Bee Venom Within Liposomes Synergistically Inhibit Atopic Dermatitis in Mice

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ABSTRACT

Background: This study was performed to determine the effects of liposome-encapsulated bee venom (BV) treatment of inflammatory factors in atopic dermatitis (AD) compared with BV treatment.

Methods: AD was induced by phthalic anhydride in mice and the effects of BV liposomes were measured. Using Leica Application Suite, thickened epidermis and dermis were measured after BV liposome treatment (0.05 and 0.1 μg/mL). The number of stained mast cells and the concentration of immunoglobulin (Ig)E were measured. Serum IgE concentration was analyzed using an enzyme-linked immunosorbent assay. The serum concentrations of interleukin (IL)-1, IL-4, and IL-6 inflammatory cytokines were measured. The levels of messenger ribonucleic acid expression of proinflammatory cytokines and chemokines were measured using reverse transcription polymerase chain reaction. Inhibition of mitogen-activated protein kinase activation, was analyzed on western blot. To measure the transcriptional activity (NF-κB inhibition by BV liposomes), western blots (p65, p-IκB, p50, and IκB) were also performed.

Results: The weight of lymph nodes, serum IgE concentrations, morphological changes in the skins from the backs of the mice, and mast cell numbers in inflamed tissues were noticeably lower in the BV liposome treatment group compared with the BV treatment group. The concentrations of pro-inflammatory cytokines (IL-1, IL-4, IL-6) and chemokines (TSLP, CCL22) were also reduced. Activation of mitogen-activated protein kinase (p-ERK and p-p38), and transcriptional activity (p65, p-IκB, p50, and IκB) was strongly suppressed in the BV liposome group.

Conclusion: BV liposomes may have a better therapeutic effect than BV for the treatment of AD.

Introduction

Atopic dermatitis (AD) is the most studied skin condition/disease [1]. Chronic recurrent eczema, itching, and a multifocal inflammatory mechanism involving hypersensitivity of organs, including the skin, and vulnerability of the skin barrier function appear as common symptoms in AD [2]. AD is a chronic relapse condition/disease [3], treated with immunosuppressants to reduce symptoms, however, risks, and side effects of treatment continue to be reported [4,5]. Therefore, safe alternative long term treatments are required [6].

Bee venom (BV) is a complex containing Apis melifera which is applied in a variety of conditions/diseases in modern medicine, as well as in ancient traditional medicine [7,8]. Anti-inflammatory, anti-bacterial, anti-cancer, anti-allergic, and pain relief effects of treatment with BV have been reported [8,9]. BV has been reported to have an anti-inflammatory effect against AD and improved skin barrier function [10].

Liposomes are effective drug delivery systems that protect drugs by enclosing them in a phospholipid bilayer to prevent chemical and
immunological breakdown, and increase intracellular absorption [11]. In particular, studies of BV liposomes have reported the potential to reduce side effects such as BV anaphylaxis, and increased drug absorption [12,13].

Studies on the synergistic effects of BV in liposome that inhibit AD-related inflammatory factors are scarce.

In AD, cytokines are important inflammatory agents, biomarkers of activation, and affect the differentiation of immune-related cells such as keratinocytes, macrophages, T and B lymphocytes during most stages of the immune response [14]. Thymic stromal lymphopoietin (TSLP) causes an immune response that induces pro-inflammatory cytokine production and inhibits the secretion of filaggrin proteins [15]. Mitogen-activated protein kinase (MAPK) is a kinase activates and responds to various immune cells [16,17].

In this study, by analyzing the levels of messenger ribonucleic acid (mRNA) expression of chemokines and pro-inflammatory cytokines, and the activity of MAPK and NF-κB, the effects of BV and BV liposomes on AD was studied. The inhibitory capacity of the BV-liposome, and BV alone, against the main inflammatory agents was investigated.

Materials and Methods

Preparation of BV liposomes

BV (You-Miel BV Ltd. Hwasoon, Jeon-nam, Republic of Korea) components were as follows: 45-50% melittin, 12% phospholipase A2, 4-5% 6-pentyl a-pyrene 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% sercinpin, 0.1% procamine, 0.1% tertiapin and 19-27% of others including protease inhibitor, glucosidase, acid phosphomonoesterase, norepinephrine, dopamine, invertase, unknown amino acids, with 99.5% purity. According to instructions to users of BV (H&M pharmachem, Bucheon, Kyoung-ki, Republic of Korea), 8.2 g of BV was dissolved using 120 mL of 95% ethanol and shaking at room temperature, and insoluble substances were removed using Whatman filter paper. The ethanol was distilled off, at room temperature, so that the volume of the solution became about half the initial volume using a vacuum distiller. The remaining solution was transferred to a plate (with a thickness of about 5 mm), and the liquid component was removed using a vacuum freezer set at -20°C for the first 3 hours, and then vacuum was applied overnight. The dry sample was collected with a spatula, ground in a mortar for 3 minutes, sealed, and stored at room temperature for later use.

Animal treatment

Chungbuk National University-Institutional Animal Care and Use Committee approved the animal experimental protocol for this study (Approval no.: 00NU-2015-0976) and strict scientific and ethical care procedures were followed. Eight-week-old, male, HR-1 mice were randomly distributed into 5 treatment groups; (1) 3.5% phthalic anhydride (PA) solution [100 μL (20 μL/cm²)], was applied topically to the dorsal area of the ears, and the back, 3 times a week, for 4 weeks (n = 3); (2) 5% PA group, 5% phthalic anhydrous solution was administered topically to the back, 3 times a week, for 4 weeks (n = 3); (3) PA+BV 0.1 μg (n = 3); (4) PA+BV 0.1 μg liposomes (n = 3); and (5) PA+BV liposomes 0.05 μg (n = 3). All groups had PA administered, and 3 hours later, 100 μL of 0.1 μg of BV, 0.05 μg, and 0.1 μg of BV liposomes were administered. The mice in the control group were of the same age (n = 3).

Measurement of body weight, lymph nodes, and thickness of ears

During the 4 weeks of the experiment, the weight of each mouse was measured once a week on electronic scales (Mettler Toledo, Greffensee, Switzerland). Following euthanasia, the weight of lymph nodes extracted from mice was also measured. Ear thickness was measured using an instrument that specifically measures thickness (Digimatic Indicator, Mitsutoyo Co., Tokyo, Japan) to determine the extent of allergic dermatitis induced by the PA solution.

Histological analysis

The skin from the ears and backs of the mice were fixed in formalin (10%), embedded in paraffin wax, and 5 μm sections were prepared which were dyed with cosin and hematoxylin. Following dehydration and paraffin removal, the skin sections were stained with toluidine blue solution (0.25% Sigma Aldrich MO, USA), and examined under light microscopy. Using the Leica Application Suite, the thickness of the dermis and epidermis was measured, and the number of mast cells per unit area was counted (Leica Microsystem, Wetzlar, Germany).

The detection of serum IgE concentration by enzyme-linked immunosorbent assay

For serum IgE concentration, the enzyme-linked immunosorbent assay kit (Gunma sibayagi Co., Ltd., Japan) instructions were followed. Antibodies were plated onto the Nunc C lower immunoplate (contained in the kit). The well was washed 3 times (pH 8.0, TWEEN 20 0.05%, 50 mM Tris, 0.14 M NaCl). Standards and serum samples were diluted with wash buffer and added to the well and cultured for 2 hours. To each well, 50 μL of biotin anti-IgE conjugated antibodies were diluted 1,000 times, administered, and incubated for 2 hours to bind with captured IgE. After washing every well, horseradish peroxidase-conjugated detection antibodies were diluted 2,000 times and were added, and incubated for 1 hour. The enzyme reaction was initiated by adding tetramethylbenzidine substrate solution (100 mM sodium acetate buffer pH 6.0, 0.006% H₂O₂) and incubating on the plate at room temperature for 20 minutes in the dark. The reaction was ended by adding an acid solution (reaction stopper, 1M H₂SO₄) and the absorbance of the yellow product was measured at 450 nm using a plate reader. The last concentration of IgE was quantified according to a standard curve.

Cytokine assay

Blood specimens were taken in the last period of the study. Levels
of serum interleukin (IL)-1β, IL-6 and IL-4 were measured using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific Inc. Meridian Road, Rockford, IL USA) according to protocol.

**RNA quantification**

The RiboEX RNA extraction kits (GeneAll Biotechnology, Seoul, Korea) were used to collect total RNA from the mice skins. The High-Capacity RNA-to-cDNA kits (Applied Biosystems, Foster City, CA, USA) was used to synthesize complementary deoxyribonucleic acid. Reverse transcription qualitative polymerase chain reaction was performed using specific primers within the StepOnePlus™ PCR System (Applied Biosystems, Foster City, CA, USA) as previously described [18]. The fold change between groups was determined for all targets by the $2^{ΔΔCt}$ method. The mRNA levels were normalized to the 18S sequence as a housekeeping control.

**Western blot analysis**

About 100 mg of skin or $1 \times 10^6$ cells were homogenized with lysis buffer [pH 8.0, 50 mM Tris, 150 mM NaCl, 0.2% sodium dodecyl sulfate, 10 μL/mL aprotinin, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 1% igepal 630 (Sigma Chem. Co. St. Louis, MO, USA), 0.5 mM EDTA, 10 mM NaF, 0.5% sodium deoxycholate, 0.1 mM EGTA]. After centrifuging at 23,000 g for 1 hour, the same amount of protein (20 μg) was separated on a sodium dodecyl sulfate/10%-polyacrylamide gel, and it was transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The nitrocellulose membrane was fixed at room temperature for 2 hours with 5% (w/v) fat free dried milk in Tris buffered saline solution (10 mM Tris, pH 8.0, 150 mM NaCl) with 0.05% TWEEN 20. The membrane was incubated for 4 hours at room temperature using specific antibodies. Mouse monoclonal antibodies directly targeting p50 (1:500, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), p-p38, p38, p-ERK, ERK, p65 (1:1,000, Cell signaling Technology, Beverly, MA, USA) were used. Rabbit polyclonal antibodies directly against IκB, IκB-α (1:500, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) were also used. The anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) were equivalently conjugated and used for the western blots' and incubated. For the detection of immunoreactive proteins, the enhanced chemiluminescent western blotting detection system was used.

**Statistical analysis**

In this study, all experiments were repeated 3 times and had similar results. Every statistical analysis was worked by GraphPad Prism 5 software (Version 5.03, GraphPad software Inc., CA, USA). The difference between groups was analyzed with one-way ANOVA followed by Tukey’s multiple comparison test. All values were shown as the mean ± standard deviation, and statistical significance was $p < 0.05$.

**Results**

**Effects of BV liposomes treatment on morphology and ear thickness**

The change in body weight was measured and no significant difference was observed between the groups of mice (Fig. 1). Changes in morphology of the skin from the backs of the mice, compared with the control group, included erythema, edema, and erosion in the PA treatment groups. These morphological changes were improved with 0.1 μg/mL of BV treatment, which were dramatically improved by BV in liposomes (0.05 and 0.1 μg/mL; Fig. 1).

**Effect of BV liposome application on IgE concentration and lymph node weight**

To investigate the inhibitory effect of BV liposomes, the weight of each auricular lymph node, and serum IgE concentrations were evaluated. PA application caused an increase in the weight of the
lymph node compared with the control group. After application of the BV liposomes, the lymph node weight reduced significantly in the 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups, as well as in the 0.1 µg/mL BV treated group (Fig. 2). Repeated local administration of PA solution caused a significant increase in serum IgE concentration in the blood of the mice as the allergic response developed. However, serum IgE concentration was significantly lower in the 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups, as well as in the 0.1 µg/mL BV treated group (Fig. 2).

Effect of BV liposome treatment on histological change caused by PA on the skins from the backs of the mice

Histology was performed on the skins from the backs of the mice and was analyzed to observe potential changes in the epidermis caused by the BV liposomes. The epidermis thickness of the PA group was greater than control group (Fig. 3). The epidermis became thinner in the BV treated group (0.1 µg/mL), and where BV in liposomes were used (0.05 µg/mL, and 0.1 µg/mL BV liposome group) this action was synergistic. Moreover, there was a significant dose-dependent decrease in epidermal thickness in the 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups (p < 0.05).

In addition, using a microscope to count the change in the number of infiltrated mast cells, the number was significantly higher in the PA treatment group compared with the control group (Fig. 3). However, the number of mast cells after treatment was observed as less in the 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups, as well as in the 0.1 µg/mL of BV treated group. Mast cell infiltration was concomitantly, synergistically, and dose-dependently inhibited in groups treated with BV in liposomes (0.05 µg/mL, and 0.1 µg/mL; Fig. 3).

Fig. 2. Changes in auricular lymph node weight and serum IgE concentration. All mice were euthanized under anesthesia. The auricular lymph nodes were collected using micro scissors and weighed. Serum IgE were quantified by enzyme-linked immunosorbent assay. Data are presented as mean ± SD (n = 3).

** p < 0.05.
*** p < 0.01.
### p < 0.001 significantly different from control.
Fig. 3. Histopathological analysis of the skins from the backs of the mice in control group, 5% PA treated group, 0.1 µg/mL of BV treated group, 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups was performed. PA solution was repeatedly applied to the backs while BV liposomes were administered. Histopathological changes were identified in the slide sections by staining with hematoxylin and eosin followed by observation at 200× magnification (scale bars, 50 μm). Infiltration of mast cells in control, 5% PA treated group, 0.1 µg/mL of BV treated group, 0.05 µg/mL, and 0.1 µg/mL BV liposome treated groups. Tissue sections were observed by staining using Toluidine blue (0.25%) by 200× magnification (scale bars, 50 μm). The number of mast cells penetrated were counted. Data are presented as mean ± SD (n = 3).

### p < 0.01.
#### p < 0.001, statistically significant compared with control (non-treated group):
* p < 0.05.
** p < 0.01.
*** p < 0.001 significantly different from PA treatment group.

* Scale bar : 50µm
Effect of BV liposome treatment on the release of inflammatory cytokines in the sera of AD induced mice

The mouse serum levels of IL-1β, IL-4 and IL-6 were measured to observe how BV liposome administration was involved in potential changes when inflammatory cytokines are released from dermatitis induced by PA in control, PA treatment, PA+BV, and PA+BV liposome treatment groups. After PA solution was applied topically and repeatedly, a significant increase of IL-1β, IL-4 and IL-6 levels was induced in serum (p < 0.05). However, those were significantly and dose-dependently decreased in the 0.05 μg/mL, and 0.1 μg/mL BV liposome treated groups, as well as in the 0.1 μg/mL of BV treated group compared with 5% PA treated group (p < 0.05; Fig. 4).

Effect of BV liposome treatment on PA-induced inflammation in the skin of the backs of the mice

To examine the production of chemokines and pro-inflammatory cytokines in the skin of the backs of the mice, real-time PCR was performed. The levels of pro-inflammatory cytokines such as IL-1β, IL-4, and IL-6, and of chemokines such as TSLP, and CCL22 increased due to the repeated application of PA solution topically. However, those were significantly and dose-dependently decreased in the 0.05 μg/mL, and 0.1 μg/mL BV liposome treated groups, as well as in the 0.1 μg/mL BV treated group by comparison with 5% PA treated group (p < 0.05; Fig. 5).

Effect of BV liposome treatment on the expression of MAPK-activated protein kinases in the skins from the backs of the mice

To observe the potential action of BV liposomes involved in MAP kinase pathway on PA-induced inflammation in mice, the MAP kinase activation (phosphorylation of p38 and ERK), induced by PA treatment, was measured in cells of the skins from the backs of the mice. The analysis of western blot from individual 3 experiments appeared that BV liposomes (0.05 and 0.01 μg/mL) as well as BV (0.1 μg/mL) strongly dose-dependently blocked PA (5%)-induced activation of p-ERK and p-p38 in cells of the skin from the backs of the mice (Fig. 6).

Effect of BV liposome treatment on expression of NF-κB signal molecules in the skins from the backs of the mice

To examine the potential involvement of signal molecules in the NF-κB signal pathway in the suppressive effect of BV liposomes on PA-induced inflammatory dermatitis in mice, the activation of p65, IκB, p50 and p-1xB induced by PA treatment was evaluated in cells of skins from the backs of the mice. The analysis of western blots from 3 different experiments showed that BV liposomes (0.05 and 0.01 μg/mL) as well as BV (0.1 μg/mL) strongly and dose-dependently blocked PA (5%)-induced activation of NF-κB in cells from the skins of the backs of the mice (Fig. 7).

Discussion

AD is a long-term condition/disease that needs to be treated [19]. AD is a systemic inflammatory response that is not limited to lesions, causes persistent itching, and dry skin, and may develop at an early age and has recurrence flare-ups [20]. Various causes of AD include genetic, socioeconomic, and environmental factors [21]. The mechanism of AD can be divided into destruction of external skin barrier function, and internal skin inflammation [22], but this is not a one-sided relationship of ‘skin wall damage - skin internal inflammation’, but rather a vicious cycle amplified through feedback mechanisms [23]. When biological or genetic factors break down skin barriers, keratin and mast cells are stimulated, creating inflammatory cytokines, and increasing a series...
Fig. 5. BV in liposomes synergistically prevents PA-induced inflammation in the skins from the backs of the mice. mRNA expression levels of chemokines such as thymic stromal lymphopoietin, and CCL22, and pro-inflammatory cytokines such as IL-1β, IL-6, IL-4 in the skins from the backs of the mice were measured using quantitative real time reverse transcription polymerase chain reaction. Data are presented as mean ± SD (n = 3).

# < 0.05.
## p < 0.01.
### p < 0.001, statistically significant compared with control (non-treated group).
* p < 0.05.
** p < 0.01.
*** p < 0.001, different from PA treated group significantly.

Fig. 6. BV liposomes inhibited expression of MAPK-activated protein kinases in skins from the backs of the mice. The expression of p-ERK and p-p38 were observed by western blot with specific antibodies in skins from the backs of the mice. The blot represents 3 experiments. Histone protein was used an internal control.
Treatment methods that reduce skin irritation are prioritized and include self-care to avoid deteriorating factors, moisturizers, and topical corticosteroids. These methods aim to improve skin barrier conditions and reduce inflammation in the skin by using drugs. In mild cases, topical corticosteroids, topical calcineurin inhibitors, and antihistamines are used first [7]. Systemic therapy and biologically targeted treatment for AD may be used in severe, uncontrollable AD cases [25]. The use of anti-inflammatory agents, such as topical corticosteroids, may temporarily show rapid symptom improvement, but a later rebound can lead to increased IgE production by B cells and many side effects [26,27].

Recent studies have reported significant relationships between AD and other conditions/diseases of organs such as chronic gut disease, and arthritis [28-30]. Even non-lesion skin in AD patients was determined to have modifications in internal keratinocyte differentiation compared with the skin of healthy people, with significant increases in cytokines, chemokines, and T helper cells[1]. This is a characteristic of AD alone compared with other dermatitis. These studies show that AD is not local but is a systemic, chronic condition taking effect throughout the body. Therefore, treatment of AD requires fundamental and systemic treatment beyond immunosuppressants.

BV contains various types of peptides, amines, enzymes, lipids, carbohydrates, and amino acids, and the main ingredients are melittin, apamin, and phosphoripase A2 [31]. BV has been used to treat conditions/diseases since ancient Egypt and ancient China [13]. The effects of BV resulting in the inhibition of inflammation in AD has been studied [32]. BV blocks the signaling pathway of MAPK and inhibits gene transcription of pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α, indicating an anti-inflammatory effect on AD [12]. The increase in the expression of pillar green due to BV treatment contributed to the improvement and maintenance of skin barrier layer function [33].

PA is an industrial compound used in plasticizers, plastics, paints, and adhesives. Exposure to PA stimulates the eyes, skin, and respiratory tract to induce IgE production and activate mast cells. It activates Th-cells, releases inflammatory cytokines, and increases irritability [34]. It was reported that AD occurs and worsens [35].

In this study, the condition of AD was produced by experimentally inducing skin inflammation and allergic reactions with PA in a murine model. Inflammatory markers such as levels of IgE, lymph node weight, dorsal ear and dorsal epidermis thickness, mast cell numbers, cytokine, and chemokine concentration were used. It was investigated whether BV liposomes inhibited inflammatory cytokines, prevented NF-κB activation in the lymph nodes and tissues, and reduced inflammation. Furthermore, BV in liposomes were determined to have a synergistic effect compared with BV alone in reducing inflammation, and the effects of the change at 2 concentrations were investigated.

In this study, an increase in ear thickness and symptoms (erythma, edema, erosion) was observed in the PA treatment group compared with the control group. These changes were reversed using BV treatment and BV in liposome was superior to BV alone. After the procedure, no significant weight difference in the mice was detected. PA treatment resulted in increased weight of lymph node and increased IgE serum concentration compared with control mice group. The weight of lymph nodes was significantly reduced in the BV treatment group, as well as in the BV liposome treatment group. Serum IgE concentrations were significantly reduced in the BV liposome treatment group.

In the histological analysis of the skins from the backs of the mice, the PA treatment group epidermal thickness was significantly more than the BV liposome treatment group, and this was dose-dependent.

Changes in the numbers of mast cells were observed in the skins from the backs of the mice which were dyed toluidine blue. Mast cell infiltration was concomitantly, synergistically, and dose-
dependently inhibited by BV in liposomes.

Levels of IL-1β, IL-4, and IL-6 were measured to determine whether BV in liposomes could synergistically alter inflammatory cytokine release in PA-induced skin inflammation. The BV liposome treatment group showed significant and dose-dependent reductions in cytokine levels.

The concentrations of pro-inflammatory cytokines such as IL-1β, IL-4, and IL-6 and chemokines such as TSLP, and CCL22 in the skins from the backs of the mice increased in the PA treatment group and significantly decreased in the BV liposome treatment group.

The activation of MAP kinase (phosphorylation of ERK and p38) induced by PA treatment in cells of the skins from the backs of the mice was blocked strongly and, in a dose-dependent manner in the BV liposome treatment group.

To investigate the potential involvement of signal molecules in the NF-xB signal pathway in the inhibitory effect of BV liposomes on PA-induced inflammation in mice, the activation of IxB, p-IxB, p50 and p65 induced by PA treatment was evaluated in the cells from the skins from the backs of the mice. BV liposomes strongly, and dose-dependent manner blocked the NF-xB signal pathway.

In conclusion, BV within liposomes synergistically showed anti-inflammatory effects by reducing pro-inflammatory cytokines, and inhibiting the activation of NF-kB and MAPK signaling pathways in vitro. Moreover, compared with the BV group, BV within liposomes inhibited inflammation in a concentration-dependent which was synergistic. Thus, BV liposome treatment presents a bright prospect for AD treatment.

Conflicts of Interest

Ho Sueb Song has been the editor in chief of Journal of Acupuncture Research since April 2011, but had no role in the decision to publish this original article. No other potential conflict of interest relevant to this article was reported.

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Ethical Statement

This research did not involve any human experiments.

References


