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Anti-Inflammatory Effects of Bee Venom on Phthalic Anhydride-Induced Atopic Dermatitis

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ABSTRACT

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Background: Atopic dermatitis (AD) is a chronic inflammatory condition which can be studied using phthalic anhydride (PA) to induce AD. Anti-inflammatory properties of bee venom (BV) were investigated to determine whether it may be a useful treatment for AD.

Methods: AD was induced by applying to pical PA to 8-week-old HR-1 mice ($N = 50$), then treating with (0.1, 0.25, and 0.5 μg) or without topical BV. Body weight, ear thickness histology, enzyme-linked immune sorbent assay (serum IgE concentrations), Western blot analysis [inducible nitric oxide synthase, cyclooxygenase-2, I κ B- α , phospho-I κ B- α , c-Jun N-terminal kinase (JNK), phospho-JNK, p38, phospho-p38, extra cellular signal-regulated kinase (ERK), and phospho-ERK], and the pull down assay for immunoblotting (p50), were used to measure inflammatory mediators.

Results: PA + BV (0.1, 0.25, and 0.5 μg) significantly decreased ear thickness without altering body weight. IgE concentrations decreased in the PA + BV (0.5 μg)-treated groups compared with PA treatment. Tumor necrosis factor- α , interleukin-1 β , inducible nitric oxide synthase, cyclooxygenase-2, phospho-I κ B- α , phospho-JNK, p38, phospho-p38, and phospho-ERK, all decreased following treatment with PA + BV compared with the PA-treatment alone. p50 was upregulated in the PA + BV-treated groups compared with the PA-treated group. Furthermore, the number of mast cells decreased in the PA + BV-treated groups compared with the PA-treated group. Epidermal thickness was significantly lower in the PA + BV-treated group compared with PA treatment alone.

Conclusion: BV maybe a useful anti-inflammatory treatment for AD.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory condition involving itchy skin (the most common type of eczema) [1], and is caused by several genetic and environmental factors [2]. Approximately 50% of patients with AD develop symptoms within their first year of life, and 95% experience symptoms by 5 years of age; however, AD can also occur in adults [3,4]. The diagnostic criteria for AD include evidence of itchy skin and 3 or more of the following: history of skin creases, hay fever or generally dry skin in the past year, onset of symptoms under 2 years, and visible flexural dermatitis [5]. AD is not curable and has multiple etiologies, therefore treatments focus on symptom relief [4,6]. The treatment options are topical treatments such as emollients, corticosteroids, and calcineurin inhibitors (Pimecrolimus cream and tacrolimus

ointment), and systemic therapies such as immunosuppressants, immune response modifiers, antihistamines, and antibiotics; however, such treatment methods have limitations [7-10].

Mast cells, macrophages, and inflammatory cells significantly affect the development of AD by producing inflammatory cytokines such as interleukin (IL)-6. In addition, tumor necrosis factor (TNF)- α increases serum IgE levels, resulting in increased levels of inflammatory chemokines and cytokines. These inflammatory effects can be reversed by treatment with anti-inflammatory candidates that inhibit signal transducer and activators of transcription (STAT) and nuclear factor (NF)- κ B signaling pathways [11-17].

Bee venom (BV) has been used for apitherapy in early civilizations in China, India, Egypt, Babylon, and Greece. BV is a complex mixture of many enzymes, including phospholipase

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(PL) A2, PLB, hyaluronidase, polypeptides including melittin and adolapin, and low-molecular-weight constituents. BV has been reported to treat various acute or chronic inflammatory diseases [18-20]. Recent studies have described how BV can induce anti-inflammatory responses mediated by the inhibition of inflammatory mediators, similar to that achieved by administration of non-steroidal anti-inflammatory drugs [21-23].

In this study, we investigated the effects of BV on anti-inflammatory therapy for the treatment of AD in vitro and in vivo.

Materials and Methods

Materials

The BV (You-Miel BV Ltd. Hwasoon, Korea) composition was as follows: 45-50% melittin, 12% PLA2, 4-5% 6-pentyl a-pyrone lipids, 4-5% carbohydrates, 2-3% amine, 2.5-3% mast cell degranulating peptide, 1% lysophospholipase A, 1.5-2% hyaluronidase, 1-1.5% histidine, 0.5% secarpin, 0.1% procamine, 0.1% teritapin, and 19-27% other components, including protease inhibitors, acid phosphomonoesterase, glucosidase, dopamine, norepinephrine, invertase, and unknown amino acids, with 99.5% purity.

Primary antibodies used for Western blot analysis were anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK (1:1,000; Cell Signaling Technology, Beverly, MA, USA), anti-p50 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-inducible nitric oxide synthase (iNOS), anti-cyclooxygenase (COX)-2, and anti-inhibitory κ Ba antibodies (1 κ B- α ; 1:500; Santa Cruz Biotechnology Inc.). The secondary antibody was horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology Inc.).

Animal treatment

The protocol for the animal experiment followed rigid scientific and ethical animal care procedures and was approved by the Chungbuk National University-Institutional Animal Care and Use Committee (approval no.: CBNU-2015-0976).

Eight-week-old male HR-1 mice (Samtako BioKorea Co. Osan, Korea) were randomly divided into 5 groups of 10 mice ($N = 50$). In the first group phthalic anhydride [PA (PA group, $n=10$)], 5% solution [100 μ L (20 μ L/cm²)] was applied on the dorsal area 3 times a week for 4 weeks. The second, third, and fourth groups were treated with BV (0.1, 0.25, and 0.5 μ g in 100 μ L; $n=10$ each) at 3 hours after treatment with PA as described in the first group. The fifth group was the control group of 8-week-old male HR-1 mice.

Measurement of body weight and ear thickness

Alterations in body weight were measured with an electric balance (Mettler Toledo, Greifensee, Switzerland) once a week for 4 weeks during the experimental procedure. Changes in ear thickness were measured using a thickness gauge (Digimatic Indicator, Matusutoyo Co., Tokyo, Japan) to determine the degree of allergic skin inflammation induced by PA.

Histological analysis

Skin was removed from the dorsal area of the mice, fixed with 10% formalin, embedded in paraffin wax, processed and sectioned into 5- μ m-thick slices. The skin sections were then stained with hematoxylin and eosin. The thickness of the epidermis was also

measured using the Leica Application Suite (Leica Microsystems, Wetzlar, Germany). Mast cells in toluidine, blue-stained sections were counted under a microscope (200 \times).

Enzyme-linked immunosorbent assay for detection of serum IgE concentrations

Serum IgE concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi Inc., Gunma, Japan) according to the manufacturer's instructions. Briefly, capture antibodies were plated in the Nunc C bottom immunoplate supplied in the kit. Wells were washed with washing solution (50 mM Tris, 0.05% Tween 20, 0.14 M NaCl, pH 8.0) 3 times. Then, serum samples and standards (diluted with buffer solution) were added to the wells, and the plate was incubated for 2 hours. The wells were washed again with washing solution, 50 μ L biotin-conjugated anti-IgE antibodies (1,000-fold dilution) was added to each well, and the plates were incubated further for 2 hours to bind the captured IgE. The wells were washed again with washing solution, after which horseradish peroxidase-conjugated detection antibodies (2,000-fold dilution) were added to each well, and the plates were incubated for 1 hour. An enzyme reaction was then initiated by adding tetramethylbenzidine substrate solution (100 mM sodium acetate buffer, pH 6.0, 0.006% H₂O₂), and the plates were incubated at room temperature in the dark for 20 minutes. The reaction was terminated by adding acidic solution (reactions stopper, 1 M H₂SO₄), and the absorbance of the yellow product was measured spectrophotometrically at 450 nm. The final concentration of IgE was calculated using a standard curve.

Cytokine assay

Serum levels of mouse TNF- α , IL-6, and IL-1 β were measured by ELISA kits (Thermo Fisher Scientific Inc., Meridian Rd, Rockford, IL, USA) according to the manufacturer's protocol.

Western blot analysis

Skin or ear tissue (100 mg) or approximately 1×10^6 cells were harvested and homogenized with lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 10 μ L/mL aprotinin, 1% igapal 630 (Sigma Chem. Co., St. Louis, MO, USA), 10 mM NaF, 0.5 mM ethylenediaminetetraacetic acid, 0.1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 0.5% sodium deoxycholate]. The extracts were centrifuged at 23,000 \times g for 1 hour. Equal amounts of protein (20 μ g) were separated on SDS/10%polyacrylamide gels and then transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 hours at room temperature with 5% (w/v) nonfat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% Tween-20. The membranes were incubated for 4 hours at room temperature with mouse monoclonal antibodies directed against JNK, phospho-JNK, p38, phospho-p38, ERK, phospho-ERK (1:1,000; Cell Signaling Technology), and p50 (1:500; Santa Cruz Biotechnology Inc.) and rabbit polyclonal antibodies against iNOS, COX-2, and I κ B- α (1:500; Santa Cruz Biotechnology Inc.). The blots were then incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with an enhanced chemiluminescent Western blotting detection system.

Pull-down assay

BV was conjugated with cyanogen bromide (CNBr)-activated Sepharose 6B (Sigma Aldrich). Briefly, BV (1 mg) was dissolved in 1 mL coupling buffer (0.1 M NaHCO₃ and 0.5 M NaCl, pH 6.0). The CNBr-activated Sepharose 6B was swelled, washed in 1 mM HCl on a sintered glass filter, and then washed with coupling buffer.

CNBr-activated Sepharose 6B beads were added to the BV-containing coupling buffer and incubated at 4°C for 24 hours. BV conjugated with Sepharose 6B were washed with 3 cycles of alternating pH wash buffers (buffer 1: 0.1 M acetate and 0.5 M NaCl, pH 4.0; buffer 2: 0.1 M Tris HCl and 0.5 M NaCl, pH 8.0). BV-conjugated beads were then equilibrated with a binding buffer (0.05 M Tris HCl and 0.15 M NaCl, pH 7.5). The control unconjugated CNBr-activated Sepharose 6B beads were prepared as described above in the absence of BV. The cell lysate or p50 recombinant protein (Abnova, Taipei, Taiwan) was mixed with BV-conjugated Sepharose 6B or Sepharose 6B at 4°C for 24 hours. The beads were then washed 3 times with TBST. The bound proteins were eluted with SDS loading buffer. The proteins were then resolved by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with antibodies against p50 (1:500 dilution; Santa Cruz Biotechnology Inc.).

Statistical analysis

The experiments were conducted in triplicate, and all experiments had similar results. All statistical analyses were performed using GraphPad Prism 5 software (Version 5.03; GraphPad software, Inc., CA, USA). Group differences were analyzed by one-way analysis of variance followed by Tukey's multiple comparison tests. All values are presented as mean \pm SD. Results with *p* values of less than 0.05 were considered significant.

Results

Effects of BV treatment on ear thickness and morphology

To determine whether BV suppressed changes in ear thickness induced by the application of PA, the morphology and thickness of the ear was assessed. Ear thickness significantly increased in PA-induced mice compared with the control, and BV-treated groups (PA+BV 0.1 μ g, PA+BV 0.25 μ g, PA+BV 0.5 μ g; Fig. 1A). To assess whether BV suppressed changes in AD, clinical scores for AD observed on the dorsal area were assigned to each group. AD clinical scores increased rapidly in PA-induced mice and significantly decreased in BV-treated mice compared with control mice (Fig. 1B). Changes to skin in the dorsal area were significantly improved upon BV treatment (Fig. 1C). To evaluate whether BV suppressed changes in body weight, measurements were taken once a week for 4 weeks. No differences in body weight were observed after any treatment (Fig. 1D).

Effects of BV treatment on IgE concentrations

To determine the inhibitory effects of BV on serum IgE concentrations, the effects of BV in the blood of mice were analyzed using ELISAs. The mice were treated with different concentrations of BV (0.1, 0.25, and 0.5 μ g). Topical application of PA induced an increase in IgE concentrations compared with the control group. IgE concentrations were detected in the BV-treated group in a concentration-dependent manner (where the highest concentration of BV treatment resulted in lower IgE serum concentrations), and PA + BV 0.5 μ g had statistically significantly

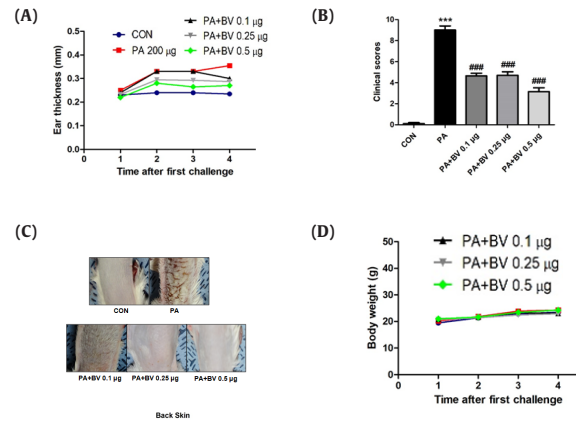


Fig. 1. Differences in ear thickness, clinical score, dorsal area of the skin, and body weight after treatment of HR-1 mice with PA and BV. (A) Changes in ear thickness after application of PA and/or BV. (B) Clinical scores after 4 weeks. Changes in the dorsal area of the (C) skin and (D) body weights. BV, bee venom; PA, phthalic anhydride.

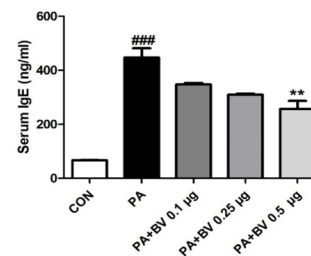


Fig. 2. Effects of BV treatment on serum IgE concentrations in HR-1 mice treated with PA. Mice were treated with BV (0.1, 0.25, and 0.5 μ g). Data are presented as mean \pm SD (*n* = 10). ###*p* < 0.001, compared with the control group. ***p* < 0.01, compared with the PA group. BV, bee venom; PA, phthalic anhydride.

lower IgE concentration than PA treatment (*p* < 0.01; Fig. 2).

Effects of BV treatment on the release of inflammatory cytokines

To assess whether BV could induce alterations in the release of inflammatory cytokines in PA-induced skin inflammation, the levels of TNF- α , IL-1 β , and IL-6 were measured in the serum from mice in the control, PA, and PA + BV (0.1, 0.25, and 0.5 μ g) groups. The levels of TNF- α , IL-1 β , and IL-6 were higher in the PA-treated group compared with the control group. In contrast, the levels of these cytokines in the BV-treated group decreased in a dose-dependent manner. The serum concentration of TNF- α was statistically significantly lower in the PA + BV 0.5 μ g (*p* < 0.001) or BV 0.25 μ g treated groups compared with the PA treated group (*p* < 0.001). The level of IL-1 β was also statistically significantly lower in the PA + BV 0.5 μ g (*p* < 0.05), or BV 0.1 μ g (*p* < 0.001) and BV 0.25 μ g treated groups compared with the PA treated group (*p* < 0.001). There were no statistically significant differences observed in IL-6 levels between the PA treated group and the PA + BV groups (Fig. 3)

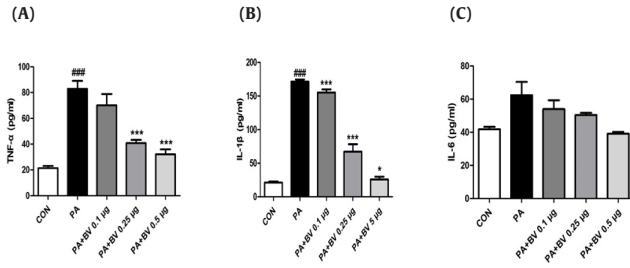


Fig. 3. Changes in serum cytokine concentrations with various doses of BV. (A) TNF- α , (B) IL-1 β , and (C) IL-6 concentrations in the serum were quantified by ELISA. Data are presented as mean \pm SD ($n = 10$). ### $p < 0.001$, *** $p < 0.001$, * $p < 0.05$. BV, bee venom.

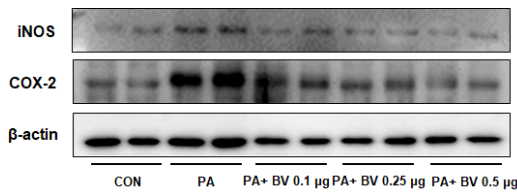


Fig. 4. Effects of BV treatment on inflammatory responses. Expression of iNOS and COX-2 was determined using Western blot analysis. BV, bee venom.

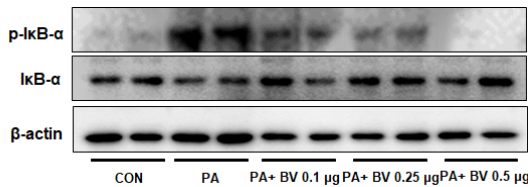


Fig. 5. Effects of BV on NF- κ B DNA binding activity in PA-induced AD. I κ B- α and phospho-I κ B- α levels were detected by Western blotting using specific antibodies. β -Actin protein was used as an internal control. AD, atopic dermatitis; BV, bee venom; PA, phthalic anhydride.

Effects of BV treatment on inflammatory responses

To determine whether BV had inhibitory effects on AD, ear and dorsal tissue were prepared for Western blotting. Protein levels of COX-2 and iNOS appeared to be higher in PA-induced AD mice compared with the control group, and lower in the PA + BV treated groups (Fig. 4).

Effects of BV on NF- κ B DNA binding activity

To examine whether BV could inhibit NF- κ B activation in PA-induced AD, nuclear extracts from ear and dorsal skin tissue were prepared to determine NF- κ B DNA binding. PA-treated mice showed an increased level of I κ B- α degradation in the cytosolic fraction when compared with the control group. In contrast, degradation in PA + BV-treated mice groups were reduced in a dose-dependent manner compared with the PA group (Fig. 5).

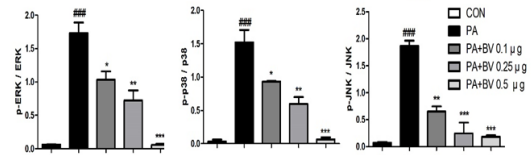
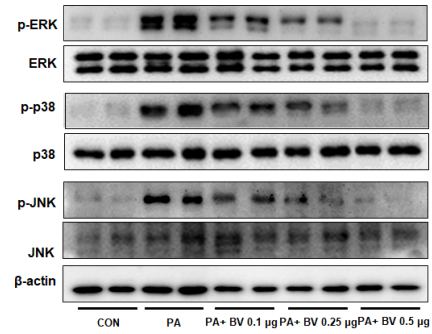


Fig. 6. Effects of BV on ERK, p38, and JNK phosphorylation. Phosphorylation of JNK, ERK, and p38 was determined following treatment with different concentrations of BV (0.1, 0.25, and 0.5 μ g). Data are presented as mean \pm SD ($n = 10$). ### $p < 0.001$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. AD, atopic dermatitis; BV, bee venom; ERK, extra cellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PA, phthalic anhydride.

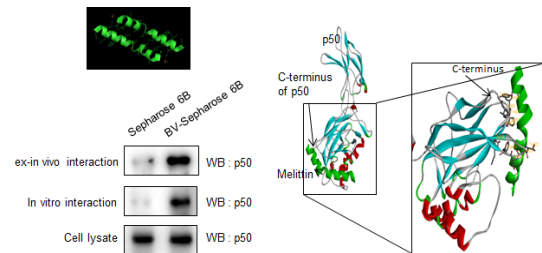


Fig. 7. Structural interaction between BV and p50. The docking model of BV with p50. The pull-down assay identified an interaction between the BV and p50. p50 was conjugated with BV-activated Sepharose 6B, and was detected by Western blotting. BV, bee venom.

Decreased ERK, p38, and JNK phosphorylation

Phosphorylation of JNK, ERK, and p38 was determined following treatment with different concentrations of BV (0.1, 0.25, and 0.5 μ g) using mouse monoclonal antibodies. The results showed a statistically significant reduction in the levels of ERK, p38, and JNK in PA + BV 0.5 μ g compared with PA ($p < 0.001$; Fig. 6).

Effects of BV on translocation of the p50 subunit of NF- κ B into the nucleus

p50 has roles in inflammation and traditionally forms a heterodimer with pro-inflammatory cytokines; p50 homodimers act as repressors of pro-inflammatory genes. To further identify the binding of p50 to BV, computational docking experiments with p50 and BV were performed using Autodock Vina software, and showed that BV bound to p50 (Fig. 7).

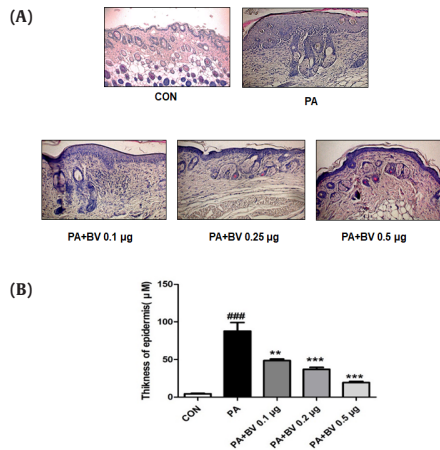


Fig. 8. Histopathological analysis of the dorsal area of the skin. Histopathological analysis of dorsal tissue and inhibition of NF- κ B DNA binding activity following topical application of BV and/or PA. Histopathological changes were evaluated by staining with hematoxylin and eosin, followed by observation at 200 \times magnification (scale bars, 100 μ m). (A) Histological images and (B) thickness of the epidermis. Data are presented as mean \pm SD ($n = 10$).

$p < 0.001$, ** $p < 0.01$, *** $p < 0.001$.
BV, bee venom; PA, phthalic anhydride.

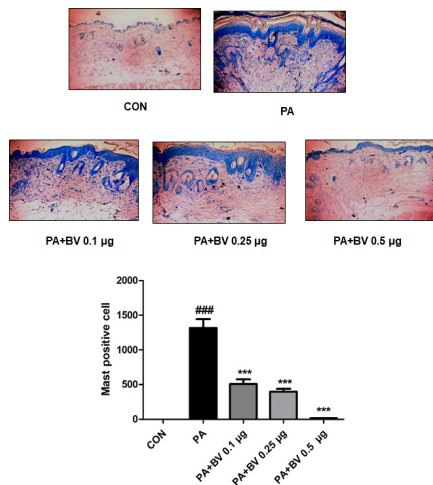


Fig. 9. Effects of BV on mast cells. Slide sections of ear tissues were evaluated by staining with 0.25% toluidine blue and observed at 200 \times magnification (scale bars, 100 μ m). The number of infiltrated mast cells per specific area was measured. Data are presented as mean \pm SD ($n = 10$).

$p < 0.001$, *** $p < 0.001$.
BV, bee venom; PA, phthalic anhydride.

Effects of BV on histological changes in the skin of the dorsal area

To investigate the suppressive effects of BV treatment on the dorsal area, skin histology was performed. The epidermis was thicker in the PA-treated group than in the control group ($p < 0.001$). However, the skin thickness was less in the PA + BV-treated group (in a dose-dependent manner) compared with the PA-treated group ($p < 0.01$; Fig. 8).

Effects of BV on mast cells

Histological analysis of ear and dorsal tissue showed that the epidermis and dermis were thicker in the PA-treated group than in

the control group. Additionally, the dorsal area showed evidence of inflammatory cell infiltration of the dermis in PA-induced mice. However, the thickness and inflammatory cell infiltration were statistically significantly decreased in the PA + BV-treated groups ($p < 0.01$). The dorsal tissue was stained with toluidine blue to determine PA-induced mast cell infiltration in the dermis. The number of mast cells in the dermis of skin in the dorsal area increased statistically significantly in PA-induced mice compared with that in mice in the control group, and this increase was blocked with a topical application of BV ($p < 0.001$; Fig. 9).

Discussion

AD is a chronic skin condition characterized by pruritus and eczematous skin lesions, and is accompanied by immune responses which are dominated by Th2 cells in the acute phase, defects in the epidermal barrier, a thickened epidermis, and IgE-mediated sensitization to several antigens [24]. The mechanism involved in AD is not fully understood. Currently, it is thought that defects in the epidermal barrier and immunity, underlie the development of AD [25], and therefore, therapies that target AD should consider not only epidermal barrier dysfunction, but also immune responses.

PA is a low-molecular-weight organic compound industrially used in the large-scale production of plasticizers, alkyl and polyester paint resins, and as a curing agent for epoxy resins owing to its high reactivity and ability to crosslink polymers [26,27]. However, exposure to PA causes occupational exposure irritation, immuno-mediated allergic conditions (including asthma, contact urticarial, rhinitis, conjunctivitis, and AD), and direct skin sensitivity [28,29]. In humans, the eye, skin, and respiratory tract are irritated and sensitized by PA in its various forms, including vapors, fumes, and dust [26,30]. Moreover, PA treatment may induce IgE production, stimulate infiltration of mast cells, promote the development of AD, and increase type IV hypersensitivity, including activation of Th cells and cytokine secretion [31-35].

NF- κ B is a main component of intracellular signaling pathways and is responsible for the upregulation of pro-inflammatory cytokines, which facilitates AD progression [36]. Activation of NF- κ B has also been detected following IgE-induced production of IL-6 and TNF- α in mast cells [37]. Although a direct relationship between NF- κ B and AD has not been reported, it has been suggested that NF- κ B may have a role in immune responses observed in AD. NF- κ B is activated immediately after aggregation of mast cells, leading to the production of inflammatory chemokines and cytokines [38]. B and T cells are stimulated through T-cell receptors, and NF- κ B is activated, indicating the essential role of NF- κ B in both cell activation and Ig production [39]. Moreover, studies have shown that reduced NF- κ B activation suppresses the symptoms of AD [40,41].

The STAT pathway plays an important role in blocking the inflammatory responses in AD [42]. Activation of STAT3 is responsible for increased expression of COX-2 and iNOS [43]. Activation of NF- κ B is involved in the induction of COX-2 and iNOS by lipopolysaccharide, and other inflammatory cytokines [44]. In this study, the aim was to determine whether BV decreased the nuclear translocation of NF- κ B and I κ B α in lymph nodes and tissues, accompanied by reduced cytokine levels.

ERK, JNK, and p38 are members of the MAPK family in humans. Members of the MAPK family are widely expressed serine/tyrosine kinases that play important roles in many signaling pathways in human cells [45]. Under certain conditions related to pain, MAPKs in glial cells help to increase the synthesis and release of pro-inflammatory cytokines [46,47]. Each MAPK signaling

pathway has a different role depending on the specific conditions [48-50]. Accordingly, MAPKs are a fundamental part of the inflammatory response [51].

BV contains several biologically active peptides, including melittin, adolapin, and apamin [52,53]. Many studies have indicated that BV can modulate systemic immune responses by inhibiting inflammatory mediators, similar to the effects of non-steroidal anti-inflammatory drugs [54]. Other in vitro studies have reported that BV has anti-inflammatory activity, which is ascribed to the transcriptional downregulation of MAPKs and NF- κ B in target tissues [55]. In a previous study, Th1 cytokine-based inflammatory symptoms in AD were alleviated by BV, and this pharmacological anti-inflammatory effect involved modulation of NF- κ B and MAPK signal transduction pathways [56].

In this current study, ear and epidermal thickness significantly increased in PA-induced mice compared with that in control mice, and this effect was reduced following treatment with BV. Notably, BV did not affect body weight, but significantly decreased the levels of IgE, TNF- α , IL-1 β , iNOS, COX-2, and IkB- α . Moreover, PA-induced increases in ERK, p38, and JNK phosphorylation were significantly reduced. BV bound to NF- κ B (p50) was observed which appeared to block PA-induced increases in mast cell numbers after topical application.

Overall, our current findings demonstrated that BV inhibited the activation of NF- κ B and contributed to the reduction in pro-inflammatory cytokines and the inhibition of iNOS and COX-2 expression in a PA-induced AD animal model. Therefore, BV maybe a promising therapeutic agent for the treatment of AD.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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