

Anti-allergic effects of So-Cheong-Ryong-Tang in ovalbumin-induced allergic rhinitis model

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Abstract Allergic rhinitis (AR) is an allergic inflammation of the nasal airways. The Korean herbal medicine, So-Cheong-Ryong-Tang (SCRT) has been typically used for the treatment of AR for hundreds of years. In the present study, we investigated whether SCRT suppresses the progression of AR in animal model. AR was induced by ovalbumin (OVA). Treatment with SCRT was assessed to study the effect of SCRT on AR in mice. Histological analysis, multiplex cytokine assay, blood analysis, cell viability assay, RT-PCR and Elisa assay were performed to verify inhibitory effect of SCRT on AR. SCRT reduced infiltration of inflammatory cells into nasal cavity. SCRT reduced infiltration of mast cells into nasal mucosa. SCRT reduced the levels of cytokines (IL-4 and LIF) in the serum. SCRT reduced the levels of leukocytes in the blood. SCRT decreased cell viability of HMC-1 cells and splenocyte. SCRT suppressed IL-4 level in HMC-1 cells and splenocyte cells in a dose-dependent manner. SCRT suppressed IL-6 level and TNF- α level in splenocyte. SCRT suppresses the progression of AR induced by OVA. SCRT might be a useful drug for the treatment of AR.

Keywords Allergic rhinitis · Ovalbumin · Mast cell · Cytokine · Leukocyte · So-Cheong-Ryong-Tang

Introduction

Allergic rhinitis (AR) is a global health problem, which is associated with life quality. 40 % of the population worldwide is suffering from AR. AR is an allergic inflammation of the nasal airways and is characterized by congestion, rhinorrhea (anterior and posterior), sneezing, and itching in any combination. In modern society, environmental pollution may have occurred; air pollution has become a serious problem day by day. The allergic diseases are an overreaction of the immune system to an inappropriate stimulus, such as dust, pollen, grass, pet dander, mold, etc. [1].

AR is associated with an IgE-mediated immune response in Th2 cell response [2]. AR stimulation increases the number of surface-bound IgE and IgE receptors. Mast cells are activated in an IgE-dependent manner and produce histamine [3, 4]. AR is caused by the triggering of mast cell degranulation resulting from the cross-linking of surface-bound IgE by the aeroallergen. The inflammation that develops over the course of an allergy season is associated with an approximately tenfold increase in the number of mast cells present in nasal epithelial and sub-mucosal tissue [5].

Mononuclear phagocytic cells, B lymphocytes, and especially dendritic cells are antigen-presenting cells. IL-1, IL-6, and TNF are associated with innate immunity. The newly activated T lymphocytes are able to produce IL-4, IL-5, IL-9, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF). These are major components of the inflammatory response in AR [6–9].

Mediators released by mast cells stimulate infiltration of the nasal mucosa with inflammatory cells, including basophils, eosinophils, neutrophils, newly synthesized mast cells, and mononuclear cells [10, 11].

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At present, antihistamines, corticosteroids, decongestants, and mast cell stabilizers are available for AR patients. However, these drugs are limited due to short duration of effect and their long-term use causes severe side effects. Hence, we need to find safer and more effective new drugs [12, 13].

The traditional herbal medicine “So-Cheong-Ryong-Tang” (SCRT) is called “Sho-seiryu-to” in Japan and “Xiao-Qing-Long-Tang” in China. SCRT contains eight species of medicinal plants. SCRT has been used for the treatment of AR, bronchitis, bronchial asthma, and symptoms for hundreds of years in Asian countries. Recent studies indicated that SCRT inhibits histamine release, degranulation of mast cells, proliferation of eosinophils and basophils and cholinergic effects for nasal gland acinar cells [14]. Several other studies have evaluated the anti-allergic effects of SCRT in animal model: in a bronchial asthma model, in a passive cutaneous anaphylactic reaction model, in a mite-sensitized bronchial asthma mouse model or in an ovalbumin (OVA)-sensitized bronchial asthma guinea pig model [15–18]. However, there were not many attempts to investigate the efficacy of SCRT in pharmacologic mechanism.

In the present study, we investigated the effects of SCRT on AR and examined the underlying mechanisms of its anti-allergic effects in a mouse model.

Materials and methods

Preparation of So-Cheong-Ryong-Tang (SCRT)

SCRT was supplied by Han-poong Pharm Co., Ltd (Jeonju, Republic of Korea). SCRT is composed of *Asiasarum sieboldi* var. *seoulense* Nakai, *Glycyrrhiza uralensis* Fischer, *Ephedra sinica* Stapf, *Cinnamomum cassia* Blume, Peony root, *Pinellia ternata* Breitenbach, *Schisandra chinensis*, *Zingiber officinale* Roscoe (Table 1). SCRT powder was dissolved in distilled water to give 20 mg/ml.

Animals

Five-week-old male BALB/c mice were purchased from Orient (Seoul, Republic of Korea). Mice were maintained for 1 week before starting the experiment. Animals were randomized and all housed under controlled temperature ($23 \pm 3^\circ\text{C}$) and humidity ($55 \pm 15\%$), with a 12-h light/12-h dark cycle. Animals were provided with a laboratory diet and water ad libitum. Body weight of animals was measured twice a week. All procedures performed on the mice were approved by the animal care center of Kyung-Hee University (Approval no. KHUASP(SE)-12-048).

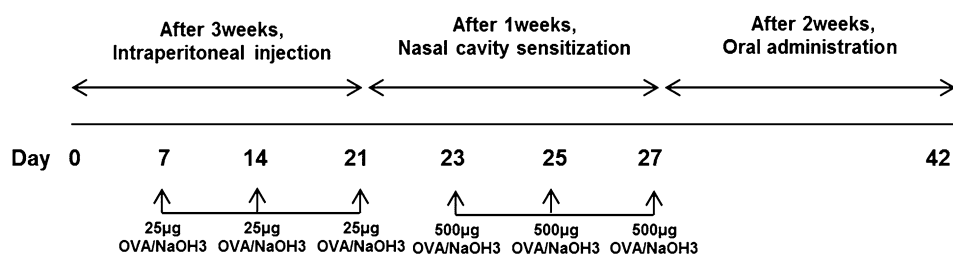
Murine AR model and treatment

The mice were divided into five groups ($n = 8$)—group 1, normal saline; group 2, OVA + saline; group 3, OVA + SCRT (78 mg/kg); group 4, OVA + SCRT (156 mg/kg); group 5, OVA + SCRT (312 mg/kg). The procedures for allergen sensitization and treatment are summarized in Table 2. For establishment of OVA-induced AR model, 6 weeks old BALB/c male mice were intraperitoneally sensitized with 25 μg of OVA twice a week for 3 weeks. After 3 weeks, OVA group of mice was sensitized by intranasal instillation of 500 μg OVA in 30 μL PBS twice a week for 1 week. Finally, mice were fed with SCRT together with OVA sensitization during 2 weeks. At the end of experiment, mice were killed by CO_2 inhalation, and samples were collected.

Table 1 The composition of herbal medicines in SCRT

Component	Volume (g)
<i>Asiasarum sieboldi</i> F. Maekawa	2
<i>Glycyrrhiza uralensis</i> Fischer	2
<i>Ephedra sinica</i> Stapf	2
<i>Cinnamomum cassia</i> Blume	2
<i>Paeonia lactiflora</i> PALL	2
<i>Pinellia ternata</i> Breitenbach	5.3
<i>Schisandra chinensis</i>	5.3
<i>Zingiber officinale</i> Roscoe	2

Table 2 Allergic rhinitis model



Histological analysis

Nasal mucosa samples were immediately fixed with 10 % formaldehyde and embedded in paraffin. The section of the nasal mucosa samples was 4 µm thick. Each section was stained with hematoxylin and eosin (H&E) for inflammatory cells or with toluidine blue (T.B) for mast cells and examined under light microscopy (Olympus). Mast cells and inflammatory cells were counted in ten parts of high-power fields (HPF) at 40×, 400× and 1000× magnification.

Multiplex cytokine assay

Blood serum was analyzed by the Bio-Plex Multiplex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA,

USA) according to the manufacturer's instructions. The assay was read using a Luminex 100 (Austin, TX, USA), and analyzed using a Bio-Plex Manager software. The mean concentration of cytokines in supernatants from OVA-stimulated cells over the unstimulated cells (background) was then calculated.

Analysis of mouse blood

Whole blood samples were collected by cardiac puncture. The blood was placed in Vacutainer™ tubes containing EDTA (BD science, USA). Anti-coagulated blood was processed to determine hematological parameters (WBC, lymphocytes, monocytes, eosinophils, basophils and neutrophils) in a HEMAVET 950 hematology analyzer

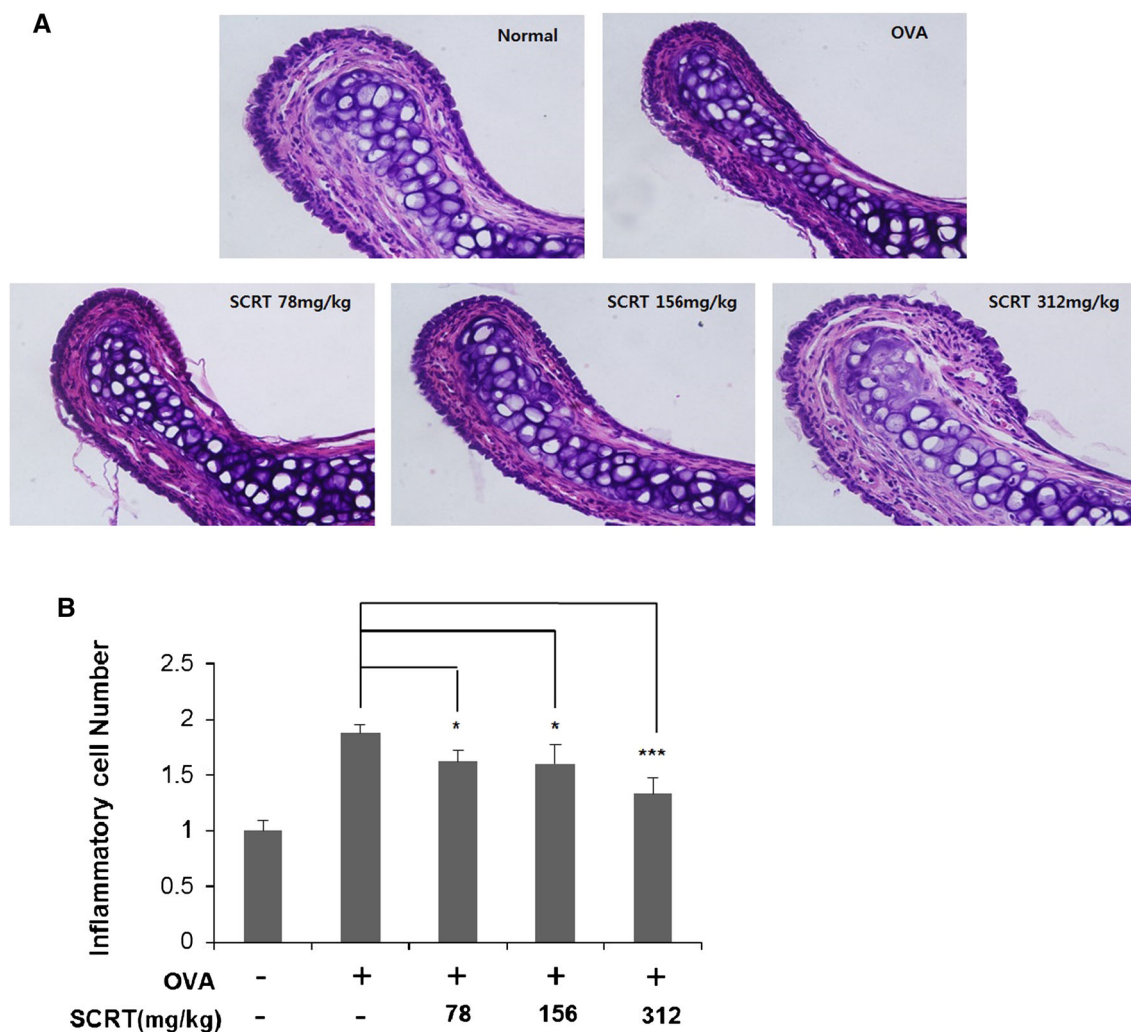


Fig. 1 SCRT reduced infiltration of inflammatory cells into nasal cavity. The nasal mucosa sections were stained with hematoxylin and eosin. Sections were evaluated using microscope at an original

magnification of ×40 and ×400. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

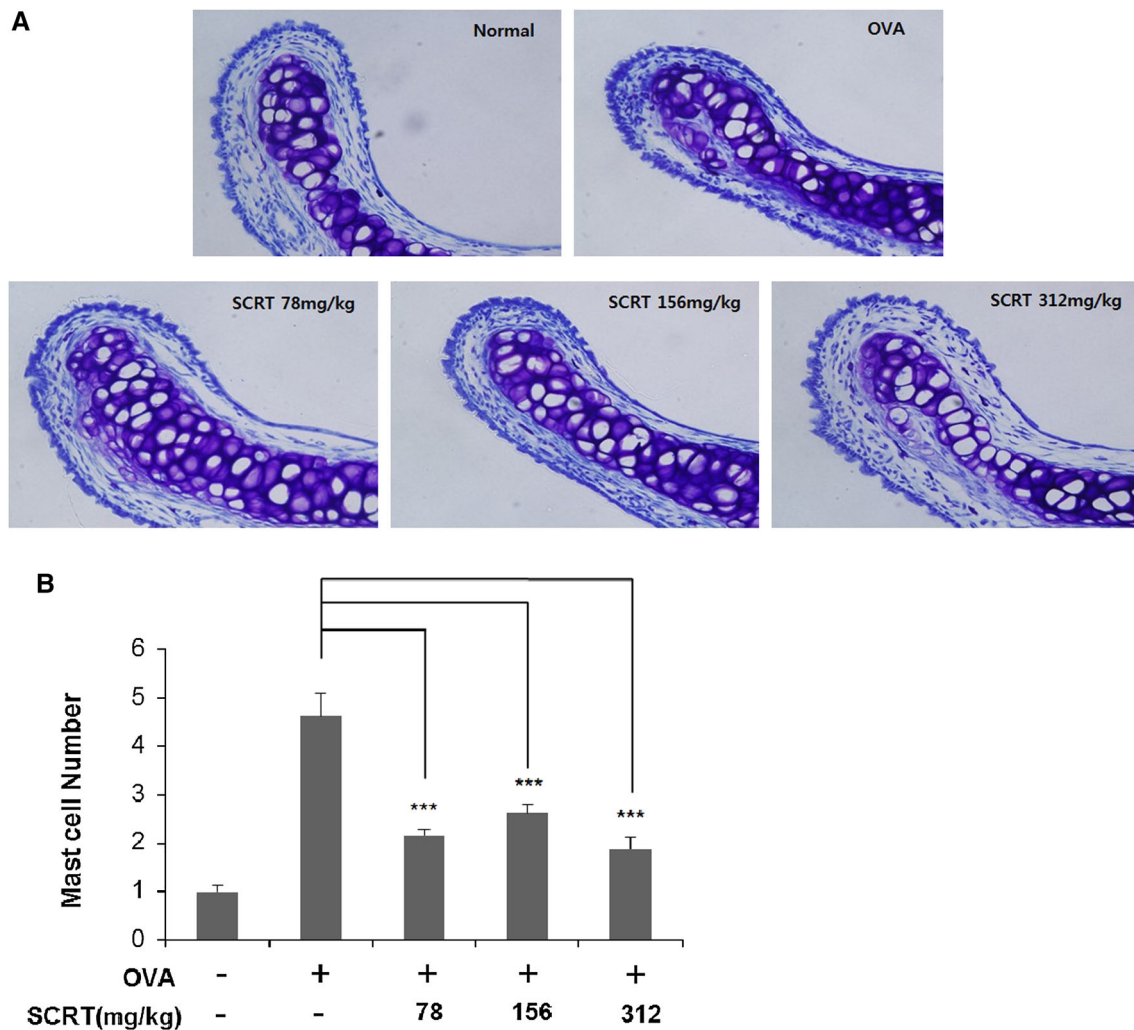


Fig. 2 SCRT reduced infiltration of mast cells into nasal mucosa. The nasal mucosa sections were stained with *toluidine blue*. Sections were evaluated using microscope at an original magnification of

$\times 200$. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

(Drew Scientific, Inc., Oxford, USA) in accordance to manufacturer' recommendation.

Splenocyte

Suspension of spleen from normal mice under aseptic condition was prepared by homogenization in RPMI-1640 medium (containing 10 % FBS, 1 % antibiotics, and 0.05 mM β -Mecaptoethanol). The suspension was centrifuged and pelleted. The contaminating red blood cells were removed using red blood cell lysis buffer (Sigma, St. Louis, MO, USA). Cells were centrifuged and suspended in

complete RPMI-1640. Cells were maintained at 37 °C in a humidified incubator with 5 %.

Cell viability assay

Cell viability was measured using MTS assay. HMC-1 cells were plated in 96-well flat bottom tissue culture plates at a density of 3×10^3 cells/well and incubated for 24 h. Cells were treated with different concentrations of SCRT (5–1,000 μ g/ml). After 24-h incubation, 10 μ L of WST solution was added to each well of the plate, and the plates were incubated in the dark at 37 °C for another 2 h. Optical

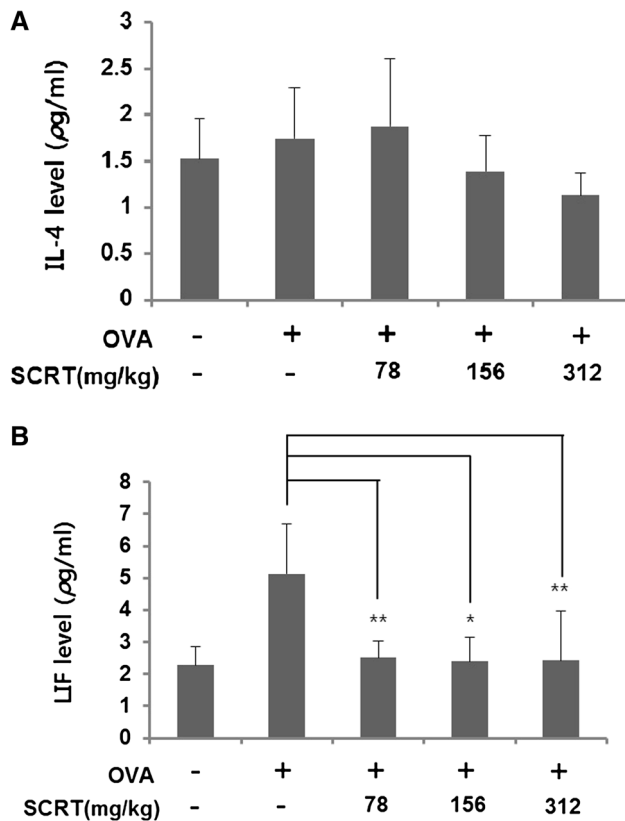


Fig. 3 SCRT reduced the levels of cytokines in the serum. The cytokines levels were measured by multiplex cytokine assay. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

density was measured at 450 nm using an ELISA plate reader (Versa Max, Molecular Devices, CA, USA).

RT-PCR

Cells were harvested by centrifugation and the pellet was washed with ice-cold PBS. Next, RNA was isolated using easy-blue RNA extraction kit (iNtRON biotech, Republic of Korea) according to the manufacturer's instructions. Isolated RNA content was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, USA). Total cellular RNA from each sample was reversely transcribed using cDNA synthesis kit (TaKaRa, Otsu, Shiga, Japan). PCR was performed using the specific primer. The primers used were as follows: human IL-4 (Forward: 5'-TGC CTC CAA GAA CAC AAC TG-3', Reverse: 5'-CTC TGG TTG GCT TCC TTC AC-3'); human GAPDH (Forward: 5'-GAG GGG CCA TCC ACA

GTC TTC-3', Reverse: 5'-CAT CAC CAT CTT CCA GGA GCG-3'); mouse IL-6 (Forward: 5'-GAT GCT ACC AAA CTG GAT ATA ATC-3', Reverse: 5'-GGT CCT TAG CCA CTC CTT CTG TG-3'); mouse TNF- α (Forward: 5'-ATG AGC ACA GAA AGC ATG ATC-3', Reverse: 5'-TAC AGG CTT GTC ACT GGA ATT-3'); and mouse GAPDH (Forward: 5'-GAG GGG CCA TCC ACA GTC TTC-3', Reverse: 5'-CAT CAC CAT CTT CCA GGA GCG-3').

Western blot analysis

Cells were harvested, incubated in one volume of lysis buffer (50 mM Tris-Cl pH 7.4, 1 % NP-40, 0.25 % sodium deoxycholate, 0.1 % SDS, 150 mM NaCl, 1 mM EDTA, and protease inhibitor) for 20 min and centrifuged at 13,000 rpm at 4 °C for 20 min. Aliquots containing 20 μ g of protein were separated by SDS-polyacrylamide gel electrophoresis using 8–12 % gels and transferred to nitrocellulose membranes (Protran nitrocellulose membrane, Whatman, UK). Membranes were blocked with 5 % nonfat milk and probed with specific primary antibodies. Membranes were then incubated with horseradish peroxidase-conjugated secondary IgG antibody (Calbiochem, San Diego, CA, USA) and visualized using the enhanced chemiluminescence detection system (Amersham ECL kit, Amersham Pharmacia Biotech Inc. Piscataway, NJ, USA).

Statistical analysis

All quantitative data derived from this study were analyzed statistically. The results were expressed as the mean $\pm$ SEM. Statistical significance at $P < 0.05$, <0.01 and <0.001 has been given respective symbols in the figures.

Results

SCRT reduced infiltration of inflammatory cells into nasal cavity

To determine whether SCRT reduces infiltration of inflammatory cells into nasal cavity, we performed H&E staining on the nasal mucosa samples. Numbers of inflammatory cells in AR mice were shown to be higher than those in the normal mice. SCRT decreased such infiltration of inflammatory cells into nasal cavity (Fig. 1a).

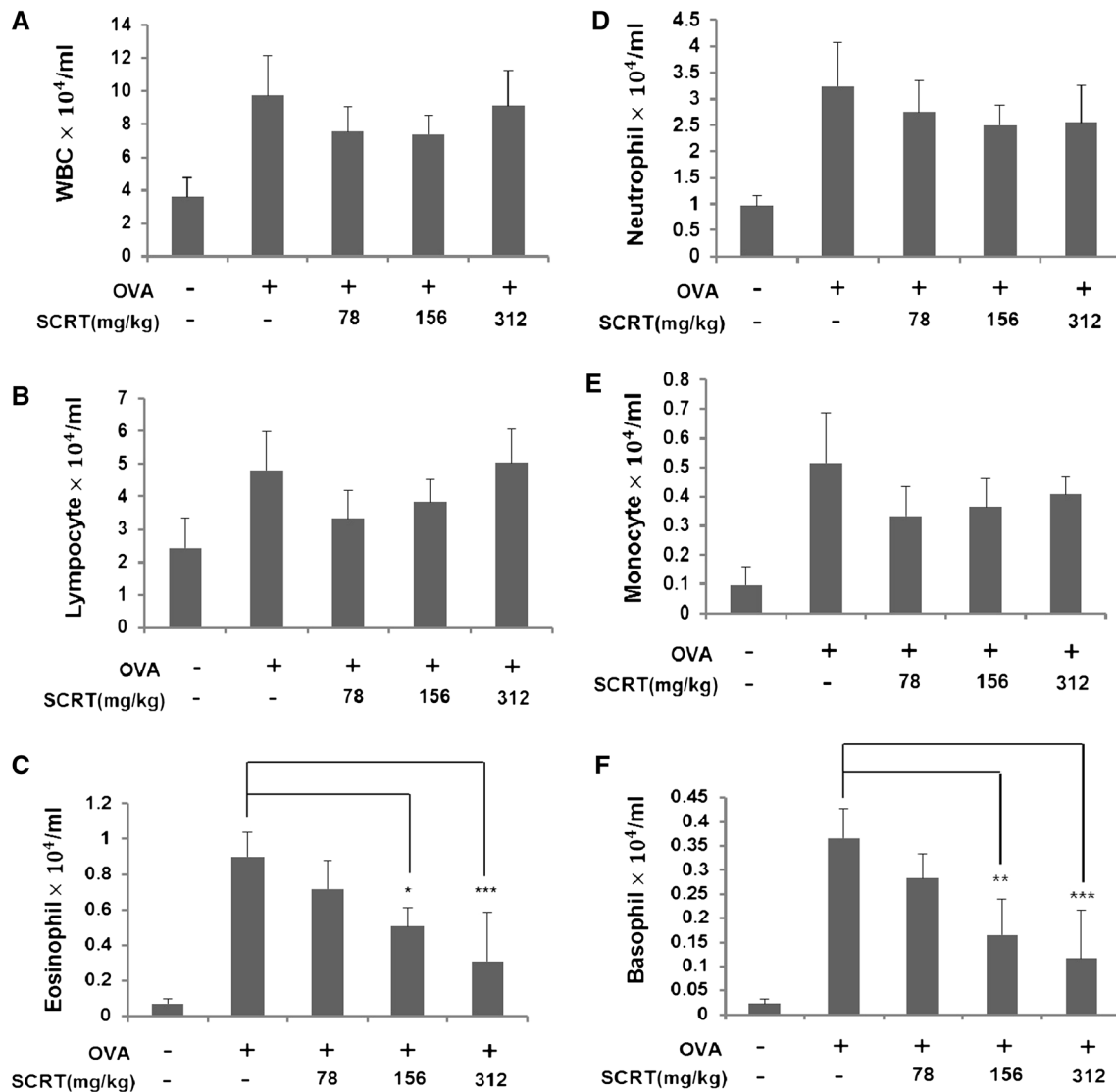


Fig. 4 SCRT reduced the levels of leukocytes in the Blood. Blood samples were analyzed using HEMAVET 950 hematology analyzer. Data are presented as mean $\pm$ SEM * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

Inflammatory cell numbers under each condition are shown in Fig. 1b.

SCRT reduced infiltration of mast cells into nasal mucosa

To determine whether SCRT reduces infiltration of mast cells into nasal mucosa, we performed T&B staining on the nasal mucosa samples. Numbers of mast cells in AR mice were shown to be higher than those in the normal mice.

SCRT decreased such infiltration of mast cells into nasal cavity (Fig. 2a). Mast cell numbers under each condition are shown in Fig. 2b.

SCRT reduced the levels of IL-4 and LIF in the serum

We measured the levels of cytokines in the blood samples by multiplex cytokine assay. We found that OVA increased the levels of IL-4 and LIF while SCRT inhibited such increase (Fig. 3a, b).

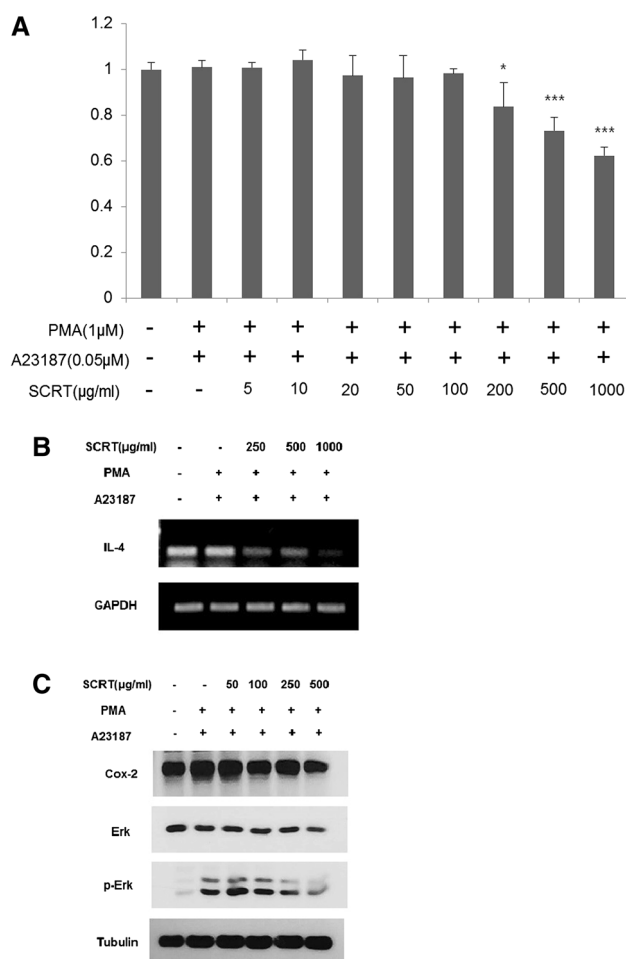


Fig. 5 Effect of SCRT in HMC-1 cells. HMC-1 cells were treated with different concentrations of SCRT (5–1,000 μg/ml) for 24 h. Then, cell viability was measured using MTS assay (**a**). IL-4 mRNA expression was measured by RT-PCR (**b**). Whole cell lysates were analyzed by western blot with anti-COX-2, anti-Erk, anti-p-ERK and anti-Tubulin antibodies (**c**). Data are presented as mean ± SEM * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

SCRT reduced the levels of leukocytes in the blood

To investigate whether SCRT suppresses inflammatory phenomenon, we measured leukocytes levels in cardiovascular blood samples using HEMAVET 950 hematology analyzer. We found that OVA increased the levels of eosinophils, neutrophils, monocytes, basophils, lymphocytes and WBC while SCRT inhibited such increases (Fig. 4a–f).

Effect of SCRT on HMC-1 cell viability

We investigated whether SCRT affects cell viability of HMC-1 cells. HMC-1 cells were treated with different concentrations of SCRT (5–1,000 μg/ml) for 24 h in the presence or absence of PMA plus A23187. Cell viability

was then measured by MTS assay. At higher concentrations (200–1,000 μg/ml), SCRT significantly suppressed HMC-1 cell growth (Fig. 5a). Moreover, we found that SCRT suppressed IL-4 level in HMC-1 cells in a dose-dependent manner (Fig. 5b). We also found that SCRT decreased the levels of p-Erk and Cox-2 in HMC-1 cells (Fig. 5c).

Effect of SCRT on splenocyte

We investigated whether SCRT affects cell viability of splenocyte. Splenocyte cells were treated with different concentrations of SCRT (10–2,000 μg/ml) for 24 h. Cell viability was then measured by MTS assay. We found that con A increases cell viability while SCRT decreases it (Fig. 6a). Elisa assay indicated that SCRT decreased the levels of IL-4 and IL-1β induced by ConA in splenocyte cells (Fig. 6b, c). RT-PCR indicated that SCRT suppressed the levels of IL-6 and IL-1β induced by LPS (Fig. 6d).

Discussion

Allergic rhinitis (AR) is the most common atopic disorder affecting between 9 and 24 % of adults and up to 42 % of children in the United States [19–21]. AR is an allergic inflammation of the nasal airways. This inflammation can cause a variety of annoying symptoms, including sneezing, itching, nasal congestion, rhinorrhea (anterior and posterior) and post-nasal drip. Hence, treatment of AR improves the quality of life.

The Korean herbal medicine, SCRT contains eight herbal plants [22]. Pinelliac acid was suggested as an active herbal component of SCRT and showed oral adjuvant activity. However, pinelliac acid alone was not sufficient to induce all the anti-allergic activities of SCRT, and some other active constituents seem to play a combinatorial role in the medicine [15]. Other ingredients such as paeoniflorin [23], α-asarone [24], Schizan [15], drin [25], 6-gingerol [26] and glycyrrhizic acid [27, 28] have also been suggested to contribute to a protection against allergy. The present study demonstrated that SCRT is effective to treat AR in a mouse model. SCRT has been used widely in traditional medicine for centuries. Its efficacy and mechanism of action about several allergic diseases have been investigated since the 1980s. Anti-allergic effects of SCRT were also tested in human studies [22].

In this study, we investigated the effects of SCRT on OVA-induced AR in mouse model. Increase of infiltration of inflammatory and mast cells into nasal cavity are known in OVA-induced AR mice. Interestingly, SCRT strongly suppressed such increase. Induction of various cytokines

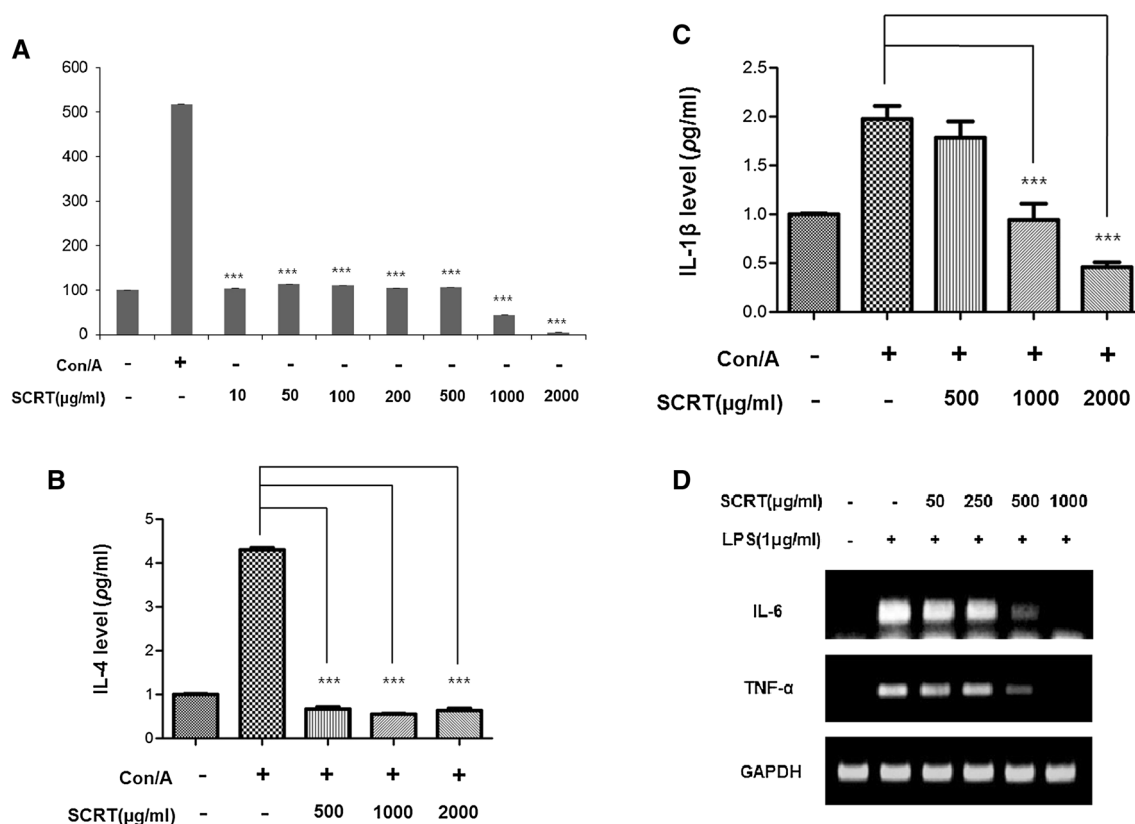


Fig. 6 Effect of SCRT on splenocyte. Splenocyte was treated with different concentrations of SCRT (10–2,000 μg/ml) for 24 h. Then, cell viability was measured using MTS assay (**a**). The release of IL-4 and IL-1β was measured by sandwich ELISA assay (**b**, **c**). IL-6 and

TNF-α mRNA expression was measured by RT-PCR (**d**). Data are presented as mean ± SEM * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

due to AR was also suppressed by SCRT in mice. The activation of eosinophils released a variety of chemicals to cause inflammation and tissue injuries. A variety of cytokines are released by inflammatory stimulation. We found that OVA increases the levels of IL-4 and LIF while SCRT inhibits such increases. We also found that OVA increases the levels of eosinophils, neutrophils, monocytes, basophils, lymphocytes and WBC while SCRT inhibits such increases. SCRT also decreased cell viability of HMC-1 cells and splenocyte. SCRT suppressed IL-4 level in HMC-1 cells and splenocyte cells in a dose-dependent manner. We found that SCRT suppressed IL-6 level and TNF-α level in splenocyte. These data clearly demonstrate that SCRT has an efficacy to treat AR.

Conclusions

SCRT suppresses the progression of AR induced by OVA. SCRT might be a useful drug for the treatment of AR.

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Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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