

## Original Article

# Bee venom ameliorates compound 48/80-induced atopic dermatitis-related symptoms

Kyung-Hyun Kim<sup>1</sup>, Woo-Ram Lee<sup>1</sup>, Hyun-Jin An<sup>1</sup>, Jung-Yeon Kim<sup>1</sup>, Hyun Chung<sup>2</sup>, Sang-Mi Han<sup>3</sup>, Myeong-Lyoel Lee<sup>3</sup>, Kwang-Gill Lee<sup>3</sup>, Sok Cheon Pak<sup>4</sup>, Kwan-Kyu Park<sup>1</sup>

<sup>1</sup>Department of Pathology, Catholic University of Daegu, College of Medicine, Daegu, 705-718, Republic of Korea;

<sup>2</sup>Department of Dermatology, Catholic University of Daegu, College of Medicine, Daegu, 705-718, Republic of Korea; <sup>3</sup>Department of Agricultural Biology, National Academy of Agricultural Science, Suwon, 441-100, Republic of Korea; <sup>4</sup>School of Biomedical Sciences, Charles Sturt University, Bathurst, NSW 2795, Australia

Received September 24, 2013; Accepted October 21, 2013; Epub November 15, 2013; Published December 1, 2013

**Abstract:** Honeybee (*Apis mellifera* L.) venom (BV) has been traditionally used for the treatment of pain and inflammatory diseases such as itchy skin problems. However, the precise mechanism of BV in ameliorating the scratching behavior is not fully understood. Objective: In order to evaluate the effect of BV on atopic dermatitis-related symptoms in mice, we used a mouse skin scratching model induced by compound 48/80. The anti-itch effect of BV was investigated in a compound 48/80-induced mouse scratching behavior model. BALB/c mice were injected intraperitoneally with vehicle (saline 0.9%) or BV (0.01 and 0.1 mg/kg). One hour after treatment, the animals received a subcutaneous injection of compound 48/80. Intraperitoneal administration of BV (0.01 and 0.1 mg/kg) attenuated compound 48/80-induced scratching behaviors. The anti-scratching behavior effect of BV was in proportional to its vascular permeability effects. Treatment with BV also inhibited the degranulation of mast cells and the production of pro-inflammatory cytokines in compound 48/80-treated skin tissues. According to these results, BV may improve atopic dermatitis-related symptoms by inhibiting the mast cell degranulation and pro-inflammatory cytokine expression.

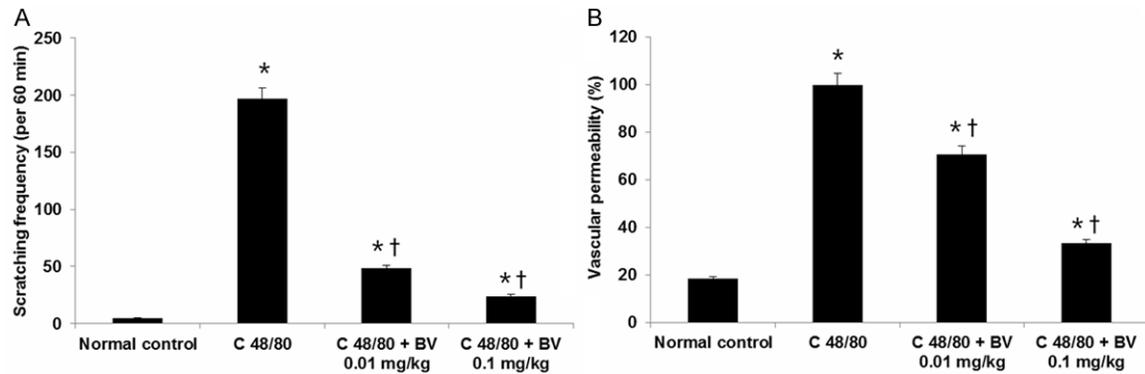
**Keywords:** Bee venom, scratching behavior, mast cell degranulation, compound 48/80, inflammation

## Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease that is accompanied by severe itching [1]. Itch, a sensation causing the urge to scratch, is the most significant outcome of AD, where insistent scratching further aggravates the skin symptoms of the disease [2]. Histamine derived from skin mast cells seems to be important mediator of itchiness [3-5]. The underlying mechanism for AD, however, is still not clearly known. Many trials of therapies intended to modulate AD have been performed with limited success [6, 7]. Temporary treatment with steroids and anti-histamines relieves the symptoms, but the possibility of side effects still remains with this kind of intervention [8]. Therefore, new therapeutic agents, which have low risk of side effects, are needed to treat AD.

Bee venom (BV), which is a complex mixture of active peptides, has long been used in China, Japan and Korea as a traditional medicine. It contains melittin, phospholipase A<sub>2</sub>, apamin, adolopin and mast cell degranulating peptide [9]. As a complementary medicine approach, BV exhibits anti-inflammatory effects as well as further activities in relieving rheumatoid arthritis, pain and in immune modulation [10]. Recent studies have shown that BV treatment can induce a significant anti-inflammatory response mediated by inhibition of inflammatory mediators, similar to what is achieved with the administration of non-steroidal anti-inflammatory drugs [11-13]. For instance, Han et al. reported that BV treatment had an anti-skin inflammatory effect and rapid cicatrizing effect of wound in rats [14]. From these findings, it is reasonable to presume that BV may also have an inhibitory effect on both skin inflammatory and allergic

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**Figure 1.** Inhibitory effect of BV on scratching behavior (A) and vascular permeability (B) induced by compound 48/80 in mice. BV (0.01 and 0.1 mg/kg) was intraperitoneally administered 1 h prior to compound 48/80 injection. Vascular permeability was indicated by the amount of Evans blue dye released. Mice in the normal control group were treated with saline instead of compound 48/80. \* $p < 0.05$  vs normal control group. † $p < 0.05$  vs compound 48/80 treated group.

diseases. Therefore, the purpose of this study is to examine the effects of BV on scratching behavior induced by compound 48/80 in mice.

### Materials and methods

#### Materials

Compound 48/80 and Evans blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Colonies of natural honeybees (*Apis mellifera* L.) used in this study were maintained at the National Academy of Agricultural Science, Suwon, Korea. BV was collected by a bee venom collecting device (Chung Jin Biotech Co., Ltd., Ansan, Korea) in a sterile manner under strict laboratory conditions. In brief, the BV collector was placed on the hive, and the bees were given enough electric shocks to cause them to sting a glass plate from which dried bee venom was later scraped off. The collected venom was purified by method of Han et al [15]. Purified BV was stored in a refrigerator for later use. BV used in the experiment was confirmed with size exclusion gel chromatography (AKTA Explorer, GE Healthcare, Pittsburgh, PA, USA) by dissolving in 0.02 M phosphate buffer with 0.25 M NaCl adjusted to pH 7.2 using a Superdex Peptide column (Amersham Biosciences, GE Healthcare, Pittsburgh, PA, USA).

#### Mice

Male BALB/c mice (6 weeks old, 20-25 g) were supplied from Orient Experimental Animal Breeding Center (Seongnam, Korea). All ani-

mals were housed in wire cages at  $22 \pm 2^\circ\text{C}$  with a 12 h light-dark cycle, fed standard laboratory chow (Orient Bio Inc., Seongnam, Korea) and allowed water *ad libitum*. The animal protocols for this study were approved by the boards of the Catholic University of Daegu and Daegu Catholic University Medical Center.

#### Scratching behavior

The scratching behavioral experiment in male BALB/c mice was performed according to the method of Sugimoto et al [16]. One day before the experiment the rostral part of the skin on the back of each mouse was clipped. On the test day, mice were put into acrylic cages (22 × 22 × 24 cm) for about 10 min for acclimatization. After that, mice were injected intraperitoneally with vehicle (n=8, saline 0.9%, 0.1 ml/10 g) or BV (n=8 for 0.01 mg/kg and n=8 for 0.1 mg/kg). One hour after treatment, the animals received a subcutaneous injection of compound 48/80 (50 µg/50 µl) in the dorsal region of the head using a 29 gauge needle. Immediately after the final injection the mice were returned to their observation cages, one mouse per cage. The scratching behavior was observed for 60 min by observers who were unaware of the treatments.

#### Vascular permeability of the skin

After intradermal injection of compound 48/80 (50 µg/50 µl) into the rostral part of skin on the back, the injected site (1 × 1 cm) was outlined with an indelible marker, and saline solution of

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2% Evans blue was injected intravenously into each animal. The animals were sacrificed 60 min later and the scratching agent-injected site (1 × 1 cm) was immediately excised. The skin specimen was dissolved with 1 ml of 1 M KOH solution by overnight incubation and 4 ml of a mixture of 0.2 M phosphoric acid solution-acetone (5:13) was added. After vigorous shaking, the precipitates were filtered off and the amount of dye was measured colorimetrically at 620 nm.

### *Histology and mast cell degranulation*

After mice were sacrificed, the scratching agent-injected site (1 × 1 cm) was excised and fixed in neutral buffered formalin overnight, embedded in paraffin and stained with hematoxylin and eosin (H&E) and toluidine blue for histological analysis. The granulation of mast cells was examined using the method of Katayama et al. [17] with slight modifications. Granulation scores of 0, 1 or 2 were allocated as follows: 2, more than eight mast cell granules were observed around the mast cells; 1, one to seven mast cell granules were observed around the mast cell; 0, no mast cell granules were observed around the mast cell. Both the degranulation scores and the numbers of mast cells were examined and counted from all fields per section. The granulation index per single mast cell was calculated by dividing the total degranulation score by the total number of mast cells.

### *Western blot analysis*

For cytosolic fractions, skins were homogenized in extraction buffer (10 mM HEPES pH 8.0, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 300 mM sucrose, 0.1% NP-40 and 0.5 mM PMSF; all from Sigma) for 15 min on ice and centrifuged at 6,000 g for 15 min. The supernatant from this step is the cytosolic fraction. The nuclear fraction was collected by extraction buffer (20 mM HEPES pH 8.0, 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT; all from Sigma) for 15 min on ice and centrifuged at 12,000 g for 10 min at 4°C. SDS-PAGE was performed on 8-12% polyacrylamide gels at 100 V for 2 h. The resolved proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA) and probed with anti-TNF-α (Abcam, USA), anti-IL-1β, anti-GAPDH (Santa Cruz Biotechnology,

Santa Cruz, CA, USA), anti-phospho-p65 NF-κB and anti-p65 NF-κB (cell signaling, USA) followed by secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK). After this, the membranes were detected with enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, UK). Signal intensity was quantified by an image analyzer (Las3000, Fuji, Japan).

### *Statistical analyses*

Results were analyzed with Duncan's test and expressed as mean ± SD. All experiments were performed at least three times.

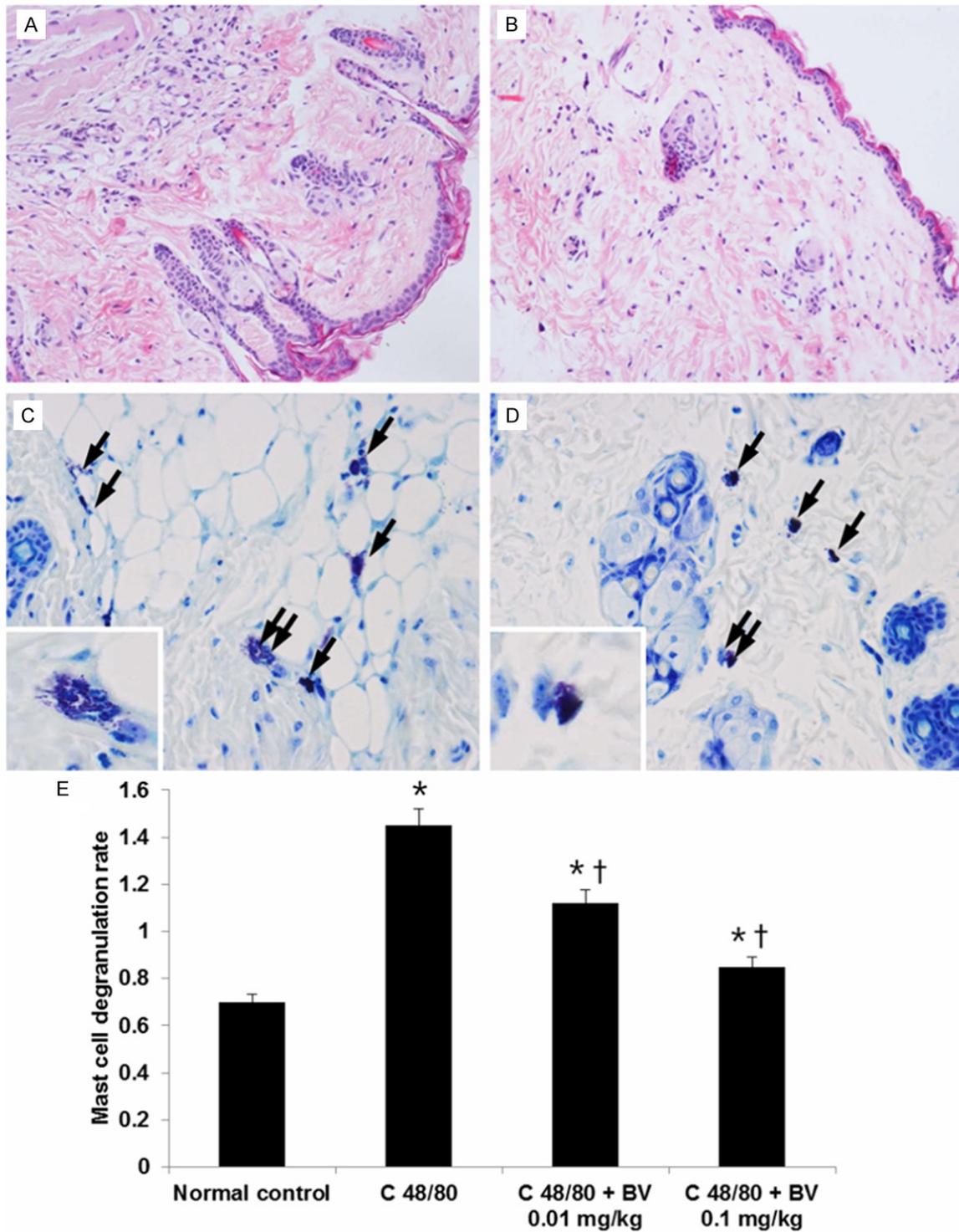
## Results

### *Effect of bee venom on scratching behavior and vascular permeability induced compound 48/80 in mice*

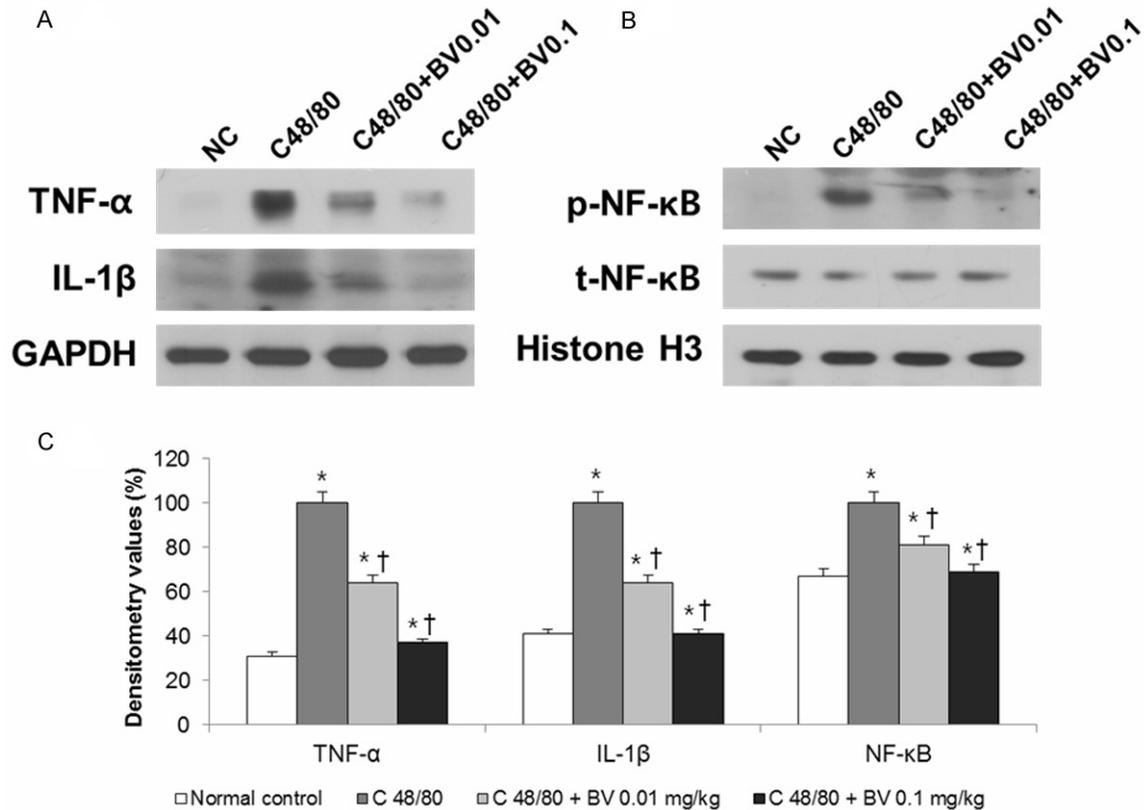
The anti-itch effect of BV was investigated in a compound 48/80-induced mouse scratching behavior model. The mice pre-treated with saline did not exhibit scratching behavior, while subcutaneous injection of compound 48/80 caused intense scratching in BALB/c mice (**Figure 1A**). Pre-treatment with BV at doses of 0.01 and 0.1 mg/kg inhibited compound 48/80-induced scratching by 75% and 87%, respectively. Injection of compound 48/80 increased vascular permeability in mice. However, administration of BV at a dose level of either 0.01 or 0.1 mg/kg reduced the vascular permeability of the skin by 33.3% and 70.7%, respectively, and it decreased dose-dependent manner by BV (**Figure 1B**).

### *Inhibitory effect of bee venom on compound 48/80-induced mast cell degranulation*

The histopathological examination of skin specimens showed that compound 48/80 caused massive edema of epidermal layers and marked cellular infiltration (**Figure 2A**). Administration of BV alleviated compound 48/80-induced edema and inflammatory symptoms (**Figure 2B**). Subsequently, we tested the ability of BV on mast cell degranulation in skin of compound 48/80-treated mice. Mast cells observed in dermis were completely degranulated following the injection of compound 48/80 (**Figure 2C**). Pre-administration of BV reduced mast cell



**Figure 2.** Inhibitory effect of BV on skin histological changes induced by compound 48/80. The skins of mice treated with compound 48/80 with or without BV were stained with hematoxylin-eosin and toluidine blue. (A) Hematoxylin-eosin stain of compound 48/80 injected skin  $\times 200$ . (B) Hematoxylin-eosin stain of 0.1 mg/kg BV pre-treatment + compound 48/80 injection  $\times 200$ . (C) Toluidine blue stain of compound 48/80 injected skin  $\times 400$ . (D) Toluidine blue stain of 0.1 mg/kg BV pre-treatment + compound 48/80 injection  $\times 400$ . Arrows: degranulated mast cells (C) and intact mast cells (D). (E) The mast cell degranulation rate that represents the degranulation score per single mast cell was determined as described in "Materials and methods". \* $p < 0.05$  vs normal control group. † $p < 0.05$  vs compound 48/80 treated group.



**Figure 3.** Effects of BV on the protein expression of pro-inflammatory cytokine and NF-κB in mouse skin tissues. A: Representative Western blotting for TNF-α and IL-1β in compound 48/80-treated mouse skin with or without BV. B: Representative Western blotting for phospho-p65 NF-κB and p65 NF-κB in nuclear extract of compound 48/80-treated mouse skin with or without BV. C: Graphical presentation of the ratio of TNF-α and IL-1β to GAPDH in various groups. Graphical presentation of the ratio of phospho-p65 NF-κB to Histone H3 in various groups. \**p* < 0.05 vs normal control group. †*p* < 0.05 vs compound 48/80 treated group. Results are representative of three independent experiments.

degranulation induced by compound 48/80 (Figure 2D). In particular, the compound 48/80-induced mast cell degranulation appeared to be more attenuated in 0.1 mg/kg of BV-treated mice (Figure 2E).

#### Effect of bee venom on compound 48/80-induced skin inflammatory changes

The effect of BV on the inflammatory changes of scratching skin was examined. The expression levels of TNF-α and IL-1β were increased in compound 48/80-treated mice (Figure 3). However, BV treatment significantly suppressed the expression of TNF-α and IL-1β as compared to those in compound 48/80-treated mice. BV also inhibited the compound 48/80-induced activation of the transcription factor, NF-κB, which regulates pro-inflammatory cytokine expression.

#### Discussion

Although it has been reported that BV has an anti-skin inflammatory effect [14], information about the effect of BV on AD was previously absent from the published literature. In this study, we demonstrated that BV suppressed experimental AD-related symptoms in compound 48/80 treated mice as evidenced by reduced degranulation rates of mast cells and the expression of pro-inflammatory cytokines at transcriptional and translational levels.

Various allergic diseases have been treated using antihistamines, steroids, non-steroidal anti-inflammatory drugs, and immunosuppressants [18-20]. Unfortunately, these agents have several side effects, such as severe nephrotoxicity and neurotoxicity [18, 21]. Therefore, natural products have emerged as therapeutic

alternatives for the treatment of immune disorders such as AD because they have potent immuno-modulatory effects, good efficacy and low risk of side effects. BV therapy, which is one of the well-known complementary interventions, has been used to relieve pain and to treat inflammatory diseases since ancient times as a traditional medicine [22]. In experimental rheumatoid arthritis, BV treatment significantly decreased the expression of inflammation-related cytokines such as COX-2, PLA<sub>2</sub>, TNF- $\alpha$ , IL1, IL-6, NO and ROS [23]. Our previous report also showed that BV suppressed the expression of pro-inflammatory cytokines in LPS-stimulated macrophages and C6 glioma cells [11, 24]. However, its anti-allergic and anti-scratching effects have not been thoroughly examined.

To understand the anti-allergic and anti-scratching properties of BV, we evaluated the effects of BV on compound 48/80-induced scratching behavior in mice. Scratching behavior in BALB/c mice was confirmed by vigorous scratching of compound 48/80-injected skin, as previously reported [25]. Compound 48/80-induced itching behavior, however, was suppressed by pretreatment with BV. It was confirmed that increased vascular permeability is one of the earliest events of acute inflammation and allergy. Mast cell mediators such as histamine and serotonin also increase vascular permeability in various species [26]. The anti-scratching behavioral effects of BV in compound 48/80-treated mice were in proportion to its inhibitory effects against the compound 48/80-induced vascular permeability.

Histologically, acute eczematous lesions in AD patients exhibit hypertrophy and hyperkeratosis of the epidermis, intracellular edema and infiltration of inflammatory cells in the dermis [27]. Similar histological changes were observed in mice exposed to compound 48/80, but these changes were diminished with BV administration.

Compound 48/80 is known to be a potent activator of skin mast cells. Cutaneous reactions stimulated by compound 48/80 may induce scratching behavior through the release of histamine from mast cells [28]. Histamine released from activated mast cells plays important role in the increase of vascular permeability elicited by compound 48/80. During itching in humans,

histamine derived from mast cells by various stimuli is also considered to be an important mediator. Therefore, inhibition of the mast cell degranulation is an important step to regulate the histamine release during the scratching behavior. In this study, compound 48/80 caused a potent activation of skin mast cells. However, pre-treatment with BV mitigated the levels of degranulation of mast cells significantly. These results suggest that the anti-scratching behavioral effects of BV might be due to decreased vascular permeability by regulating mast cell degranulation.

The hallmark of AD is a chronic, relapsing form of skin inflammation [29]. In the acute phase of AD, the recruited monocytes differentiate into inflammatory dendritic epidermal cells and produce pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , which are also important for regulating T-cell activation [30]. TNF- $\alpha$  is a member of a group of cytokines that stimulate the acute phase reaction. It also can induce the release of IL-1 $\beta$  and IL-6 which, in turn, enhance the sensitivity of histiocytes to TNF- $\alpha$  [31, 32]. Treatment of compound 48/80 induced the expression of pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  following activation of transcription factor NF- $\kappa$ B. NF- $\kappa$ B is an important signal in immune responses of allergic diseases [33]. Various reports showed that BV regulates the activation of NF- $\kappa$ B during inflammatory reactions [9, 34, 35]. BV pretreatment inhibited TNF- $\alpha$  and IL-1 $\beta$  expression as well as activation of transcription factor NF- $\kappa$ B in compound 48/80 treated skin tissues. These results suggest that BV may inhibit TNF- $\alpha$  and IL-1 $\beta$  expression by regulating the activation of transcription factor NF- $\kappa$ B.

In summary, this study suggests that BV has potential effects in ameliorating compound 48/80-induced AD symptoms by reducing scratching behavior and inhibiting degranulation of mast cells. The inhibitory effects of BV are mainly due to an inhibition of pro-inflammatory cytokines via NF- $\kappa$ B activation. We conclude that BV may be a good therapeutic agent for AD. Furthermore, BV may be applicable in the development of AD-reducing or dermatitis-preventing therapies.

### Acknowledgements

This work was supported by a grant from the Next-Generation BioGreen 21 Program (No.

PJ009519), Rural Development Administration, Republic of Korea.

**Disclosure of conflict of interest**

The authors declare no conflict of interest.

**Address correspondence to:** Dr. Kwan-Kyu Park, Department of Pathology, Catholic University of Daegu, College of Medicine, 3056-6 Daemyung 4-Dong, Nam-Gu, Daegu, 705-718, Republic of Korea. Tel: +82-53-650-4149; Fax: +82-53-650-4834; E-mail: kkpark@cu.ac.kr

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