



Original Article

Effect of Bee Venom Pharmacopuncture on Inflammation in Mouse Model of Induced Atopic Dermatitis



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ABSTRACT

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Background: This study was designed using a mouse model of atopic dermatitis [phthalic anhydride (PA)-treated mice], to investigate the anti-inflammatory effect of bee venom pharmacopuncture (BVP) in keratinocytes.

Methods: Western blot analysis was performed to investigate inflammation related protein expression of iNOS, COX-2, phospho-ERK (p-ERK), and ERK, in LPS (1 µg/mL)-activated keratinocytes, following BVP treatment, and in PA-treated mice, after BVP treatment. Griess reaction was performed to investigate NO concentration. Enzyme-linked immunosorbent assays were used to determine the concentrations of interleukin (IL)-4, IL-17A, IL-13 and IL-4 in PA-treated mice after BVP treatment. In addition, monocyte, macrophage, neutrophil, and eosinophil counts were measured to observe the changes in white blood cell infiltration.

Results: The keratinocytes of the BVP-treated group showed a decreased expression of iNOS, COX-2, ERK at 5 OX-2, ERK E, and p-ERK at 1, 2 and 5 RKRK ERK ERK, and a dose-dependent decrease in NO concentration at 2 and 5 ntrationof s. In the BVP-treated groups (0.1 µ.1-trea µ.1-treated gr), PA-treated mice showed recovery after 4 weeks which was dose-dependent, showing a significant decrease in clinical scores for AD, and a decreased concentration of IL-13 and IL-4 with BV treatment. There was a dose-dependent decrease in the infiltration of eosinophils, neutrophils, monocytes, macrophages, and a decreased thickness of the epidermis due to inflammation, and decreased expressions of iNOS, COX-2, p-ERK, ERK, especially in the 0.1 µ0/mL BVP-treated group.

Conclusion: These results suggest that BVP may be an effective alternative treatment for atopic dermatitis.

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Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common, chronic skin disease/condition characterized by itchy, dry, cracked skin that may become sore and inflamed and may form scaly rashes [1]. AD has been reported to affect up to 20% of children and 1-3% of adults in developed countries and is thought to be derived from genetic factors concerned with skin barrier dysfunction and is triggered by multiple environmental factors [2,3].

There are several studies that suggest AD is caused by dysregulation of immune system which is induced by the abnormal differentiation of skin epithelial cells and extrinsic

stimulatory factors [1]. The main role in the pathogenesis of AD is reportedly the Th2 cell response which produces IL-4, IL-5 and IL-13 [1]. Increased production of IL-4, IL-5 and IL-13 by Th2 cells is followed by an increased level of serum immunoglobulin (IgE) and circulating eosinophils which are key cells triggering AD in most patients [4,5]. AD can be studied in a mouse model which was developed by using a topical application of 2,4-dinitrochlorobenzene giving rise to an increased concentration of serum IgE and Th2 cytokines such as IL-4, IL-5, and IL-13. These cytokines directly affect epidermal keratinocytes which in turn produce pro-inflammatory cytokines that are involved in the recruitment of immune cells which infiltrate the skin lesion site. The activation of immune cells and inflammation caused at the site

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of the skin lesion is thought to be an important factor associated with AD [1].

Bee venom (BV) is a natural toxin produced by honeybees which has been used to treat various diseases. BV is composed of many peptides, including melittin, apamin, adolapin and mast cell degranulating peptide. Among them, melittin is the key component which accounts for 50% of dry weight of BV and it has been investigated to determine its biological and pharmacological activities [6]. A recent review of treatment with BV reported anti-inflammatory, anti-apoptotic, antibacterial, and anticancer effects [7]. BV treatment for AD-like skin lesions has been reported to reduce serum immunoglobulin E levels and inhibit the infiltration of eosinophils and mast cells [8].

In this current study the aim was to determine whether treatment with BV could have an anti-inflammatory effect on LPS-activated keratinocytes and AD-like skin lesions induced by PA in a mouse model.

Materials and Methods

Materials

BV (You-Miel Bee Venom Ltd., Hwasoon, Korea) composition was as follows: 45-50% melittin, 12% phospholipase A2, 4-5% 6-pentyl a-pyrone lipids, 4-5% carbohydrate, 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 ingredients including protease inhibitor, acid phosphomonoesterase, glucosidase, dopamine, norepinephrine, invertase and unknown amino acids (99.5% purity).

Cell cultures

Mouse keratinocytes were harvested from mouse ears (100 mg ear tissue) and grown in 24-well plates (Costar) in RPMI 1640 (MEM alpha) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA), 100 U/mL penicillin (Gibco Life Technologies) and 100 µg/mL streptomycin (Gibco Life Technologies), at 37°C, in a humidified 5% CO₂ incubator.

Animal treatment

The protocol for the animal experiment followed rigid scientific and ethical 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. In the first group 5% PA solution [100 µL (20 µL/cm²)] was applied on the dorsal skin of the back 3 times a week for 4 weeks. The second group (BV 0.1 µg, *n* = 10), third group (BV 0.25 µg, *n* = 10) and fourth group (BV 0.5 µg, *n* = 10) had PA applied. After 3 hours, 100 µL of BVP (0.1, 0.25, and 0.5 µg) was delivered to the PA applied groups respectively. Age-matched HR-1 mice were used for the control group (control, *n* = 10).

Measurement of clinical score

The development of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The total clinical dermatitis severity score for each mouse was defined as the sum of the individual scores [9,10].

Blood cell number measurements

Mice blood was taken by heart puncture. Blood cell number were measured in the laboratory animal research center at Chungbuk National University.

Cytokine assay

Serum levels of mouse IL-4, and IL-13 were measured using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific Inc., Meridian Rd, Rockford, IL USA) according to the manufacturer's protocol.

Histological analysis

Ear skins were removed from mice, fixed with 10% formalin, embedded in paraffin wax, routinely processed, and then sectioned into 5 µm 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). The sections were incubated in 10% bovine serum albumin in PBS at room temperature for 1 hour and then stained with rat anti-Ly6G antibody (RB6-8C5, 1:250 dilution; Abcam), rabbit anti-F4/80 antibody (D2S9R, 1:100 dilution; Cell Signaling Technology) overnight at 4°C, followed by biotinylated anti-rat IgG antibody or anti-rabbit IgG antibody (1:500; Vector Laboratories, Burlingame, California, USA) and Vectastain ABC reagent (Vector Laboratories, Burlingame, California, USA) at room temperature for 60 minutes and 30 minutes, respectively. Finally, the sections were stained with DAB Peroxidase Substrate Kit before imaging (Vector Laboratories, Burlingame, California, USA). For analyses of epidermal thickness or cell number, 18 randomly selected sites were evaluated by using microscopy (MZ-X710, Keyence, Osaka, Japan) and its associated software.

Western blot analysis

Keratinocytes or 100 mg ear tissues, or about 1 x 10⁶ 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 (PMSF), 10 µL/mL aprotinin, 1% igapel 630 (Sigma Chem. Co. St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate]. The extracts were centrifuged at 23,000 g for 1 hour. Equal amount of protein (20 µg) was separated on SDS/ 10%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 hours at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was incubated for 4 hours at room temperature with specific antibodies: Mouse monoclonal antibodies were used at 1:1,000 and were directed against ERK, p-ERK (Cell signaling Technology, Beverly, MA, USA), and against rabbit polyclonal antibodies against iNOS, COX-2 (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA). The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the enhanced chemiluminescent Western blotting detection system.

Nitrite quantification assay

Nitric oxide (NO) concentrations were measured indirectly by measuring the nitrite level in the cell culture media. The nitrite accumulation in the supernatant was assessed by Griess reaction. The keratinocytes were seeded at 1×10^6 cells/well into 6-well plates in 2 mL of cell culture media, and incubated for 24 hours. The old culture media was discarded and replaced with new media to maintain the cells. The same pretreatment concentrations of BV (1, 2 and 5 $\mu\text{g/mL}$) were used to treat the keratinocytes. LPS (1 $\mu\text{g/mL}$)-activation of keratinocytes for 24 hours was performed for all samples except for the control group. Then, 100 μL of the collected supernatants was added with 100 μL of Griess reagent (0.1% NED, 1% sulphanilamide, and 2.5% phosphoric acid) and incubated at room temperature for 10 minutes in the dark. The absorbance was determined by using a microplate reader (Thermo Fisher Scientific Inc.) at 540 nm wavelength. The NO concentration was determined by comparison to the standard curve.

Statistical analysis

The experiments were conducted in triplicate, and all experiments were repeated at least 3 times with similar results. All statistical analysis was performed with GraphPad Prism 5 software (Version 5.03; GraphPad software, Inc., CA, USA). Group differences were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. All values are presented as mean \pm SD. Significance was set at $p < 0.05$ for all tests.

Results

Effect of BVP on iNOS, COX-2, p-ERK and ERK expression and NO concentration in keratinocytes

To assess the anti-inflammatory effect of BVP, keratinocytes were prepared and assayed using Western blot analysis. Protein expressions of COX-2, iNOS, p-ERK and ERK were significantly decreased, compared to LPS activated control keratinocytes (Fig. 1). NO concentration measured by Griess reaction, was significantly lower in the BVP-treated group (2 and 5 $\mu\text{g/mL}$ compared with the control (Fig. 1).

Effects of BVP on morphology of the skin on the back and clinical scores

To investigate whether BVP treatment suppressed changes in the skin induced by PA treatment, morphology of the skin was observed. Compared to control, PA induced erosion and edema was reduced by BVP in BVP-treated group, and the clinical scores measuring the severity of AD also decreased significantly and dose-dependently (Fig. 2).

Effect of BVP on eosinophils, neutrophils and monocytes in serum and histological changes of F4/80 positive cells and Ly6G positive cell in the ear skin

Infiltration of eosinophils, neutrophils, macrophages and monocytes were counted in serum samples to determine whether BVP treatment caused changes. BVP treatment showed a dose-dependent decrease in eosinophil, neutrophil and monocyte numbers compared with the PA-treated group (Fig. 3).

Histological analysis of the thickness of the epidermis of the ear skin decreased in BVP-treated mice compared with the PA-treated group.

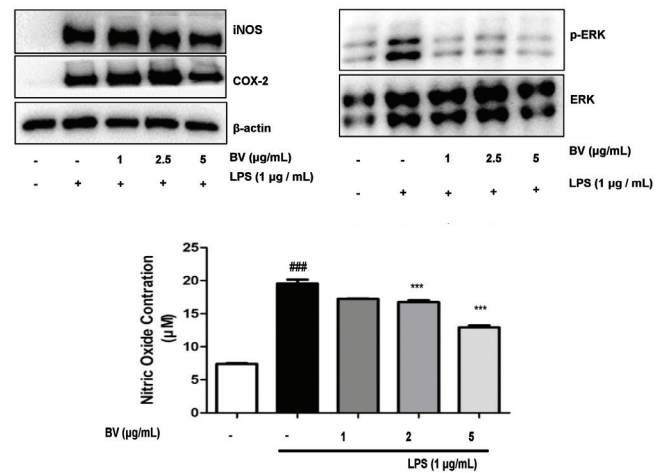


Fig. 1. Effect of BVP treatment of keratinocytes in the expression of NO, iNOS, COX-2, p-ERK and ERK. Expression of proteins related with inflammation, iNOS, COX-2, p-ERK and ERK were determined using Western blot analysis. Control values were obtained in the absence of LPS. BV and LPS represents bee venom and lipopolysaccharide respectively. Data are presented as mean \pm SD.

$p < 0.001$, significantly different from non LPS treated group.

*** $p < 0.001$, significantly different from control (LPS treated group).

BV, bee venom; BVP, bee venom pharmacopuncture; p-ERK, phospho-ERK.

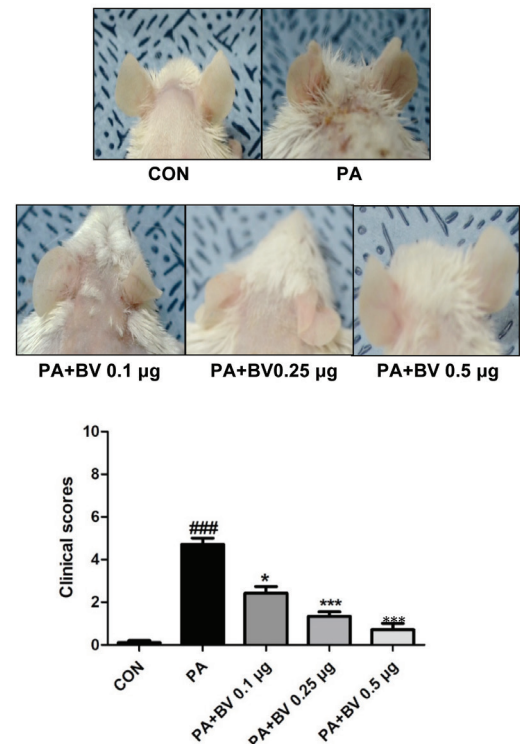


Fig. 2. Effect of BVP on Morphological Difference and clinical scores of Back Skin of Mice. PA was applied on the dorsum of the back skin 3 times a week for 4 weeks. Data are presented as mean \pm SD ($n = 10$).

$p < 0.01$, significantly different from non-PA-treated group (control).

* $p < 0.05$, *** $p < 0.001$, significantly different from PA-treated group.

BV, bee venom; BVP, bee venom pharmacopuncture; CON, control; PA, phthalic anhydride.

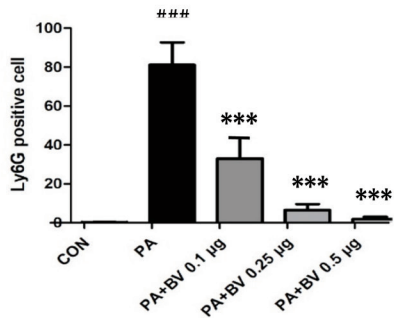
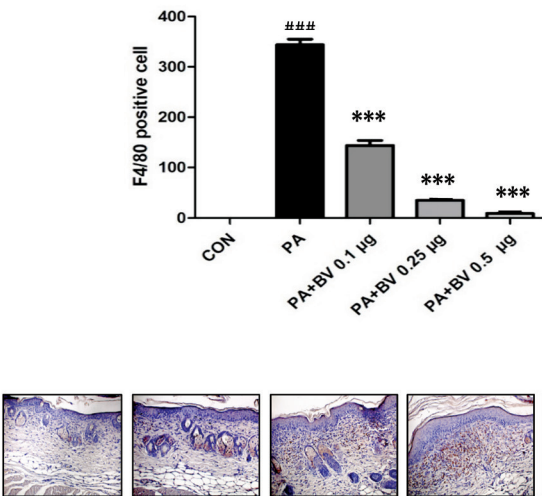
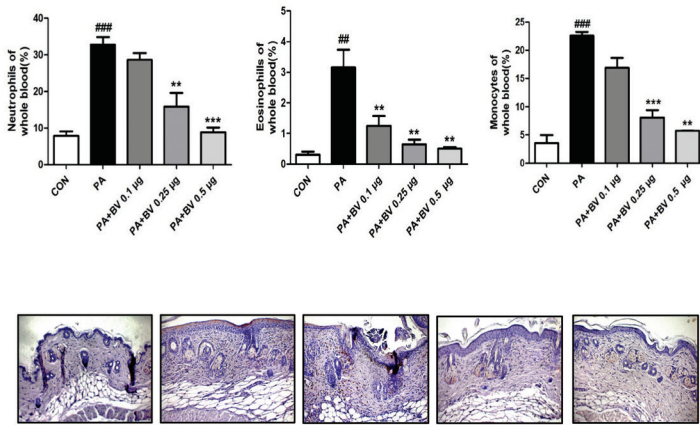


Fig. 3. Changes in histopathology of ear tissue as well as serum eosinophils, neutrophils, and monocytes with various doses of BVP. Data are presented as mean \pm SD ($n = 10$).
 $## p < 0.01$.
 $### p < 0.001$, significantly different from control (non-PA-treated group).
 $*** p < 0.001$.
 $** p < 0.01$ significantly different from PA-treated group.
 BV, bee venom; BVP, bee venom pharmacopuncture; CON, control; PA, phthalic anhydride.

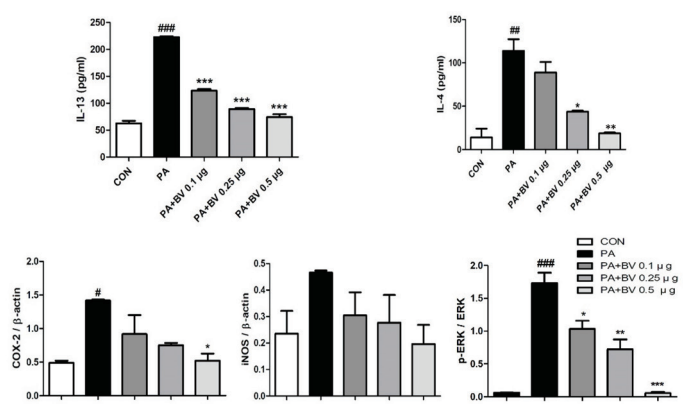


Fig. 4. Effect of BVP on protein expression of COX-2, iNOS and p-ERK/ERK and serum cytokine concentrations.

$# p < 0.05$.
 $## p < 0.01$.
 $### p < 0.001$, significantly different from control (non-PA-treated group).
 $*** p < 0.001$.
 $** p < 0.01$.
 $* p < 0.05$ significantly different from PA-treated group.
 BV, bee venom; BVP, bee venom pharmacopuncture; CON, control; PA, phthalic anhydride; p-ERK, phospho-ERK.

F4/80 and Ly6G positive cell infiltration into the dermis showed that the numbers of both were significantly lower in the BVP-treated groups compared with the PA-treated group (Fig. 3).

Effect of BVP treatment on the release of inflammatory cytokines and protein expressions

To investigate whether BVP induced a change in the release of the inflammatory cytokines, the levels of IL-4 and IL-13 were measured in mouse serum of the control group, PA-treated group, and PA + BVP (0.1, 0.25, and 0.5 μ g) treated group. The levels of IL-4 and IL-13 were significantly lower and decreased in a dose-dependent manner in the BVP-treated group (0.1, 0.25, 0.5 μ g/mL and 0.25, 0.5 μ g/mL respectively), compared with the PA-treated group (Fig. 4). In addition, Western blot analysis showed protein expression for COX-2 (0.5 μ g/mL), iNOS and p-ERK/ERK (0.1, 0.25, 0.5 μ g/mL), significantly decreased in BVP-treated groups compared with the PA-control (Fig. 4).

Discussion

Atopic dermatitis (AD) is the most prevalent inflammatory skin condition observed in children and may continue to affect them as they get older [9]. AD is clinically characterized by its itchy, inflamed skin, and susceptibility to skin infections caused by skin barrier abnormalities [9-11]. AD is caused by various factors such as genetic factors, and is triggered by environmental pollutants, stress, resulting in cell mediated immune responses which may cause dysfunction with the skin's function as a barrier [12]. Several studies have suggested that AD is related with a Th1/Th2 imbalance, but recently Th17 and Th22 cells have been reported to also play a significant role in the disease. In all the stages of AD, Th2 cells play a dominant role as the increase of the Type 2

cytokines and chemokines such as IL-4, IL-5, IL-10, IL-13, IL-31 are evident. These mediators are involved in allergic responses impairing keratinocyte differentiation [12]. In particular, Th17 cells which are defined by production of IL-17 and IL-22, show an increase in number but decrease in activity [12,13].

BV treatment has been used to treat various inflammatory diseases. There are several recent studies about the biological and pharmacological activities of BV, proposing that BV inhibits the increase of inflammatory and pro-inflammatory cytokines from the human keratinocytes [6-8]. This study investigated whether BV treatment had anti-inflammatory effects on AD using LPS-activated murine keratinocytes and PA-treated mice.

Expression of iNOS, COX-2, p-ERK and ERK were observed using a Western blot which showed the LPS-activated group had higher levels compared with the BVP-treated groups which showed low levels of expression of these proteins, indicating that treatment with BVP had anti-inflammatory effects. BVP treatment of keratinocytes also resulted in a lower NO concentration compared with the LPS-activated group.

The in vivo effect of BVP was determined in a mouse model of AD where the mice had PA applied to dorsal skin on their back. The morphology of the treated skin and tissue from their ears showed a dramatic change in clinical scores of severity of AD due to BVP treatment. Furthermore, BVP treatment inhibited the effect of Th2 and Th17 cell activity, as observed by IL-4+ and IL-17+ cell concentrations which decreased. BVP treatment acted in a dose-dependent way reducing the infiltration of eosinophils, neutrophils, macrophages and monocytes. Histopathological analysis showed the thickness of the epidermis of ear skin was reduced, and infiltration of F4/80 positive and Ly6G positive cells decreased due to BVP treatment. In addition, BVP inhibited the release of inflammatory cytokines IL-4 and IL-13. Moreover expression of COX-2, iNOS was inhibited by BVP treatment and phosphorylation of ERK was down regulated.

Conclusion

BVP had an anti-inflammatory effect on keratinocytes in vitro by reducing the expression of iNOS, COX-2 and p-ERK which all play an important role in inflammation. In addition, in vivo, BVP reduced the concentration of cytokines, and reduced the infiltration of white blood cells, and the thickness of the epidermis. Though there are undefined causes leading to AD, this study suggests that BVP could be an effective and promising intervention for the treatment of AD.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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