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Bee Venom Reduces Fungi Induced Bronchial Epithelial Cells Activation Through Down Regulation of NF- κ B

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Abstract: Bee Venom (BV) has been used as treatment against a variety of inflammatory diseases. Studies have demonstrated anti-inflammatory effect of BV. This study aimed to clarify the potential clinical efficacy of BV concerning the anti-inflammatory effect on bronchial epithelial cell. BEAS-2B cells were exposed to *Alternaria alternata* and *Aspergillus nigr*a with or without BV. IL-6, IL-8 and granulocyte macrophage colony-stimulating factor were measured to determine the activation of epithelial cells. Nuclear factor kappa B (NF- κ B) and activator protein 1 expression and activity were determined with Western blot analysis and ELISA. Cytotoxicity of BV was measured using a CellTiter-96[®] aqueous cell proliferation assay kit. Cell survival was significantly decreased concentrations at 10 μ g mL⁻¹. Fungi induced IL-6 and IL-8 production was effectively inhibited by BV. BV suppressed fungi induced NF- κ B expression and *Alternaria* induced NF- κ B activation. BV seems relatively safe and is of potential value for the treatment of airway inflammatory diseases.

Key words: Bee venom, *Alternaria*, *Aspergillus*, bronchial epithelial cell, nuclear factor κ B

INTRODUCTION

Bee Venom (BV) has been used to treat chronic inflammatory diseases and malignant tumors for long time in Oriental medicine (Hider, 1988). BV contains enzymes, biologically active amines, peptides and nonpeptide components with various pharmacological characteristics. Studies in animal models has demonstrated the capability of BV to modify the immune function in the body with anti-inflammatory and anti-bacterial activities in the absence of side effects (Han *et al.*, 2007). Anti-inflammatory activity is due to the ability of BV to inhibit the expressions of cyclooxygenase-2 and phospholipase A2. BV also inhibits the release of inflammatory mediators similar to nonsteroidal anti-inflammatory drugs.

Fungal spores are continuously inhaled and deposit in normal and patient's airway mucosa without harmful effect. But fungi that are commonly involved in asthmatic reaction with atopic response belong to *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium* and *Candida* (Knutsen *et al.*, 2012). Fungal elements like proteins and enzymes induce immunologic and inflammatory responses. Proteases from fungi activate epithelial cells through surface receptors, resulting in generation of

cytokines and facilitating antigen access either by proteolytic attack or cell desquamation (Kauffman *et al.*, 1995; Shin and Lee, 2010).

The respiratory epithelium is the first mucosal defense organ exposed to the environmental stimulants associated with airway disease. Respiratory epithelial cells provide mechanical barrier to prevent infection and produce chemokines, cytokines and antimicrobial components to eliminate pathogenic microorganisms. These cells also play an active role in inflammation through the production of a variety of proinflammatory cytokines and chemokines for the recruitment of inflammatory cells (Matsuwaki *et al.*, 2012).

Humans are constantly exposed to various fungi, but only a few, especially *Alternaria* and *Aspergillus*, are associated with respiratory disease. The proteolytic enzymes released by fungi induce the production of chemoattractive and proinflammatory cytokines by respiratory epithelial cells (Shin and Lee, 2010; Matsuwaki *et al.*, 2012). Until now, no studies have been performed to investigate the anti-inflammatory effect of BV on fungi-activated bronchial epithelial cells. The aim of this study was to evaluate the effect of BV on the production of chemical mediators from activated bronchial epithelial cells and their anti-inflammatory mechanisms.

MATERIALS AND METHODS

Bronchial epithelial cell culture and activation with fungi:

The human bronchial epithelial cell line BEAS-2B, which was derived from human bronchial epithelium transformed by adenovirus 12-SV40, was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured with DMEM/F12 medium supplemented with heat-inactivated 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin at 37°C and 5% CO₂. Cells were seeded at 5×10⁴ cells/well in a 24 well tissue culture plate and grown until 80% confluence. At this stage, the BEAS-2B cells were stimulated for up to 24 h with endotoxin free *Alternaria alternata* (50 µg mL⁻¹) and *Aspergillus nigra* (25 µg mL⁻¹) (Greer Lab, Lenoir, NC, USA). In our previous experiments with nasal polyp epithelial cells, maximal cytokine production was found at 50 µg mL⁻¹ of *Alternaria* and 25 µg mL⁻¹ of *Aspergillus* (Shin and Lee, 2010). The same concentrations of these allergens were used currently. Supernatants and cells were harvested and stored at -70°C until they were assayed. Production of interleukin (IL)-6, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF) was quantified using commercially available enzyme-linked immunosorbant assay (ELISA) kits (R and D Systems, Minneapolis, MN, USA).

Preparation and cytotoxicity assay of BV: Pure honeybee (*Apis mellifera*) venom was obtained from the National Institute of Agricultural Science and Technology, Suwon, Korea. BV was collected using a specialized collector without damaging honeybees by an established electric shock method. BV was dissolved in distilled water and centrifuged at 12,000×g for 10 min to remove insoluble materials. The BV was lyophilized by freeze drying and stored (Han *et al.*, 2007).

BEAS-2B cells were incubated at 37°C, 5% CO₂ in 96 well plates in the presence of 0, 0.03, 0.15, 0.5, 1, 5 and 10 µg mL⁻¹ BV. Cytotoxicity was measured using a CellTiter-96[®] aqueous cell proliferation assay kit (Promega, Madison WI, USA). For this assay, the tetrazolium compound and Owen's reagent were added to each well and incubated for 4 h at 37°C in a humidified 5% CO₂ chamber. Color intensities were assessed with a microplate reader at 490 nm.

Expression and activation of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1): To perform Western blot analysis for NF-κB and AP-1, NE-PER nuclear extraction reagents (Pierce Chemical, Rockford, IL, USA) was used to isolate proteins. The protein was

quantified using a BCATM protein assay reagent kit (Pierce Chemical). The resulting protein was separated on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with membrane blocking solution (Invitrogen) and probed with anti-human NF-κB and c-Jun antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. After washing, the membranes were incubated with secondary goat anti-rabbit antibody coupled with horseradish peroxidase (HRP) for 1 h at room temperature and developed with SuperSignal West Pico Chemiluminescent substrate (Pierce Chemical). The band intensities were expressed as a percentage of treated versus untreated cells.

Nuclear NF-κB and AP-1 activities were quantified using an ELISA-based TransAM kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, the nuclear protein extracts were added to wells of a 96-well plate that was immobilized with oligonucleotide containing NF-κB and AP-1. NF-κB and AP-1 contained in the nuclear extracts bound specifically to the oligonucleotide during incubation for 1 h at room temperature. NF-κB antibody (1:1000 dilution) and AP-1 antibody (1:500 dilution) were then added to each well for 1 h followed by HRP-conjugated secondary antibody for 1 h. After adding 100 µL of detection reagent for up to 15 minutes, the colorimetric reaction was stopped. NF-κB and AP-1 activities were determined by reading the absorbance on a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

Statistical analysis: All experiments were performed at least five times and produced comparable results. Results are presented as the Mean±SD. Statistical significance was determined using one-way Analysis of Variance (ANOVA) followed by a Dunnett's test for multiple comparisons to determine the overall effect of BV pretreatment on BEAS-2B cells. One factor repeated measure analysis was performed to determine the cytotoxic effect and cytokine production effect of BV (SPSS ver. 14.0; SPSS Inc., Chicago, IL, USA). A p-value of 0.05 or less was considered significant.

RESULTS

Cytotoxic effect of BV: In order to determine the optimal dose and treatment time of cytotoxic effect of BV on BEAS-2B, an established viability assay utilizing MTT was carried out. The cells were treated with BV at concentration of 0.03, 0.15, 0.5, 5 and 10 µg mL⁻¹ for 24,

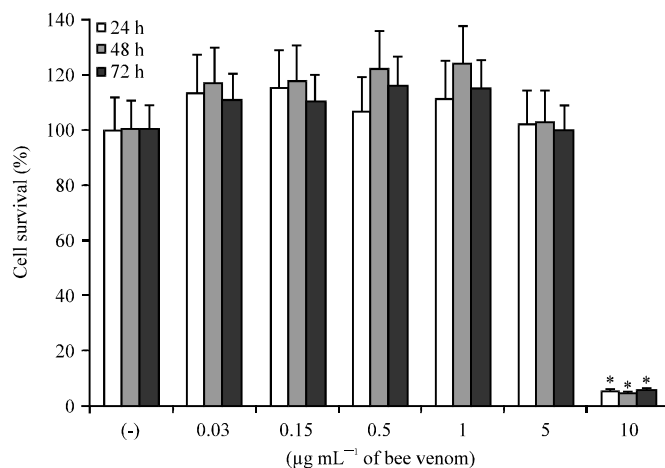


Fig. 1: Cytotoxic effect of bee venom (BV) on bronchial epithelial cells (BEAS-2B) at various concentrations and times with CellTiter-96[®] aqueous cell proliferation assay. Cell survival was significantly decreased at BV concentrations at 10 µg mL⁻¹, *p<0.05 compared to negative control, n = 5

48 and 72 h. The viability of cells treated with BV at a concentration of 10 µg mL⁻¹ were 5.8±2.3% for 24 h, 4.43±1.4% for 48 h and 5.8±2.2% for 72 h. Although the concentration of BV influenced the survival of epithelial cells, BV exposure time did not significantly influence the cell survival (Fig. 1).

Effects of BV on the stimulants induced cytokine production:

When the BEAS-2B cells were stimulated with *Alternaria* (IL-6; 413.7±117.1 pg mL⁻¹, IL-8; 2736.2±148.8 pg mL⁻¹ and GM-CSF; 383.3±149.2 pg mL⁻¹) and *Aspergillus* (IL-6; 506.4±142.7 pg mL⁻¹, IL-8; 2947.1±422.58 pg mL⁻¹ and GM-CSF; 452.6±133.3 pg mL⁻¹), cytokine production was significantly increased. IL-6 and IL-8 production were significantly inhibited by BV. However, GM-CSF production was not inhibited (Fig. 2). For example, 24.1-36.7% of *Alternaria* induced IL-6 production was inhibited by BV concentrations of 1, 0.5, 0.1 and 0.01 µg mL⁻¹ and 22.1-24.1% of *Alternaria* induced IL-8 production using BV concentrations of 0.1 and 0.01 µg mL⁻¹.

Effects of BV on the fungi induced expression and activation of NF-κB and AP-1:

When BEAS-2B cells were stimulated with *Alternaria* and *Aspergillus*, nuclear NF-κB expression was significantly increased for 24 h. The increased NF-κB expression was significantly inhibited by 0.1 and 0.01 µg mL⁻¹ BV. *Alternaria* induced NF-κB expression was inhibited at 3 and 24 h after stimulation and *Aspergillus* induced NF-κB expression was inhibited at 3, 6, 12 and 24 h. When the BEAS-2B cells were treated with fungi, AP-1 expression was not significantly increased (Fig. 3).

After stimulation with fungi for 24 h, NF-κB and AP-1 activations were evaluated in an ELISA-based assay kit using positive control (Jukat nuclear extract for NF-κB and K-562 nuclear extract for AP-1). *Alternaria* induced nuclear NF-κB activity was significantly inhibited when BEAS-2B cells were treated with BV. However nuclear AP-1 activity was not influenced by fungi and BV (Fig. 4).

DISCUSSION

Asthma is the lower airway inflammatory disease with airway hypersensitivity and airflow limitation. Their recent incidence, prevalence and severity have continuously increased. Although the etiology of asthma is complex and multifactorial, fungi play an important role in the pathogenesis of asthma. Severe asthma and acute exacerbation of asthma have been associated fungal sensitivity or increased concentration of fungi (Zureik *et al.*, 2002). Airborne fungi and their elements induce immune and inflammatory response from respiratory epithelial cells. The interaction of fungi with respiratory epithelial cell receptors, by protease-activated receptor or toll-like receptors, leads to the production of inflammatory chemical mediators, induction of respiratory burst and inflammatory cell recruitment.

In this study, *Alternaria* and *Aspergillus* were used to activate the bronchial epithelial cells, because these organisms are common pathogens found in respiratory secretions and in respiratory tract diseases. *Alternaria* and *Aspergillus* extracts have a high protease dependent activation and are highly potent in inducing cytokine production from upper and lower airway epithelial cells (Kauffman *et al.*, 1995; Shin and Lee, 2010). *Alternaria*

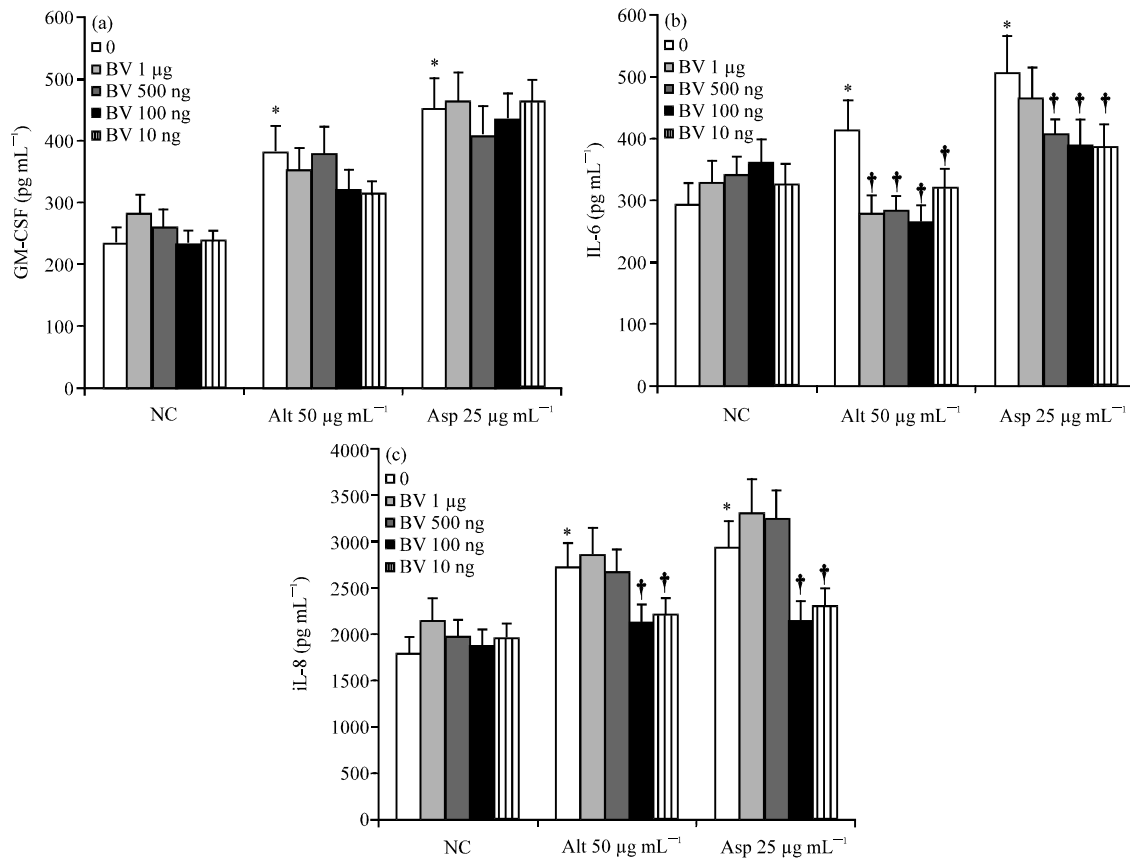


Fig. 2(a-c): Influence of bee venom on fungi induced cytokine production by bronchial epithelial cells (BEAS-2B). When BEAS-2B cells were treated with various concentrations (0.01, 0.1, 0.5 and 1 $\mu\text{g mL}^{-1}$) of BV, *Alternaria* 50 $\mu\text{g mL}^{-1}$ (Alt 50) and *Aspergillus* 25 $\mu\text{g mL}^{-1}$ (Asp 25) induced IL-6 and IL-8 production was significantly inhibited. NC: negative control, Alt: *Alternaria*, Asp: *Aspergillus*, BV: bee venom, * $p < 0.05$ compared with non-stimulated group † < 0.05 compared to *Alternaria* or *Aspergillus* stimulated groups, $n = 7$

and *Aspergillus* induced IL-6, IL-8 and GM-CSF production from BEAS-2B cells. These proinflammatory cytokine influence the pathogenesis and symptom development of asthma. The expressions of inflammatory mediators are regulated by the activation of protein kinase C, mitogen-activated kinase, NF- κ B and AP-1 (Fujioka *et al.*, 2004). When BEAS-2B cells were stimulated with fungi, cytokine productions were significantly increased with up-regulation of intranuclear NF- κ B. NF- κ B regulates the expression of cytokines, growth factors and effector enzymes in response to ligation of many receptors involved in immune responses. However, presently, AP-1 expression was not influenced by exposure to the fungi.

The data concerning transcription factor expression and activation in respiratory epithelial cells initially prompted the consideration, that NF- κ B might be the most

important factor associated with cytokine production. *Alternaria* induced NF- κ B expression was stronger than *Aspergillus* and continuously increased for 24 h. NF- κ B activity was significantly increased by *Alternaria* but not by *Aspergillus*. *Alternaria* might more strongly induce the production of chemical mediators and might be associated with the aggravation of respiratory symptoms.

BV has anti-inflammatory, anti-nociceptive and anti-cancer activities (Jung *et al.*, 2007; Han *et al.*, 2007; Lee *et al.*, 2001; Shin *et al.*, 2013). The anti-inflammatory effect is particularly well established and various mechanisms have been suggested. Peptides of BV have anti-inflammatory effects due to their ability to inhibit prostaglandin synthesis system or stabilize mast cells. BV also inhibits the activity of cyclooxygenase (COX-2) and expression of COX-2 mRNA in a dose-dependent manner,

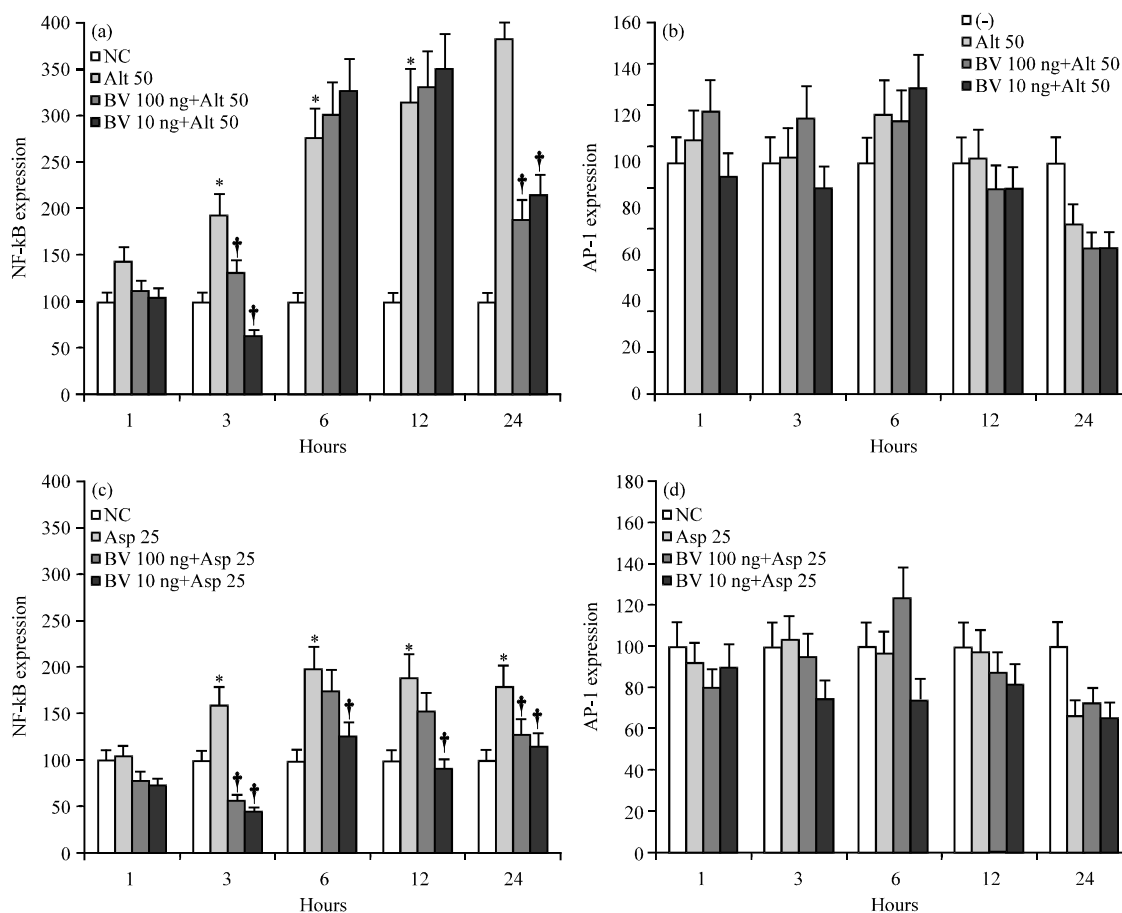


Fig. 3(a-d): Expression of NF-κB and AP-1 in bronchial epithelial cells (BEAS-2B) stimulated by *Alternaria* 50 μg mL⁻¹ (Alt 50) and *Aspergillus* 25 μg mL⁻¹ (Asp 25). NF-κB expression was significantly increased by fungi at various time intervals and Bee Venom (BV) significantly inhibited NF-κB expression. Although fungi did not influence the AP-1 expression, BV tended to inhibit AP-1 expression. NC: Negative control, *p<0.05 compared with non-stimulated group, †<0.05 compared to *Alternaria* or *Aspergillus* stimulated group, n = 5

without having any cytotoxic effects (Nam *et al.*, 2003). When BEAS-2B cells were pre-treated with various concentration of BV, the fungi induced production of IL-6 and IL-8 were significantly inhibited. The anti-inflammatory effect was stronger at lower concentrations of BV (10 and 100 ng mL⁻¹) than at higher concentrations (1 μg mL⁻¹ and 500 ng mL⁻¹). These results were similar to previous experiment with nasal epithelial cells (Shin *et al.*, 2013). Lower than 100 ng mL⁻¹ of BV had anti-inflammatory property on upper and lower airway inflammation. At higher concentrations of BV, enzymatic components that enhance inflammatory responses might more actively or strongly influence the immune response of bronchial epithelial cells. For the clinical use of BV, the optimal concentration that contains maximal anti-inflammatory effects without harmful or cytotoxic effects should be determined.

NF-κB and AP-1 are key transcription factors associated with induction and regulation of chemical mediators involved in inflammation. Although NF-κB and AP-1 transcription factors are regulated by different mechanisms, the signaling pathways leading to activation of NF-κB and AP-1 overlap, which suggests that they work cooperatively (Fujioka *et al.*, 2004). At lower concentrations, BV inhibited fungi induced IL-6 and IL-8 production and NF-κB expression and activity. These inhibitory effects are consistent with the decrease in the release of IκB detected in the cytosol through the suppression of IκB phosphorylation and the decrease in the translocation of the p50 subunit of NF-κB (Park *et al.*, 2007). This means that BV inhibits the DNA binding activity of NF-κB by inhibiting IκB phosphorylation, thereby preventing p50 translocation, resulting in a decrease in the expression of the inflammatory gene.

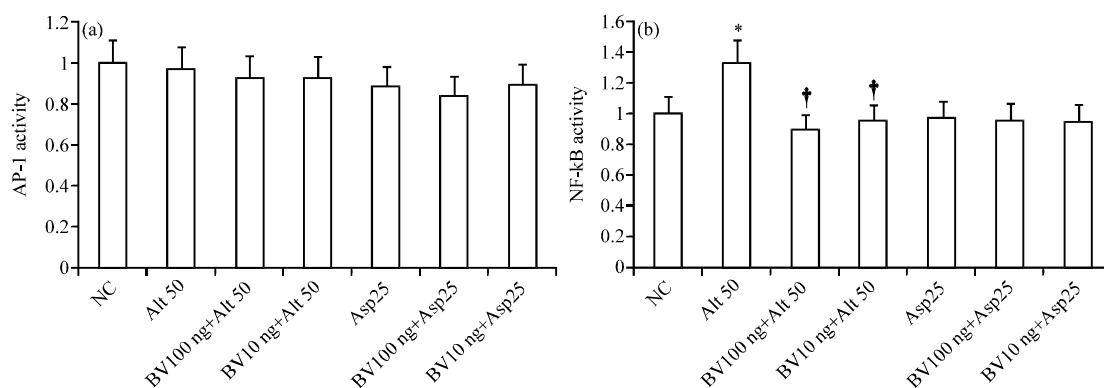


Fig. 4(a-b): Intensity of NF-κB and AP-1 activity in bronchial epithelial cells (BEAS-2B) stimulated by *Alternaria* 50 $\mu\text{g mL}^{-1}$ (Alt 50) and *Aspergillus* 25 $\mu\text{g mL}^{-1}$ (Asp 25). Bee venom (BV) significantly inhibited *Alternaria* induced NF-κB activation. AP-1 activity was not significantly changed with fungi and BV treatment. NC: Negative control, * $p < 0.05$ compared with non-stimulated group, † $p < 0.05$ compared to *Alternaria* stimulated group, $n = 5$

However, GM-CSF production was not inhibited with BV. Thus, NF-κB may be involved in the IL-6 and IL-8 release, with another transcription factor, such as mitogen-activated protein kinase, being associated with the production of GM-CSF. These results were similar to previous experiment with nasal epithelial cells (Shin *et al.*, 2013). Lower concentrations of BV inhibited intranuclear NF-κB expression and activity in upper and lower airway inflammation. Fungi did not influence the AP-1 expression; however, BV tends to suppress the AP-1 expression. Lee *et al.* (2009) suggested that BV abolishes the expression and translation of PKC- α , phosphorylation of MEK/ERK and expression and DNA binding activity of AP-1/NF-κB (Lee *et al.*, 2009).

CONCLUSION

The present findings elucidate the anti-inflammatory mechanism of activated bronchial epithelial cells with BV. *Alternaria* and *Aspergillus* can induce cytokine production from BEAS-2B cells and enhance the expression and activity of NF-κB. BV suppresses the production of cytokines, especially IL-6 and IL-8, with inhibition of the expression and activity of NF-κB. Although further study is needed for determination the more detail anti-inflammatory mechanism of BV, an optimal effect convention of BV and the effects in-vivo, BV contributes to the development of a new therapeutic strategy for airway inflammatory diseases.

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