



Pulmonary, gastrointestinal and urogenital pharmacology

Evaluation of the effect of kaempferol in a murine allergic rhinitis model

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ABSTRACT

Kaempferol (KP) is a major compound of *Naju Jjok* (*Polygonum tinctorium* Lour.). The effect of KP on allergic rhinitis (AR) has not been elucidated. Here, we report the effects and mechanisms of KP on new and predominant mediators of AR using an eosinophil cell line, Eo1 and an ovalbumin (OVA)-induced AR mouse model. KP significantly inhibited the production of interleukin (IL)-32 and IL-8 and activation of caspase-1 in Eo1 cells. Allergic symptoms and predominant mediators (IgE and histamine) in the KP-administered group were significantly lower than in the AR group. The levels of interferon- γ were enhanced while the levels of IL-4 were reduced in the KP group. KP significantly reduced the levels of IL-32 and thymic stromal lymphopoietin (TSLP) compared with the AR mice. KP reduced the levels of inflammation-related proteins. In the KP-administered groups, the infiltrations of eosinophils and mast cells increased by OVA were decreased. In addition, KP significantly reduced caspase-1 activity in nasal mucosa tissue of AR mice. Our findings indicate that KP has an anti-allergic effect through the regulation of the production of IL-32 and TSLP and caspase-1 activity in allergic diseases including AR.

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1. Introduction

Allergic rhinitis (AR) is a global health problem. Patients, clinicians, and other health care professionals worldwide are faced with the relative merits and downsides of the various treatment options (International Rhinitis Management Working Group, 1994). AR is characterized by the symptoms of sneezing, itchiness, rhinorrhea, and nasal congestion. Patients with AR present an inflammatory IgE-mediated response characterized by a Th2 immunological pattern with mast cells and eosinophil activation and release of inflammatory mediators in response to exposure to allergens (Durham et al., 1992).

Eosinophils are prominent in Th2-driven immune responses, including asthma and allergic and parasitic diseases (Melvin and Ramanathan, 2012). As inflammatory cells, eosinophils contribute to the pathogenesis of allergic inflammation by secreting toxic granule proteins and lipid mediators (Isobe et al., 2012). In addition to their roles in acute inflammation, eosinophils are recognized as immuno-modulatory cells because of interaction

with T and B lymphocytes (Bandeira-Melo and Weller, 2005). In particular, granulocyte-macrophage colony-stimulation factor (GM-CSF) is a major survival and activating factor for hematopoietic cells that primes mature macrophages, eosinophils, and neutrophils, and is known as a pleiotropic and pro-inflammatory cytokine (Tai and Spry, 1990). In addition, inflammatory cells in tissues are dependent on adhesion of blood-borne inflammatory cells to endothelial cells before migration to the inflammatory site (Pilewski and Albelda, 1995). Upon stimulation, eosinophils release the tumor necrosis factor (TNF)- α and interleukin (IL)-4, which can modulate adhesion molecules on endothelial cells (Bandeira-Melo and Weller, 2005; Bacon et al., 1998). Activated endothelial cells express the intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on their cell surfaces (Bacon et al., 1998). Macrophage-inflammatory protein (MIP)-2 is a pivotal chemoattractant for immune cells (Gupta et al., 1996).

IL-32 is a cytokine produced mainly by T lymphocytes, natural killer cells, epithelial cells, mast cells, keratinocytes, eosinophils, and blood monocytes and it has the properties of a classical pro-inflammatory mediator (Kim et al., 2005; Netea et al., 2005). IL-32 induces the overexpression of cyclooxygenase (COX)-2 (Lee et al., 2011). In addition, IL-32 induces the production of proinflammatory cytokines by activation of nuclear factor (NF)- κ B and caspase-1 (Kim et al., 2005; Netea et al., 2005; Jeong et al., 2011). Recently, (Jeong et al., 2011) reported that IL-32 released from eosinophils

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significantly increased the levels of IgE and production of inflammatory cytokine, including IL-1 β , IL-18, and GM-CSF in AR.

Thymic stromal lymphopoietin (TSLP) has been linked to the pathogenesis of asthma, atopic dermatitis, and AR (Ying et al., 2008; Soumelis et al., 2002; Bunyavanich et al., 2011). TSLP is an important cytokine for the initiation of allergic inflammatory diseases (Song et al., 2012; Allakhverdi et al., 2007). TSLP has been produced through caspase-1 in mast cells (Moon and Kim, 2011). Recently, we reported that IL-32 induced TSLP production by activation of NF- κ B and caspase-1 in monocytes (Jeong et al., 2012).

Kaempferol (KP) is a major component of Naju Jjok (*Polygonum tinctorium* Lour.). In this study, we examined the anti-allergic effects of KP on GM-CSF-stimulated Eol-1 cells and ovalbumin (OVA)-induced AR mice.

2. Materials and methods

2.1. Materials

KP, dexamethasone (DEX), OVA, O-phthalaldehyde (OPA), avidin peroxidase (AP), 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) tablets substrate (ABTS), purchased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and bichoninic acid (BCA) other reagents were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640, and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). Anti-mouse IgE/TSLP/IL-4/interferon (IFN)- γ /MIP-2/ICAM-1 antibody (Ab), biotinylated anti-mouse IgE/TSLP/IL-4/IFN- γ /MIP-2/ICAM-1 Ab, recombinant mouse IgE/TSLP/IL-4/IFN- γ /MIP-2/ICAM-1, anti-human IL-8 Ab, biotinylated anti-human IL-8 Ab, and recombinant human IL-8/GM-CSF were purchased from Pharmingen (San Diego, CA, USA). IL-32 Abs were obtained from BioLegend (San Diego, CA), YbDY Biotech (Seoul, Korea), and Acris (Herford, Germany). Ab for COX-2, caspase-1, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Caspase-1 assay kit was supplied by R&D Systems Inc. (Minneapolis, MN, USA).

2.2. OVA-induced AR mouse model

We maintained 6-week-old female BALB/c (Charles River Laboratories, Inc., Wilmington, MA, USA) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the animal care committee of Kyung Hee University [KHUASP(SE)-11-037]. We sensitized mice on days 1, 5, and 14 by intraperitoneal (i.p.) injection of 100 μ g OVA emulsified in 20 mg aluminum hydroxide (Sigma) and then mice were challenged by intranasal (i.n.) injection with 1.5 mg OVA according to previous reports (Choi et al., 2006; Jeong et al., 2011; Pellaton-Longaretti et al., 2011). KP (0.2, 2, or 20 mg/kg) and DEX (5 mg/kg) administrated orally before i.n. OVA challenge for 10 days. The dose of DEX was determined according to the previous reports (Richter et al., 2003; Masferrer et al., 1990). Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation at 10 days after challenge. The numbers of mice in each group are 5.

2.3. Culture of Eol-1 cells

Eol-1 cells, human eosinophilic leukemia cell line, are a useful in vitro model for studying human eosinophil functions. The Eol-1 cells have cytological features of myeloblasts under normal culture conditions and differentiate functionally into eosinophils by various stimuli (Mayumi, 1992). Human Eol-1 cells were a kind gift from Dr. Bae H. (Kyung Hee University). The Eol-1 cells were grown in RPMI1640 supplemented with 100 unit/ml penicillin, 100 mg/ml

streptomycin and 10% heat-inactivated FBS at 37 °C 5% CO₂ and 95% humidity. Eol-1 cells (3×10^5 cells/ml) were treated with KP (0.2, 2, or 20 μ g/ml) for 1 h prior to stimulation with GM-CSF.

2.4. Histamine assay

Histamine of serum was measured by the OPA spectrofluorometric procedure. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Eol-1 cells (3×10^5) were treated with KP (0.2, 2 or 20 μ g/ml) for 1 h prior to stimulation with GM-CSF incubated for 24 h. Cytokines of serum, nasal mucosa tissue, and spleen tissue and supernatant were measured by ELISA. ELISA was performed by coating 96-well plates with 1 μ g/well of capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with $1 \times$ PBS containing 0.05% tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, and AP, and ABTS substrate solution containing 30% H₂O₂. The plates were read at 405 nm. IL-32 was analyzed according to the manufacturers' specifications. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine level in spleen and nasal mucosa was divided according to the total protein. Protein was determined using a BCA.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Eol-1 cells (3×10^6) were treated with KP (0.2, 2 or 20 μ g/ml) for 1 h prior to stimulation with GM-CSF incubated for 4 h. Total RNA was isolated from cells and nasal mucosa according to the manufacturer's specification using easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final elutes was determined by spectrophotometry. Total RNA (2.5 μ g) was heated at 65 °C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37 °C using cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with the following primers for mouse TSLP (5'-TAT GAG TGG GAC CAA AAG TAC CG-3'; 5'-GGG ATT GAA GGT TAG GCT CTG G-3'), mouse GAPDH (5'-TTC ACC ACC ATG GAG AAG GC-3'; 5'-GGC ATG GAC TGT GGT CAT GA-3'), human IL-8 (5'-CGA TGT CAG TGC ATA AAG ACA-3'; 5'-TGA ATT CTC AGC CCT CTT CAA AAA-3'), human IL-32 (5'-TGA CAT GAA GAA GCT GAA GGC-3'; 5'-CAT GAC CTT GTC ACA AAA GCT C-3') and human GAPDH (5'-TCG ACA GTC AGC CGC ATC TTC TTT-3'; 5'-ACC AAA TCC GTT GAC TCC GAC CTT-3') was used to verify whether equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 60 °C for TSLP, IL-8, IL-32, and GAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.7. Western blot analysis

Western blot analysis was used nasal mucosa tissue extracts was prepared by detergent lysis procedure. Samples were heated at 95 °C for 5 min, and briefly cooled on ice. Following the centrifugation at 15,000g for 5 min, 50 μ g aliquots were resolved by 12% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerol, 20% methanol at 25 V. Blots were blocked for at least 2 h with PBST containing

5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

2.8. Histological examination

Tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. Section of the nasal mucosa sample is 4 μ m thick. Each section was stained with hematoxylin and eosin (H&E, for eosinophils) before dewaxing and dehydration. The number of eosinophils and mast cells on both sides of the septal mucosa was counted. Sections were coded and randomly analyzed by two blinded observers.

2.9. Confocal laser scanning microscopy

Tissue samples were fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with bovine serum albumin followed by 60 min of incubation with an anti-mouse c-Kit (Santacruz, CA, USA) at a concentration of 1 μ g/ml. The secondary antibody, tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated anti-mouse IgG (invitrogen), was added to the incubation for 30 min. The mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA) was used to counterstain DNA. All specimens were examined with a confocal laser-scanning microscope. The numbers of mast cells on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

2.10. Caspase-1 assay

Eol-1 cells (3×10^6) were treated with KP (0.2, 2 or 20 μ g/ml) for 1 h prior to stimulation with GM-CSF incubated for 2 h. Caspase-1 assay was used nasal mucosa tissue and cell extracts. Caspase-1 activity was measured according to the manufacturer's specification using caspase assay kit (R & D system). Equal amount of total protein was quantified by BCA protein quantification kit (Sigma) in each lysate. Catalytic activity of caspase-1 from cell lysate was measured by proteolytic cleavage of WEHD-pNA for 4 h at 37 °C. The plates were read at 405 nm.

2.11. MTT assay

Eol-1 cell aliquots (2×10^5 cells/ml) were cultured in microplate wells for 8 h after treatment by KP (0.2, 2 or 20 μ g/ml) and incubated with 20 μ l of an MTT solution (5 mg/ml) for additional 4 h at 37 °C under 5% CO₂ and 95% air. Consecutively, 250 μ l of DMSO was added to extract MTT formazan and the absorbance of each well at 540 nm was read by an automatic microplate reader.

2.12. Statistical analysis

The experiments shown are a summary of the data from at least three experiments and statistical analyses were performed using SPSS statistical software (SPSS 11.5, USA). Treatment effects were analyzed by one-way ANOVA, offered by Tukey's multiple range tests, and $P < 0.05$ was used to indicate significance.

3. Results

3.1. KP reduced the levels of IL-32, IL-8, and caspase-1 in Eol-1 cells

We previously reported that IL-32 was increased in AR patients and a mouse model and was expressed via the activation of caspase-1 by GM-CSF in eosinophils (Jeong et al., 2011). Thus, we examined the effects of KP on IL-32 levels in GM-CSF-stimulated Eol-1 cells. The dose of KP was determined according to a previous report (Gong et al., 2012). The protein and mRNA levels of IL-32 were significantly inhibited by the treatment of KP (Fig. 1A and C). KP significantly inhibited the GM-CSF-induced IL-8 production and mRNA expression (Fig. 1B and C). In addition, the treatment of KP inhibited the GM-CSF-induced caspase-1 activation in Eol-1 cells (Fig. 1D). KP had no effect on cell viability (Fig. 1E).

3.2. KP reduced the clinical symptoms, spleen weight, IgE, histamine, and IL-4 levels in the AR mice

To confirm the regulatory effect of KP in the AR mouse model, KP was administrated orally before an i.n. OVA challenge for 10 days. DEX was used as a positive control. Clinical symptoms were significantly inhibited in KP-administrated mice (Fig. 2A). The spleen weight was significantly elevated in the AR mice compared with normal mice. This increased spleen weight was reduced by KP administration (Fig. 2B). Serum IgE levels were significantly elevated in the AR mice compared with the normal mice. IgE levels increased by OVA were reduced by KP administration (Fig. 2C). Serum histamine levels were also reduced by KP administration (Fig. 2D). To identify the Th1/Th2 immune reactions in KP-administered mice, we measured IL-4 and IFN- γ levels in the spleen. As shown in Fig. 2E, the levels of IL-4 in the AR mice were significantly increased compared to those in the normal mice. Increased IL-4 levels were significantly decreased in the KP-administered mice. However, IFN- γ levels decreased by OVA were significantly increased in the KP-administered AR mice (Fig. 2F).

3.3. KP reduced the levels of IL-32 and TSLP in the AR mice

Repeated nasal exposure to IL-32 significantly increases the inflammatory reactions in AR nasal epithelium (Jeong et al., 2011), so we measured the IL-32 levels in the AR mice. IL-32 levels in the serum and nasal mucosa tissues of the AR mice were increased more than in normal mice (Fig. 3A). Increased IL-32 levels were significantly reduced by KP administration (maximal inhibition rate in serum $\approx$ 83.95%, maximal inhibition rate in nasal mucosa tissue $\approx$ 75%). The OVA-induced AR mice induced the TSLP expression in the AR nasal mucosa (Miyata et al., 2008). Thus, we measured TSLP levels in the AR mice. As shown in Fig. 3B, TSLP levels in the serum and nasal mucosa tissues of AR mice were increased more than in normal mice. In the KP-administrated mice the levels of TSLP were significantly inhibited compared with in the AR mice (maximal inhibition rate in serum $\approx$ 85.04%, maximal inhibition rate in nasal mucosa tissue $\approx$ 65%). To evaluate the regulatory effects of KP on TSLP mRNA expression, we measured the mRNA levels of TSLP in the nasal mucosa tissue. TSLP mRNA levels were also significantly decreased by KP administration (Fig. 3C).

3.4. KP reduced the inflammation-related protein levels in the AR mice

To evaluate the regulatory effects of KP on inflammation-related protein levels, we analyzed the MIP-2, ICAM-1, and COX-2 levels in the AR mice. The levels of MIP-2, ICAM-1, and COX-2 in the AR mice were significantly higher than those in the nasal mucosa tissues of the normal mice (Fig. 4). Increased protein levels were reduced by KP administration (Fig. 4).

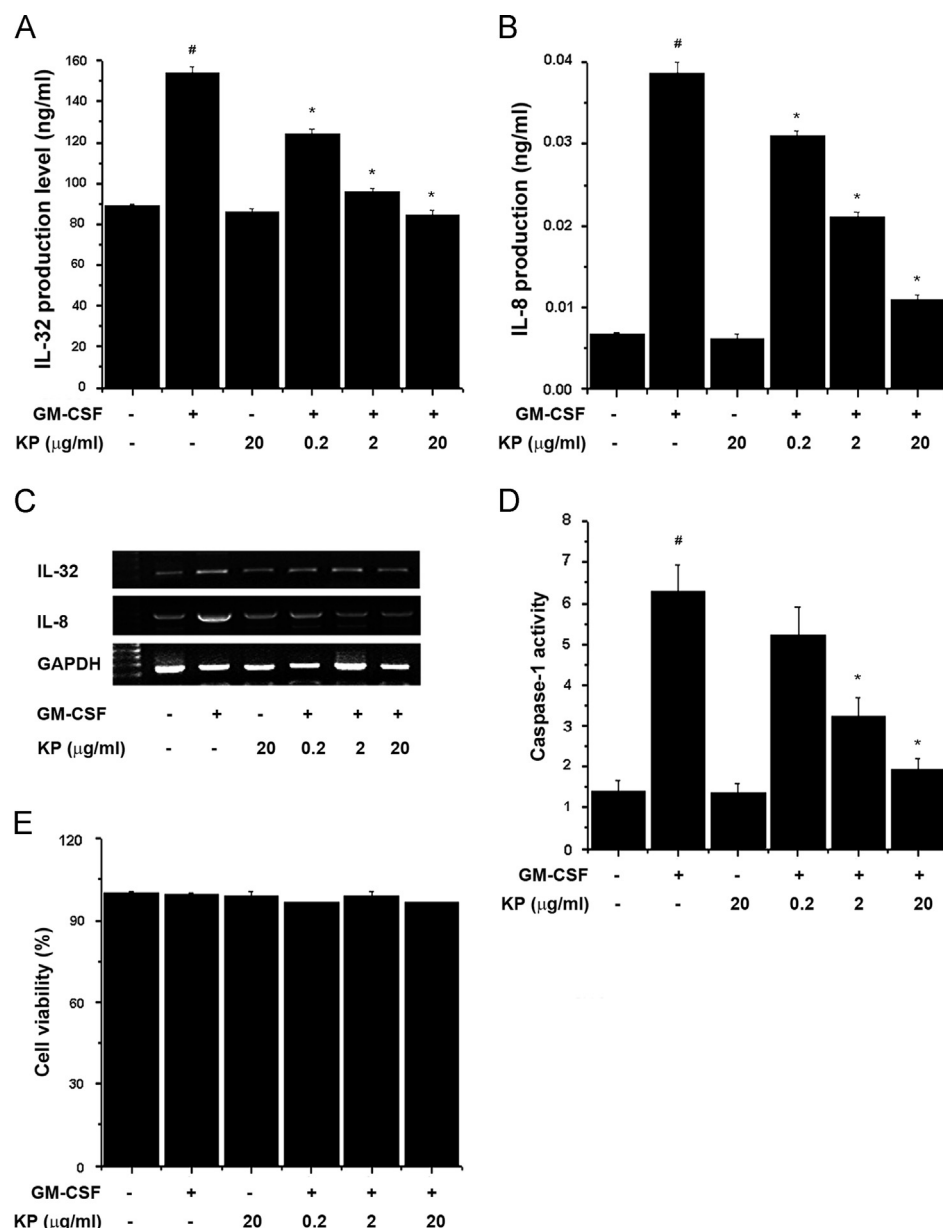


Fig. 1. Effects of KP on levels of IL-32 and IL-8 and activity of caspase-1 in Eo1-1 cells. Eo1-1 cells were treated with KP (0.2, 2 or 20 μg/ml) and then stimulated with GM-CSF. (A and B) IL-32 and IL-8 was measured by the ELISA method. (C) Messenger RNA was measured using the RT-PCR method. (D) The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. (E) Cell viability was evaluated by an MTT assay. [#]*P* < 0.05; significantly different from the unstimulated cells. ^{*}*P* < 0.05; significantly different from the GM-CSF-stimulated cells. KP, kaempferol.

3.5. KP reduced the caspase-1 activation in the AR mice

Jeong et al. reported that IL-32 induced TSLP production by increasing NF-κB and caspase-1 activation (2012). To investigate the effects of KP on caspase-1 activation, we performed Western blot analysis for caspase-1 and caspase-1 assay with the nasal mucosa tissues. KP reduced the active form of caspase-1 in the nasal mucosa tissue. OVA-induced caspase-1 activity was decreased by KP administration (Fig. 5).

3.6. KP reduced the eosinophil and mast cell infiltration in the AR mice

The respective numbers of inflammatory cells (eosinophil and mast cell) in the nasal mucosa of the AR mice were significantly higher than those in the normal mice. In the KP-administered

mice, eosinophil and mast cell infiltration increased by OVA was significantly decreased (Fig. 6).

4. Discussion

AR is symptomatic of the nose induced by an IgE-mediated inflammation of the nasal mucosa after allergen exposure (Bousquet et al., 2001). The repeated exposure to allergens such as OVA causes repeated or continuing inflammation such as eosinophilia, Th2 cytokine induction, or airway hyper-responsiveness (Henderson et al., 1996; Nials and Uddin, 2008). In mice challenged with OVA, IL-4 and IL-5 producing cells (Th2-type cells) infiltrated into the spleens (Chung et al., 2012). OVA-sensitized mice increased spleen weight and spleen index (Wang et al., 2011). Reduction in the weight of the spleen can be indicative of the relative immune status, such as

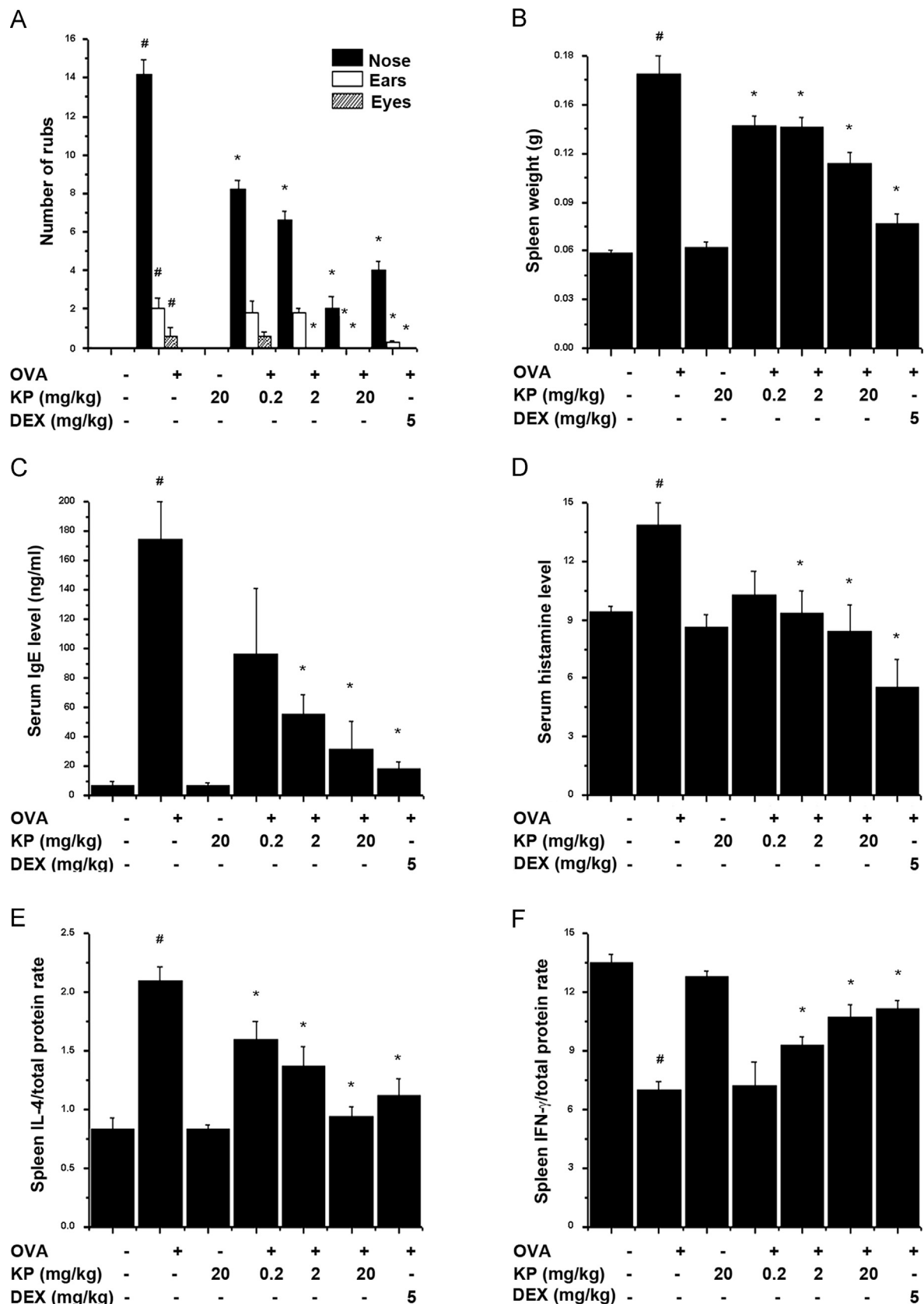


Fig. 2. Effect of KP on clinical symptoms, IgE, histamine, spleen weight, IL-4, and IFN- γ level in AR model. We sensitized the mice on days 1, 5, and 14 by i.p. injections of 100 μ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged the mice with 1.5 mg OVA. Mice received KP before the intranasal OVA challenge for 10 days. (A) The number of the nasal, ear, and eye rubs that occurred in the 10 min after the OVA i.n. provocation. Mice received KP before the intranasal OVA challenge for 10 days. (B) IgE in serum. (C) Serum was isolated from blood and then assayed about histamine. (D) Spleen weight. (E) IL-4 and (F) IFN- γ were measured by the ELISA method. All parameters measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. # $P < 0.05$; significantly different from the OVA-unsensitized mice. * $P < 0.05$; significantly different from the OVA-sensitized mice. OVA, ovalbumin; KP, kaempferol; DEX, dexamethasone. $N = 5$.

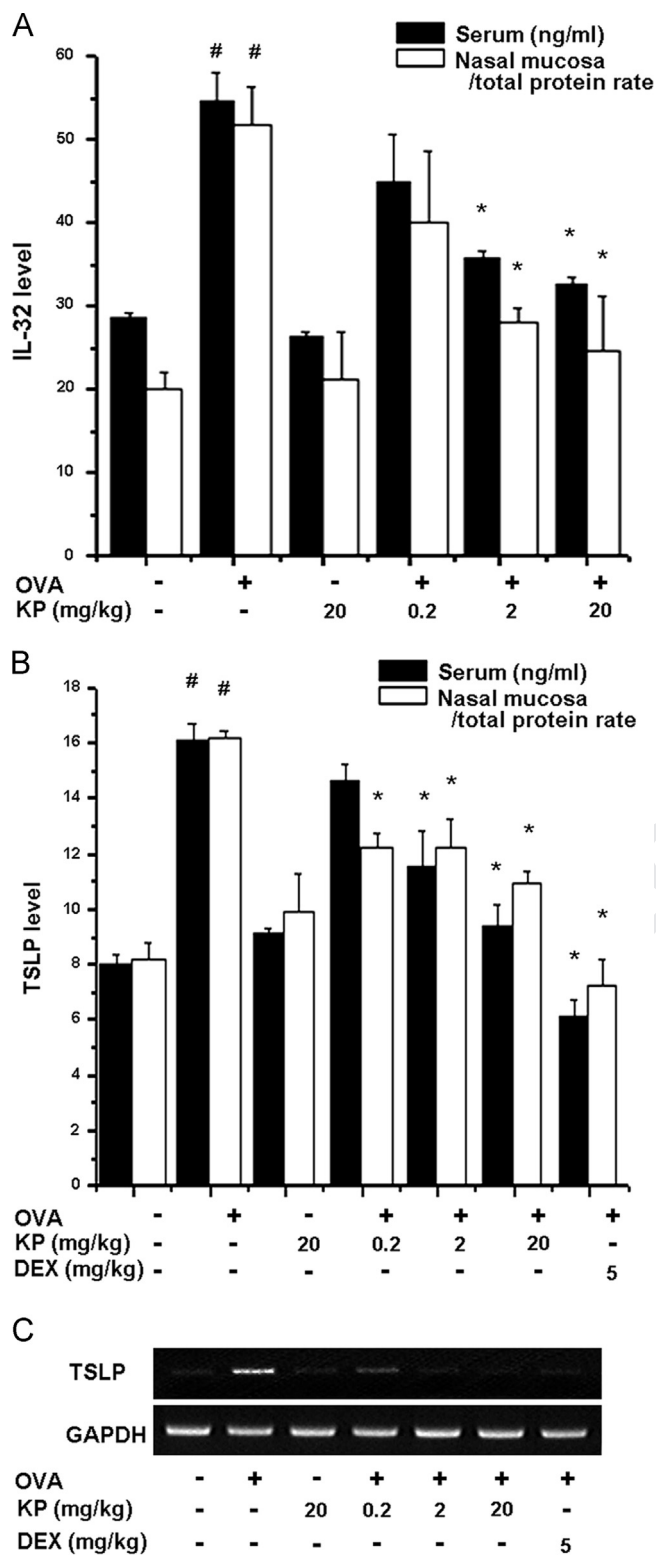


Fig. 3. Effects of KP on IL-32 and TSLP expressions in the AR mice. (A and B) IL-32 and TSLP were measured by ELISA method in the serum and nasal mucosa tissue. (C) Messenger RNA was measured using the RT-PCR method. All parameters measured in the tissue homogenate were presented as a ratio to the total protein levels in the tissue. $^{\#}P < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}P < 0.05$; significantly different from the OVA-sensitized mice. OVA, ovalbumin; KP, kaempferol; DEX, dexamethasone. $N=5$.

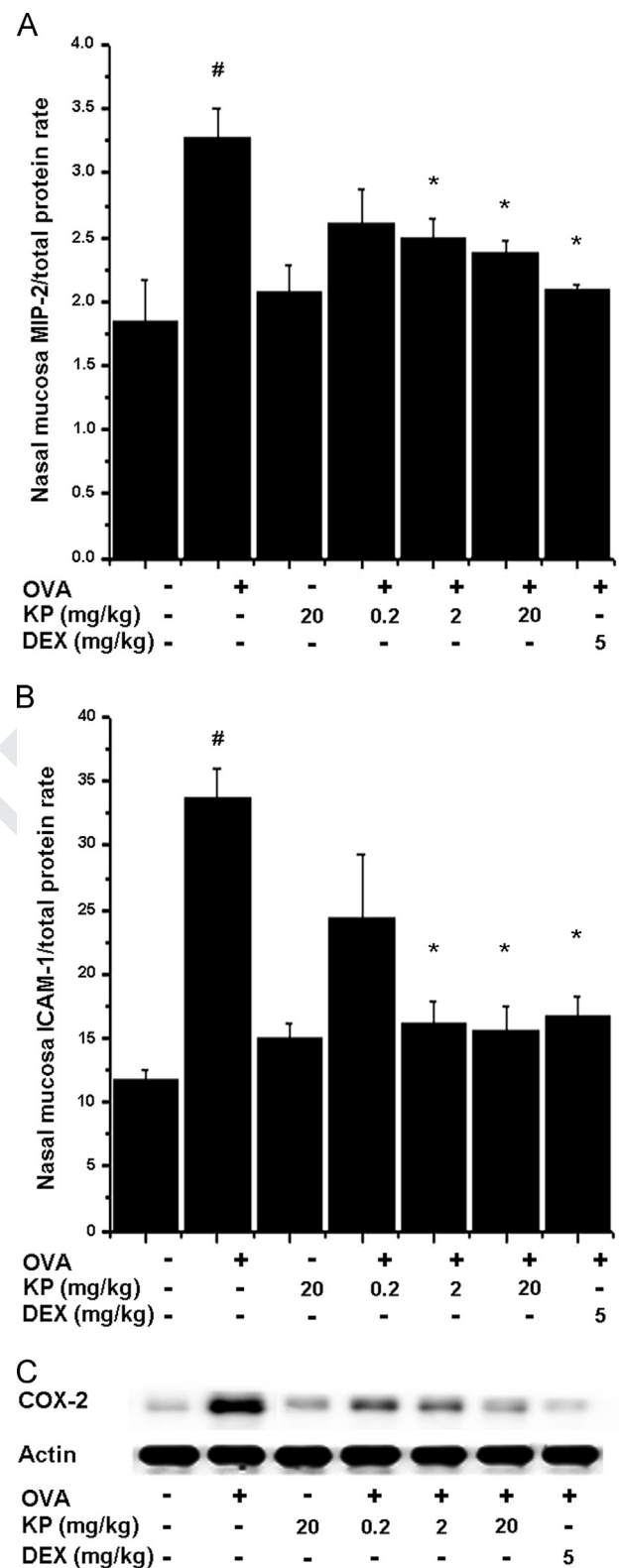


Fig. 4. Effects of KP on MIP-2, ICAM-1, and COX-2 levels in the nasal mucosa of the AR mice. (A) MIP-2 and (B) ICAM-1 were measured by the ELISA method in nasal mucosa tissue. (C) COX-2 protein expression was evaluated by Western blot analysis. All parameters measured in the tissue homogenate were presented as a ratio to the total protein levels in the tissue. $^{\#}P < 0.05$; significantly different from the OVA-unsensitized mice. $^{*}P < 0.05$; significantly different from the OVA-sensitized mice. OVA, ovalbumin; KP, kaempferol; DEX, dexamethasone. $N=5$.

cell-mediated immune response (Aggarwal et al., 2008). A number of different models of chronic allergic pulmonary inflammation have been developed by increasing the number of OVA challenges

(Nials and Uddin, 2008). An AR mice model can be induced by repeated intranasal challenge with OVA (Saito et al., 2002; Jeong et al., 2011). However, it cannot exclude the possibility that intranasal

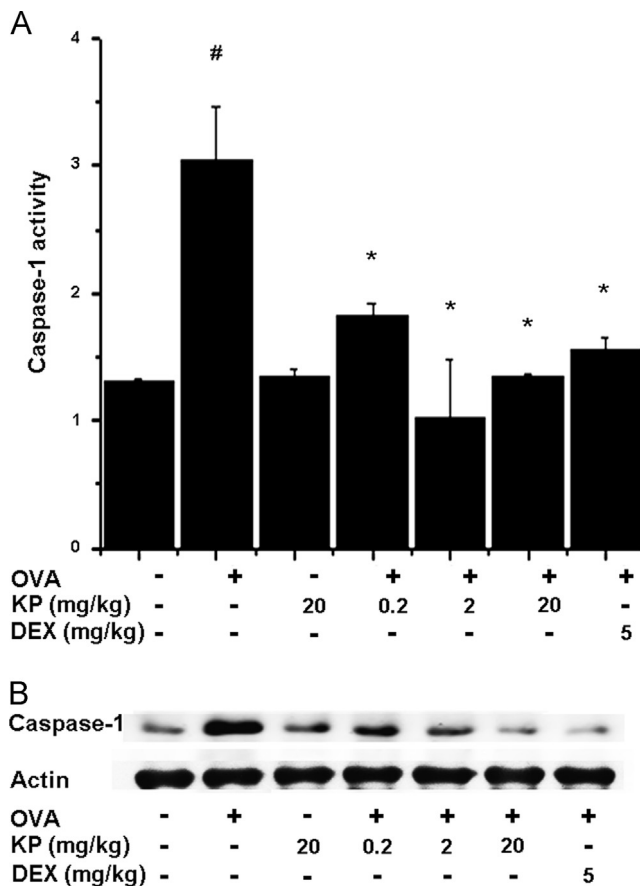


Fig. 5. Effects of KP on caspase-1 activation in the nasal mucosa of the AR mice. (A) The enzymatic activity of caspase-1 was tested by a caspase-1 colorimetric assay. (B) Caspase-1 protein expression was evaluated by Western blot analysis. [#] $P < 0.05$; significantly different from the OVA-unsensitized mice. ^{*} $P < 0.05$; significantly different from the OVA-sensitized mice. $N = 5$.

administration of OVA according to doses of OVA might induce systemic anaphylactic reactions. (Daheshia et al., 2001) challenged mice with a high dose of OVA and induced systemic anaphylactic reactions.

The various cytokines, chemokines, or histamine released from inflammatory cells, such as eosinophils or mast cells, are predominant mediators in AR (Howarth et al., 2000). IL-32 expression was induced by GM-CSF through the activation of caspase-1 in eosinophils (Jeong et al., 2011). We have also reported that IL-32 significantly increased the TSLP production in monocytes (Jeong et al., 2012). Blocking of the signal pathway of TSLP inhibited the TSLP-driven Th2 inflammatory cell infiltration, cytokine secretion, and IgE production in mouse lung (Seshasayee et al., 2007). TSLP was expressed by the caspase-1 and NF- κ B signaling pathways in mast cells (Moon and Kim, 2011). In addition, mast cells express the functional TSLP receptor and respond to TSLP in the presence of IL-1 and TNF- α (Allakhverdi et al., 2007). Furthermore, IL-32 expression is up-regulated in the nasal mucosa of AR patients (Jeong et al., 2011). TSLP played a role in the pathogenesis of AR in children with asthma (Bunyanich et al., 2011). In addition, the levels of IL-32 and TSLP were significantly increased in serum and nasal mucosa of the AR mouse model in this study. Thus, we suggest that IL-32 and TSLP are new mediators of AR in humans and mice. In this study, we showed that KP inhibited the levels of new mediators, IL-32 and TSLP as well as predominant mediators such as histamine and IgE. Therefore, we deduce that KP has an anti-allergic effect.

IL-32 had the capacity to provoke histamine release in mast cells (Daheshia et al., 2001). TSLP also induced the histamine release from mast cells (Ito et al., 2012). All effects of the histamine were abolished by the presence of the caspase-1 inhibitor, which indicated that histamine action is dependent on cytokines mediated by caspase-1 (Nishibori et al., 2001). In this study, KP significantly inhibited the level of serum histamine. Thus, we postulate that KP might inhibit clinical symptoms through down-regulation of histamine by regulating IL-32/TSLP/caspase-1 signaling pathways.

Nasal biopsies of patients with AR have shown accumulations of inflammatory cells, including mast cells, eosinophils, and basophils in the epithelium (Fuentes-Beltrán et al., 2009). Eosinophils are innate effector cells that are important in immune responses against helminth parasitic infections and contribute to the pathology associated with allergic inflammatory conditions (Melvin and Ramanathan, 2012; Isobe et al., 2012). The eosinophil granule proteins play a central role in the development of rhinitis and chronic rhinosinusitis (Bystrom et al., 2012). IL-32 released from eosinophils exaggerated the AR (Jeong et al., 2011). TSLP produced from mast cells plays a role in allergic inflammation (Moon and Kim, 2011). Mast cells contribute to the induction and maintenance of eosinophilic inflammation by IgE-dependent and IgE-independent processes (Pawankar et al., 2007). The recruitment of these mast cells to inflammatory sites occurs in response to chemotactic and activation signals (Boumazou et al., 2010). Inflammation is a physiopathological phenomenon referring to the presence of an inflammatory cell infiltrate (eosinophils and neutrophils) associated with the expression of ICAM-1 in the epithelial cells of the mucosa exposed to the allergen, in the absence of clinical symptoms. ICAM-1 is expressed in the mucosal epithelial cells of allergic patients. ICAM-1 has been considered a marker of allergic inflammation (Montoro et al., 2007). The COX-2 pathways have also been implicated in allergic and inflammatory reactions (Montuschi et al., 2007). In this study, we observed that KP inhibited the levels of inflammation-related protein and infiltration of inflammatory cells. An anti-allergic effect by KP was associated with inhibition of the infiltrated inflammatory cell number. Therefore, KP provides affirmative proof of a possible therapeutic application of this agent in allergic inflammatory diseases.

Naju Jjok (*Polygonum tinctorium* Lour.) has been known for several thousand years to have anti-bacteria, anti-cancer, and anti-atopic effect (Iwaki et al., 2006; Satomi et al., 2001; Katoka et al., 2001; Kunikata et al., 2000). KP, tryptanthrin, and indirubin are components of the Naju Jjok. (Lee et al., 2010) reported that KP as a flavonoid inhibited the production of IL-8 and TNF- α on the IgE-stimulated intestinal epithelial cell line. KP has anti-oxidant and anti-inflammatory properties through the blocking of leukotriene B4 and lipoxigenase (Loke et al., 2008). KP and tryptanthrin have anti-bacterial action (Katoka et al., 2001). Tryptanthrin has an anti-cancer effect (Satomi et al., 2001). Indirubin inhibited inflammatory reaction (Kunikata et al., 2000). Our previous study reported that hesperidin as a flavonoid has an anti-allergic effect through the regulation of caspase-1 activity by interaction with the active site of caspase-1 (Song et al., 2012). In this study, we showed that KP inhibited the activity of caspase-1 in GM-CSF-stimulated Eo1-1 cells and the nasal mucosa tissue of an AR mouse model. Thus, we assume that KP has an anti-allergic activity by targeting caspase-1 as hesperidin. However, further study is necessary to clarify the reaction mechanism between KP and caspase-1.

5. Conclusion

KP has not been elucidated to understand its inhibitory effects and mechanism on new mediators of AR such as IL-32 and TSLP. In this study, for the first time, we demonstrated that KP

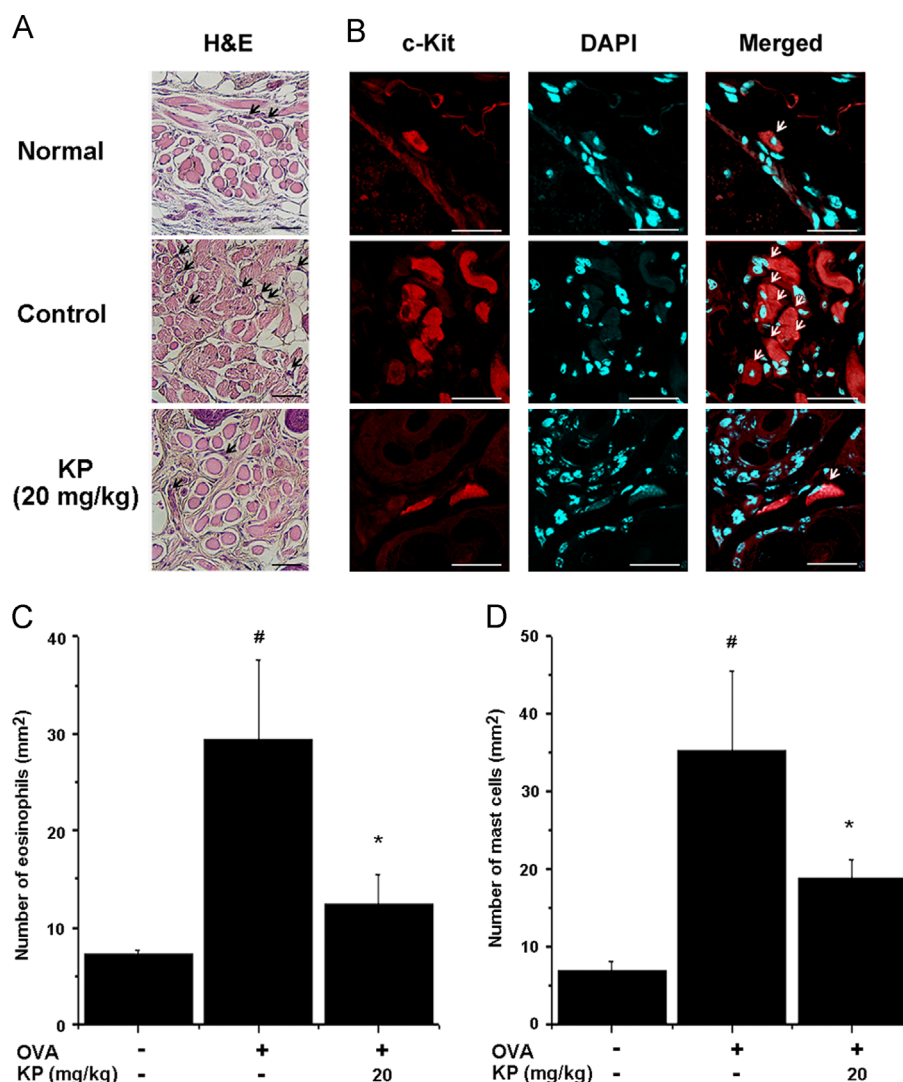


Fig. 6. Effect of KP on eosinophil and mast cell infiltration in AR nasal mucosa tissue. (A) Nasal mucosa tissue was stained with hematoxylin and eosin (H&E) for eosinophils (black arrow). (B) Mast cells were stained by a primary anti-c-Kit for 1 h and then incubated by secondary TRITC-conjugated IgG for 1 h (white arrow). (C and D) Eosinophils and mast cells were counted by three individuals. The five randomly selected tissue sections per mouse were counted. The absolute number of cells was counted as the mean $\pm$ S.E.M. [#] $P < 0.05$; significantly different from the OVA-unsensitized mice. ^{*} $P < 0.05$; significantly different from the OVA-sensitized mice. $N=5$. Normal; OVA-unsensitization, Control; OVA-sensitization; KP, OVA-sensitization and KP administration. (Original magnification $\times 400$ for H&E; $\times 600$ for c-Kit, scale bar = 50 μ m).

significantly reduced the early phase response to allergen exposure such as nasal rubs, IgE production, and histamine release as well as the late phase responses such as expression of inflammation markers (IL-32, TSLP, IL-4, IL-8, ICAM-1, MIP-2, and COX-2). KP also decreased caspase-1 activation. Therefore, we hypothesize that KP can be helpful in treating allergic inflammatory diseases, including AR.

Q2 Uncited references

(Lee et al. (2010); Takano et al. (2004)).

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