GMHGYGT on behavior in rhinitis mouse model

From Day 21, the frequency of rubbing was measured in the OVA-induced rhinitis model. The result indicated that there were 150 ± 5.79 events in the OVA group; a significant increase from the 100.4 ± 7.22 events observed in the Control group. The rubbing value of the Cet group was 55.8 ± 6.13, while that of the GMHGYGT group was 76.6 ± 6.31. The GMHGYGT group demonstrated a significant decrease compare with the OVA group (Fig. 1A and B).

The number of sneezing events was counted for 10 days. In the Control group, 31.4 ± 4.08 events occurred, while 88.2 ± 9.05 were observed in the OVA group. Again, the number in the OVA group was significantly higher than in the Control group. The number of sneezes was only 16.4 ± 0.94 in the Cet group and 25.8 ± 3.61 in the GMHGYGT group; also representing significant decreases compared to the OVA group (Fig. 1C and D).

As a preliminary study to determine the most effective dose of GMHGYGT on the OVA-induced AR murine model, we tested the inhibitory effect of GMHGYGT in two doses (13.4 mg/kg and 134 mg/kg). The number of sneezing or nasal rubbing events was reduced by GMHGYGT treatment in a dose-dependent manner (Fig. S3).

3.2. Effect of GMHGYGT on IgE and OVA-specific IgE levels in serum of the AR model

The total serum IgE level of the OVA group (2652.3 ± 154.6 ng/ml) was higher than in the Control group (1076.5 ± 144.4 ng/ml). The group administered cetirizine showed a significant decreased compared to the OVA group. IgE production was inhibited in the GMHGYGT group (2341.7 ± 160.7 ng/ml; Fig. 2A). The serum OVA-specific IgE level was 12.10 ± 1.46 Unit/ml in the OVA group, which was significantly higher than in the Control group (4.84 ± 0.83 Unit/ml). The serum IgE level in the Cet group (7.11 ± 0.14 Unit/ml) and GMHGYGT group (7.38 ± 1.16 Unit/ml) both decreased significantly compared with the OVA group (Fig. 2B).

3.3. Effect of GMHGYGT on cytokine and chemokine in serum of AR model

The serum concentrations of IL-5, IL-6, IL-1β, MCP-1, and MIP-2 in the OVA group were also significantly higher than the Control group (Table 2). IL-5, IL-6, IL-1β, MCP-1, and MIP-2 levels of the GMHGYGT group were significantly reduced compared with the OVA group. For the Cet group, only the levels of MCP-1 and MIP-2 were significantly lower than in the OVA group.

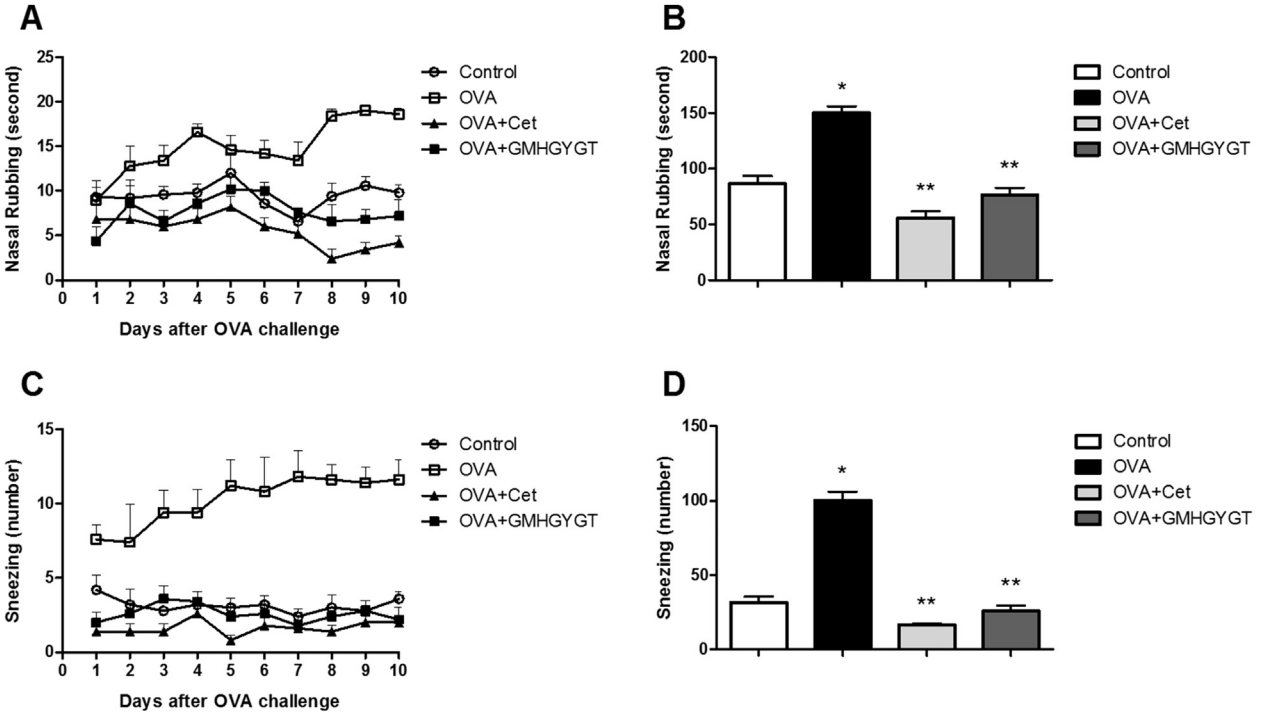

Fig. 1. Time course of the development of nasal rubbing and sneezing induced by antigen in mice sensitized with OVA and aluminum hydroxide. Clinical scores such as sneezes and nasal rubs were measured for 10 minutes after the last intranasal challenge from Day 21 to Day 30. (A) Time of nasal rubbing each day. (B) Total time of nasal rubbing for 10 days. (C) Number of sneezes each day. (D) Total number of sneezes for 10 days. Data represent the mean ± standard error. *$p < 0.01$, significantly different from the Control group. **$p < 0.01$, significantly different from the OVA group. OVA = ovalbumin.

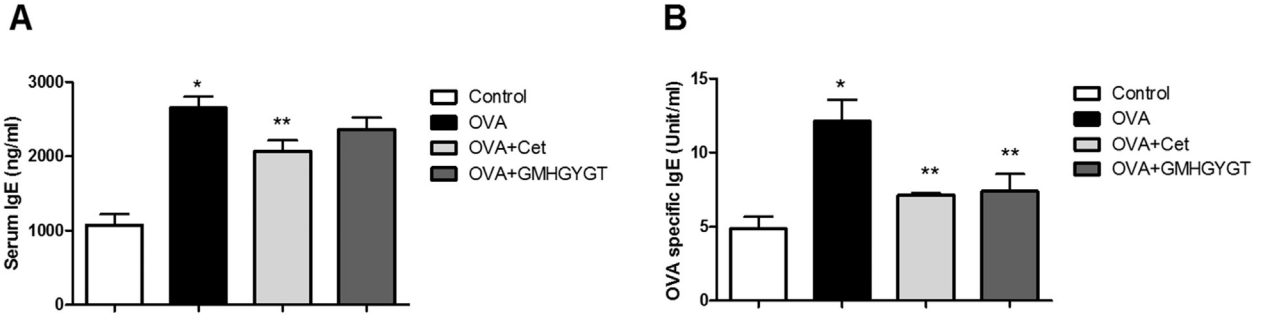

Fig. 2. Effect of Gami-hyunggyeyeongyotang on IgE and OVA-specific IgE levels in serum of the allergic rhinitis model. (A) Total serum IgE levels. (B) OVA-specific IgE levels in serum. Total IgE and OVA-specific IgE were measured by enzyme-linked immunosorbent assay. Columns and error bars represent the mean ± standard error. *$p < 0.01$, significantly different from the Control group. **$p < 0.05$, significantly different from the OVA group. IgE = immunoglobin E; OVA = ovalbumin.

Table 2
Effects of GMHGYGT on cytokine serum levels of allergic rhinitis model.

	Control	OVA	OVA+Cet	OVA+GMHGYGT
IL-5 (pg/mL)	12.81 ± 1.81	26.55 ± 2.64[a]	42.30 ± 4.78	11.71 ± 71[c]
IL-6 (pg/mL)	7.52 ± 1.45	26.75 ± 2.52[a]	32.75 ± 5.54	16.14 ± 2.85[b]
IL-1β (pg/mL)	4.26 ± 0.43	6.24 ± 0.60[a]	5.11 ± 0.60	4.47 ± 0.45[b]
MCP-1 (pg/mL)	19.71 ± 2.74	33.50 ± 3.03[a]	21.33 ± 3.19[c]	15.22 ± 2.02[c]
MIP-2 (pg/mL)	44.87 ± 7.59	78.45 ± 5.23[a]	57.21 ± 6.73[b]	60.24 ± 6.32[b]

Data represent mean ± standard error.
[a]$p < 0.01$, significantly different from the Control group. [b]$p < 0.05$ and [c]$p < 0.01$, significantly different from the OVA group.

3.4. Histological changes of the nasal mucosa and infiltration of eosinophils

The number of infiltrated eosinophils in the OVA group was significantly higher than in the Control group. Regarding the Cet and GMHGYGT groups, the numbers were significantly reduced compared with the OVA group (Fig. 3A and B). The nasal mucosa thickness of the OVA group was 17.8 ± 2.7 μm; also demonstrating a significant increase compared with the Control group (10.8 ± 1.3 μm). The Cet group demonstrated a thickness of 10.8 ± 1.5 μm, while the GMHGYGT group had a thickness of 9.8 ± 1.8 μm. Both groups showed significantly decreased nasal mucosa thickness (Fig. 3A and C).

3.5. Immunohistochemistry of nasal mucosa and expression of caspase-1 and IL-1β

Immunohistochemistry was performed to measure the levels of caspase-1 and IL-1β expression in the OVA-induced AR mouse model. Caspase-1 expression in the nasal septum of the GMHGYGT group was significantly lower than in the OVA group (Fig. 4), while the decrease in IL-1β expression was not significant.

3.6. Effect of GMHGYGT on PMA plus A23187-stimulated MAPK activation

To measure the MAPKs of mast cells treated with GMHGYGT, western blotting was performed using HMC-1 cells. Phosphorylated-ERK and phosphorylated-JNK were inhibited

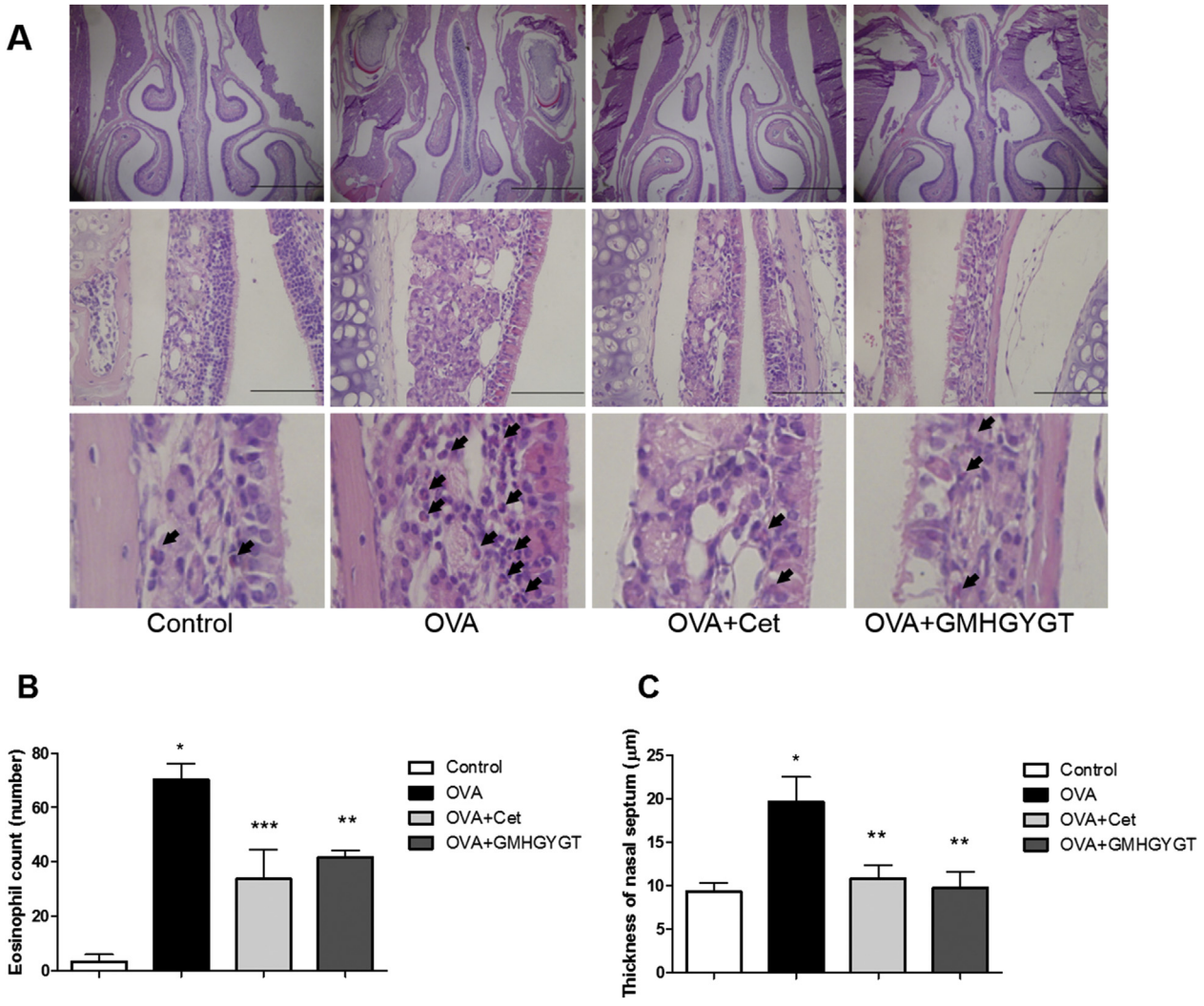

Fig. 3. Histological changes of nasal mucosa and infiltration of eosinophils (arrows) in the nasal mucosa of the allergic rhinitis model. (A) Thickness of the nasal septum in nasal mucosa (magnification: 40×, 400×, and 1000×). Lane 3 marked eosinophil infiltration. Eosinophil infiltration to the nasal mucosa was determined by staining with Harris hematoxylin. OVA + Cet and OVA +GMHGYGT groups were orally treated with cetirizine hydrochloride (10 mg/kg body weight) or GMHGYGT (134 mg/kg), respectively, 1 hour before intranasal challenge with OVA. Control and OVA groups were treated with distilled water. Columns and error bars represent the mean ± standard error. *$p < 0.01$, significantly different from the Control group. **$p < 0.05$ and ***$p < 0.01$, significantly different from the OVA group. Cet = cetirizine; GMHGYGT = Gami-hyunggyeyeongyotang; OVA = ovalbumin.

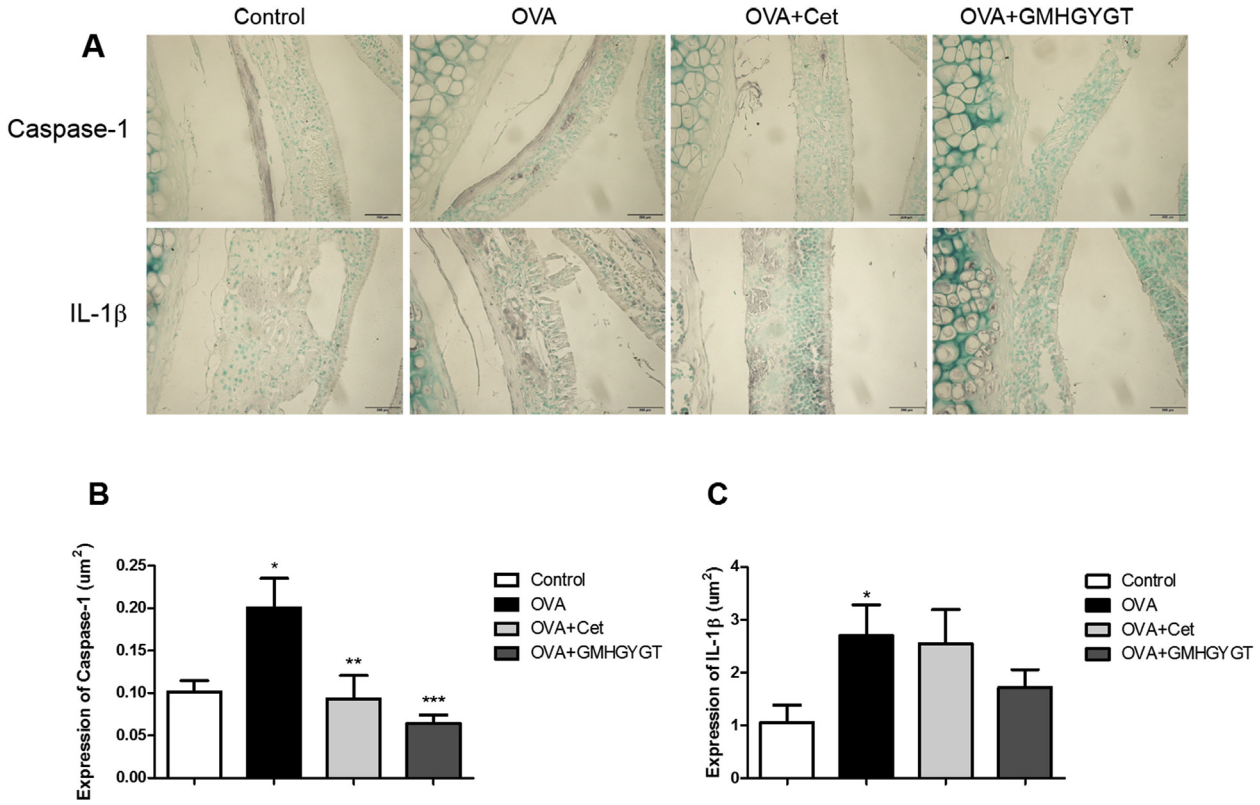

Fig. 4. Inhibitory effects of Gami-hyunggyeyeongyotang on caspase-1 and IL-1β in the nasal mucosa of the allergic rhinitis mouse model. The areas of the nasal septum stained violet (A: magnification: 400×) indicate caspase-1 and IL-1β expression. Nasal mucosa was stained with diaminobenzidine and counter stained with methyl green. Expression of caspase-1 and IL-1β in the nasal tissue was measured using Image J software. Columns and error bars represent the mean ± standard error. *$p < 0.01$, significantly different from the Control group. **$p < 0.05$ and ***$p < 0.01$, significantly different from the OVA group. IL = interleukin.

by the GMHGYGT concentration of 100 μg/mL (Fig. 5). In contrast, phosphorylated-p38 had no significant change.

3.7. Effect of GMHGYGT on PMA plus A23187-stimulated NF-κB activation and IκB-α degradation

To measure the NF-κB activation and IκB-α degradation in mast cells treated with GMHGYGT, western blotting was performed with HMC-1 cells. GMHGYGT inhibited the degradation of IκB-α in a concentration-dependent manner. GMHGYGT significantly decreased IκB-α degradation compared with the OVA group at a concentration of 100 μg/mL. Moreover, GMHGYGT also demonstrated concentration-dependent inhibition of NF-κB, with significant reduction at a concentration of 100 μg/mL compared to the OVA group (Fig. 6).

4. Discussion

In the present study, the effect of GMHGYGT treatment on an OVA-induced AR mouse model was examined. GMHGYGT effectively ameliorated the allergic symptoms and suppressed the production of total and OVA-specific serum IgE. It also inhibited the expression of IL-5, IL-6, IL-1β, MCP-1, and MIP-2 in serum of AR mice. In addition, suppression of the NF-κB signaling pathway was observed in HMC-1 cells treated with GMHGYGT.

The allergic inflammatory response is initiated within minutes of allergen exposure and is primarily due to the release of mediators by mast cells, including cytokines (IL-4, IL-5, and IL-6), chemotactic factors, and enzymes.[9] The net effect of these mediators is to produce the early symptoms of AR, and to stimulate the production and adhesion of circulating leukocytes, especially eosinophils, as well as their infiltration into the local tissue.[20] OVA sensitization and challenge in animal models leads to an increase in the OVA-specific IgE in plasma and infiltration of inflammatory cells in epithelium and subepithelium of the nasal mucosa.[21] In the present study, GMHGYGT remarkably inhibited the symptoms of rhinitis including the nasal rubbing time, number of sneezing events, and the increase in thickness of the nasal septum. In addition, the number of infiltrated eosinophils and the nasal mucosa thickness were significantly lower in the GMHGYGT group compared with the OVA group. These results demonstrated that GMHGYGT alleviated the allergic immune response in the AR model by reducing the infiltration of eosinophils.

Exposure to allergen initiates a cascade of biochemical and cellular events, resulting in the synthesis and release of IgE. This IgE-mediated response is marked by the T helper 2 immunological response, with mast cell and eosinophil

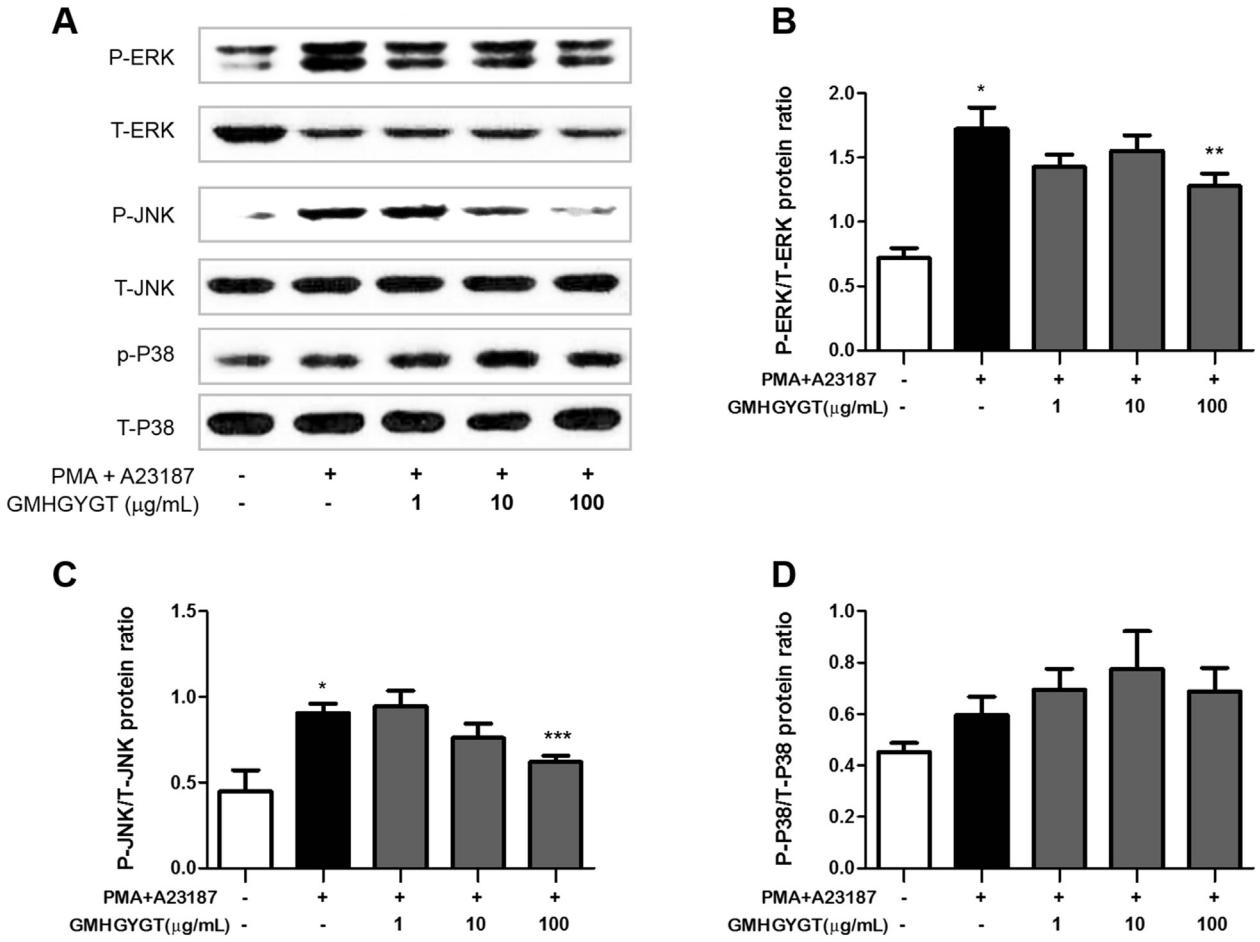

Fig. 5. Effect of GMHGYGT on PMA plus A23187-stimulated MAPK activation. HMC-1 cells were pretreated with GMHGYGT for 1 hour and then stimulated by 30 minutes incubation with PMA and A23187. The relative levels of expression of the MAPKs were measured using Image J. Columns and error bars represent the mean ± standard error. *$p < 0.01$, significantly different from normal. **$p < 0.05$ and ***$p < 0.01$, significantly different from PMA and A23187 alone. GMHGYGT = Gami-hyunggyeyeongyotang; MAPK = mitogen-activated protein kinase; PMA = phorbol 12-myristate 13-acetate.

expression.[20] In this study, GMHGYGT decreased the total IgE, with significant reduction of the OVA-specific IgE. This indicated that GMHGYGT inhibited the allergic response, especially through the reduction of OVA-specific IgE.

T helper 2 cells produce cytokines such as IL-5, IL-6, MCP, and MIP, which induce hypersensitivity of eosinophils and mast cells, and the production and differentiation of B lymphocytes.[22] IL-5 regulates the growth and differentiation of eosinophils, and serves as an essential signal for their movement into inflammatory tissue upon antigen exposure.[23] IL-6 has the important role of inducing cell generation, growth, survival, and migration during inflammatory reaction.[24] MCP and MIP are the key factors that affect the movement or activation of eosinophils and neutrophils.[25,26] In this study, the GMHGYGT group appeared to have significant reduction in the serum levels of IL-5, IL-6, MCP-1, and MIP-2 compared to the OVA-induced group. These results suggest that GMHGYGT might influence the production of IL-5, IL-6, MCP-1, and MIP-2 during the process of antigen-specific IgE production.

The activation of caspase-1 can induce inflammation. Caspase-1 contains an N-terminal caspase recruitment domain that is capable of activating NF-κB associated with inflammatory responses.[27] Caspase-1 contributes to the activation of IL-1β by separating IL-1β from precursors.[28] IL-1β plays a role as a highly inflammatory cytokine, and is considered the host immune defensor.[29] In the present study, GMHGYGT considerably decreased the expression of caspase-1 and IL-1β in the nasal mucosa tissue, as observed through immunohistochemistry analysis. These results suggest that GMHGYGT can attenuate allergic inflammation by suppressing the expression of IL-1β and caspase-1.

Mast cells are activated by the synthesis of cytokines that can damage the target inflammatory tissue.[9] In this study, the MAPKs cascade and NF-κB pathway were investigated via protein levels in HMC-1 cells. MAPKs including ERK, JNK, and p38 are responsible for various functions including the proliferation, differentiation, and apoptosis of cells, cellular response to cytokines and stress control.[30] ERK is essential for cell proliferation and differentiation in the signaling pathways. JNK and p38 are induced by environmental stresses and growth factors, and are known to promote cell growth inhibition, inflammatory response, and apoptosis.[31–33] In the

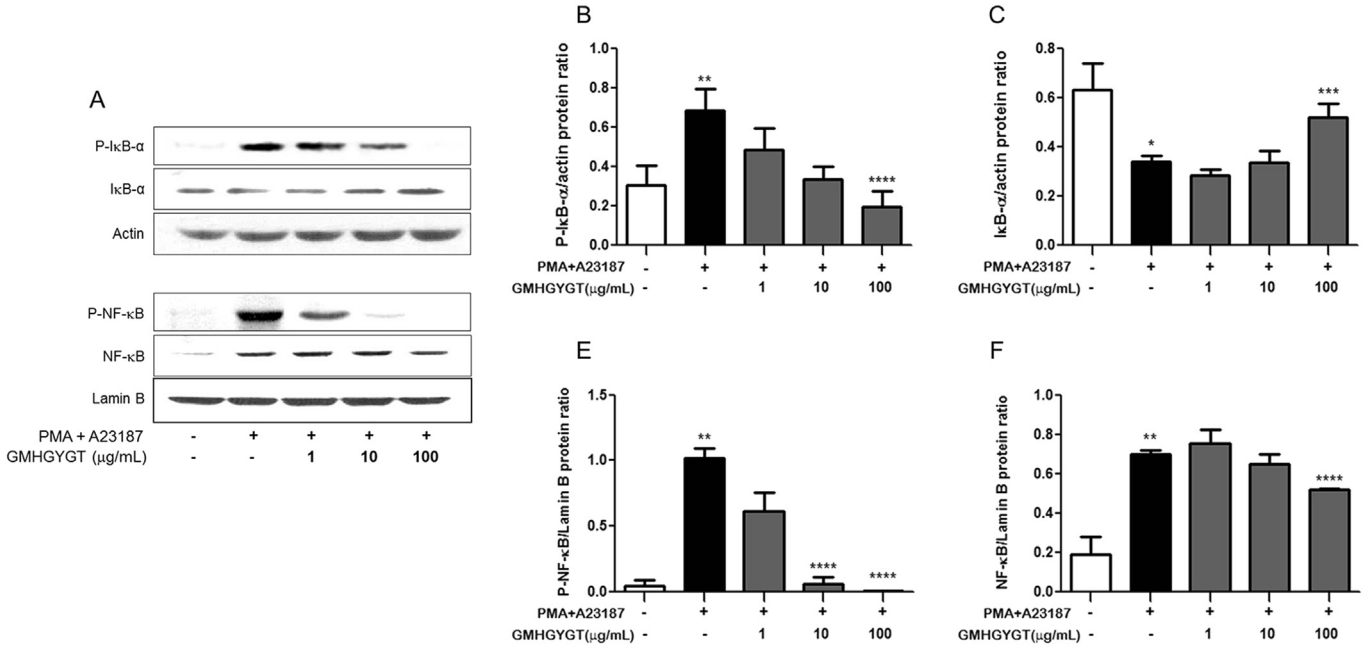

Fig. 6. Effect of GMHGYGT on PMA plus A23187-stimulated NF-κB and phosphorylated-NF-κB activation, along with IκB-α and p-IκB-α degradation. HMC-1 cells were pretreated with GMHGYGT for 1 hour and stimulated by 2 hours incubation with PMA and A23187 (A, D). The relative expression levels of IκB-α, phosphorylated-IκB-α, NF-κB and phosphorylated-NF-κB were measured using Image J (B, C, E, F). Columns and error bars represent the mean ± standard error. $*p < 0.05$ and $**p < 0.01$, significantly different from normal. $***p < 0.05$ and $****p < 0.01$, significantly different from PMA and A23187 alone. GMHGYGT = Gami-hyunggyeyeongyotang; IκB = inhibitor of NF-κB; NF-κB = nuclear factor-κB; PMA = phorbol 12-myristate 13-acetate.

present study, GMHGYGT inhibited expression of phosphorylated-ERK and phosphorylated-JNK. This means that GMHGYGT inhibited the inflammatory reaction in mast cells involved in the production of inflammatory cytokines through blocking the phosphorylation of ERK and JNK. In addition, GMHGYGT inhibited the phosphorylation of NF-κB. These results indicate that GMHGYGT could regulate the expression of proinflammatory cytokines through the inactivation of phosphorylated-IκB and translocation of NF-κB into the nucleus.

In conclusion, our study demonstrated GMHGYGT to have an antiallergic effect by improving the symptoms of rhinitis, and inhibiting the release of allergic mediators in the OVA-induced AR model. In addition, GMHGYGT has anti-inflammatory activity by suppressing the production of inflammatory cytokines such as IL-5, IL-6, IL-1β, MCP-1, and MIP-2, via the ERK, JNK, and NF-κB pathways in HMC-1 cells. These results suggest that GMHGYGT might be a good therapeutic drug for the treatment of allergic rhinitis.

Acknowledgments

This work was supported by Hamsoa Pharmaceutical Co. R&D Center (Seoul, South Korea) (20121084).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcma.2015.08.012.

References

1. Oh HA, Kim HM, Jeong HJ. Beneficial effects of chelidonic acid on a model of allergic rhinitis. *Int Immunopharmacol* 2011;**11**:39−45.
2. Shin JH, Kang JM, Kim SW, Cho JH, Park YJ, Kim SW. Effect of oral tolerance in a mouse model of allergic rhinitis. *Otolaryngol Head Neck Surg* 2010;**142**:370−5.
3. Ren J, Deng Y, Xiao B, Wang G, Tao Z. Protective effects of exogenous surfactant protein A in allergic rhinitis: a mouse model. *Ann Otol Rhinol Laryngol* 2013;**122**:240−6.
4. Lee NP, Arriola ER. How to treat allergic rhinitis. *West J Med* 1999;**171**:31−4.
5. Pawankar R, Bunnag C, Khaltaev N, Bousquet J. Allergic rhinitis and its impact on asthma in Asia Pacific and the ARIA update 2008. *World Allergy Organ J* 2012;**5**:S212−7.
6. Shinmei Y, Yano H, Kagawa Y, Izawa K, Akagi M, Inoue T, et al. Effect of Brazilian propolis on sneezing and nasal rubbing in experimental allergic rhinitis of mice. *Immunopharmacol Immunotoxicol* 2009;**31**:688−93.
7. Howarth PH, Persson CG, Meltzer EO, Jacobson MR, Durham SR, Silkoff PE. Objective monitoring of nasal airway inflammation in rhinitis. *J Allergy Clin Immunol* 2005;**115**:S414−41.
8. Hayashi T, Fujii T. Acute urticaria[corrected]-like lesions in allergen-unexposed cutaneous tissues in a mouse model of late allergic rhinitis. *Int J Exp Pathol* 2008;**89**:188−200.
9. Church MK, Levi-Schaffer F. The human mast cell. *J Allergy Clin Immunol* 1997;**99**:155−60.
10. Lee BJ, Naclerio RM, Bochner BS, Taylor RM, Lim MC, Baroody FM. Nasal challenge with allergen upregulates the local expression of vascular endothelial adhesion molecules. *J Allergy Clin Immunol* 1994;**94**:1006−16.
11. Meltzer EO, Bukstein DA. The economic impact of allergic rhinitis and current guidelines for treatment. *Ann Allergy Asthma Immunol* 2011;**106**:S12−6.

12. Jung HW, Jung JK, Park YK. Antiallergic effect of Ostericum koreanum root extract on ovalbumin-induced allergic rhinitis mouse model and mast cells. *Asian Pac J Allergy Immunol* 2011;**29**:338−48.
13. Jung HW, Jung JK, Park YK. Comparison of the efficacy of KOB03, ketotifen, and montelukast in an experimental mouse model of allergic rhinitis. *Int Immunopharmacol* 2013;**16**:254−60.
14. Gong JH. *Wan Bing Hui Chun*. Seoul: Bubin; 2007. p. 624−31.
15. Shin SY, Park EJ. Effects of Hyunggyeyungyotang and Kami-hyunggyeyungyotang administration on the anti-inflammation, analgesia and anti-allergic reaction in mice. *J Pediatr Korean Med* 1997;**11**:249−73.
16. Yu TS, Jin YS, Jung GM. Study of the effects of Hyunggaeyeungyotang on the anti-allergic effect in rats and mice. *J Pediatr Korean Med* 1990;**4**:19−30.
17. Lehman JM, Blaiss MS. Selecting the optimal oral antihistamine for patients with allergic rhinitis. *Drugs* 2006;**66**:2309−19.
18. Kim SH, Jun CD, Suk K, Choi BJ, Lim H, Park S, et al. Gallic acid inhibits histamine release and pro-inflammatory cytokine production in mast cells. *Toxicol Sci* 2006;**91**:123−31.
19. Balletta A, Lorenz D, Rummel A, Gerhard R, Bigalke H, Wegner F. Human mast cell line-1 (HMC-1) cells exhibit a membrane capacitance increase when dialysed with high free-Ca(2+) and GTPgammaS containing intracellular solution. *Eur J Pharmacol* 2013;**720**:227−36.
20. Quraishi SA, Davies MJ, Craig TJ. Inflammatory responses in allergic rhinitis: traditional approaches and novel treatment strategies. *J Am Osteopath Assoc* 2004;**104**:S7−15.
21. Bahekar PC, Shah JH, Ayer UB, Mandhane SN, Thennati R. Validation of guinea pig model of allergic rhinitis by oral and topical drugs. *Int Immunopharmacol* 2008;**8**:1540−51.
22. Aversa G, Punnonen J, Cocks BG, de Waal Malefyt R, Vega Jr F, Zurawski SM, et al. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. *J Exp Med* 1993;**178**:2213−8.
23. Kim YH, Park CS, Lim DH, Son BK, Kim JH, Ahn SH, et al. Antiallergic effect of anti-Siglec-F through reduction of eosinophilic inflammation in murine allergic rhinitis. *Am J Rhinol Allergy* 2013;**27**:187−91.
24. Hirano T. Interleukin 6 and its receptor: ten years later. *Int Rev Immunol* 1998;**16**:249−84.
25. Curran CS, Bertics PJ. Lactoferrin regulates an axis involving CD11b and CD49d integrins and the chemokines MIP-1alpha and MCP-1 in GM-CSF-treated human primary eosinophils. *J Interferon Cytokine Res* 2012;**32**:450−61.
26. Kaplan AP, Kuna P, Reddigari SR. Chemokines as allergic mediators − relationship to histamine-releasing factors. *Allergy* 1994;**49**:495−501.
27. Lamkanfi M, Kalai M, Saelens X, Declercq W, Vandenabeele P. Caspase-1 activates nuclear factor of the kappa-enhancer in B cells independently of its enzymatic activity. *J Biol Chem* 2004;**279**:24785−93.
28. Boost KA, Hoegl S, Hofstetter C, Flondor M, Stegewerth K, Platacis I, et al. Targeting caspase-1 by inhalation-therapy: effects of Ac-YVAD-CHO on IL-1 beta, IL-18 and downstream proinflammatory parameters as detected in rat endotoxaemia. *Intensive Care Med* 2007;**33**:863−71.
29. Kleinnijenhuis J, Joosten LA, van de Veerdonk FL, Savage N, van Crevel R, Kullberg BJ, et al. Transcriptional and inflammasome-mediated pathways for the induction of IL-1beta production by *Mycobacterium tuberculosis*. *Eur J Immunol* 2009;**39**:1914−22.
30. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;**298**:1911−2.
31. Caivano M. Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. *FEBS Lett* 1998;**429**:249−53.
32. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 1995;**270**:1326−31.
33. Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 1997;**275**:90−4.