

Original Article

KGC3P attenuates ovalbumin-induced airway inflammation through downregulation of p-PTEN in asthmatic mice

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ABSTRACT

Background: The roots of Korean red ginseng (*Panax ginseng* C.A.Mey.; KGC) have been used as an herbal supplement to enhance vital energy and immune capacity. *Salvia plebeia* R.Br. has been used to treat inflammatory diseases.

Purpose: The aim of this study was to examine the anti-asthmatic effects of a mixture of Korean red ginseng and *Salvia plebeia* R.Br. (KGC3P), its component nepetin, and their modes of action in alleviating ovalbumin (OVA)-induced asthma in mice.

Method: BALB/c mice were sensitized with OVA then subjected to intratracheal, intraperitoneal, and aerosol challenges. KGC3P and nepetin were administered orally for four weeks. Airway hyperresponsiveness (AHR), OVA-specific IgE levels, and Th2 cytokine- and gene expression levels in bronchoalveolar lavage fluid (BALF) and splenocytes were measured. Histological and immune cell subtype analyses were performed. PTEN and Akt phosphorylation levels were also evaluated.

Results: KGC3P reduced OVA-induced AHR, serum IgE levels, histological changes, and eosinophils infiltration but also the absolute number of immune cell subtypes including CD3⁺/CD4⁺, CD3⁺/CD8⁺, CD4⁺/CD69⁺, and Gr-1⁺/CD11b⁺ in the lungs, BALF, and mesenteric lymph nodes (MLN). KGC3P also lowered the Th2 cytokines IL-4, IL-5, and IL-13 in the BALF and splenocytes and downregulated the IL-4, IL-13, IL-17, TNF- α , and MUC5AC genes in the lung. KGC3P upregulated the peroxisome proliferator-activated receptor (PPAR) γ gene but downregulated the p-Akt and p-PTEN phosphorylation. Similar results were obtained with nepetin treatment.

Conclusion: KGC3P and nepetin are anti-asthmatic because they reduce various immune cells such as eosinophils and Th2 cell as well as Th2 cytokines. These mechanisms may be accompanied by the regulation of PPAR γ expression and the PTEN pathway. Taken together, our results indicate that KGC3P and nepetin may potentially prevent and treat asthma.

Abbreviations: AHR, airway hyperresponsiveness; Akt, protein kinase B; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; IL, interleukin; INF- α , interferon alpha; KGC3P, Korean red ginseng-*Salvia plebeia* mixture; MLN, mesenteric lymph nodes; MUC5AC, mucin 5AC; NF- κ B, nuclear factor kappa B pathway; OVA, ovalbumin; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PPAR γ , peroxisome proliferator-activated receptor; PTEN, phosphatase-tensin protein; RQ, relative quantity; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; Th2, T-helper 2 cells; TNF- α , tumor necrosis factor alpha; UPLC-PDA, ultraperformance liquid chromatography-photodiode array detection

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Introduction

Asthma is a chronic allergic inflammatory disease characterized by airway hyperresponsiveness (AHR), airway obstruction with bronchial eosinophilic inflammation, mucus hyperproduction, and airway wall remodeling (Kim et al., 2014). Its prevalence has increased worldwide over the last few decades. The pathogenesis of asthma is associated with cytokines, chemokines, and inflammatory cells (mast cells, eosinophils, basophils, neutrophils, and Th2 cells) (Gleich, 2000; Schuijs et al., 2013). Th2 cells contribute to the pathogenesis of allergic asthma by secreting cytokines such as interleukin (IL)-4, 5, 13, and 17. These induce eosinophilic inflammation, activate immunoglobulin E (IgE) and goblet cell hyperplasia and increase airway mucus secretion (Gleich, 2000; Schuijs et al., 2013). Airway eosinophilic inflammation is linked to the initiation and maintenance of allergic asthma (Schuijs et al., 2013). Suppressing these Th2 cell effects could prevent and treat the allergic asthma cascade.

The roots of Korean red ginseng (*Panax ginseng* C.A.Mey. KGC) have been used in Korea as herbal supplements to enhance vital energy and immune capacity (Lee et al., 2018). Ginseng extracts and their major constituents known as ginsenosides are anti-inflammatory and therapeutic for asthma (Babayigit et al., 2008; Chen et al., 2015). According to a clinical trial, Korean red ginseng may also prevent acute respiratory illness (Lee et al., 2012). *Salvia plebeia* R.Br. is an annual or biennial plant distributed worldwide and has been used to treat inflammatory diseases such as coughs, hepatitis, and hemorrhoids (Choi et al., 2015). According to pharmacological reports, it exhibited anti-inflammatory, anti-asthma, anti-oxidant, anti-cancer, and hepatoprotective effects (Choi et al., 2015; Jang et al., 2016). Phytochemical studies of *S. plebeia* indicated that it contains hispidulin, homoplantagin, nepetin, nepitrin, luteolin, luteoloside, and eupatorin (Jin et al., 2008). Nepetin, a natural flavonoid compound, had anti-inflammatory activity in various cells (Chen et al., 2018). These findings suggest that KGC and *S. plebeia* may improve dysregulated inflammation and could be used to treat inflammatory diseases. To the best of our knowledge, the anti-inflammatory and anti-asthmatic effectiveness of the combination of KGC, *S. plebeia*, and nepetin have not been previously assessed. In this study, then, our objective was to determine whether the combination of KGC and *S. plebeia* (KGC-03-PS, KGC3P) and nepetin could improve inflammation and asthma in an ovalbumin (OVA)-induced asthma model.

Materials and methods

KGC-03-PS (KGC3P)

Korean red ginseng (Araliaceae family, *Panax ginseng* C. A. Meyer) extract powder was acquired from Korea Ginseng Corporation (Seoul, Republic of Korea). Dried *Salvia plebeia* R.Br. (Labiatae family, Voucher number: KGC530) was purchased from Dongjin Farm (Buan, Jeonbuk, Republic of Korea) and was identified by Professor Young-Cheol Lee (Department of Herbology, College of Korean Medicine, Sangji University, Republic of Korea). Dried *S. plebeia* was extracted twice with a 15 volume of 30% v/v ethanol at 80 °C for 4 h. The extracts were then passed through a 1- μ m pore filter, concentrated in an evaporator and pulverized by spray-drying. KGC3P was prepared as a 1:3 mixture of Korean red ginseng and *S. plebeia* R.Br.

Analysis of ginsenosides

Rg1, Rb1, and 20(S)-Rg3 ginsenoside standards were purchased from Chromadex (Irvine, CA, USA). 2 g of KGC3P powder was extracted with 10 ml of 30% methanol for 30 min and centrifuged (Legend Mach 1.6R; Thermo Fisher Scientific GmbH, Dreieich, Germany) at 3000 $\times$ g for 10 min. The supernatant was passed through a 0.2- μ m pore Acrodisc® filter (Sigma Corp., USA) and injected into the UPLC-PDA

system (Waters Corp., USA). Chromatographic separation was achieved with a BEH C18 column (100 mm $\times$ 2.1 mm, 1.7 μ m; Waters Corp., USA) and the column temperature was 40 °C. The binary gradient elution system consisted of 0.001% phosphoric acid in water (A) and 0.001% phosphoric acid in acetonitrile (B). Separation was realized using the following protocol: 0–25.0 min (15% B), 35.0 min (20% B), 39.5 min (30% B), 40.5 min (32% B), 42.5 min (40% B), 46.0 min (65% B), 47.0–49.0 min (90% B), and 51.0–53.0 min (15% B). The flow rate was 0.6 ml min⁻¹ and the sample injection volume was 2.0 μ l. The ginsenosides were determined with a photodiode array detector (Waters Corp., USA) at a UV wavelength of 203 nm.

Analysis of nepetin

0.5 g of KGC3P powder was extracted with 20 ml methanol in an ultrasonic chamber for 30 min. The extract was diluted to 50 ml with methanol. After extraction, the solution was passed through a 0.2- μ m pore Acrodisc® filter (Sigma Corp., USA) and injected into the HPLC-PDA system (Waters Corp., USA). Chromatographic separation was achieved with a Zorbax eclipse XDB C18 column (250 mm $\times$ 4.6 mm, 5 μ m; Agilent Technologies, USA). The binary gradient elution system consisted of 0.05% acetic acid in water (A) and methanol (B). Separation was realized using the following protocol: 0.0 min (38% B), 14.0 min (42% B), 17.0 min (45% B), 17.1 min (48% B), 32.0 min (50% B), 40.0 min (85% B), and 45.0 min (38% B). The flow rate was 1.0 ml min⁻¹ and the sample injection volume was 10.0 μ l. Nepetin was determined with a photodiode array detector (Waters Corp., USA) at a UV wavelength of 342 nm.

Animal experiments

Male BALB/c mice (seven weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed at 21 $\pm$ 2 °C, 50 $\pm$ 5% relative humidity, and a 12 h:12 h light/dark cycle. They had free access to a commercial diet and water. The animal protocol was approved by the Committee for Animal Welfare at Daejeon University, Republic of Korea (DJUAR2017-009). This study was carried out in accordance with the Guidelines of the Institutional Animal Care and Use Committee of the South Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

OVA sensitization and inhalation

The mice were immunized by intraperitoneal injection with alum-precipitated antigen containing 12.5 μ g ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO, USA) and 0.26 mg aluminum hydroxide (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) on the first days of the first and second weeks and one day before inhalation exposure. They were then administered intratracheal injections of 2 mg OVA in PBS at days 3 and 10 after the first sensitization. The mice were then exposed by inhalation to 1% OVA in saline aerosolized with the ME-U12 ultrasonic nebulizer (Omron, Tokyo, Japan) for 30 min d⁻¹ and 3 d wk⁻¹ from days 21 to 42. On day 45, the mice were exposed by inhalation to aerosolized 2% OVA in saline. The mice were separated into six groups (n = 10 per group) as follows: (1) Normal control, (2) OVA-control, (3) OVA-montelukast (Mon; Sigma-Aldrich, St. Louis, MO, USA) 10 mg kg⁻¹, (4) OVA-KGC3P 200 mg kg⁻¹, (5) OVA-KGC3P 100 mg kg⁻¹, and (6) OVA-nepetin (Nep; Sigma-Aldrich, St. Louis, MO, USA) 20 mg kg⁻¹. KGC3P (200 and 100 mg kg⁻¹), nepetin and montelukast were orally administered daily for 4 wks (from days 21 to 46). Montelukast was used as a positive control. The normal control mice were sensitized with 0.9% saline. The experimental scheme is presented in Fig. 1A.

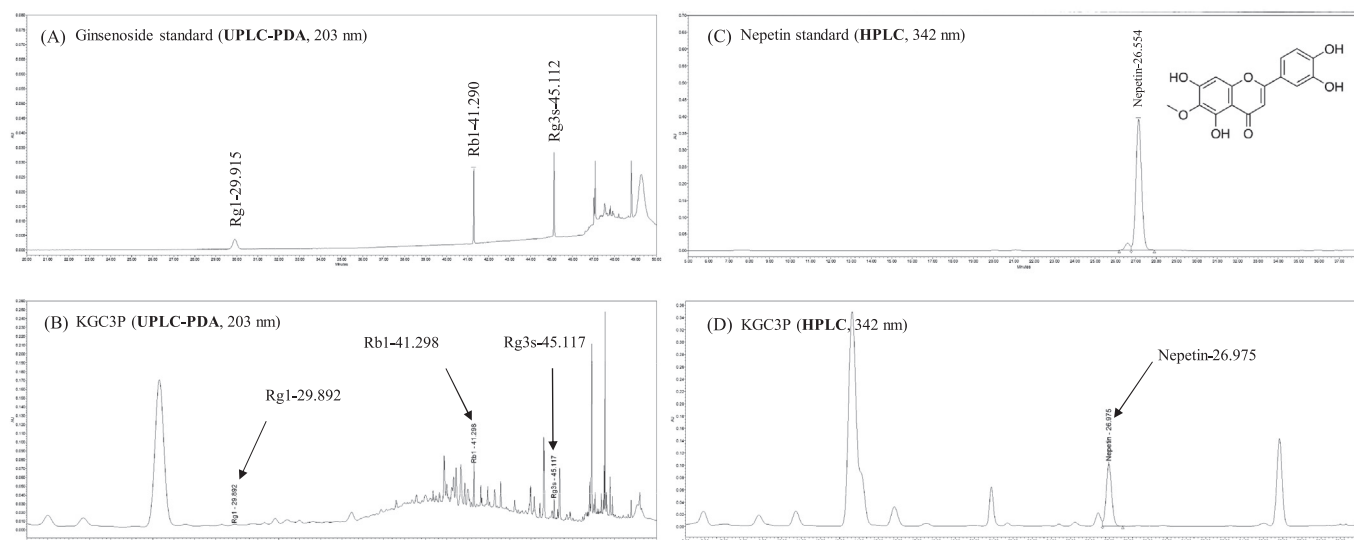


Fig. 1. Chromatogram and structure of four compounds purified from KGC-03-PS mixture (KGC3P).

Rg1, Rb1, Rg3s, and nepetin were identified as marker compounds of the Korean red ginseng/*Salvia plebeia* R.Br. mixture (KGC3P). UPLC-PDA chromatograms of three standard ginsenoside mixtures (A) at $\lambda = 203$ nm and KGC3P mixture (B) and HPLC chromatograms of standard nepetin (C) at $\lambda = 342$ nm and KGC3P mixture (D). Rg1, Rb1, Rg3s, and nepetin appeared at retention times of ~ 29.8 , 41.2, 45.1, and 26.9 min, respectively. UPLC-PDA: ultra-performance liquid chromatography-photodiode array detector, HPLC: high-performance liquid chromatography.

Penh values measurement

One day after the last 2% OVA inhalation (day 46), the mice were given aerosolized saline followed by increasing doses of aerosolized methacholine (6.25, 12.5, and 25 mg ml⁻¹). Airway hyperresponsiveness was then monitored for 30 min using a Buxco System (Biosystem XA; Data Sciences International Inc., St. Paul, MN, USA) and respiratory curves were converted into Penh values.

Collection of BALF, lung, and mesenteric lymph node (MLN) cells

Tracheotomies were performed on the mice and the BALF was obtained by airway lamina and lung lavage by tracheal cannulation. The BALF was then centrifuged at 400×g and 4 °C for 5 min. The pellets were resuspended in PBS and the total cell number was counted. Lungs and MLN were removed from the mice, rinsed, sectioned and incubated in 1 mg ml⁻¹ collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 40 min. The cell suspension was then filtered and re-centrifuged at 450×g for 20 min. The pellet was collected and the total cell number was counted.

Flow cytometric analysis

BALF, lung and MLN cells were incubated with anti-CD3, anti-CD4, anti-CD8, anti-CD69, anti-CD11b, and anti-Gr-1 antibodies (BD

Biosciences Pharmingen, San Diego, CA, USA) in staining solution (PBS with 1% fetal bovine serum (FBS) and 0.01% NaN₃) on ice for 10 min. The stained cells were analyzed on a BD FACSCalibur™ two-color flow cytometer interfaced to CellQuest software (BD Biosciences, Mountain View, CA, USA). The absolute cell number was calculated as a percentage of the total cell number.

Serum IgE and cytokines in BALF and splenocytes measurements

The serum IgE, IL-4, IL-5, IL-13, and IFN- γ levels in the BALF and splenocytes were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Spleen cells were cultured in OVA (10 μ g ml⁻¹) coated well plates for 16 h and cytokines levels were measured.

Real-time quantitative RT-PCR

Total RNA from the lung tissue was isolated with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocols. 5 μ g total RNA was reverse-transcribed into cDNA with a First Strand cDNA synthesis kit (Amersham Pharmacia, Piscataway, NJ, USA). Gene expression was analyzed with Taqman Universal PCR Master Mix (ABI) using an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Primers and probe sequences are shown in Table 1.

Table 1
Primers and probe sequence used in real-time PCR analysis.

IL-4	NM_021283.2	Forward	5'-ACAGGAGAAGGGACGCCAT-3'
		Reverse	5'-GAAGCCCTACAGACGAGCTCA-3'
IL-13	NM_008355.3	Forward	5'-ATGCCCAACAAGCAGAGAC-3'
		Reverse	5'-TGAGAGAACCAAGGAGCTGT-3'
IL-17	NM_010552.3	Forward	5'-TCTCATCCAGCAAGAGATCC-3'
		Reverse	5'-AGTTTGGGACCCCTTTACAC-3'
TNF- α	NM_013693.3	Forward	5'-GGCTTTCCGAATTCACCTGGAGCCT-3'
		Reverse	5'-CCCCGGCCTTCCAAATAAATACATTGATA-3'
MUC5AC	NM_010844.1	Forward	5'-AGAATATCTTTCAGGACCCCTGCT-3'
		Reverse	5'-ACACCACTGCTGAGCATACTTTT-3'
PPAR γ	NM_001127330.2	Forward	5'-ATGCCATTCTGGCCCACTT-3'
		Reverse	5'-CCCTTGCATCCTTCAAGCATG-3'
GAPDH	NM_001289726.1	VIC	5'-CATCTGCACCACTGCTTAGCC-3'

Histological analysis

Lung and tracheal tissues were fixed in 10% (v/v) formalin solution, embedded in paraffin and cut into 4- μ m-thick sections. The lung sections were stained with H&E and M-T (Sigma-Aldrich, St. Louis, MO, USA) to assess inflammatory cell infiltration and collagen deposition, respectively. The tracheal sections were stained with AB-PAS (Sigma-Aldrich, St. Louis, MO, USA) and examined under a light microscope for goblet cell hyperplasia and mucus secretion. To determine the severity of inflammatory cell infiltration, peribronchial cell counts and the extent of mucus production and goblet cell hyperplasia in the airway epithelium were blindly quantified with a five-point grading system (Tanaka et al., 2001).

Western blotting analysis

Lung proteins were extracted with a protein extraction kit (Intron Biotechnology Inc., Seoul, Republic of Korea). The proteins were separated by SDS polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blocked for 1 h with blocking solution (EzBlockChemi; ATTO Corporation, Tokyo, Japan), followed by incubation overnight at 4 °C with primary antibodies against p-Akt, p-PTEN, and β -actin (Cell Signaling Technology, Beverly, MA, USA) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were washed and developed by electrochemiluminescence (Amersham, GE Healthcare, Uppsala, Sweden). The relative expression levels of p-Akt, p-PTEN, and β -actin were quantified for each band. For protein quantification, the relative expression levels were calculated as a ratio against those for the normal control group.

Cell culture

LA-4 murine lung epithelial cells were cultured in RPMI 1640 medium (Gibco, Big Cabin, OK, USA) supplemented with 10% FBS (Gibco) and antibiotics (Gibco) in 5% CO₂ incubator at 37 °C. For western blot analysis, the cells were treated with DMSO (8 μ l ml⁻¹), KGC3P (200 and 100 μ g ml⁻¹), or nepetin (5 and 2.5 μ g ml⁻¹) for 24 h.

Statistical analysis

All data were presented as means $\pm$ SEM. Differences between groups were assessed by ANOVA followed by Duncan's multiple range test in Prism v. 7.0 (GraphPad Inc., San Diego, CA). Differences were considered significant at $p < 0.05$.

Results

Chromatogram analysis of KGC3P mixture

Chromatograms of the KGC3P mixture are shown in Fig. 1. Three ginsenosides (Rg1, Rb1, and Rg3s) and nepetin were identified by UPLC and HPLC system in the KGC3P mixture, respectively. The concentrations of Rg1, Rb1, Rg3s, and nepetin were 0.22 ± 0.03 , 2.06 ± 0.06 , 0.51 ± 0.05 , and 5.42 ± 0.39 mg g⁻¹, respectively.

Inhibitory effects of KGC3P and nepetin on airway inflammation

The effects of KGC3P and nepetin on AHR were determined from their impact on the increases in Penh value in response to a methacholine challenge. Compared to the normal control, the Penh value of the OVA-control group increased with methacholine dose. However, the KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups exhibited significantly lower Penh values than the OVA-control group (Fig. 2B). OVA sensitization induced OVA-specific IgE production which is associated with inflammatory cells

involved in the pathogenesis of allergic asthma. As shown in Fig. 2C, OVA-specific IgE levels were markedly higher in the OVA-control group than the normal group but comparatively lower in the KGC3P (200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups. As shown in Fig. 2D and E, PAS staining revealed that relative to the normal control, the OVA-control group presented with fibrosis, elevated inflammatory cell filtration into the lung tissue, goblet cell hyperplasia, thickened smooth muscle, narrow lumens, and mucus hyperproduction in the trachea. However, KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) reduced these histopathological changes in the lung and trachea and inflammatory cell infiltration.

Effects of KGC3P and nepetin on the cell number in lung, MLN, and BALF

The numbers of total lung- and MLN cells were increased in the OVA-control group compared with those in the normal group. The number of total lung cells was decreased by KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) (Fig. 3A). The number of total MLN cells was decreased by KGC3P (200 mg kg⁻¹) (Fig. 3B). Compared with the normal control, the numbers of cells and eosinophils in the BALF were significantly higher in the OVA-control group. However, the numbers of these cells were markedly reduced in the KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups (Fig. 3C).

Effects of KGC3P and nepetin on the absolute number of immune cell subtype in lung, BALF and MLN

The absolute numbers of CD3⁺/CD4⁺, CD3⁺/CD8⁺, CD4⁺/CD69⁺, and Gr-1⁺/CD11b⁺ double-positive cells in the lungs, BALF, and MLN were elevated in the OVA-control group. The absolute numbers of these cells were significantly lower in the lungs of the KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups than in those of the OVA-control group. In the BALF and MLN, the absolute numbers of CD3⁺/CD4⁺, CD3⁺/CD8⁺, and Gr-1⁺/CD11b⁺ were also comparatively lower for the KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups (Fig. 3D and Table 2).

Effects of KGC3P and nepetin on inflammatory cytokine levels in BALF and splenocytes

As shown in Fig. 4, the IL-4, IL-5, and IL-13 levels were elevated in the BALF and splenocytes of the OVA-control group compared to those in the normal control. In contrast, the levels of these cytokines were significantly lower in the KGC3P (100 and 200 mg kg⁻¹), nepetin (20 mg kg⁻¹), and montelukast (10 mg kg⁻¹) groups. On the other hand, compared with the OVA-control group, KGC3P, nepetin, and montelukast did not affect the INF- γ levels in BALF but KGC3P and nepetin increased them in splenocytes.

Effects of KGC3P and nepetin on gene expression of inflammatory cytokines levels in the lung

IL-4, IL-13, IL-17, TNF- α , and MUC5AC mRNA were upregulated in the lungs of the OVA-control group compared with those of the normal control group. KGC3P and montelukast significantly downregulated IL-4, IL-13, IL-17, TNF- α , and MUC5AC mRNA and nepetin downregulated IL-17 and MUC5AC mRNA (Fig. 5).

Effects of KGC3P and nepetin on the PPAR-PTEN signal pathway in the lungs

PPAR γ mRNA was upregulated in the OVA-control group compared to that of the normal control. PPAR γ mRNA was significantly

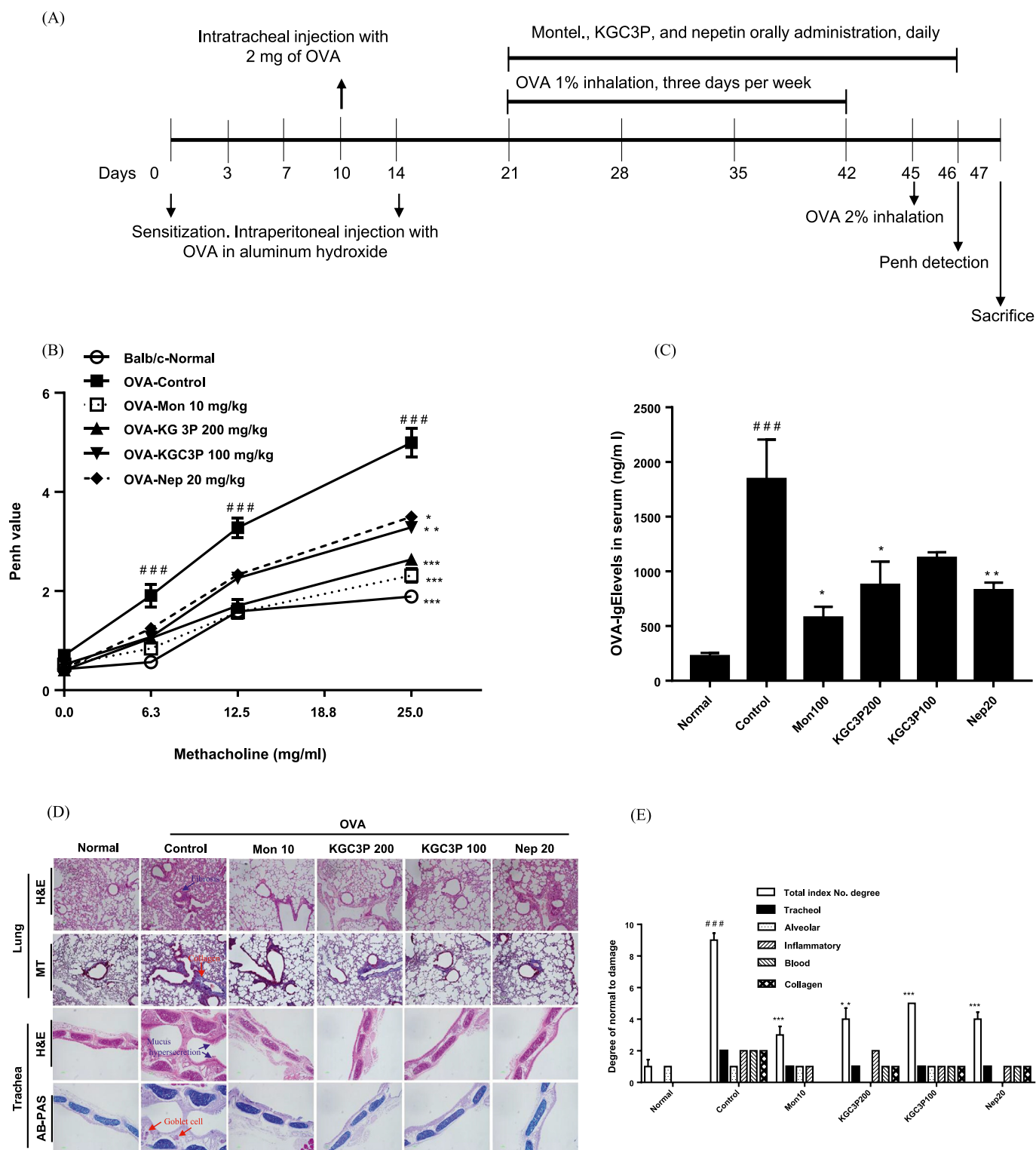


Fig. 2. Effects of KGC3P on airway inflammation in OVA mice.

(A) Experimental scheme, (B) Airway responsiveness to aerosolized methacholine (Penh), (C) OVA-specific IgE levels in serum, (D) Histopathology of lung and trachea tissues, and (E) Quantitative analyses of the degree of lung tissue damage. Normal: normal control; OVA-control: OVA-sensitized control; KGC3P: a 1:3 mixture of Korean red ginseng and *Salvia plebeia* R.Br.; Mon 10: 10 mg kg⁻¹ montelukast; Nep 20: 20 mg kg⁻¹ nepetin; H&E: Hematoxylin-eosin; MT: Masson's trichrome; AB-PAS: Alcian blue-Periodic acid Schiff. Data are expressed as means $\pm$ SEM ($n = 10$ /group). $###p < 0.005$ vs. normal control group; $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$ vs. OVA-control group.

upregulated in the KGC3P (100 and 200 mg kg⁻¹) and nepetin (20 mg kg⁻¹) groups but downregulated in the montelukast (10 mg kg⁻¹) group (Fig. 5A). KGC3P (100 and 200 mg kg⁻¹) and montelukast (10 mg kg⁻¹) downregulated both the phosphorylation

levels of AKT and PTEN (Fig. 6B–D). Consistent with the data in the lung tissues, the phosphorylation levels of AKT and PTEN were also reduced by treatment of LA-4 cells with KGC3P (100 and 200 μ g ml⁻¹) or nepetin (2.5 and 5 μ g ml⁻¹) (Fig. 6E–G).

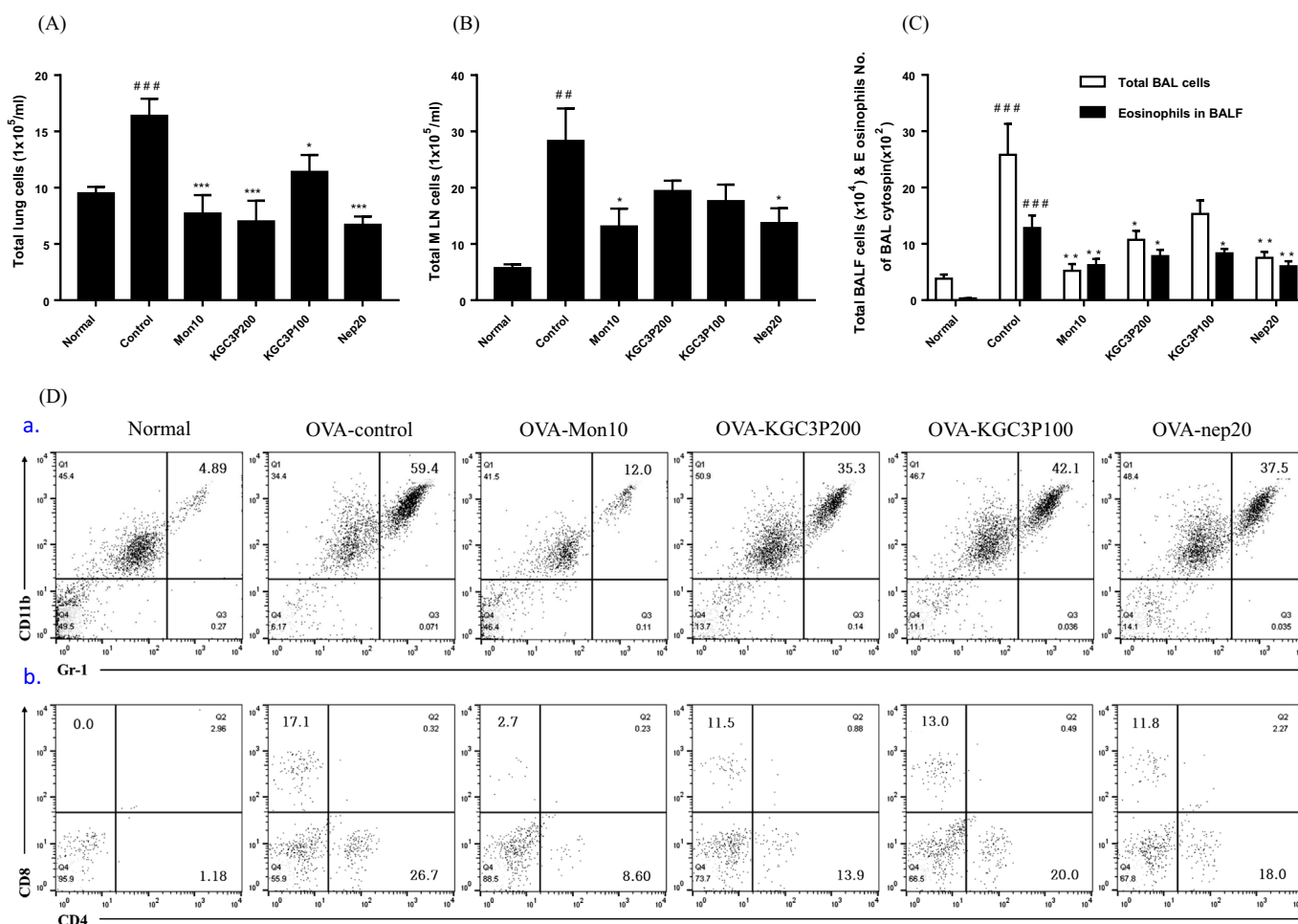


Fig. 3. Effects of KGC3P on cell numbers in lungs, MLN, and BALF in OVA mice.

(A) Lung, (B) MLN, (C) BAL cells and eosinophil numbers in BALF, (D) Dot plot of representative flow cytometry profiles on gated immune cells in BALF. Normal: normal control; OVA-control: OVA-sensitized control; KGC3P: a 1:3 mixture of Korean red ginseng and *Salvia plebeia* R.Br.; Mon 10: 10 mg kg⁻¹ montelukast; Nep 20: 20 mg kg⁻¹ nepetin. Data are expressed as means $\pm$ SEM ($n = 10$ /group). ### $p < 0.005$ vs. normal control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ vs. OVA-control group.

Discussion

Asthma is a chronic inflammatory disorder characterized by AHR, inflammation, airway obstruction, and airway wall remodeling (Kim et al., 2014). In the present study, we found that KGC3P decreased AHR and inflammatory cell infiltration. Histological analysis of the lung

and trachea of an OVA-induced mouse asthma model revealed that KGC3P also inhibited airway remodeling such as fibrosis, goblet cell hyperplasia, and mucus hypersecretion. Similar results were obtained for the nepetin group. Our finding indicates that KGC3P and nepetin may attenuate allergic asthma in an OVA-induced mouse asthma model.

Table 2

Quantification by FACS analysis of immune cell subtypes in lung, BALF, and MLN. Each point represents the mean $\pm$ SEM values for 6 mice.

Cell phenotypes in Lung, BALF, MLN		OVA-induced airway inflammation murine asthma model (absolute No.)					
		Normal	Control	Mon 10	KGC3P 200	KGC3P 100	Nep 20
CD3+/CD4+ ($\times 10^6$ cells)	Lung	2.0 $\pm$ 0.47	3.5 $\pm$ 0.36 ^{##}	1.7 $\pm$ 0.39 ^{**}	1.6 $\pm$ 0.55 ^{**}	2.3 $\pm$ 0.33 [*]	1.5 $\pm$ 0.18 ^{***}
CD3+/CD8+ ($\times 10^5$ cells)		1.7 $\pm$ 0.22	2.9 $\pm$ 0.45 ^{##}	1.4 $\pm$ 0.42 [*]	1.1 $\pm$ 0.39 ^{**}	2.0 $\pm$ 0.24	1.3 $\pm$ 0.34 ^{***}
CD4+/CD69+ ($\times 10^5$ cells)		1.2 $\pm$ 0.47	4.1 $\pm$ 0.23 ^{###}	1.7 $\pm$ 0.47 ^{***}	1.7 $\pm$ 0.61 ^{**}	2.6 $\pm$ 0.58 [*]	1.1 $\pm$ 0.11 ^{***}
Gr-1+/CD11b+ ($\times 10^5$ cells)		1.2 $\pm$ 0.08	2.6 $\pm$ 0.13 ^{###}	1.3 $\pm$ 0.30 ^{**}	1.2 $\pm$ 0.44 ^{**}	1.5 $\pm$ 0.18 ^{***}	1.1 $\pm$ 0.13 ^{***}
CD3+/CD4+ ($\times 10^4$ cells)	BALF	0.1 $\pm$ 0.02	4.2 $\pm$ 1.22 ^{##}	0.9 $\pm$ 0.40 ^{**}	1.6 $\pm$ 0.65 [*]	2.7 $\pm$ 0.96	1.6 $\pm$ 0.18 [*]
CD3+/CD8+ ($\times 10^4$ cells)		0.1 $\pm$ 0.03	1.6 $\pm$ 0.26 ^{###}	0.3 $\pm$ 0.13 ^{***}	0.8 $\pm$ 0.08 ^{**}	1.3 $\pm$ 0.42	0.6 $\pm$ 0.27 ^{**}
Gr-1+/CD11b+ ($\times 10^4$ cells)		0.7 $\pm$ 0.23	3.0 $\pm$ 0.95 ^{##}	0.5 $\pm$ 0.29 [*]	0.8 $\pm$ 0.23 [*]	1.8 $\pm$ 0.26	0.9 $\pm$ 0.27 [*]
CD3+/CD4+ ($\times 10^6$ cells)	MLN	2.3 $\pm$ 0.09	11.0 $\pm$ 2.83 ^{##}	3.3 $\pm$ 0.82 ^{**}	4.4 $\pm$ 0.61 [*]	5.4 $\pm$ 1.48	5.0 $\pm$ 0.72 [*]
CD4+/CD69+ ($\times 10^5$ cells)		0.3 $\pm$ 0.04	3.6 $\pm$ 0.44 ^{###}	1.3 $\pm$ 0.42 ^{**}	1.1 $\pm$ 0.21 ^{***}	1.9 $\pm$ 0.91	1.3 $\pm$ 0.35 ^{***}

Absolute numbers of immune cell subtypes in lung, BALF, MLN were counted (described in Section "Materials and methods"). Lung, BALF, and MLN cells were stained with anti-CD3, CD4, CD8, CD69, CD11b, Gr-1 mAb, etc. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ (compared with WT), * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (compared with OVA-CTL) as determined by unpaired Student's *t*-tests and analyses of variance (ANOVA) followed by Duncan's multiple range tests. WT: BALB/c normal; OVA-CTL: OVA-sensitized control, Mon 10: 10 mg/kg montelukast, OVA_KGC3P: 200 mg/kg & 100 mg/kg KGC3P-treated OVA-sensitized mice, and OVA_nepetin: 20 mg/kg nepetin-treated OVA-sensitized mice. BALF: bronchoalveolar lavage fluid, MLN: Mediastinal lymph node.

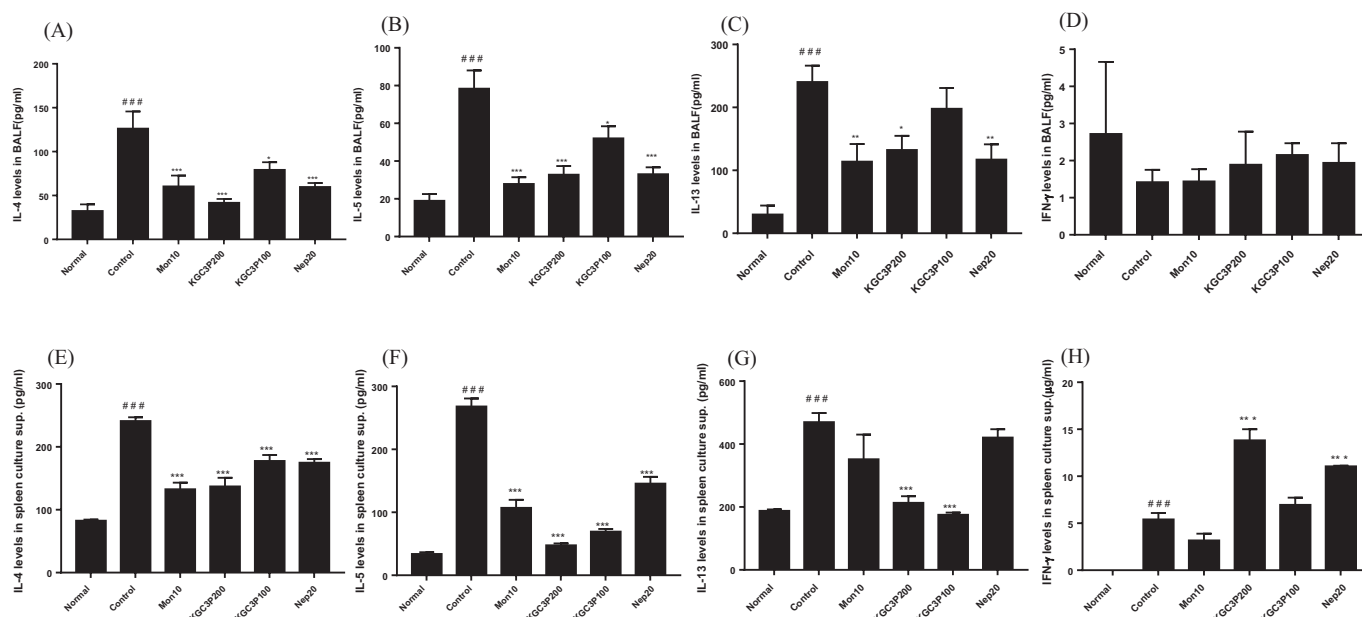


Fig. 4. Effects of KGC3P on inflammation cytokines levels in OVA mice.

(A) IL-4, (B) IL-5, (C) IL-13, and (D) IFN- γ in BALF. (E) IL-4, (F) IL-5, (G) IL-13, and (H) IFN- γ in spleenocyte. Normal: normal control; OVA-control: OVA-sensitized control; KGC3P: a 1:3 mixture of Korean red ginseng and *Salvia plebeia* R.Br.; Mon 10: 10 mg kg⁻¹ montelukast; Nep 20: 20 mg kg⁻¹ nepetin. Data are expressed as means $\pm$ SEM ($n = 10$ /group). ### $p < 0.005$ vs. normal control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ vs. OVA-control group.

We investigated the effects of KGC3P and nepetin on the infiltration of total leukocytes, eosinophils, Gr-1⁺/CD11b⁺ granulocytes, and T cells. In the airway mucosa, eosinophils are the major regulators of inflammation and remodeling in the pathogenesis of allergic asthma and they induce AHR (Gleich, 2000). Furthermore, allergic asthma is the result of an imbalance in the T-cell immune response which plays a

critical role in the pathogenesis of airway inflammation (Mojtabavi et al., 2002). Relative to the OVA-control group, the KGC3P and nepetin treatments markedly reduced the total leukocytes numbers in the lungs, MLN, and BALF and the numbers of eosinophils and Gr-1⁺/CD11b⁺ granulocytes. Eosinophils comprise a substantial portion of the Gr-1⁺/CD11b⁺ cells. Therefore, the decrease in the number of

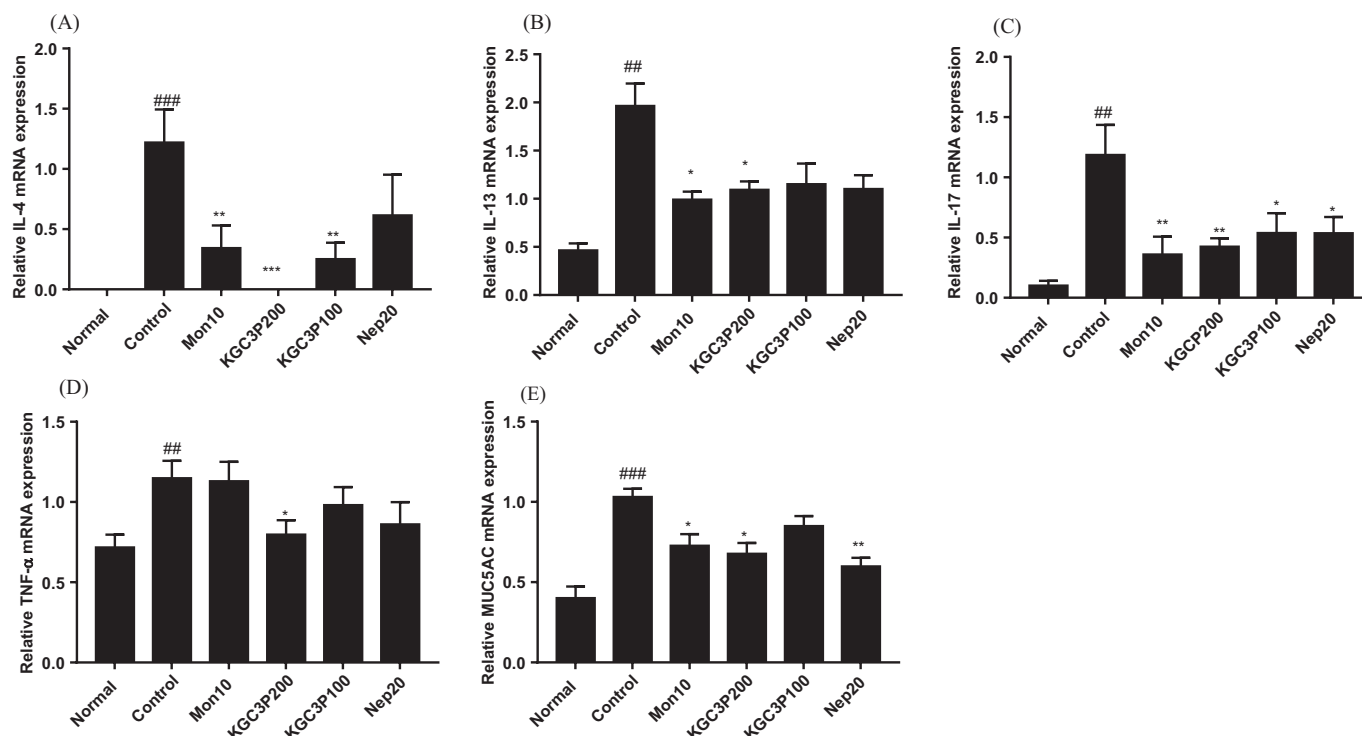


Fig. 5. Effects of KGC3P on gene expression levels of inflammation cytokines in lung of OVA mice.

(A) IL-4, (B) IL-13, (C) IL-17, (D) TNF- α , (E) MUC5AC 3. Normal: normal control; OVA-control: OVA-sensitized control; KGC3P: a 1:3 mixture of Korean red ginseng and *Salvia plebeia* R.Br.; Mon 10: 10 mg kg⁻¹ montelukast; Nep 20: 20 mg kg⁻¹ nepetin. Data are expressed as means $\pm$ SEM ($n = 10$ /group). ## $p < 0.01$ and ### $p < 0.005$ vs. normal control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$ vs. OVA-control group.

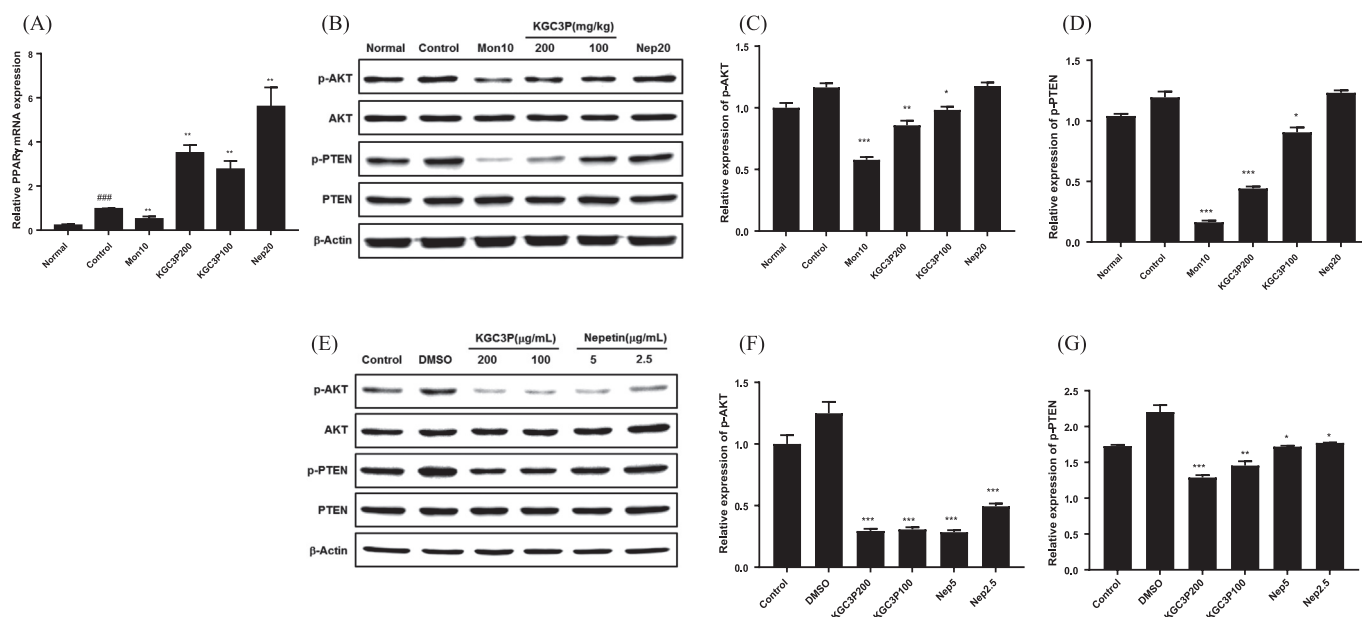


Fig. 6. Effects of KGC3P on PPAR γ expression and PTEN signal pathway in the lungs of OVA mice and LA-4 lung epithelial cells. (A) PPAR γ , (B) representative protein expression in the lung tissue of OVA mice. (C) Densitometric analyses of p-Akt and (D) p-PTEN levels were quantitatively analyzed by normalization with non-phospho forms of AKT and PTEN using a densitometry. (E) Representative protein expression of LA-4 lung epithelial cells. (F) Densitometric analyses of p-Akt and (G) p-PTEN levels in LA-4 cells. Normal: normal control; OVA-control: OVA-sensitized control; KGC3P: a 1:3 mixture of Korean red ginseng and *Salvia plebeia* R.Br.; Mon 10: 10 mg kg⁻¹ montelukast; Nep 20: 20 mg kg⁻¹ nepetin; DMSO: DMSO-treated control. Data are expressed as means $\pm$ SEM ($n = 10$ /group, $n = 3$ /group cells) ### $p < 0.005$ vs. normal control group; * $p < 0.05$ and ** $p < 0.01$, *** $p < 0.005$ vs. OVA-control group or DMSO-control group respectively.

Gr-1⁺/CD11b⁺ cells achieved by KGC3P and nepetin was the result of a reduction in the number of eosinophils in the lungs and BALF. In addition, KGC3P and nepetin decreased the absolute numbers of the CD3⁺/CD4⁺ T-cells, CD3⁺/CD8⁺ T-cells and CD4⁺/CD69⁺ T-cells in the lungs, BALF, and MLN of OVA-induced mice. Therefore, KGC3P and nepetin inhibit eosinophil, inflammatory cell infiltration and immune cell subtypes into the airway and alleviate the airway inflammation induced by OVA sensitization.

Th2-derived cytokines play important roles in amplifying the allergic inflammation of asthma by activating eosinophils and inflammatory cell infiltration, IgE secretion by B cells, and mucus overproduction (Schuijs et al., 2013). In our study, KGC3P and nepetin downregulated IL-4, IL-5, and IL-13 in the BALF, splenocytes and lungs. These results suggest that GC3P and nepetin decreased Th2 cytokine levels and, by extension, lowered the number of eosinophils and reduced IgE production in mice with OVA-induced asthma mice. KGC3P and nepetin also downregulated TNF- α and MUC5AC mRNA in the lungs. These results and histological analyses confirm that KGC3P and nepetin can inhibit airway mucus hypersecretion. KGC3P and nepetin can also improve allergic responses in the BALF (lung) by reducing Th2 cytokines. This effect is partially explained by the decrease in the number of immune cell subtypes in the BALF of OVA-induced asthma mice.

The nuclear peroxisome proliferator-activated receptor (PPAR γ) plays important roles in glucose and lipid metabolism. However, recent evidence suggests that PPAR γ participates in asthma pathogenesis by regulating immune and inflammatory responses in the airway (Liu et al., 2015). PPAR γ agonists downregulated various pro-inflammatory cytokines such as TNF- α , IL-4, IL-5, and IL-13 via the nuclear factor kappa-light-chain-enhancer (NF- κ B) in activated B-cells. They also significantly reduced the numbers of eosinophils in the BALF and AHR of OVA-induced asthmatic mice (Lee et al., 2005). On the other hand, PTEN is associated with asthma development. It blocked eosinophil survival, chemotaxis, and airway inflammation (Adachi et al., 2007) and opposed the action of the PI3K/Akt pathway by dephosphorylating the signaling lipid phosphatidylinositol 3,4,5-

triphosphate (Lee, 2004; Lee et al., 2005). The PI3K pathway attracts inflammatory mediators and activates eosinophils (Kwak et al., 2003). In asthma, PI3K inhibitors reduce eosinophilic inflammation and airway hyperresponsiveness (Tigani et al., 2001). Therefore, pharmacological targeting of PPAR γ and PTEN may be a new strategy for the treatment of allergic asthma. Our study found that KGC3P significantly upregulated the gene expression of PPAR γ and downregulated p-PTEN and p-Akt. Our results indicate that KGC3P may inhibit the development of allergic asthma by downregulating Th2 cytokines and reducing eosinophil infiltration and airway remodeling via PPAR γ upregulation and the PTEN-PI3K/Akt pathway.

Conclusion

KGC3P and nepetin exerted anti-asthmatic effects possibly accompanied by the suppression of Th2 cytokines and IgE and the downregulation of the genes encoding Th2 cytokines, TNF- α , and MUC5AC via the regulation of PPAR γ and the PTEN-Akt pathway.

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Conflict of interest

The authors have no competing interest to declare regarding this manuscript.

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