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Resveratrol: a potential new drug for the treatment of eosinophilic rhinosinusitis with nasal polyps

by

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논문제목: 비용종을 동반한 호산구성 비부비동염에 대한 resveratrol 의 치료효과 (Resveratrol: a potential new drug for the treatment of eosinophilic rhinosinusitis with nasal polyps)

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서울대학교총장 귀하

Abstract

Resveratrol: a potential new drug for the treatment of eosinophilic rhinosinusitis with nasal polyps

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Background: Patients with chronic rhinosinusitis often suffer from nasal stuffiness, olfactory dysfunction, and the decline of quality of life. Chronic rhinosinusitis is frequently accompanied by nasal polyps. Although systemic administration of corticosteroids is efficient medical treatment for nasal polyps, it cannot be used for long-term periods because of its detrimental side effects such as osteoporosis, depression and diabetes mellitus. Therefore, search for safe and effective novel drugs for nasal polyps is needed. Recently, a murine model of eosinophilic rhinosinusitis with nasal polyps was established by using *Staphylococcus aureus* enterotoxin B. Using the same protocols, therapeutic effects of resveratrol on eosinophilic rhinosinusitis with nasal polyps were examined, and the mechanism of actions were investigated in this study.

Materials and Methods: Mice in the experimental groups were sensitized with ovalbumin whereas those in the control group received phosphate-buffered saline. Experimental groups were challenged intranasally with *Staphylococcus aureus* enterotoxin B weekly during the late 8 weeks of experiments, and divided into 4 subgroups according to the administered drugs: a mixture of dimethyl sulfoxide and phosphate-buffered saline, triamcinolone acetonide, low-dose and high-dose resveratrol. Histopathologic changes were observed using hemotoxylin and eosin for overall inflammation and polyp-like lesions, Sirius red for eosinophils, Giemsa for mast cells, Masson's trichrome for collagen, and alcian blue staining for secretory cells. The expression of cyclooxygense-2 and 5-lipooxygense were evaluated by immunohistochemical staining and Western blot analysis. The levels of interleukin (IL)-4, IL-5, prostaglandin D synthase, and leukotriene C_4 synthase transcripts were determined by quantitative real-time PCR. The differences in histologic and immunologic findings were compared between groups.

Results: The degree of eosinophilic infiltration and subepithelial fibrosis, the proportion of eosinophils in total inflammatory cells were significantly decreased by administration of high-dose resveratrol, and its potency was similar to that of triamcinolone acetonide. By means of immunohistochemical staining and Western blot analysis, it was identified that 5-lipooxygenase expression was strongly inhibited by high-dose resveratrol. The gene expression of IL-4, IL-5, prostaglandin D synthase, and leukotriene C_4 synthase was highest in mice with eosinophilic

rhinosinusitis with nasal polyps, and administration of low- or high-dose resveratrol lowered their expression significantly. The number of polyp-like lesions also decreased, but this change was not statistically significant. Although low-dose resveratrol did not show definite anti-inflammatory effects, it reduced the proportion of eosinophils in total inflammatory cells and the degree of subepithelial fibrosis.

Conclusion: Resveratrol, particularly in a high dose, exhibited apparent anti-inflammatory and polyp-reducing effects in a murine model of eosinophilic rhinosinusitis with nasal polyps, and a key mechanism of its action is believed to be the inhibition of the lipooxygenase pathway. Resveratrol appears to be a new potential drug for the treatment of eosinophilic rhinosinusitis with nasal polyps although a further human study is needed to confirm it.

Keywords: resveratrol, rhinosinusitis, nasal polyp, mouse model, eosinophil, lipooxygenase.

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SAEs: *Staphylococcus aureus* exotoxins

SEB: *Staphylococcus aureus* enterotoxin B

OVA: Ovalbumin

COX-2: cyclooxygenase-2

5-LO: 5-lipooxygenase

DMSO: dimethyl sulfoxide

PBS: phosphate-buffered saline

IHC: Immunohistochemistry

qPCR: quantitative real-time PCR

IL: interleukin

PGDS: prostaglandin D synthase

LTC_4S: leukotriene C_4 synthase

Sir2: silent information regulator 2

NO: nitric oxide

PMN: polymorphonuclear leukocytes

DMBA: 7,12-dimethylbenz(α)anthracene

TGF-β: transforming growth factor-β

Introduction

The prevalence of chronic rhinosinusitis is known to reach approximately 12.5% in general population and it is frequently accompanied by nasal polyps which occur in 1% - 4% of general population.[1,2] Nasal polyps often bring about nasal stuffiness and olfactory dysfunction resulting in the decline of quality of life. Furthermore, it has propensity to recur after surgical removal. In addition, nasal polyp-related medical cost is increasing along with the increase of allergic diseases and air pollution.[3] Systemic administration of corticosteroids has been the most efficient medical treatment thus far, and its crucial mode of action is known to be an anti-inflammatory effect. However, it cannot be used for long-term periods because prolonged use of them result in detrimental side effects such as diabetes mellitus, osteoporosis, depression, and peptic ulcer.[4] Thus, it is mandatory to search for safe and effective novel drugs for the treatment of rhinosinusitis with nasal polyps.

Understanding the pathomechanism of rhinosinusitis with nasal polyps is of paramount importance in order to discover a new potential drug for it. Previous studies elucidated that nasal polyps are not simple mucosal edema, but benign inflammatory growth consisting of cystically dilated glands which are totally different from the seromucinous glands of normal turbinal mucosa.[5] Numerous factors have been reported to be associated with nasal polyp formation, including infection,[6,7] allergy,[8-11] superantigens,[12-15] and genetic predisposition,[16,17] yet the precise mechanism of nasal polyp formation is still unknown. Since infiltration of

inflammatory cells, particularly eosinophils, and tissue remodeling are consistently found in nasal polyps, nontheless, the molecules which have anti-inflammatory and/or remodeling-preventing effects are assumed to inhibit nasal polyp formation.[18-21]

Resveratrol was first extracted from a nonedible Peruvian legume, *Cassia quinquangulata* Rich. Although resveratrol is ubiquitous in nature, it is found in only a few edible substances, most notably the grape. [22] Resveratrol has been found to have various beneficial effects thus far: strong inhibition of cyclooxygenase (COX), cancer chemoprevention, and even life extension. The molecular targets of resveratrol, which mediate its diverse cellular effects, are the subject of ongoing investigations.[22,23] In terms of safety, several animal studies have been performed, and no significant toxicity such as genotoxicity and carcinogenicity was identified. Moreover, nephrotoxicity was found only at very high dosages (2,000 - 3000 mg/kg/day). On the basis of these studies, an acceptable daily intake of resveratrol in food has been defined as 450 mg/day.[24]

Since a mouse model of eosinophilic rhinosinusitis with nasal polyps was established recently, research for the development of potential new drugs and elucidation of the mechanism of their effect has been feasible.[25] In brief, the model was induced by systemic sensitization with ovalbumin (OVA) followed by intranasal instillatioin of OVA and *Staphylococcus aureus* enterotoxin B (SEB). Using this murine model, therapeutic effects of resveratrol on eosinophilic rhinosinusitis with nasal polyps, and the mechanism of actions were investigated in this study.

Materials and Methods

Experimental Animals

BALB/c mice (4 weeks of age) were purchased from Koatech Laboratory Animal, Inc. (Pyeongtaek, Korea). These animals were kept in a special pathogen-free biohazard containment facility maintained at 22℃ to 24℃ and 50% to 60% humidity. All experimental protocols complied with the Guidelines of the National Institute of Health and the Declaration of Helsinki, and were approved by the Committee on the Use and Care of Animals at Gyeongsang National University.

Experimental Protocols

Mice were categorized into a single control and 4 experimental groups, and eosinophilic inflammation in the nasal and sinus mucosa was induced in each mouse according to the protocols which were previously established.[25] In brief, OVA (Grade V; Sigma, St. Louis, MO, USA) was used to obtain systemic sensitization and local stimulation, followed by intranasal instillation of SEB in experimental groups (Figure 1). Instead, in the control group (group A), phosphate-buffered saline (PBS) was applied systemically as well as locally. The experimental groups were designated as follows: intraperitoneal injection of vehicle, which means a mixture of dimethyl sulfoxide (DMSO) and PBS with a ratio of 1:9 (group B), intraperitoneal injection of triamcinolone acetonide with a concentration of 1 mg/kg (group C), intraperitoneal injection of resveratrol with a

concentration of 0.5 mg/kg (group D) and 5 mg/kg (group E). Mice in the experimental groups were systemically sensitized with 25 μg OVA dissolved in 300 μL PBS in the presence of 2 mg aluminum hydroxide gel as adjuvants by intraperitoneal injection on days 0 and 5. One week after the second intraperitoneal injection, mice were challenged intranasally with 3% OVA diluted in 40 μL of PBS daily for 1 week. Thereafter, continual local stimulation was maintained in the same fashion three times a week for 4 consecutive weeks. Finally, 3% OVA diluted in 40 μL of PBS was applied intranasally accompanied by intraperitoneal injection of drugs including triamcinolone acetonide and resveratrol with the same intervals for eight consecutive weeks. During that period, 10ng SEB diluted in 20 μL of PBS was challenged intranasally, subsequent to the instillation of OVA once a week. Twenty-four hours after the final nasal challenge, mice were euthanized and decapitated. Each experimental and control group contained 20 and 10 mice, respectively; half were prepared for histologic examination while nasal mucosa was obtained in the remaining half for Western blot analyses and quantitative real-time PCR (qPCR). Since a single mouse in group B died during the experiments, nine mice were finally prepared for histologic analysis.

Histologic Analyses

The skin and soft tissues of the mice were removed from the skull. Heads were immediately fixed in 2% paraformaldehyde and decalcified in 5% nitric acid for 4 - 5 days at 4°C. The specimens were excised from the

second palatal ridge to the first upper molar teeth. The tissues were dehydrated and processed according to standard paraffin-embedding procedures, and then were cut in coronal sections with a thickness of 4 μm. Several stains were conducted in order to compare the characteristics between groups: hematoxylin and eosin (H&E) for overall inflammation, Sirus red for eosinophils, Giemsa for mast cells, alcian blue for secretory cells, and Masson's trichrome staining for collagen in the subepithelial layer.

An atlas of normal murine sinonasal anatomy was used to standardize the anatomic locations being examined. First, the vomeronasal organ was identified. The superior and inferior maxillary turbinelles were identified for anatomic orientation. The true maxillary sinus and ethmoidal labyrinths were identified at the lesions posterior to the two maxillary turbinelles. Three coronal sections which were similar to the sinus cavity were chosen for evaluation, according to the previous study. The number of polyp-like lesions, inflammatory and secretory cells was counted under high-power fields (10 X 40) by an examiner blinded to the groups. Polyp-like lesions were defined as distinct mucosal bulges with eosinophilic infiltration and microcavities. The thickness of the subepithelial layer was measured by using an image analysis system (NIS-Elements BR 3.0 system; Nikon Eclipse, Tokyo, Japan). Three consecutive slides were reviewed in order to exclude processing errors.

Immunohistochemistry (IHC)

Other sections were immunostained for COX-2 and 5-lipooxygenase (LO) using the avidin-biotinylated-horseradish peroxidase-complex kits (ABC; Vector Laboratories, Burlingame, CA). After deparaffinization in xylene, sections were rehydrated with ethanol. After washing in PBS, the sections were blocked with 1% normal goat serum and then treated with each primary polyclonal antibody for COX-2 (Cell Signaling Tech, Beverly, MA) and 5-LO (Cell Signaling Tech) at 4°C overnight in a humidified chamber. After washing in PBS, they were incubated for 90 minutes at room temperature with secondary antibody (biotin-conjugated goat anti-rabbit immunoglobulin G, 1:200). Finally, the sections were incubated with ABC for 60 minutes at room temperature, rinsed in PBS, and then developed by 0.027% 3, 3-diaminobenzidine tetrahydrochloride (Sigma) with 0.003% hydrogen peroxide. Finally, the sections were counterstained with hematoxylin (Sigma). Each section was examined under high-power field (10 × 40) by an independent researcher blinded to the experiments. Cells containing positive signals were counted at the transition zone of the olfactory and respiratory epithelium.

Protein Extraction and Western blot

Protein was obtained from the nasal mucosa of each mouse 1 day after the final nasal challenge and homogenized in lysis buffer (1% Triton X-100 and 1mM EDTA in 1x PBS [pH 7.4]) which contained 10μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. Cell lysates were sonicated several times for 3-5 minutes each and centrifuged at 12,000 rpm for 20

minutes at 4℃. Following the collection of supernatant, protein concentration of each lysate was determined by using bicinchoninic acid kit (Pierce, Rockford, IL) using bovine serum albumin as standard in accordance with the manufacturer's instructions. Equal amount of protein (60 μg) was loaded to 10% to 12% sodium dodecylsulfate-polyacrylamide gel. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), and the membranes were washed in Tris-buffered saline containing 0.1% Tween-20. Subsequent to the incubation with each primary rabbit polyclonal antibody for COX-2 (Cell Signaling Tech) and 5-LO (Cell Signaling Tech), the membrane was incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, 1:10,000; Pierce). Then, the blots were visualized by using ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Quantitative Real-time PCR

The levels of key cytokines interleukin (IL)-4, IL-5, and eicosanoid pathway-related enzymes prostaglandin D synthase (PGDS), and leukotriene C_4 synthase (LTC_4S) transcripts were determined by qPCR. Nasal mucosa was resuspended in 1ml of Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) and total RNA was prepared according to the manufacturer's instructions. Ten microgram of purified RNA was subsequently reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) and oligo-dT primers.

After reverse transcription, total DNA was diluted by ddH2O for quantitative real-time PCR. Quantitative cDNA amplification was performed using a CFX-96^{TM} Real-Time System (Applied Biosystems Inc., Foster City, CA), and PCR conditions as specified by the manufacturer. Each of the reaction mixtures contained: 9 µl of template cDNA, 10 µl of universal KAPA probe fast qPCR master mix (KAPA Biosystems, Woburn, MA), and 1 µl of 20× TaqMan gene expression Assay Mix for the genes of interest (Applied Biosystems Inc.) to a final volume of 20 µl. Samples were normalized using mouse GAPDH expression. Thermal cycle conditions were as follows: denaturation at 95°C for 3 minutes, followed by 50 cycles of denaturation at 95°C for 10 seconds, and annealing and extension at 60°C for 30 seconds.

Ct values representing the number of cycles at which the fluorescence value for each sample exceeded the threshold value were recorded with Bio-Rad CFX Manger 2.0 (Applied Biosystems Inc.). The expression level in each sample was compared with a calibrator. The following formula was applied: gene expression = $2^{-\Delta\Delta Ct}$

In brief, relative quantification was performed using $2^{-\Delta\Delta Ct}$ method. Reference gene for GAPDH was used. GAPDH as internal control was used for the normalization of the quantity of RNA. For normalization of the results Ct value for GAPDH was subtracted from Ct value for each of target genes (IL-4, IL-5, PGDS, and LTCS).

$\Delta Ct = \text{Average } Ct_{\text{Target gene}} - \text{Average } Ct_{GAPDH}$

The obtained difference (ΔCt) was then used to calculate ΔΔCt with the

formula:

$\Delta\Delta Ct = \Delta Ct_{experimental\ group} - \Delta Ct_{control\ group}$

The relative gene expression for cytokines normalized to an endogenous reference and relative to a calibrator was expressed as:

Gene expression level = $2^{-\Delta\Delta Ct}$

Relative gene expression for targeted genes in group A equals 1 (since $2^0 = 1$). Relative gene expression for measured cytokines and enzymes was presented as the fold change comparing to the control group.

Statistical Analyses

Data were expressed as mean ± SEM (standard error of the mean). The Mann-Whitney *U*-test and Kruskal-Wallis test generated by SPSS version 18.0 (SPSS, Chicago, IL) were used to determine the difference in the number of polyp-like lesions, inflammatory and secretory cells, the proportion of eosinophils in total inflammatory cells, the thickness of the subepithelial layer, the production of COX-2 and 5-LO, and the gene expression level of IL-4, IL-5, PGDS and LTC_4S between groups. A value of $P < 0.05$ was considered significant.

Results

Histologic Analyses

Polyp-like lesions

Polyp-like lesions were not observed in the nasal or sinus mucosa in group A. Sixteen polyp-like lesions were noted in seven out of nine mice in group B. Similarly, 12 polyp-like lesions were shown in seven out of ten mice in group D. However, only five polyp-like lesions were demonstrated in 5 out of 10 mice in group C, which seemed definitely fewer than in group B. Nontheless, statistical significance could not be obtained presumably due to the lack of number of polyp-like lesions. Similarly, the number of polyp-like lesions was also reduced in mice in group E compared to that in group B, but the difference was not statistically significant (Figure 2).

Eosinophilic inflammation

The number of eosinophils was largest in group B whereas mice in group A showed no apparent infiltration of inflammatory cells in the nasal and sinus mucosa. The degree of eosinophilic infiltration seemed less severe in group D than that in group B, yet the difference was not statistically significant. In contrast, mice in both groups C and E demonstrated much lower number of eosinophils than in group B ($P <$

0.001 and P = 0.003, respectively). Likewise, group E showed fewer eosinophilic infiltration in the nasal mucosa than group D (P = 0.006). Finally, the degree of eosinophilic inflammation was similar between group C and E (Figure 3).

The percentage of eosinophils in total inflammatory cells (mean ± SEM) was also compared between groups: 55.0 ± 1.9%, 42.0 ± 3.5%, 41.6 ± 2.7%, and 41.5 ± 3.3% in groups B, C, D, and E, respectively. The proportion of eosinophils was significantly smaller in groups C, D, and E compared with that in group B (P = 0.017, P < 0.001, and P = 0.006, respectively; Figure 4).

Distribution of mast cells

Mast cells were scarcely observed in the nasal mucosa even in group B while they were easily found in the mucosa of the maxillary sinus. Thus, mast cells were counted (mean ± SEM) in the maxillary sinus and compared between groups: 20.2 ± 2.8, 17.0 ± 1.0, 20.3 ± 2.2, and 19.9 ± 1.4 in groups B, C, D, and E, respectively. In group A, mast cells were not noted in the mucosa of maxillary sinus. The degree of mast cell infiltration seemed less severe in group C compared to other groups, but there was no significant difference between experimental groups (P = 0.39; Figure 5).

Secretory cell hyperplasia

The number of secretory cells (mean ± SEM) was calculated at the

transition zone of olfactory and respiratory epithelium: 1.0 ± 0.6, 6.1 ± 1.3, 3.8 ± 1.2, 4.3 ± 0.8, and 4.1 ± 1.5 in groups A, B, C, D, and E, respectively. A limited number of secretory cells were found in group A. Their number was largest in group B, and smaller in groups C, D, and E compared to that in group B. However, the difference was statistically insignificant (P = 0.43; Figure 6).

Subepithelial fibrosis

The thickness of subepithelial layer (mean ± SEM) was measured at the inferior end of nasal septum: 14.1 ± 2.1 μm, 55.8 ± 5.1 μm, 45.4 ± 3.1 μm, 42.6 ± 2.8 μm, and 43.1 ± 3.1 μm in groups A, B, C, D, and E, respectively. The subepithelial layer was considerably thick in group B compared to that in group A. It was apparently thinner in groups D and E than in group B (P = 0.02 and P = 0.024, respectively). Similarly, group C demonstrated thinner subepithelial layer than group B, but the difference was not statistically significant (P = 0.09; Figure 7).

Immunohistochemistry for COX-2 and 5-LO

To elucidate the mechanism of anti-inflammatory effects by high-dose resveratrol, IHC was performed for COX-2 and 5-LO. In group A, no definite positive signals were observed for COX-2 in the nasal mucosa. They were also scarcely noted in experimental groups B - E. Cells containing positive signals for COX-2 were counted at the transition zone of the olfactory and respiratory epithelium: 3.0 ± 0.8, 1.8 ± 0.5, 2.6 ± 0.9,

and 1.4 ± 0.4 in groups B, C, D, and E, respectively. It appeared that the number of positive signals is smaller in groups C and E than in groups B and D. However, there was no statistically significant difference between groups probably due to the scarcity of positive signals in all groups (P = 0.36; Figure 8).

For 5-LO, no definite positive signals were observed in the nasal mucosa in group A. However, there was strong expression of 5-LO in group B. The number of cells with positive signals was as follows: 64.6 ± 13.8, 26.6 ± 4.8, 28.0 ± 4.3, and 22.0 ± 3.1 in groups B, C, D, and E, respectively. The degree of positive signals was apparently reduced in groups C, D, and E compared with that in group B (P = 0.03, P = 0.014, and P = 0.01, respectively). Finally, there was no definite difference in 5-LO expression among groups C, D, and E (Figure 9).

Western blot Analysis

The protein levels of COX-2 and 5-LO were measured using Western blot analysis. The expression level of COX-2 was strongest in group B while it was undetectable in group A. Mice in group C exhibited the weakest expression level of COX-2, which was similar in group E. In contrast, there was no apparent decrease in COX-2 expression in group D.

Similar inter-group differences were identified in the expression level of 5-LO. Groups B and D exhibited strong expression of 5-LO, but it was weakened in groups C and E (Figure 10).

Quantitative Real-time PCR

The qPCR was used to determine the mRNA expression of key cytokines IL-4, IL-5 in the murine sinonasal mucosa. The relative expression level of IL-4 (mean ± SEM) was calculated: 1.0 ± 0.1, 79.4 ± 8.1, 40.3 ± 8.2, 48.4 ± 12.8, and 35.8 ± 6.8 in groups A, B, C, D, and E, respectively. Group B showed highest expression level of IL-4, which was significantly reduced in group C, D, and E compared with that in group B ($P = 0.009$, $P = 0.035$, and $P = 0.002$, respectively). There was no apparent difference in the expression level of IL-4 among groups C, D, and E (Figure 11). The relative expression level of IL-5 (mean ± SEM) was as follows: 1.0 ± 0.1, 2.4 ± 0.2, 0.7 ± 0.1, 0.5 ± 0.1, and 0.5 ± 0.2 in groups A, B, C, D, and E, respectively. Group B showed highest expression level of IL-5, which was considerably diminished in group C, D, and E compared with that in group B ($P < 0.001$, $P < 0.001$, and $P = 0.002$, respectively). There was no definite difference in the expression level of IL-5 among groups C, D, and E (Figure 11).

The mRNA expression of eicosanoid pathway-related enzymes PGDS and LTC_4S was also determined using the qPCR. The relative expression level of PGDS (mean ± SEM) was as follows: 1.0 ± 0.1, 4.7 ± 1.5, 4.1 ± 0.9, 0.6 ± 0.2, and 0.6 ± 0.1 in groups A, B, C, D, and E, respectively. Group B showed highest expression level of PGDS, which was significantly reduced in groups D and E compared with that in group B ($P = 0.001$ and $P < 0.001$, respectively). Groups D and E also showed significantly lower expression level of PGDS than that in group C ($P <$

0.001 and P < 0.001, respectively). There was no apparent difference in the expression level of PGDS between groups B and C (P = 0.91; Figure 11). The relative expression level of LTC_4S (mean ± SEM) was as follows: 1.0 ± 0.1, 2.1 ± 0.2, 1.2 ± 0.1, 0.5 ± 0.1, and 0.9 ± 0.2 in groups A, B, C, D, and E, respectively. Group B showed highest expression level of LTC_4S, which was considerably decreased in group C, D, and E compared with that in group B (P = 0.009, P < 0.001, and P < 0.001, respectively). Group D showed significantly lower expression of LTC_4S than group C (P = 0.02), while there was no definite difference between groups C and E (P = 0.32; Figure 11).

Discussion

Animal models: Useful tools for the exploration of new drugs

Animal models are instrumental for the clarification of pathomechanisms of the disease, the investigation into the biomarkers, and the exploration of novel treatment modalities. Thus, animal models have been widely used in the various fields in medicine such as allergy, cancer, cardio- or cerebro-vascular diseases, and psychiatric diseases.[27-31] Several trials have been made to establish an animal model of rhinosinusitis accompanied by nasal polyps. First, an agent containing bacteria including *Streptococcus pneumoniae* serotype 3, *Bacteroides fragilis* NCTC 9343, or *Staphylococcus aureus* V8 was applied to New Zealand White rabbits, and unilateral sinusitis with mucosal polyps was identified irrespective of inducing agent.[6] Thereafter, the same researchers identified that, besides bacterial infection, the deposition of agarose in the sinus cavity or a chemotactic peptide such as N-formyl-methionyl-leucyl-phenylalanine can also contribute to the formation of nasal polyps.[7] Additionally, they analyzed detailed structural changes: epithelial disruption and the migration of immature branching epithelium were key features of polyp formation. Some branches of migrating epithelia eventually covered the mucosal defect, or spread into the intraepithelial microcavities which lied in the connective tissue, resulting in the separation of polyp body from the adjacent mucosa. Although those reports provided useful information on the histologic features of nasal

polyp formation, the tissues obtained from the rabbits did not show characteristic eosinophilic infiltration, which are typically noted in human nasal polyps. In recent years, there were two reports on an animal model of nasal polyps associated with eosinophilic infiltration. In the preceding study, rabbits received valine-glycine-serine-glutamine or poly-L-arginine in their maxillary sinuses after repeated exposure to OVA.[9] Consequently, apparent eosinophilic infiltration, thickened lamina propria, and polyp formation were identified. Poly-L-arginine is a synthetic cationic polypeptide which is known to increase vascular permeability and induce airway hyperresponsiveness.[32,33] Given that human nasal polyps are closely related to the eosinophilic inflammation, and eosinophilic cationic protein is one of the major components in eosinophils, it seems reasonable to make an animal model of nasal polyps by using a cationic protein. Nontheless, there are only limited data on the relationship between poly-L-arginine and nasal polyps. On the other hand, the latest study utilized a mouse for the establishment of an animal model of nasal polyps.[25] The reason why mice were chosen was as follows: mice can be readily genetically manipulated, and a wide array of murine reagents is available and their housing costs are lower than other animals. Additionally, SEB was used to induce the formation of eosinophilic nasal polyps. Numerous previous studies have provided the information on a link between *Staphylococcus aureus* exotoxins (SAEs) and nasal polyps; SEB is one of the SAEs commonly detected in nasal polyps.[15,34-38] It was added on the mice with pre-existing allergic inflammation caused by OVA.

Serial changes in histology were examined monthly and it was found that prolonged stimulation with OVA/SEB is mandatory to induce definite nasal polyps. The current study was conducted based on the protocols established in that study and similar findings were confirmed: the proliferation of secretary cells, the amplification of inflammatory cells such as eosinophils and mast cells, increased subepithelial fibrosis, and characteristic findings of nasal polyps including elevated lesions with eosinophilic infiltration and microcavities. Using these findings as parameters for the degree of inflammation and polyp formation, the therapeutic effect of resveratrol on eosinophilic rhinosinusitis with nasal polyps was investigated.

Versatile actions of resveratrol

Mediterranean diets are known to be rich in resveratrol. In a previous population-based study named 'Lyon Diet Heart Study', patients who suffered from first myocardial infarction were followed up for an average of 46 months. Interestingly, Mediterranean dietary pattern significantly reduced the rate of recurrence even after the adjustment of traditional risk factors such as high blood cholesterol and blood pressure.[39] Subsequently, a number of studies were reported on the cardioprotective effect of resveratrol and its mechanism.[23] Based on the structural similarity of resveratrol to diethylstilbestrol, resveratrol is characterized as a phytoestrogen.[40] Given this structural similarity, the cardioprotective benefits of resveratrol was first assumed to be modulated by activation of

the estrogen receptor.[23] Yet, more recently there have been a number of studies that suggest that the estrogen receptor is not the main cellular target of resveratrol in the vasculature. Instead, SIRT 1, a mammalian homolog of the Saccharomyces cerevisae silent information regulator 2 (Sir2) protein, has been recognized as the main target of resveratrol.[41,42]

Resveratrol is also known to induce major cellular anti-oxidant enzymes such as glutathione peroxidase and superoxide dismutase.[43,44] In a previous study, resveratrol prevented H_2O_2-mediated apoptotic cell death in cultured aortic segments of rat, and its effect was attenuated by inhibition of glutathione peroxidase and heme oxygenase-1.[43] Furthermore, resveratrol treatment upregulated the expression of glutathione peroxidase, catalase, and heme oxygenase-1 in cultured arteries. In another study, resveratrol increased nitric oxide (NO) production by enhancing endothelial NO synthase expression, and reduced $O2^-$ production by inhibiting NAD(P)H oxidase activity in mice. Additionally, the anti-oxidant effects of resveratrol led to improved cardiac function by increasing the left ventricular diastolic peak filling rate.[44]

Of note, some studies showed that resveratrol has a lifespan extension effect although it was not proven in the upper vertebrates.[45,46] In a prior study using *Caenorhabditis elegans* and *Drosophila melanogaster*, resveratrol extended the lifespan of these animals without reducing fecundity by activating sirtuins, a family of NAD^+-dependent deacetylases conserved from *Escherichia coli* to humans.[45] In another study, resveratrol mimicked calorie restriction by stimulating Sir2, leading to

increased DNA stability and extended lifespan by 70% in yeast.[46]

Anti-inflammatory effects of resveratrol

In the present study, resveratrol showed definite anti-inflammatory effects, particularly in a high dose. The formation of nasal polyp-like lesions was reduced along with the decrease in the overall thickness of nasal mucosa. The degree of eosinophilic infiltration was also declined by administration of high-dose resveratrol, which was similar to the effect of triamcinolone acetonide. In the present study, tissue inflammation in mice was initiated by OVA, which increases total and OVA-specific IgE production through elevating IL-4 production.[47] Thus, IL-4 can be a good marker for evaluating OVA-induced tissue inflammations. In the current study, the gene expression level of IL-4 was approximately 80 times higher in mice with eosinophilic rhinosinusitis with nasal polyps than in control group, and a marked decrease in the IL-4 expression level was observed in mice treated with low- or high-dose resveratrol. Their effect was similar with that of triamcinolone acetonide. Furthermore, the gene expression level of PGDS and LTC_4S was remarkably inhibited by low- or high-dose resveratrol. PGDS catalyzes the isomerization of the 9,11-endoperoxide group of PGH_2, a common precursor of various prostanoids, to produce PGD_2 which is an end product of the COX pathway.[48] LTC_4S conjugates LTA_4 with reduced glutathione to form LTC_4, the parent compound of the cysteinyl leukotrienes.[49] Accordingly, the decline of gene expression of PGDS and LTC_4S by administration of resveratrol indicates that

resveratrol can exert an inhibitory action on both the COX and LOX pathway. The inhibitory effect of resveratrol on the LOX pathway, particularly in a high dose, was reconfirmed by IHC and Western blot analysis of 5-LO production. 5-LO is highly expressed in leukocytes such as neutrophils, eosinophils, and mast cells.[50] Considering that eosinophils highly express 5-LO for the pro-inflammatory action, blockage of the LOX pathway by resveratrol is believed to a key mechanism of inhibition of eosinophilic inflammation. The inhibitory action of resveratrol on the LOX pathway was also identified in some previous studies.[51-54] In a prior study, human polymorphonuclear leukocytes (PMN) were isolated from venous blood of healthy subjects and the effects of resveratrol on arachidonate metabolism were investigated. Resveratrol was found to inhibit several 5-LO products such as 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (5,12-diHETE) and LTC_4.[52] Similar effects of resveratrol were confirmed by some ensuing papers. In a study which examined anti-apoptotic activity of resveratrol using human erythroleukemia K562 cells, resveratrol was found to act as a competitive inhibitor of purified 5-LO and 15-LO and PDH synthase; as a consequence, LTB_4 and PGE_2 was reduced.[53] In another study on the anti-cancer effect of resveratrol, it potently inhibited the 5-LO expression and LTB_4 production in 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary cancer developments in rats. Moreover, resveratrol normalized the expression of transforming growth factor (TGF)-β1 which had been down-regulated in DMBA-challenged rat mammary tissue.[51] In addition to

the anti-5-LO effect, resveratrol was found to have strong inhibitory effects on LTA_4 hydrolase activity, which stimulates the production of pro-inflammatory cytokines and mediators by catalyzing the hydrolysis of LTA_4 to LTB_4. By means of small hairpin RNA-mediated knockdown of LTA_4 hydrolase, the reduction of inhibitory effects of resveratrol was identified.[54]

In addition to the inhibitory actions on the LOX pathway, anti-inflammatory effects of resveratrol were ascertained in each type of leukocytes.[55-57] In a previous study, PMN were isolated from venous blood of healthy volunteers, and stimulated with formyl methionyl leucyl phenylalamine or calcium ionophore subsequent to the treatment with resveratrol. Resveratrol not only inhibited the production of reactive oxygen species, but also prevented the release of inflammatory mediators including elastase, β-glucuronidase, and LTB_4 from PMN.[55] In another *in vitro* study, eosinophils were obtained from venous blood of healthy non-atopic volunteers, and following reactions were significantly inhibited by resveratrol: eosinophil peroxidase release after activation with IL-5 or C5a, the production of LTC_4 following stimulation with calcium ionophore, and eosinophil chemotaxis in response to eotaxin.[56] In the current study, high-dose resveratrol not only reduced overall eosinophilic inflammation, but also decreased the proportion of eosinophils in total inflammatory cells. Additionally, the expression level of IL-5 mRNA was remarkably decreased by administration of a low- or high-dose resveratrol, which had similar effects with triamcinolone acetonide. It is well established that IL-5

induces terminal maturation of eosinophil precursors, prolongs eosinophil survival, possesses eosinophilic chemotactic activity, and enhances eosinophilic effector function.[58] Moreover, using the anti-IL-5 antibody, named mepolizumab, a significant decrease in blood eosinophil counts was ascertained.[59] Taken together, it appears that resveratrol may inhibit the production and activation of eosinophils not only by inhibiting the LOX pathway, but also by suppressing the production of IL-5. On the other hand, inhibitory actions of resveratrol on mast cells were also reported. Bone marrow-derived murine mast cells were triggered by IgE or calcium ionophore, and the effect of resveratrol was identified. The release of inflammatory mediators including histamine, tumor necrosis factor-α, LTs and PGD_2 was inhibited.[57] In histological analyses in the present study, however, no definite inhibitory action of resveratrol was observed. A further study will be needed to confirm the effect of resveratrol on mast cells.

Although low-dose resveratrol did not show a definite inhibitory action on 5-LO in Western blot analysis, on the other hand, it decreased the proportion of eosinophils in total inflammatory cells, and the subepithelial fibrosis. Subepithelial fibrosis is known to be prominent in patients with asthma, in particular, accompanying tissue eosinophilia.[60] In addition, multiple cytokines, growth factors, and adhesion molecules, including IL-13 and TGF-β, are implicated in the pathophysiology of subepithelial fibrosis in asthma.[61] Several studies have been conducted regarding the inhibition of fibrosis by resveratrol. In a prior study using *ex vivo* human

lung fibroblasts, resveratrol prevented TGF-β-induced proliferation and differentiation of fibroblasts into myofibroblasts.[62] In another study, resveratrol not only inhibited the production of pro-fibrogenic factors such as IL-6 and TGF-β, but also strongly activated the nuclear factor erythroid 2-related factor 2 which is known as a critical regulator of cellular defense against oxidative stress.[63] Although the anti-oxidant effect of resveratrol is deemed to decrease subepithelial fibrosis, a further study will be needed to elucidate the precise mechanism of anti-fibrogenic effects of resveratrol. In summary, it was ascertained that resveratrol exerts anti-inflammatory effects, particularly on eosinophils, and prevents subepithelial fibrosis in a murine model of eosinophilic rhinosinusitis accompanied by polyp-like lesions. Inhibition of 5-LO appears to be one of the key mechanisms of anti-inflammatory effects of resveratrol. Consequently, resveratrol may be a new potential drug for the treatment of eosinophilic rhinosinusitis with nasal polyps although a further human study is needed to confirm it.

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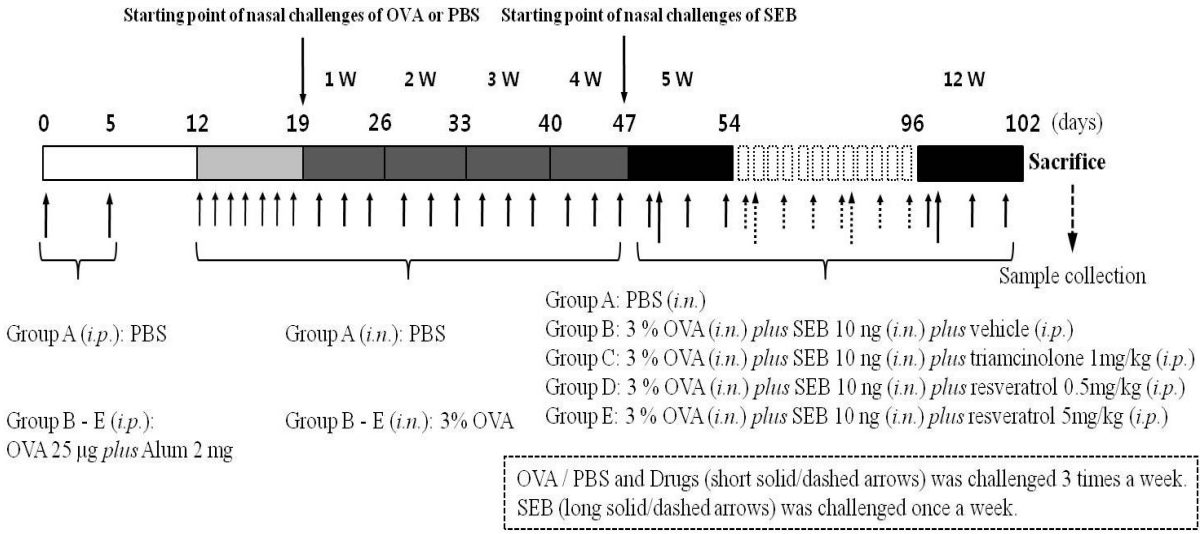

Figure 1. Protocols for the development of eosinophilic rhinosinusitis with nasal polyps in mice. Ovalbumin was used to obtain systemic sensitization and local stimulation, followed by intranasal instillation of *Stayphylococcus aureus* enterotoxin B (SEB) in experimental groups B - E. Instead, in the control group (group A), phosphate-buffered saline (PBS) was administered via a local or systemic route. The experimental groups were designated as follows: intraperitoneal injection of vehicle, which means a mixture of dimethyl sulfoxide (DMSO) and PBS with a ratio of 1:9 (group B), intraperitoneal injection of triamcinolone acetonide with a concentration of 1 mg/kg (group C), intraperitoneal injection of resveratrol with a concentration of 0.5 mg/kg (group D), and 5 mg/kg (group E). *i.p.*, intraperitoneal injection; *i.n.*, intranasal instillation.

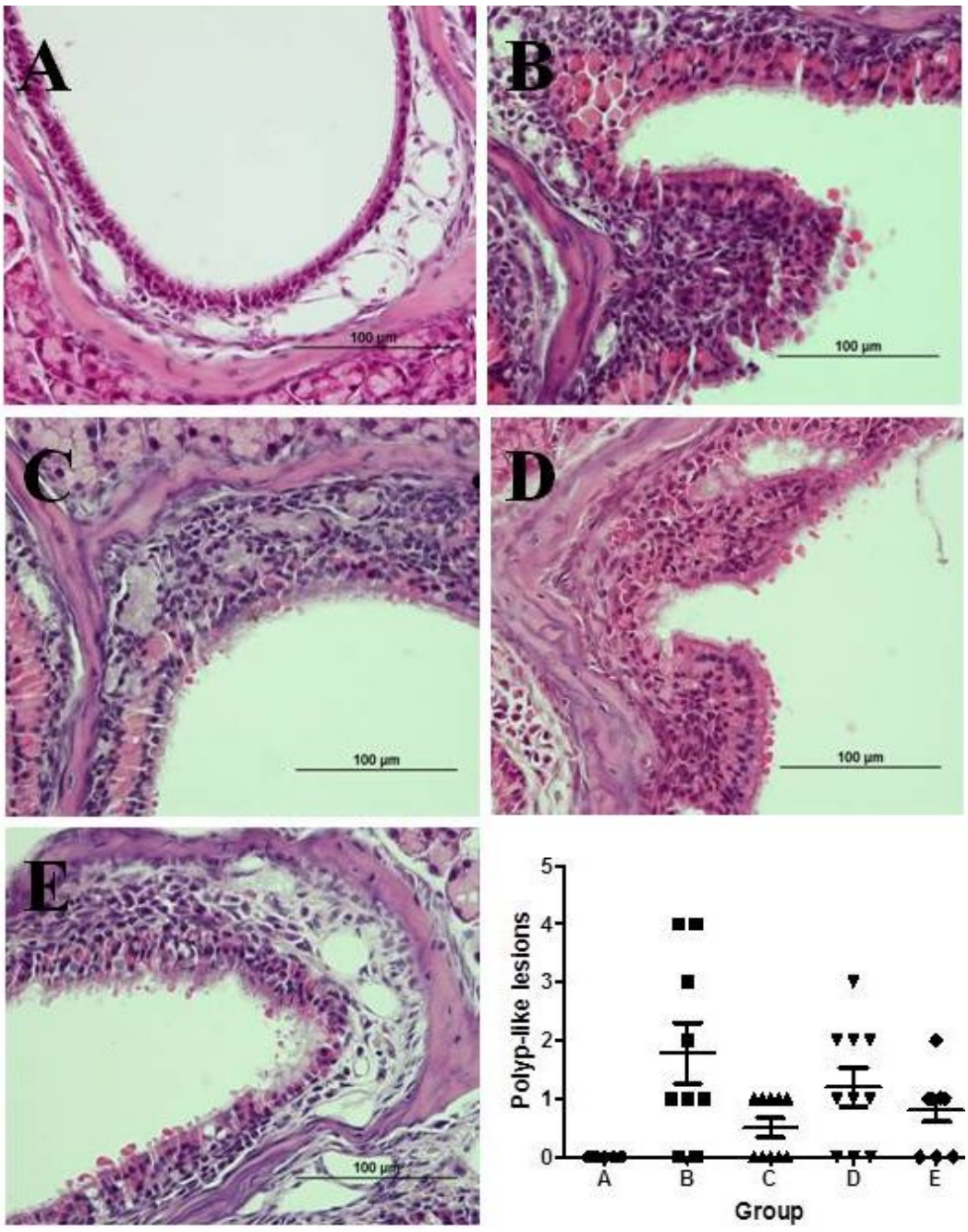

Figure 2. Comparison of the count of polyp-like lesions between groups. The same experiments were performed twice, and the representative data are depicted. The number of polyp-like lesions was highest in group B, followed by group D. Group C showed lowest number of polyp-like lesions, and group E demonstrated similar outcomes. However, these differences between groups were not statistically significant (Hematoxylin and eosin stain, ×400).

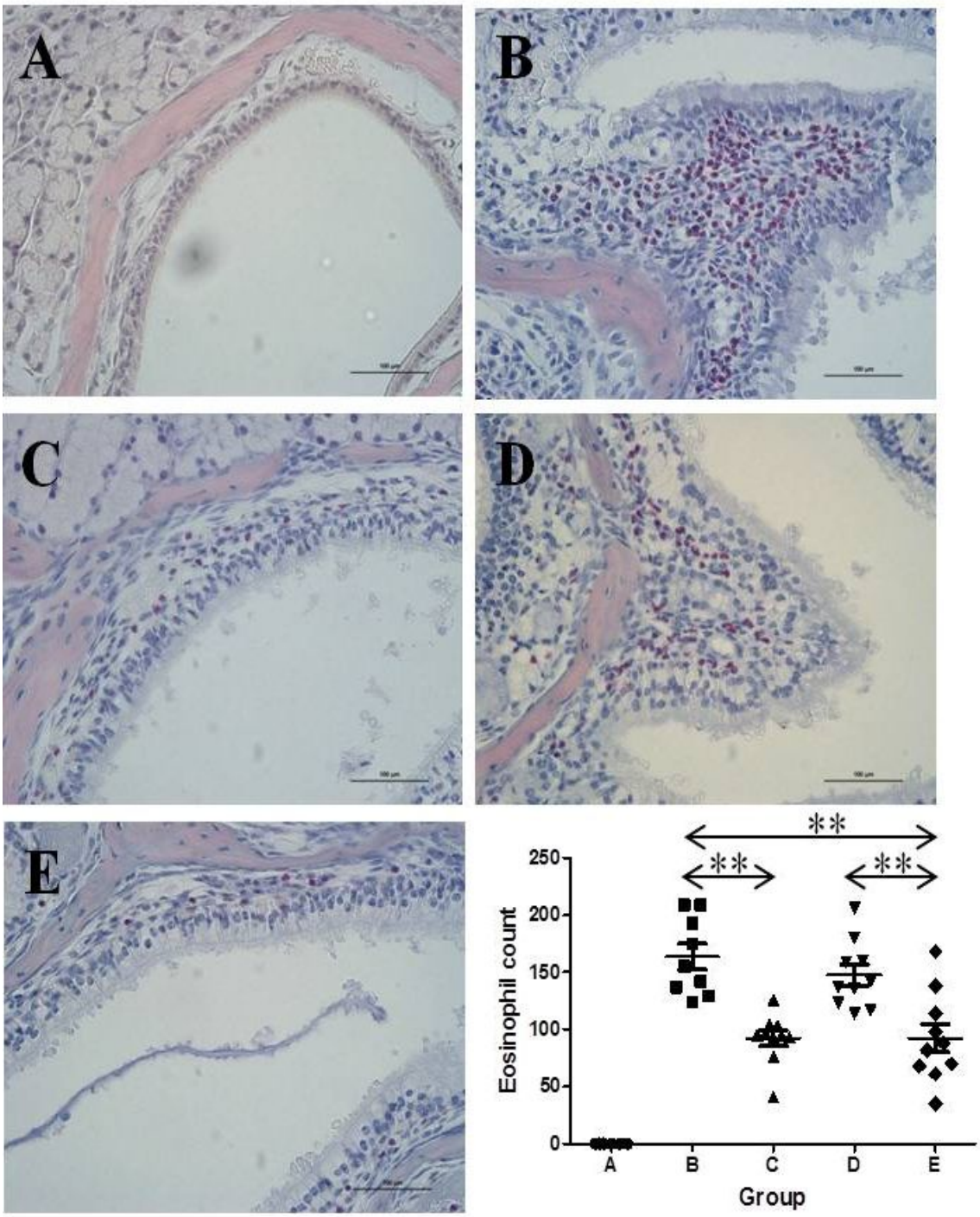

Figure 3. Comparison of the eosinophil count between groups. The same experiments were performed twice, and the representative data are depicted. The number of eosinophils was highest in group B whereas mice in groups A showed no definite infiltration of inflammatory cells in the nasal and sinus mucosa. Both groups C and E demonstrated much lower

number of eosinophils than group B. The degree of eosinophilic inflammation was similar between group C and E (**P < 0.01, Sirius red stain, ×400).

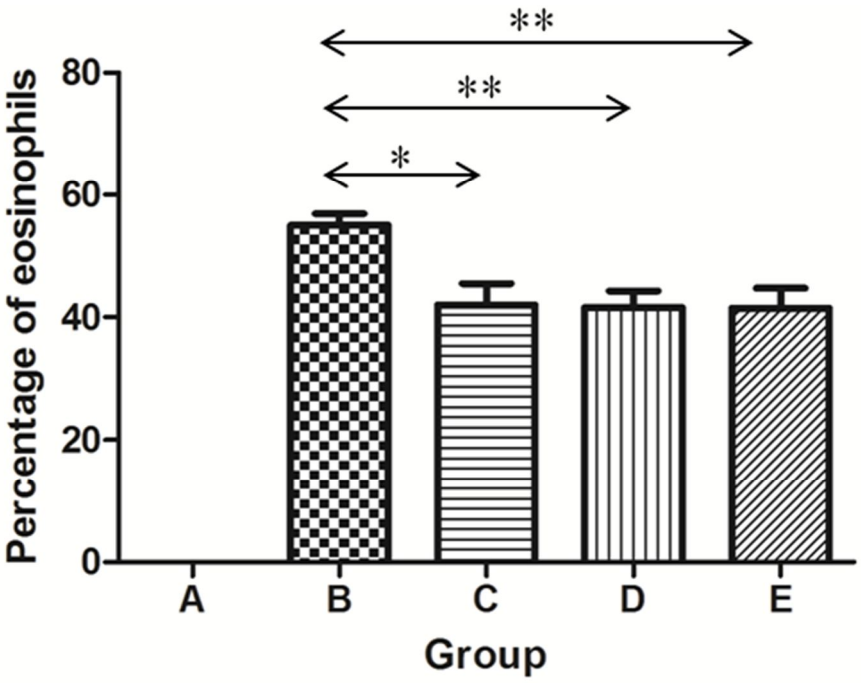

Figure 4. Differences in the proportion of eosinophils in total inflammatory cells between groups. Eosinophils were not found in group A. The percentage of eosinophils in total inflammatory cells was significantly diminished in groups C, D, and E compared with that in group B (*P < 0.05, **P < 0.01).

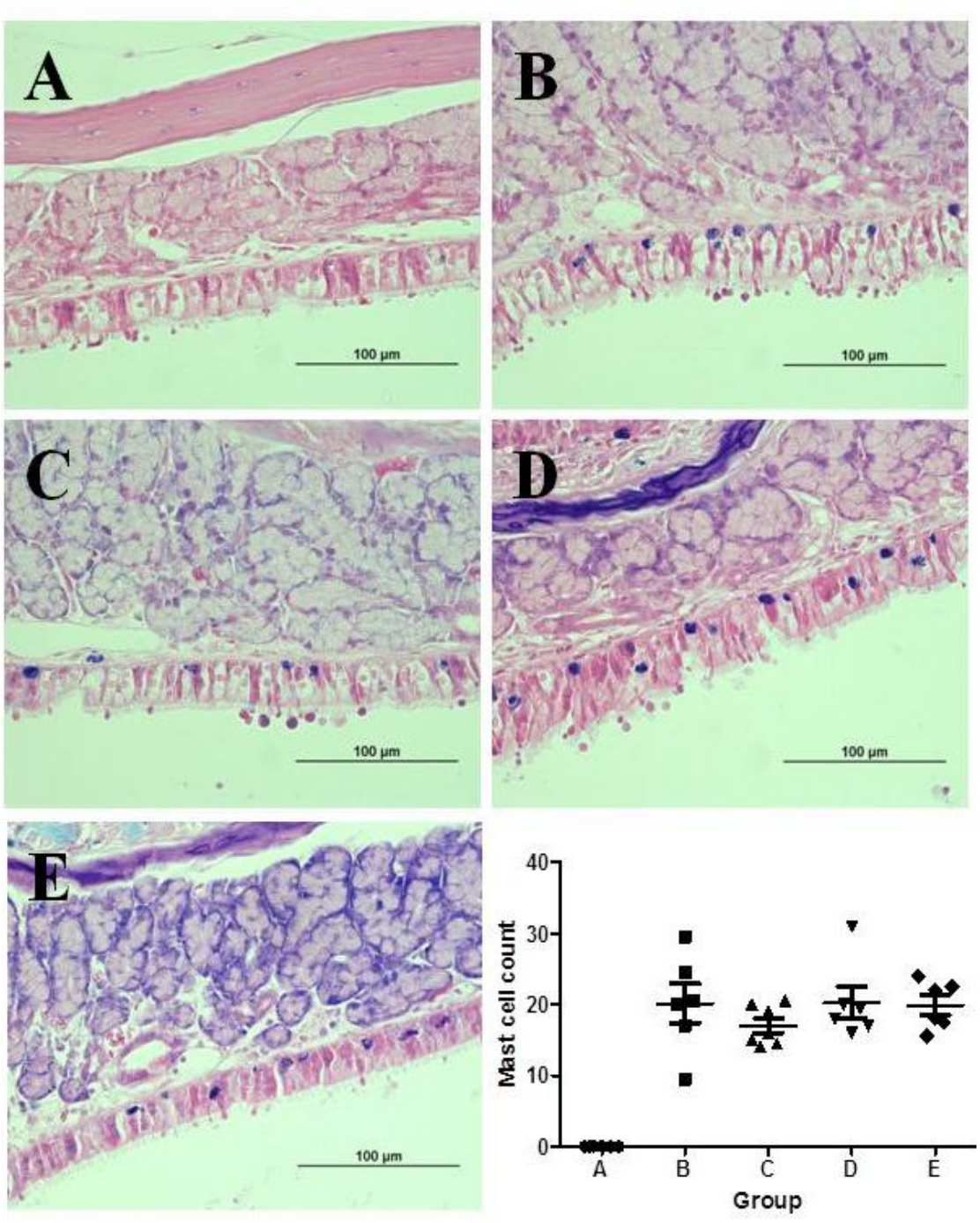

Figure 5. Comparison of the mast cell count between groups. The same experiments were performed twice, and the representative data are depicted. In group A, mast cells were rarely distributed in the sinus mucosa. The degree of mast cell infiltration seemed less severe in group C, yet there was no significant difference between experimental groups (Giemsa stain, ×400).

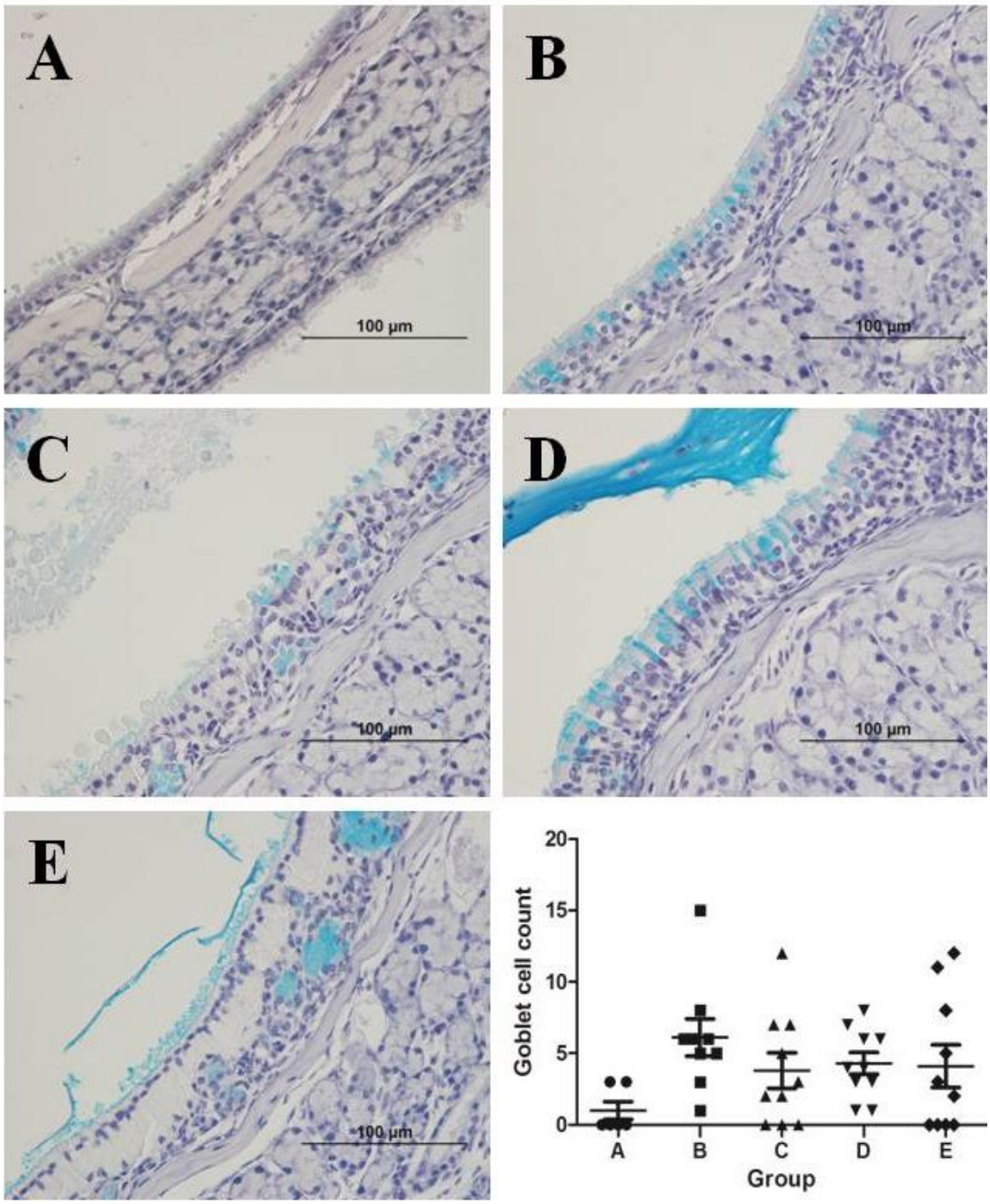

Figure 6. Comparison of the secretory cell count between groups. The same experiments were performed twice, and the representative data are depicted. A limited number of secretory cells were found in group A. Their number was largest in group B, and smaller in groups C, D, and E compared to that in group B. However, the difference was statistically insignificant (Alcian blue stain, ×400).

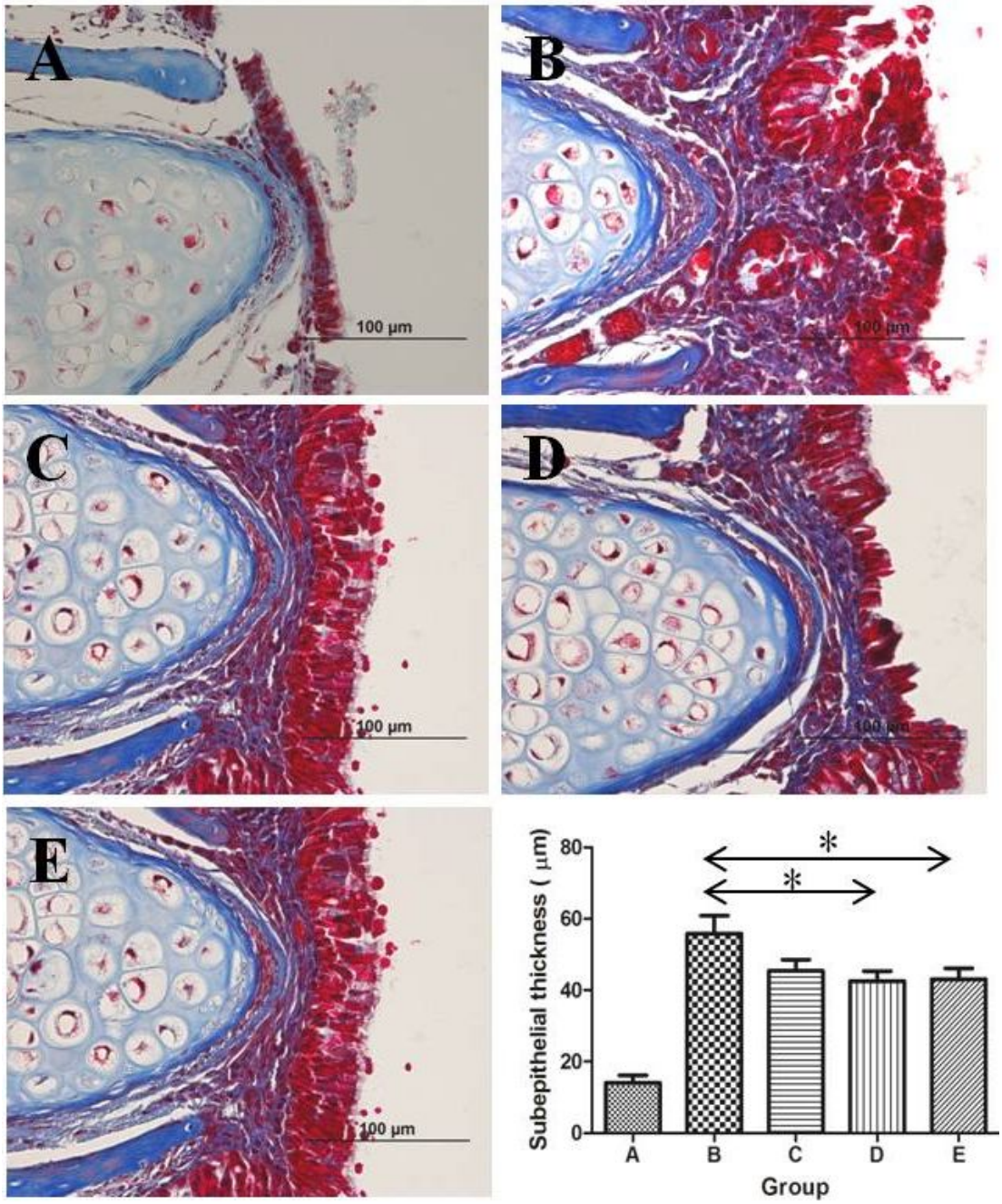

Figure 7. Comparison of the subepithelial thickness between groups. The same experiments were performed twice, and the representative data are depicted. The subepithelial layer was considerably thick in group B compared to that in group A. It was apparently thinner in groups D and E than in group B. Similarly, group C demonstrated thinner subepithelial layer than group B, but the difference was not statistically significant (*P < 0.05, Masson's trichrome stain, ×400).

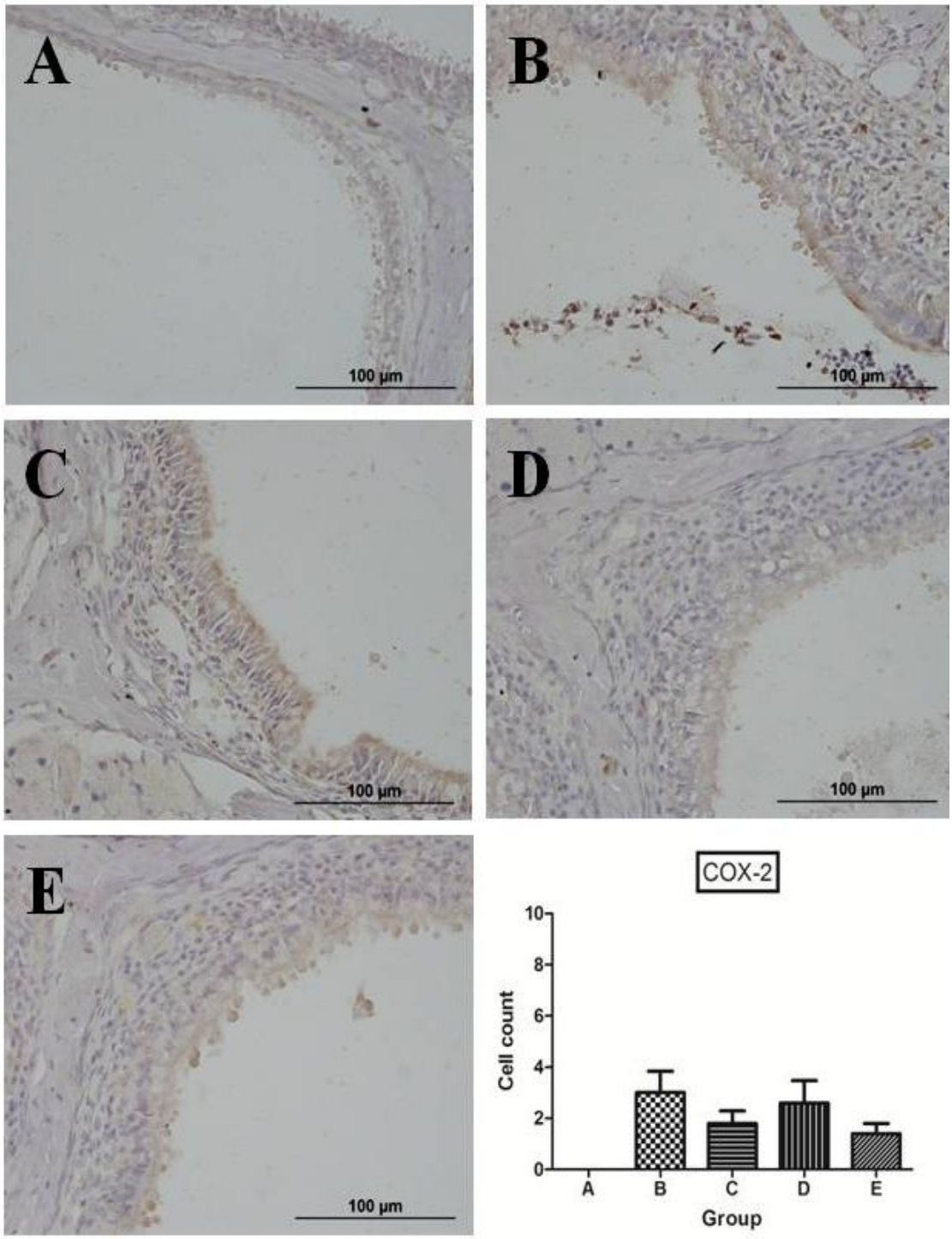

Figure 8. Representative microphotographs of immunohistochemistry and quantitative analysis of positive cells for cyclooxygenase-2. In group A, no definite positive signals were observed for COX-2 in the nasal mucosa. They were also scarcely noted in experimental groups B - E. The number of positive signals appeared to be smaller in groups C and E than

in groups B and D, but there was no statistically significant difference between groups.

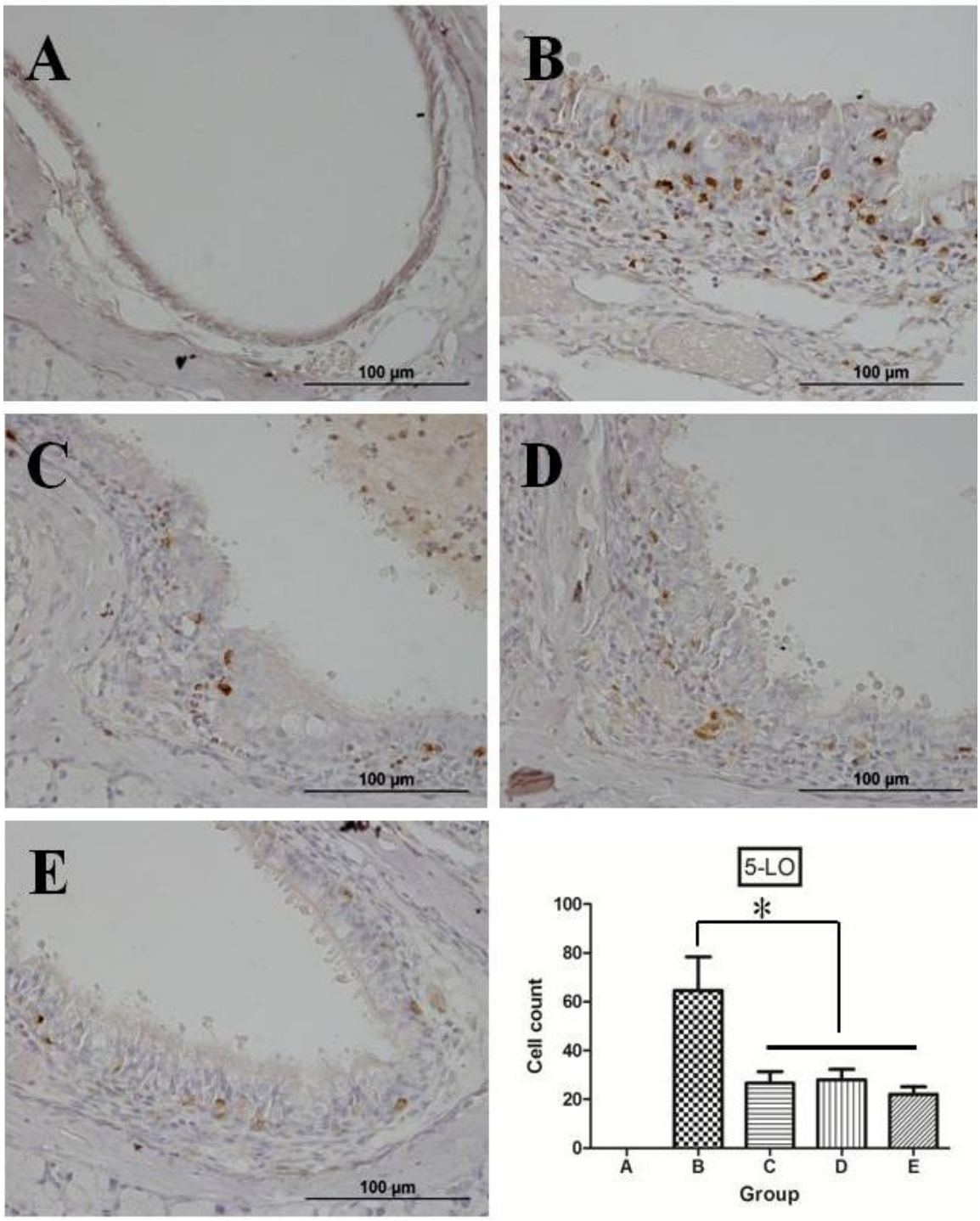

Figure 9. Representative microphotographs of immunohistochemistry and quantitative analysis of positive cells for 5-lipooxygenase. No definite positive signals were observed in the nasal mucosa in group A. However, there was strong expression of 5-LO in group B. The degree of positive signals was apparently reduced in groups C, D, and E compared with that in group B. There was no definite difference in 5-LO expression among

groups C, D, and E (*P < 0.05, ×400).

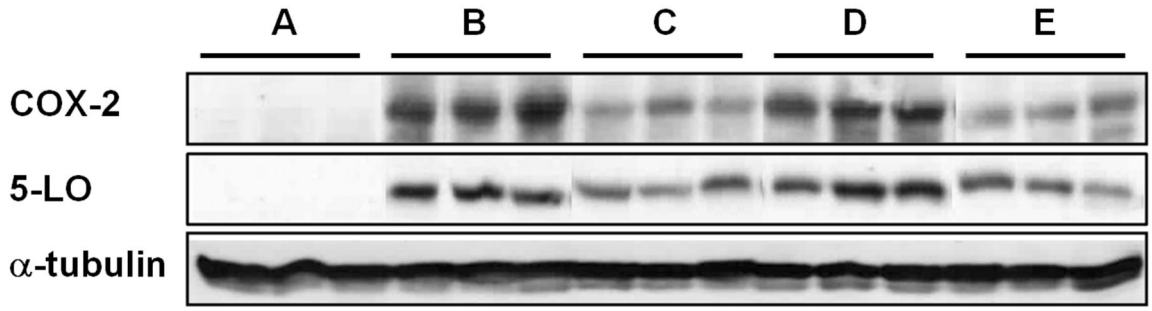

Figure 10. Western blot analysis of cyclooxygenase-2 and 5-lipooxygenase expression. The expression level of COX-2 was strongest in group B while it was undetectable in group A. Mice in group C exhibited the weakest expression level of COX-2, which was similar in group E. In contrast, there was no apparent decrease in COX-2 expression in group D. Similar inter-group differences were identified in the expression level of 5-LO. Groups B and D exhibited strong expression of 5-LO, but it was weakened in groups C and E.

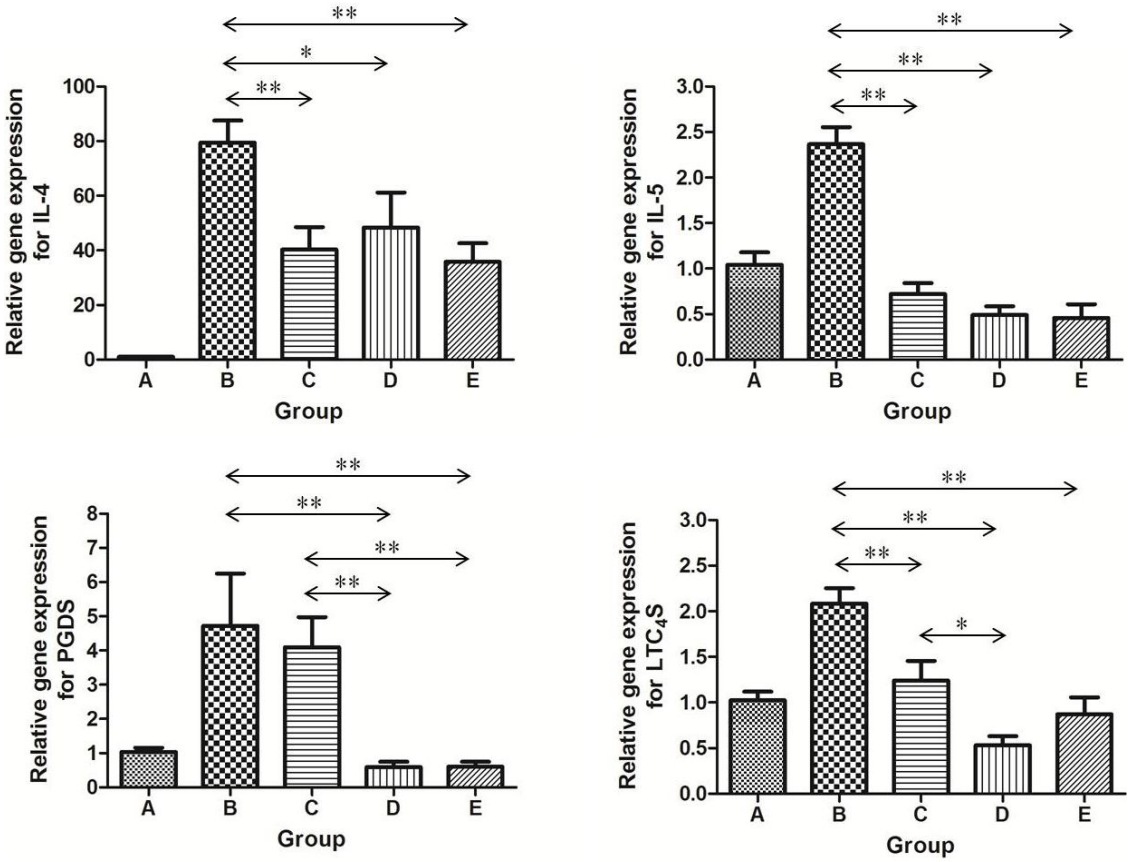

Figure 11. mRNA expression of key cytokines IL-4, IL-5, and eicosanoid pathway-related enzymes PGDS and LTC_4S in the murine sinonasal mucosa. Each value represents the mean ± SEM of relative mRNA values standardized to GAPDH housekeeping gene.

국문 초록

서론: 만성 비부비동염은 코막힘과 후각장애 등의 증상을 유발하고 삶의 질을 떨어뜨리는 질환이며 흔히 비용종을 동반한다. 스테로이드 제제의 전신적 투여는 비용종에 대한 매우 효과적인 약물 치료이나 장기간 사용하게 되면 골다공증, 우울증 및 당뇨 등의 합병증을 유발하므로 제한적으로 사용할 수 밖에 없다. 최근 포도상구균 장독소 B형을 이용한 호산구성 비부비동염 마우스 모델이 확립되었는데 본 연구에서는 resveratrol이 비용종을 동반한 호산구성 비부비동염에 미치는 치료 효과와 그 작용 기전에 대해서 탐구하였다.

대상 및 연구 방법: 실험군은 난알부민으로 감작시키고 음성대조군에는 PBS를 투여하였다. 실험군은 실험 후반 8주간 매주 한 번씩 포도상구균 장독소 B형으로 비강내 자극을 시행하였으며 주입한 약물에 따라 4그룹으로 나누었다. 양성대조군에는 DMSO와 PBS의 혼합물을 주입하였고 나머지 세 치료군에는 각각 triamcinolone acetonide, 저농도 resveratrol, 고농도 resveratrol을 투여하였다. 전반적인 염증 상태와 비용종 관찰을 위한 Hemoxylin eosin 염색, 호산구 침윤 관찰을 위한 Sirius red 염색, 비반세포 관찰을 위한 Giemsa 염색, 상피하 섬유화 확인을 위한 Masson's trichrome 염색, 분비세포의 형성을 관찰하기 위해 Alcian blue 염색을 시행하였다. 또한, 면역조직화학염색과 Western blot을 시행하여 그룹간 cyclooxygense-2와 5-lipooxygense의 발현 정도를 비교하였으며, 실시간 PCR을 시행하여 IL-4, IL-5, prostaglandin D synthase 및 leukotriene C4 synthase 유전자의 발현 정도를 그룹간에 비교하였다.

결과: 고농도 resveratrol을 투여한 실험군에서 조직내 호산구 침윤, 상피하 섬유화 및 전체 염증 세포 중 호산구의 비율 등이 양성 대조군에 비해 의미있게 감소하였으며 triamcinolone acetonide와 비슷한 효과를 보여주었다. 또한, 통계적인 유의성을 얻을 수는 없었지만 비용종의 갯수도 감소하는 경향을 보였다. 한편, 면역조직화학염색과 Western blot에서도 고농도 resveratrol이 triamcinolone acetonide와 비슷한 5-lipooxygense 발현 억제 효과를 보였다. 또한, IL-4, IL-5, prostaglandin D synthase 및 leukotriene C4 synthase의 유전자 발현은 양성 대조군에서 가장 높게 나타났으며, 저농도 및 고농도 resveratrol을 투여한 군 모두에서 유의하게 감소하였다. 저농도 resveratrol은 전체적인 호산구 염증을 뚜렷하게 줄이지는 못했지만 전체 염증 세포 중 호산구의 비율과 상피하 섬유화 정도를 감소시키는 효과를 보였다.

결론: 호산구성 비부비동염 마우스 모델에서 resveratrol을 고농도로 투여하는 경우 뚜렷한 항염증 작용과 비용종 감소 효과를 확인할 수 있었으며, lipooxygense pathway 의 발현 억제가 중요한 기전으로 생각된다. Resveratrol은 비용종을 동반한 호산구성 비부비동염의 새로운 후보 치료제로 향후 환자에 대한 임상 연구가 필요할 것으로 보인다.

주요어: resveratrol, 비부비동염, 비용종, 마우스 모델, 호산구, lipooxygense

학 번: 2010-30501

감사의 글

제가 학위를 무사히 마칠 수 있었던 것은 여러 선생님들과 선후배님들을 비롯한 지인들께서 도와주셨기에 가능했던 일인 것 같습니다. 이 자리를 빌어 감사의 말씀을 전합니다. 우선 논문 심사를 맡아주신 민양기, 한성구, 조상헌, 동헌종, 이재서 선생님께 감사 드립니다. 지도교수님이신 민양기 선생님께서는 연구에 문외한이던 제가 연구의 기본을 쌓을 수 있게 석사학위 지도를 해주신 데 이어 이번 박사학위 연구에 있어서도 늘 학문적 관심과 따뜻한 조언으로 저를 이끌어 주셨습니다. 또한 제가 박사논문을 무난히 작성할 수 있었던 건 민양기 선생님께서 후학들에게 연구 결과를 영어 논문으로 작성하는 방법을 끊임없이 전수해 주셨기 때문에 가능했습니다. 탁월한 임상가로서, 뛰어난 연구자로서, 그리고 엄격하면서도 자상한 스승으로서 제 인생의 본보기가 되어주신 민양기 선생님께 다시 한 번 깊이 감사드립니다. 또한 심사위원장을 흔쾌히 맡아 주신 한성구 선생님께서는 많이 부족했던 초기 연구결과를 보다 나은 결과로 발전시킬 수 있도록 끊임없는 격려를 아끼지 않으셨습니다. 조상헌 선생님께서는 제가 깨닫지 못했던 연구의 맹점에 대해 항상 일깨워 주시고 세세한 지적을 통해 보다 완성된 연구가 될 수 있도록 많은 가르침을 주셨습니다. 동헌종 선생님께서는 부족한 논문을 세심히 살펴주시고 자그마한 흠까지 수정해 주셨으며 향후 연구방향에 대해서도 아낌없는 조언을 주셨습니다. 마지막으로 이재서 선생님께서는 제가 전공의 시절부터 늘 학문적 관심을 잃지 않도록 이끌어 주셨으며 이번 학위 연구에도 깊은 관심을 가지고 세세한 연구 방법까지도 꼼꼼히 지도해 주셨습니다. 특히

바쁘신 와중에도 이른 아침에 심사를 위해 시간을 내주신 다섯 분의 심사위원 선생님께 다시금 감사의 말씀을 올립니다.

제가 근무하고 있는 경상대학교병원 교수님의 배려가 없었다면 이 논문은 완성되지 못했을 것입니다. 전시영 선생님께서는 어려운 환경 속에서도 경상대학교 실험실을 이끌어 오셨고 제가 학위 연구를 수행할 수 있도록 아낌없는 조언과 격려를 주셨습니다. 지금은 근무지를 옮기셨지만 김대우 선생님께서는 제 박사연구의 시작부터 끝까지 연구 방법의 구체적인 노하우를 아낌없이 전수해 주시고 변함없는 관심과 조언으로 제가 연구를 끝마칠 수 있게 도와주셨습니다. 근무와 병행하며 박사과정을 마칠 수 있게 배려해주신 김진평, 안성기, 박정제, 허동구, 우승훈 선생님께도 이 자리를 빌어 감사를 드립니다.

또한 다른 많은 선생님들의 아낌없는 도움과 격려가 없었다면 이 논문은 완성되지 못했을 것입니다. 로자 연구원의 헌신적인 노력이 있었기에 제가 무사히 박사연구를 끝마칠 수 있었고, 또한 함께 실험하면서 많은 것을 배웠습니다. 감사합니다. 임상의학연구소 김진현 선생님과 정명희 연구원께도 많은 신세를 졌습니다. 연구의 방향에 대해 관심어린 조언을 아끼지 않으셨으며 실험에 직접적인 도움을 주신 점 깊이 감사드립니다. 카이스트 면역학교실의 신의철 선생님과 장동엽 선생님께도 감사의 말씀을 전하고 싶습니다. 실험을 카이스트에서 진행할 수 있게 배려해 주시고 실험이 난관에 부딪쳤을 때 헌신적으로 도와주셨습니다. 감사합니다. 실험을 진행할 수 있게 시작 단계에서 실질적인 도움을 준 서울대학교 약리학교실 신현우 선생님에게도 이 자리를 빌어 감사의 말씀을 전합니다.

마지막으로, 저를 낳아서 길러주시고 지금까지도 늘 자식의 앞길을

걱정하며 기도하는데 당신의 인생을 헌신하는 부모님께 너무나 감사하고 사랑한다는 말씀을 전하고 싶습니다. 늘 저를 믿고 지켜봐 주시며 지지와 격려를 아끼지 않으시는 장인, 장모님께도 이 자리를 빌어 감사의 말씀을 전합니다. 끝으로 항상 의지하고 힘이 되어주며 실험에도 직접적인 도움을 준 아내 정민과 늘 아빠에게 행복한 기운을 불어넣어 주는 아들 윤재에게 사랑한다는 말을 전하며, 이 학위 논문을 바칩니다.