

Preventive Effect of *Bupleurum chinense* on Nasal Inflammation via Suppressing T Helper Type 2, Eosinophil and Mast Cell Activation

Thi Tho Bui,^{*} Chun Hua Piao,[†] Eunjin Hyeon,[†] Yanjing Fan,[†]
Dae Woon Choi,[‡] Sun Young Jung,[‡] Byung-Hyun Jang,[§] Hee Soon Shin,^{‡,¶}
Chang Ho Song^{†,¶} and Ok Hee Chai^{†,¶}

^{*}Faculty of Biology & Environmental Science, University of Science and Education
The University of Danang
Danang 555940, Vietnam

[†]Department of Anatomy, Chonbuk National University Medical School
Jeonju, Jeonbuk 54896, Republic of Korea

[‡]Food Biotechnology Program, Korea University of Science and Technology
Daejeon 34113, Republic of Korea

[§]Research Division of Food Functionality

[¶]Division of Nutrition and Metabolism Research
Korea Food Research Institute

Wanju-gun, Jeonbuk 55365, Republic of Korea

[¶]Institute for Medical Sciences, Chonbuk National University
Jeonju, Jeonbuk 54896, Republic of Korea

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Abstract: *Bupleurum chinense* is distributed in East Asia and has been used as a traditional herbal medicine for more than a thousand years. Though *B. chinense* has been reported to have immunomodulatory, anti-inflammatory, anti-oxidant, hepato-protective, antipyretic, analgesic and antifibrotic effects, its specific effect on allergic rhinitis disease has not been clarified. In this study, we investigated the anti-allergic and anti-inflammation effects of *B. chinense* extract (BCE) in an ovalbumin (OVA)-induced allergic rhinitis (AR) mouse model. Oral administration of BCE in a dose-independent manner regulated the balance of Th1/Th2/Treg cell differentiation in AR mice. Accordingly, BCE attenuated the expression of Th2-related cytokines such as IL-4, IL-5 and IL-13 in nasal lavage fluid (NALF) and nasal tissue and up-regulated the secretion of Th1/Treg cells including IL-10, IL-12 and IFN- γ .

Correspondence to: Prof. Ok Hee Chai, Department of Anatomy, Chonbuk National University Medical School, Jeonju, Jeonbuk 54896, Republic of Korea. Tel: (+82) 63-270-3109, Fax: (+82) 63-274-9880, E-mail: okchai1004@jbnu.ac.kr

Also, BCE inhibited the formation and migration of eosinophils to the nasal mucosa and NALF, as well as suppressed CCL24, an eosinophil-specific chemoattractant in NALF. The levels of anti-OVA specific IgE and anti-OVA specific IgG1 were decreased, and as a result, the allergic response was attenuated by BCE via inhibiting mast cells accumulation in nasal mucosa and serum histamine release. The nasal allergy symptoms, nasal mucosal swelling, epithelial barrier disruption and mucus hyperplasia were obviously ameliorated. These results suggest that BCE may have therapeutic potential for treating allergic rhinitis through modulating the accumulation and activation of important leukocytes in the immune system such as Th1, Th2, Treg, eosinophils and mast cells.

Keywords: *Bupleurum chinense*; Allergic Rhinitis; Eosinophils; Mast Cells; Th1/Th2/Treg Cytokines; Nasal Epithelium.

Introduction

Allergic rhinitis (AR) is a common inflammatory disease characterized by nasal itching, sneezing, stuffy nose and nasal congestion. AR is also an important risk factor for asthma (Bachert *et al.*, 2008). The previous data showed that AR affects up to 50% of the world's population (Strachan *et al.*, 1997), that over 500 million people suffer from AR worldwide and that the incidence of AR is increasing (Brozek *et al.*, 2010).

Allergic rhinitis is characterized by an accumulation of eosinophils and mast cells in the nasal mucosa. Previous studies have shown that both eosinophils and mast cells increase the present in asthmatic airways and nasal fluid of allergic rhinitis. In allergic disorders, mast cells degranulate in response to IgE-mediated allergic stimulation. Mediators released locally recruit eosinophils to cause further damage (Jarjour *et al.*, 1991; Rasp and Hochstrasser, 1993), and these interactions generate eosinophil and mast cell couplets or pairs. They were found to influence each other in a paracrine/physical pathway, using human and murine cells *in vitro*; the two cells form stable conjugates, and clear membrane contact is established between them. Eosinophils were more viable when mast cells were present, depending on soluble mediators and on physical cell contact (Kovalszki and Weller, 2014). By interacting with each other, these two cells have the capacity to influence the tissue microenvironment. On the other hand, allergic rhinitis is also associated with the imbalance of CD4+ T cell subsets, which can be classified into type 1 helper T (Th1), Th2, Th17 and regulatory T (Treg) cells based on the expression of specific cell surface markers. Th1 and Th2 subsets mediate various inflammations mainly via secretion of cytokines such as interferon (IFN)- γ , interleukin (IL)-10, IL-12, IL-4 and IL-5 (Kidd, 2003). Furthermore, AR was believed to result from the up-regulation of Th2 cells and a lack of Th1 cells (Malmhall *et al.*, 2007). On the other hand, histamine plays an important role in the differential regulation of T helper lymphocytes as the major secretory product of mast cells. Histamine influences cytokine production via H2 receptors and through PKA activation. Histamine enhances the secretion of Th2 cytokines, such as IL-4, IL-5 and IL-13 and inhibits the production of Th1 cytokines IL-12 and IFN- γ (Packard and Khan, 2003). These cytokines,

including IL-4, IL-5 and IL-13, are crucial for promoting IgE based responses and for potentiating the development and maturation of mast cells and eosinophils (Renauld, 2001).

It is well known that antihistamines, antileukotriene and intranasal corticosteroids are current therapeutic drugs for ameliorating allergic symptoms and AR treatment (Takahashi *et al.*, 2009; Jung *et al.*, 2013). However, corticosteroids do not address all issues for every patient (Alvarez *et al.*, 1992). Additionally, long-term use of steroids has undesirable adverse effects such as headaches, throat irritation, and nasal dryness, particularly when high doses are used (Lipworth and Jackson, 2000). In recent years, traditional Eastern herbal medicine has been widely used in Western medicine because of its safety, effectiveness and lack of undesirable side effects. *Bupleurum chinense* is known as “Bei Chaihu” in China and has been one of the most important traditional herbal medicines for over a thousand years. It has been reported to have anti-inflammatory, anti-oxidant, hepatoprotective, antipyretic, antifibrotic, analgesic and immunomodulatory effects (Sun, 2006; Xie *et al.*, 2012). Some research has reported that saikosaponin, the main pharmacodynamic active ingredient of *B. chinense*, has anti-inflammatory effects through the regulation of nicotinate, nicotinamide metabolism and arachidonic acid metabolism (Ma *et al.*, 2016). Saikosaponin A also has anti-inflammatory and anti-fibrotic actions on CCL₄-induced liver damage in rats, and it significantly inhibited the hepatic proinflammatory cytokines TNF- α , IL-1 β and IL-6 (Wu *et al.*, 2010). *B. chinense* was reported to have anti-asthmatic effects via reduction of the Th2 and Th17 cytokines (Bui *et al.*, 2017). However, the effect of *B. chinense* on allergic rhinitis is still unclear. This study aims to investigate the anti-allergic effects of *B. chinense* extract (BCE) on an ovalbumin (OVA)-induced allergic rhinitis mouse model.

Materials and Methods

Animals

Six-week-old male BALB/c mice were obtained from the Damool Science (Dae-jeon, Korea). The mice were housed under standard laboratory conditions for 1 week before experiments and were maintained at a temperature of $23 \pm 3^{\circ}\text{C}$, relative humidity of $50 \pm 10\%$ and 12-h light/dark cycles. All procedures and experiments were approved in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School (CBN 2016-37) and were approved by the National Institutes of Health.

Preparation of BCE

BCE samples were extracted with 10 $\times$ volumes of distilled water under a heating condition ($80 \pm 5^{\circ}\text{C}$) for 8 ± 2 h and then the extract supernatants were collected using 5 μm cartridge filter. This process was repeated again. Next, the collected supernatants were concentrated (> 20 brix) and filtered using 100 mesh. The BCE was mixed with dextrin at a ratio of 1:1 and then dried using spray dry under an inlet air temperature of 180°C and an outlet air temperature of 95°C . After filtering, the BCE powders were kept at room

temperature and managed with a specimen number (KFRI-SL-2147) at the Korea Food Research Institute. For an AR mice model treatment, BCE powder was dissolved in saline at concentrations of 50, 100 and 200 mg/kg and was prepared fresh daily before each treatment. Cromolyn disodium salt hydrate (DSGC) was used as a control group and compared to BCE treatment groups.

Allergic Rhinitis Model Establishment and Treatment

BALB/c mice were divided into six groups ($n = 6$ each group): (1) naive group, (2) OVA group, (3) BCE 50 group, (4) BCE 100 group, (5) BCE 200 group and (6) DSGC group. The OVA-induced AR mouse model and treatment were established as described in Fig. 1. Briefly, OVA-induced AR mice were sensitized by intraperitoneal injection with 200 μ L saline along with 50 μ g OVA (Grade V, Sigma, St. Louis, MO, USA) adsorbed to 1 mg aluminum hydroxide (Thermo Scientific, Rockford, MD, USA) on days 0, 7 and 14. One week after the last sensitization, on days 21 to 27, mice received an intranasal challenge with 1 mg/mL OVA, 20 μ L in each nasal cavity. Mice were treated 1 hour before the intranasal challenge of OVA. Once daily from days 15 to 27, mice in the BCE groups and DSGC group were orally administered BCE and DSGC at a dose of 50, 100, 200 mg/kg and 2.5 mg/kg, respectively; mice in the OVA group were given saline. Mice were sacrificed 24 h after the last OVA challenge. Six mice in each group were evaluated on day 27. The frequency of nasal rubbing and sneezing were counted for 15 min after the OVA intranasal challenge.

Nasal Lavage Fluid Collection and Cell Count

Mice were sacrificed, and the blood was harvested by cardiac puncture and centrifuged to obtain the serum. Nasal lavage fluid (NALF) was collected by an 18-gauge catheter. The

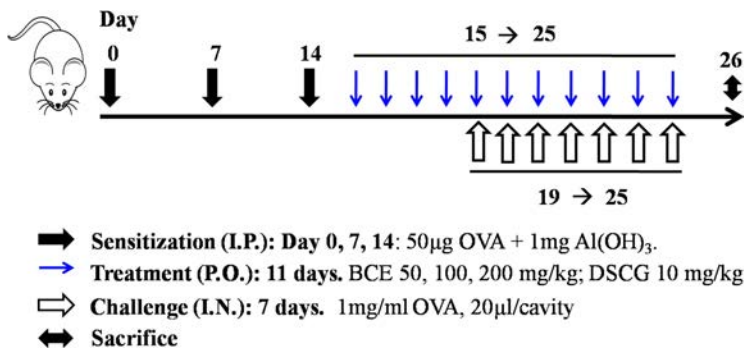


Figure 1. Experimental protocol for allergic rhinitis mouse model and treatment with BCE. Mice were sensitized on days 0, 7 and 14, and challenged on days 21 to 27 with ovalbumin (OVA). Mice in the BCE or DSGC groups were administered orally once daily at 50, 100, 200 mg/kg BCE or 10 mg/kg DSGC for 11 days, respectively.

trachea was partially resected, and the catheter was inserted into the trachea in the direction of the upper airway into the nasopharynx in order to gently perfuse the nasal passages with 1 mL PBS. After that, NALF was centrifuged (10,000 RPM/minutes for 10 min at 4°C) to collect the supernatant for determining cytokines. The cell pellets were resuspended in PBS, and the total cell numbers were counted using a hemocytometer. To determine differential cell counts, 150 µL of NALF was centrifuged onto the slides using a cytospin device (Centrifuge 5403, Eppendorf, Hamburg, Germany) (1000 rpm, 10 min, 4°C). Diff-Quik Staining reagent (1-5-1 Wakinohama-Kaigandori, Chuo-Ku, Kobe, Japan) was used for cell staining according to the protocols from the manufacturer.

Histological Examination

For nasal histological examination, the mouse heads were fixed in 10% neutral buffered formalin for 3 days and then decalcified in an ethylenediaminetetracetic acid decalcifying solution for 5 days at room temperature. The specimen was dehydrated with ethyl alcohol and xylene and embedded in paraffin. The sections were cut at 5 µ thickness and stained with hematoxylin and eosin (H&E) (Sigma, St. Louis, MO) for general morphology, periodic acid-Schiff (PAS) (Sigma, St. Louis, MO) for goblet cell hyperplasia and Giemsa (Sigma, St. Louis, MO) to assess eosinophil and mast cell infiltration. Eosinophils and mast cells were counted in the nasal septum and lateral process. Epithelial damage analysis was randomly selected using high-power fields at 400× magnification.

Measurement of Immunoglobulins and Cytokine Levels Using ELISA

The NALF supernatant was collected by centrifugation. The blood was collected from the vena cava to obtain the serum and stored at -70°C until use. Nasal tissue was collected and immediately stored at -70°C after sacrifice; it was then homogenated and centrifugated to collect the supernatant for the cytokines test. The levels of anti-OVA specific Ig (immunoglobulin) E, anti-OVA specific IgG1 and anti-OVA specific IgG2a were quantified using ELISA kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturers' instructions. Cytokine quantitation kits (BD Biosciences, San Diego, CA, USA) were used to measure cytokine levels including IL-4, IL-5, IL-13, IFN-γ, IL-10 and IL-12 according to the manufacturer protocols.

Statistical Analysis

The results were analyzed using Graph Pad Prism software (v5.0, La Jolla, CA, USA). The statistical significance of the differences among the groups was analyzed by one-way ANOVA, followed by the Tukey's test. The data were presented as means ± standard error (SE) of independent experiments. Significance was considered at the 95% confidence level ($P < 0.05$).

Results

BCE Inhibited the Allergic Nasal Symptoms Induced by OVA

To determine the effect of BCE on the early-phase allergic symptoms, the frequencies of rubbing and sneezing were counted for 15 min after OVA intranasal challenge on day 27. The results showed that rubbing and sneezing scores were significantly increased in the OVA-induced AR group compared to the control group. Nevertheless, BCE administration dose-independent manner significantly alleviated allergic nasal symptoms compared to OVA group. Similarly, the DSGC treatment also displayed a significant inhibition effect on allergic symptoms in AR mice model (Figs. 2A and 2B).

BCE Reduced the Infiltration of Differential Inflammatory Cells in NALF and Epithelial Cells Disruption

The number of total cells and differential inflammatory cells in NALF were quantified for expressing the anti-inflammation effect of BCE in the nasal mucosa. In the OVA group, the total cell numbers had strongly increased, including eosinophils, neutrophils and macrophages, compared to the naive group. However, BCE at doses of 50, 100 and 200 mg/kg and DSGC treatments significantly decreased these inflammatory cells in NALF compared to the OVA group (Fig. 3A). In addition, the epithelial cells number in NALF was over-expressed in the OVA-exposed mice but significantly decreased after BCE treatment in a dose-independent manner (Fig. 3B). The Diff-Quik staining showed that epithelial cells (black arrows) and eosinophils (red arrows) were over-expressed in the OVA group and were clearly reduced in BCE and DSGC-treated mice (Fig. 3C). These results indicated that BCE ameliorated an exacerbation of nasal epithelial barrier disruption.

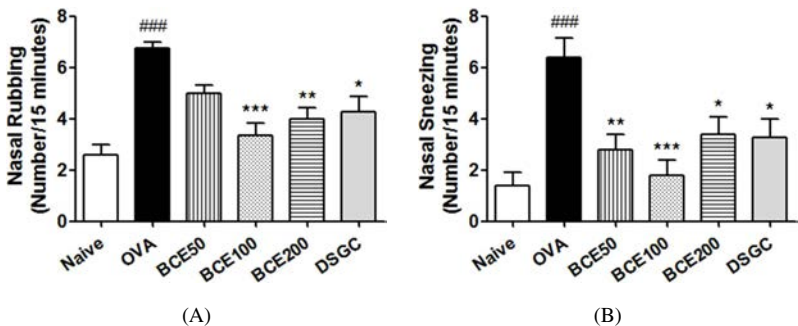


Figure 2. BCE inhibited the nasal allergic symptoms induced by OVA. (A) Rubbing score. (B) Sneezing score. Rubbing and sneezing events were counted during 15 min after the OVA challenge on day 27. BCE reduced the rubbing and sneezing frequencies in OVA-induced AR mice model. The values represent the mean $\pm$ SE ($n = 6/\text{group}$). Significant differences at # $P < 0.05$, ### $P < 0.001$ compared with the Naive group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the OVA group.

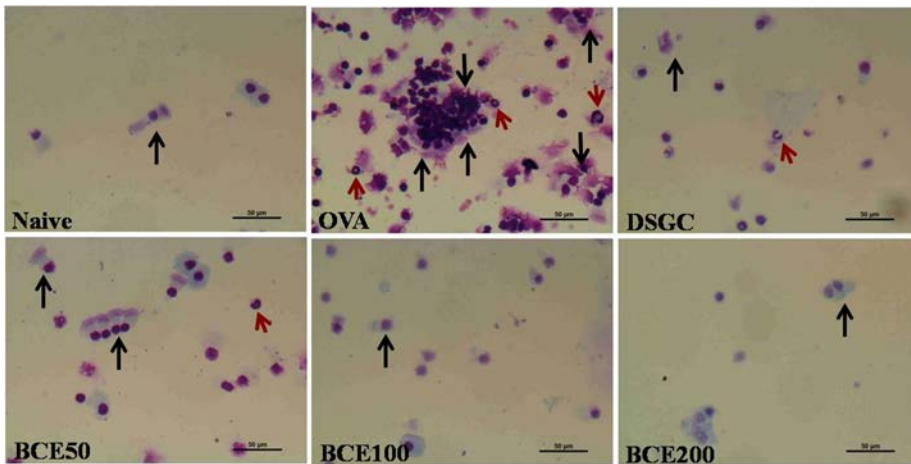
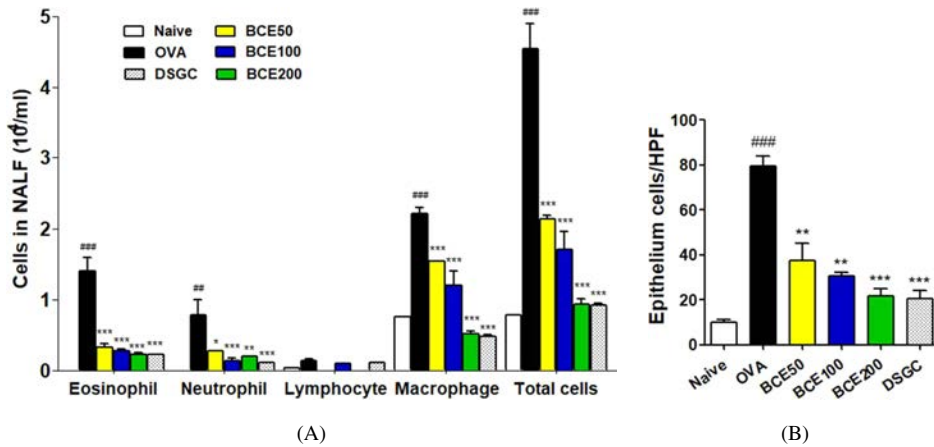


Figure 3. BCE reduced the infiltration of differential inflammatory cells in NALF and prevented epithelial cells disruption. (A) The number of differential cells and total cells in NALF. (B) Epithelial cells number in NALF. (C) Differential cells were stained with Diff-Quik. NALF was collected immediately after sacrifice; the cells were isolated by cytospin. Red arrows indicate eosinophils. Black arrows indicate epithelium cells that lost from the inflammatory tissue. Scale bar: 50 μ m, $\times 400$ magnification. The values represent the mean $\pm$ SE ($n = 6$ /group). Significant differences at $##P < 0.01$, $###P < 0.001$ compared with the naive group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the OVA group.

BCE Ameliorated Mucosa Thickness and Mucus Secretion in Nasal Mucosa

H&E staining was performed to identify the general structure of nasal mucosa. Morphological abnormalities were observed in the nasal mucosa of OVA-induced AR mice, and the nasal septum showed an increased accumulation of inflammatory cells in the subepithelium that correlated with a significant increase in mucosa thickness when

compared to the naive mice (double-headed arrow, Figs. 4A and 4B). The nasal mucosa layers in BCE and DSGC-treated mice were considerably thinner than those in the OVA group, indicating that BCE administrations had a protective effect on nasal mucosa structure. Furthermore, BCE also ameliorated the mucus hyperplasia, which was observed as a violet color, from goblet cells in epithelium compared to the OVA group (black arrows, Fig. 4C).

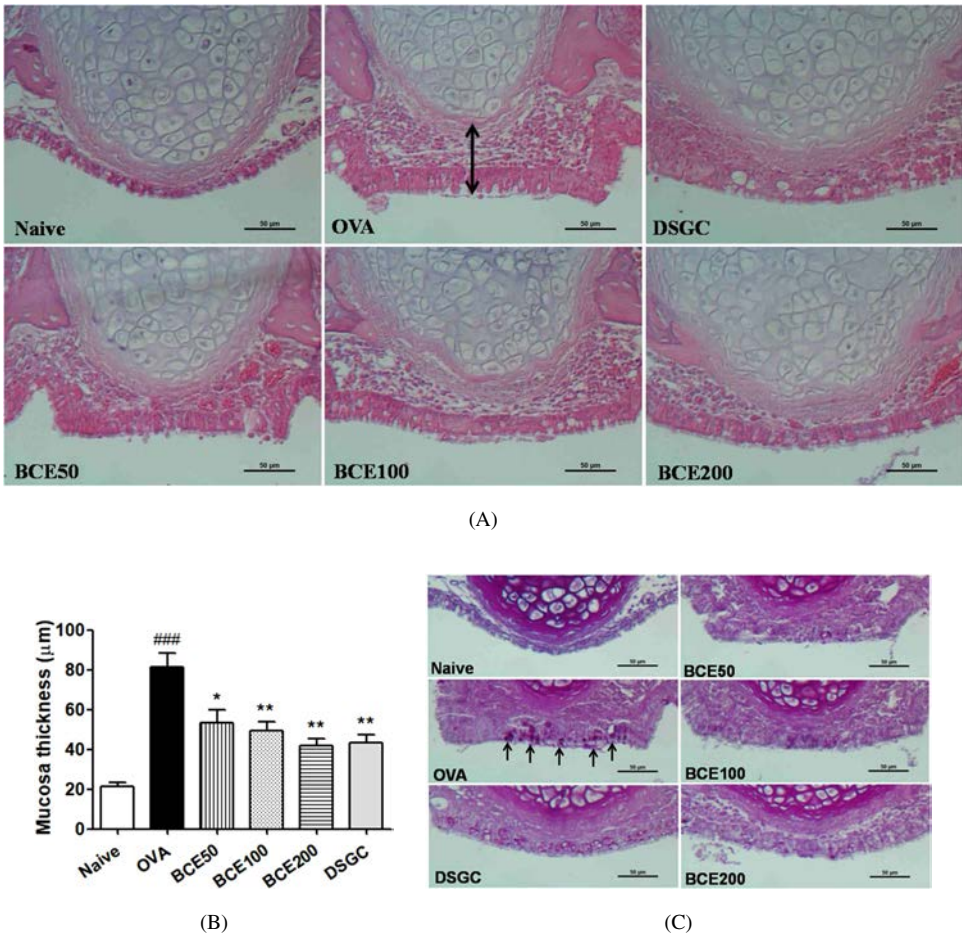


Figure 4. BCE ameliorated mucosa thickness and mucus secretion in the nasal mucosa. (A) Histological features of the nasal mucosa were identified by H&E staining. (B) Mucosa thickness of nasal septum. The mucosa swelling that was observed in the OVA group (double-headed arrow) was ameliorated by BCE treatment in dose-independent manners. (C) Goblet cells were identified by PAS staining. BCE inhibited mucus hyperplasia in epithelium cells that were observed as violet and marked by black arrows. Scale bar: 50 μm , $\times 400$ magnification. The values represent the mean $\pm$ SE ($n = 6/\text{group}$). Significant differences at ### $P < 0.001$ compared with the Naive group. * $P < 0.05$, ** $P < 0.01$ compared with the OVA group.

BCE Inhibited the Mast Cell Infiltration into Nasal Mucosa and Allergic Responses

It is well-known that antibodies play a key role in the immune system. The anti-OVA specific IgE, anti-OVA specific IgG1 and anti-OVA specific IgG2a in serum were determined to evaluate the effect of BCE on allergic inflammatory responses. There was a significant up-regulation of anti-OVA-specific IgE (Fig. 5A) and IgG1 (Fig. 5B) and down-regulation of the anti-OVA specific IgG2a level (Fig. 5C) in OVA group compared with those in the naive group. In contrast, dose-dependent BCE administration resulted in lower levels of anti-OVA IgE and anti-OVA IgG1 and higher levels of anti-OVA IgG2a than the levels observed in the OVA group, which acted similarly in DSGC-treated mice (Fig. 5).

Giemsa staining revealed that the administrations of BCE and DSGC suppressed mast cell infiltration into the nasal mucosa, where cytoplasm was stained a blue color and marked with a red circle (Fig. 6A). Additionally, there was a significantly higher number of mast cells in the nasal mucosa from the OVA group than in the naive group. However, both mast cells and degranulated mast cells were decreased after BCE and DSGC treatments (Fig. 6B). Accordingly, histamine release from activated mast cells was also significantly suppressed in serum and NALF at the dose-independent manner of BCE-treated mice (Fig. 6C).

BCE Inhibited The Eosinophil Accumulation in Nasal Mucosa

The histopathological study revealed that the administrations of BCE and DSGC suppressed eosinophil infiltration into the nasal mucosa, which resulted in cells with red-stained cytoplasm and over-expression in the OVA group (black arrows, Fig. 7A). In AR mice, the presence of eosinophils in the subepithelium increased significantly compared with the presence in the naive mice; eosinophil presence was markedly decreased after BCE and DSGC treatment in a dose-independent manner (Fig. 7B).

CCL24 is known as eosinophil chemotactic protein 2 and is significantly elevated in both serum, nasal tissue homogenate and NALF in OVA-exposed mice. Because of the

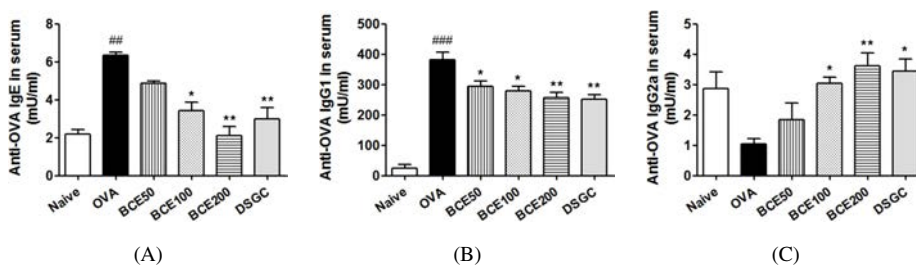


Figure 5. BCE regulated the levels of serum anti-OVA specific antibodies. BCE-treated mice down-regulated the levels of anti-OVA specific IgE (A) and anti-OVA specific IgG1 (B), meanwhile, up-regulated the anti-OVA specific IgG2a (C). The values represent the mean $\pm$ SE ($n = 6/\text{group}$). Significant differences at ### $P < 0.01$, ### $P < 0.001$ compared with the Naive group. * $P < 0.05$, ** $P < 0.01$ compared with the OVA group.

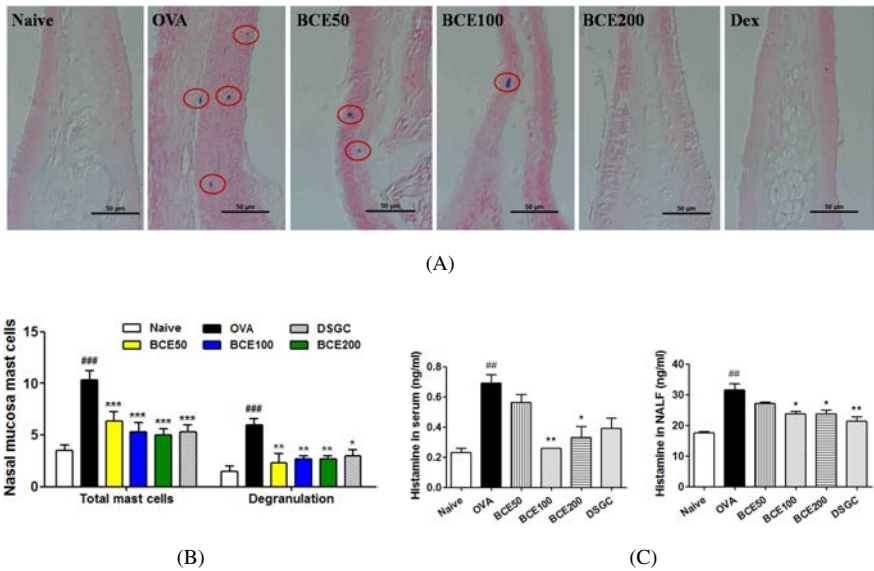


Figure 6. BCE reduced mast cell infiltration into nasal mucosa and mast cell histamine release. (A) Mast cells were identified by Giemsa staining. The red circles were used to mark the mast cells. (B) The mast cells number was counted in nasal mucosa in a high power field of view. (C) Histamine level was examined in serum and NALF. Scale bar: 50 μ m, $\times$ 400 magnification. The values represent the mean $\pm$ SE ($n = 6$ /group). Significant differences at $##P < 0.01$, $###P < 0.001$ compared with the Naive group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the OVA group.

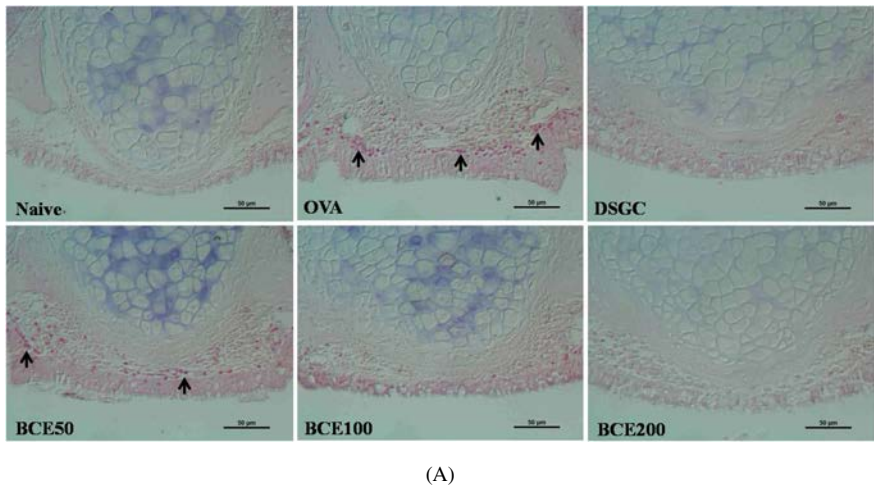


Figure 7. BCE inhibited the accumulation and activation of eosinophils in the nasal mucosa. (A) Eosinophils were identified by Giemsa staining. The black arrows show eosinophils, which can be observed as red. (B) The number of eosinophils was counted in nasal mucosa in a high power field of view. (C) The levels of CCL24 (eotaxin-2) were measured in NALF, serum and nasal tissue. Scale bar: 50 μ m, $\times$ 400 magnification. The values represent the mean $\pm$ SE ($n = 6$ /group). Significant differences at $##P < 0.01$, $###P < 0.001$ compared with the nNaive group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the OVA group.

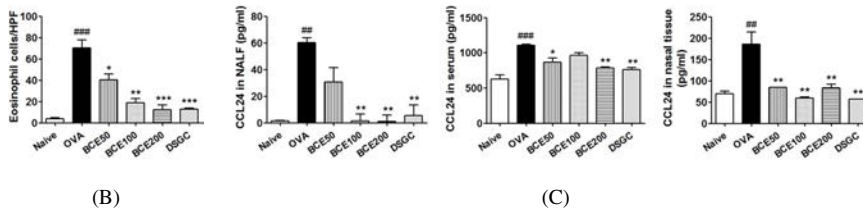


Figure 7. (Continued)

decreased number of eosinophils, CCL24 was also significantly inhibited in both serum and nasal tissue homogenate, especially in NALF at the dose-independent manner of BCE treatment (Fig. 7C).

BCE Modulated the Balance of Th1/Th2/Treg-Associated Cytokines in NALF and Nasal Tissue

We examined the levels of Th1 (IFN- γ , IL-12), Th2 (IL-4, IL-5, IL-13) and Treg (IL-10) cytokines in NALF and nasal tissue homogenate to investigate the effect of BCE on the modulation of T helper cells responses. In NALF and nasal tissue of OVA group, the levels of IL-4, IL-5 and IL-13 were significantly elevated; IL-10, IL-12 and IFN- γ levels had no statistical difference compared to those in the naive group. In contrast, BCE oral administrations in a dose-independent manner significantly down-regulated IL-4, IL-5 and IL-13 levels compared to the levels observed in the OVA group, especially IL-13 strongly decreased in all doses in both NALF and nasal tissue. Also, BCE notably decreased the relative expression of IL-4 mRNA in nasal tissues in a dose-dependent manner (Supplementary Fig. 1). In addition, BCE-treated mice had significantly up-regulated IL-10 cytokine in both NALF and nasal tissue, IL-12, INF- γ in NALF and INF- γ mRNA in nasal tissues in a dose-dependent manner (Fig. S1). Similarly, DSGC-treated mice demonstrated significantly decreased levels of IL-4, IL-5 and IL-13 and increased levels of IL-10 and IL-12 compared to the OVA group (Figs. 8 and S1). We investigated whether BCE regulates Th2-associated cytokine in the splenocytes via OVA-recall response. BCE enhanced cell proliferation and significantly inhibited the secretion of IL-5 and IL-13 (Fig. S2). These results indicated that BCE oral administration could reverse an abnormal allergic immune response and regulate the balance of Th1/Th2/Treg responses by enhancing the Th1 and Treg responses and by suppressing the Th2 response.

Discussion

In the present study, we used an OVA-induced AR mouse model to investigate the anti-allergic effects of BCE. To demonstrate the contributions of BCE in allergic inflammation responses, we evaluated nasal symptoms, OVA-specific antibodies, the infiltration of inflammatory cells into nasal mucosa such as mast cells, eosinophils and goblet cells.

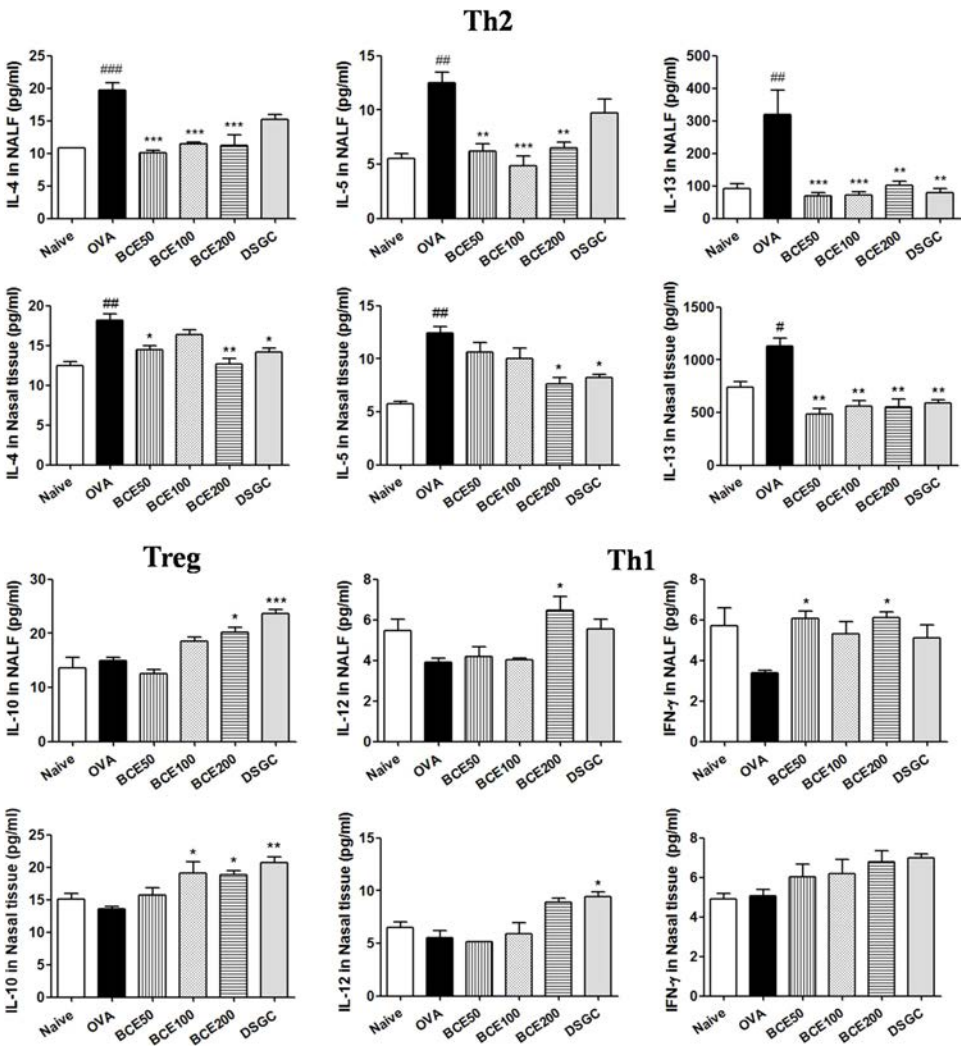


Figure 8. BCE modulated the balance of Th1/Th2/Treg-associated cytokines in NALF and nasal tissue. The Th2-related cytokines were down-regulated, while the Th1 and Treg-related cytokines were up-regulated in NALF and nasal tissue. The values represent the mean $\pm$ SE ($n = 6/\text{group}$). Significant differences at $##P < 0.01$, $###P < 0.001$ compared with the Naive group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with the OVA group.

In addition, the levels of Th1/Th2-mediated cytokines were examined to investigate the effect of BCE on T cells and inflammatory cells under AR disease progression.

Allergic inflammation often is classified into three temporal phases: early-phase reactions, late-phase reactions and chronic allergic inflammation. Early-phase reactions occur within minutes of allergen challenge. In the early-phase of AR, mast cells immediately secrete histamine, which induces early-phase symptoms including sneezing, itching and a runny nose (Skoner, 2001; Galli *et al.*, 2008; Wallace *et al.*, 2008). In our study, the

rubbing and sneezing symptom scores were examined for 15 min immediately after the OVA nasal challenge. BCE oral administrations significantly inhibited nasal allergic symptoms, indicating that BCE may prevent the inflammation reactions in early-phase of AR. Besides, mast cell degranulation following IgE-mediated crosslinking of the membrane-bound IgE high-affinity receptor (FcεRI) released granules stored mediators in mast cells within minutes (Turner and Kinet, 1999). Therefore, the IgE level and mast cells number illustrated a clear increased BCE effect in early-phase reactions. In the present study, the mast cells and mast cells degranulation numbers, which were observed in nasal histology, decreased after BCE treatments compared to the OVA group. The levels of anti-OVA specific IgE and anti-OVA specific IgG1 in serum were down-regulated at BCE in a dose-independent manner. According to these results, the histamine releases were also inhibited significantly. These data indicate that BCE oral administrations may ameliorate allergic inflammation responses in AR early-phase by targeting the modulation of OVA-specific antibodies level and mast cells activation.

Late-phase reactions typically develop within 2 to 9 h after allergen exposure, and chronic allergic inflammation is a persistent inflammation that occurs at sites of repeated allergen exposure. Inflammatory cells like eosinophils infiltrate the nasal cavity during the late-phase reactions and increase in number as allergen exposures repeat. Eosinophils-derived mediators induce epithelial damage, resulting in nasal mucosal swelling (Skoner, 2001; Galli *et al.*, 2008; Wallace *et al.*, 2008). In this study, eosinophils in the nasal mucosa were counted in a high power field and their number markedly decreased in BCE treated-mice. Additionally, we found the level of CCL24, known as eosinophil chemotactic protein 2 (eotaxin-2), to be lower in NALF serum and nasal tissue homogenate than the levels found in the OVA group. These results indicate that BCE may reduce eosinophil accumulation in nasal mucosa, prevent eosinophil activation and release eosinophil mediators, including chemokine CCL24. Therefore, BCE could protect the nasal cavity from mucosal swelling, eosinophilia and mucus hyperplasia in the OVA-induced AR mouse model.

During an allergic response, Th2 cells play a potential role via secretion of Th2-associated cytokines and activation of IgE-producing B-cells. Once released from B-cells, IgE coats the surface of mast cells and triggers the release of histamines (Amin, 2012). Among Th2-related cytokines, IL-4, IL-5 and IL-13 have been reported to regulate the growth and differentiation of eosinophils (Kim *et al.*, 2013). IL-13 induces inflammation, mucus hypersecretion, subepithelial fibrosis and eotaxin production (Renauld, 2001). In this study, the oral administrations of BCE in a dose-independent manner effectively suppressed the secretion of IL-4, IL-5 and IL-13 in both NALF and nasal tissue; this suppression may result in the reduction of eosinophil, neutrophil and macrophage numbers in NALF as well as the reduction of eosinophils, mast cells and goblet cell activation in the nasal mucosa. Furthermore, recent studies reported that IL-4 and IL-13 disrupted the junctional structure and epithelial barrier function of nasal epithelial cells via diverse mechanisms (Georas and Rezaee, 2014). Our study shows that the loss of epithelial cells in the OVA group was over-expressed in the nasal cavity and is represented as resulting in an exacerbated inflammation of nasal mucosa. BCE oral treatments in a dose-dependent

manner inhibited cell loss from epithelial layers, indicating that BCE protects the nasal epithelial barrier function via down-regulating Th2-associated cytokines. On the other hand, IFN- γ and IL-12, which are known as Th1-related cytokines, promote Th1 differentiation and prevent Th2 development in OVA-induced AR mice (Fulkerson *et al.*, 2004; Roever *et al.*, 2003). IL-10 is an inflammation-inhibiting cytokine, shown to be produced by Th1 and Tregs (Li and Flavell, 2008; Ogawa *et al.*, 2008). Our data show that IFN- γ , IL-12 and IL-10 were up-regulated after BCE treatments at high doses. These results suggested that BCE could inhibit the activation of inflammatory cells via modulating Th1/Th2/Treg dynamic balance.

Conclusion

Collectively, the oral administrations of BCE attenuated allergic responses via suppression of mast cell accumulation and serum histamine level, down-regulation of serum anti-OVA IgE and anti-OVA IgG expression and up-regulation of anti-OVA IgG2a. BCE ameliorated the nasal inflammation by reducing eosinophils infiltration along with its active production (CCL24), and other inflammation cells such as neutrophils, macrophages, goblet cells in both NALF and nasal mucosa. Furthermore, a dynamic balance among Th1/Th2/Treg-associated cytokines was established under BCE treatments that led to prevent inflammatory cells activation. The results obtained in this study suggest that BCE may provide a promising strategy for immunotherapy such as allergic rhinitis treatment.

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Supplementary Figures

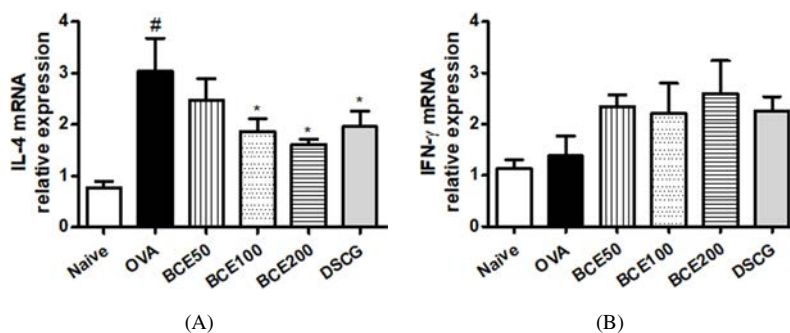


Figure S1. BCE modulated the balance of IL-4 mRNA (A) and IFN- γ mRNA (B) in nasal tissues. The mRNA was extracted and purified with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands), and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed in a Rotor-Gene Q 2 plex System (Qiagen) as follows: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 25 s at 60°C and 25 s at 72°C. Transcript levels were normalized to an internal control GAPDH. The IL-4 mRNA was down-regulated, meanwhile, the IFN- γ mRNA was up-regulated in nasal tissue. The values represent the mean $\pm$ SE ($n = 4$ /group). Significant differences at [#] $P < 0.05$ compared with the Naive group. ^{*} $P < 0.05$ compared with the OVA group.

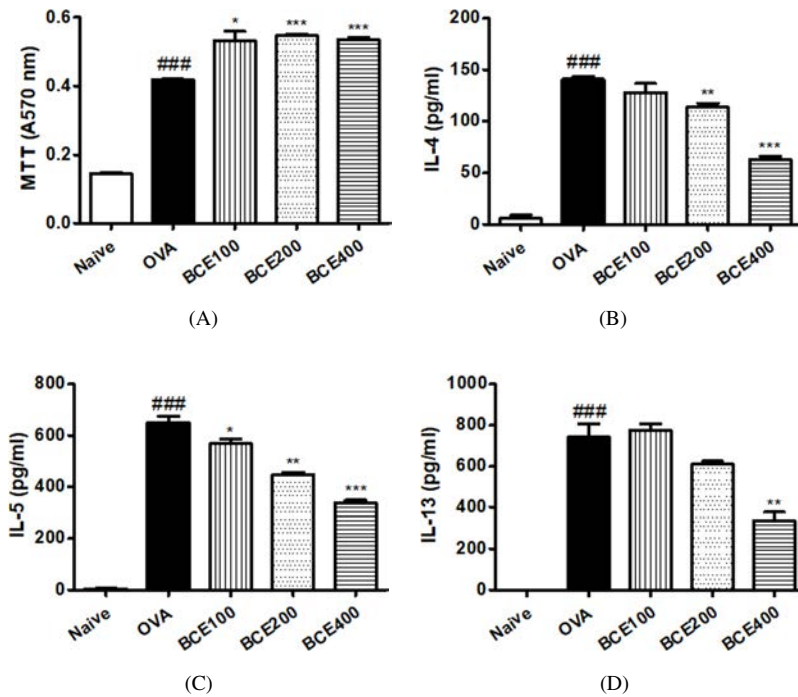


Figure S2. BCE regulated cell proliferation and OVA-specific recall response. Splenocytes were isolated from immunized mice by OVA. The cells were cultured for 72 h with OVA and BCE. (A) Cell proliferation was measured by MTT assay using splenocytes. BCE down-regulated the levels of IL-4 (B), IL-5 (C) and IL-13 (D). The values represent the mean $\pm$ SE ($n = 6$ /group). Significant differences at ### $P < 0.001$ compared with the Naive group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the OVA group.