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Original Article

Zanthoxylum Piperitum Attenuates the Allergic Inflammation *in vivo* and *in vitro*

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Natural products have received revived interest *via* traditional remedies or alternative medicine used for the treatment of various diseases. Zanthoxylum piperitum (ZP) has been utilized in traditional medicine for various medicinal purposes. The present study was conducted to evaluate whether ZP modulates allergic inflammation both *in vivo* and *in vitro*. We examined the pharmacological effects of ZP on 2, 4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD) symptoms in mice. Additionally, in order to clarify the anti-inflammatory mechanisms of ZP, we elucidate the effect of ZP on the expression levels of inflammatory cytokines and nuclear factor-κB (NF-κB) in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells (HMC-1). The results demonstrated that ZP attenuated AD clinical symptoms such as erythema, edema and dryness as well as histamine and IgE serum levels in DNCB-induced AD model mice. Additionally, ZP suppressed the expression of inflammatory cytokines and activation of NF-κB in AD-like skin lesions and stimulated HMC-1. These results provide experimental evidence that ZP may be useful candidate for treating allergic inflammation including AD.

Key Words: Zanthoxylum piperitum; Allergic inflammation; Inflammatory cytokines; Nuclear factor-κB; Mast cells

INTRODUCTION

Allergic inflammation plays a central role in allergic diseases and involves the action of multiple factors within a complex network (Hawiger, 2001). Recently, allergic inflammatory diseases have become a global health problem. Atopic dermatitis (AD), a chronic allergic inflammatory disease, is characterized by erythema, edema and severe pruritus (Waldman et al., 2018). Pathogenesis and progression of AD have been associated with a complex interrelationship between genetic, environmental, and immunologic factors as well as skin barrier dysfunction. Generally, AD is treated with corticosteroids (Berke et al., 2012), but long-term treatments can cause serious side effects such as immunosuppression and epidermal barrier dysfunction (Vatti et al., 2014). Consequently, there is a need to develop effective anti-atopic agents that cause fewer side effects.

Mast cells contribute to allergic inflammation, such as AD (Modena et al., 2016). Mast cells are important effector cells of IgE-mediated allergic inflammatory reactions and IgE levels are related to AD severity (Siraganian, 2003). In response to various stimuli, mast cells generate a variety of inflammatory cytokines including interleukin (IL)-6 and tumor necrosis factor (TNF)-α that contribute to the infiltration of immune cells to sites of inflammation (Trefzer et

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al., 2003). It was previously reported that mast cells are present in larger numbers in AD lesional skin. Therefore, the suppression of cytokine production by mast cells is a useful therapeutic strategy for allergic inflammation.

Nuclear factor-kappa B (NF-κB), transcription factor, regulates the transcription of numerous genes involved in allergic inflammation (Lee et al., 2020). Increased NF-κB activity associated with the secretion of high levels of IL-6 and TNF-α was shown to be involved in AD (Gilmore and Garbati, 2011). It was reported that inhibition of NF-κB activation diminished the influx of inflammatory cells and reduce the allergic inflammation (Birrell et al., 2005). These results have suggested the NF-κB activation is an attractive target for the treatment of allergic inflammatory diseases.

Traditional medicines are commonly used as complementary and alternative therapies for various diseases. Although traditional medicines have long been employed in effectively treating diseases, the pharmacologic mechanisms of them are not completely understood. Zanthoxylum piperitum (ZP) has been used in herbal medicine for various medicinal purposes including stomachic, toothache and anthelmintic problems (Choi et al., 2008; Park et al., 2008). Despite previous studies demonstrating the pharmacological effects of ZP, information on the pharmacologic mechanism of ZP on allergic inflammation including AD remains limited. The present study was conducted to evaluate the beneficial effects of ZP on 2, 4-dinitrochlorobenzene (DNCB)-induced AD symptoms in mice. Additionally, to find a possible explanation for the anti-allergic mechanisms of ZP, we investigate the effect of ZP on the expression of inflammatory cytokines as well as activation of NF-κB in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated human mast cells (HMC-1).

MATERIALS AND METHODS

Reagents

DNCB, PMA, calcium Ionophore A23187, avidin peroxidase (AP), dimethyl sulfoxide (DMSO) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iscove's Modified Dulbecco's Media (IMDM) was procured from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) and enhanced chemiluminescence kit were obtained from Thermo Fisher Scientific Inc. (Somerset, NJ, USA). ELISA kits human TNF-α/IL-6 and mouse TNF-α/IL-6/IgE was procured from BD Biosciences (San Diego, CA, USA). NF-κB and histone antibodies (Abs) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation of ZP

Dried roots of ZP were bought from the Human herb (Gyeongbuk, Korea). ZP (200 g) was pulverized into fine powder and 2 L of 70% aqueous ethanol solution was extracted at room temperature for 24 h and then concentrated under vacuum. The ethanol extract was next filtered, concentrated, and lyophilized (yield: 15.2%).

Animals

BALB/c mice (6 weeks, 19~20 g) were purchased from the Hyochang Science (Daegu, Korea). The animals were housed and allowed spontaneous intake of food and water ad libitum. Moreover, the animals were kept under a 12/12-h light/dark cycle at room temperature 24±2℃ and humidity 56±10%. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care, as described in the Daegu Haany university guidelines.

Induction of AD-like skin lesions

DNCB (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in vehicle (3:1 acetone:olive oil) and used as a sensitizer for inducing AD-like skin lesions in mice (Chan et al., 2013). The dorsal skin of mice was shaved with depilatory and gauzed a day before sensitization. Mice were randomized divided into 4 groups (n=7/group): vehicle, DNCB, and DNCB plus treatment of ZP (5 mg/kg) or DNCB plus treatment of ZP (50 mg/kg). Exposed skin was treated with vehicle or 200 μL of a 1% DNCB for 4 days. After sensitization, the dorsal skin was challenged with a 0.5% DNCB (200 μL) solution three times per week. This procedure was repeated for 3 weeks and ZP was orally administrated every day for 2 weeks.

Evaluation of skin dermatitis severity

The severity of dermatitis was evaluated according to the Eczema Area and Severity Index scoring system: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. The sum of the individual scores was defined as the dermatitis score for erythema/haemorrhage, edema, excoriation/erosion and scaling/dryness (Lee et al., 2010).

Histamine assay

Blood samples of mice were collected, and serum was separated by centrifugation at 4,000xg for 20 min at 4℃. Histamine concentration in the serum was measured using a specialized ELISA kit according to the manufacturer's instructions (Neogen, Lexington, USA).

Cell culture

HMC-1 was maintained in IMDM containing with 100 IU /mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37℃ in 5% CO_2 atmosphere at 95% humidity. HMC-1 was stimulated with of PMA (50 nM) plus A23187 (1 μg/mL).

MTT assay

To investigate cell viability by ZP, the MTT colorimetric assay was performed. Briefly, cells were incubated with ZP (0.05, 0.5 and 1 mg/mL) for 12 h and 50 μL of MTT (5 mg/mL) solution was subsequently added and was incubated for 4 h. Then, the crystallized formazan was dissolved in DMSO and the absorbance of plate was read at 540 nm.

Cytokine assay

The level of TNF-α and IL-6 was measured by modification of an enzyme-linked immunosorbent assay (ELISA) as previously described (Kim et al., 2010). Briefly, 96-well plates were coated with monoclonal Abs and incubated overnight at 4℃. After additional washes, sample or standard solution of TNF-α and IL-6 were added and incubated for 2 h. Plates were next exposed to biotinylated Abs was added and incubated for 2 h. After washing the plates, AP and ABTS substrate containing H_2O_2 was sequentially added. Color development was evaluated at 405 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Nuclear extracts were prepared by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Thermo Scientific, Rockford, USA). After bicinchoninic acid protein quantification, the supernatant was mixed with a sample buffer, separated by gel electrophoresis, and transferred to membranes. The membranes were then blocked with 5% skimmed milk and subsequently reacted with primary Abs. After washing, membranes were then incubated with secondary Abs for 1 h. After washing with 0.1% PBST, protein bands were visualized using an ECL detection system.

Luciferase reporter gene assay

Cells were transiently transfected with NF-κB-luc DNA and refreshed with completed media. The transfected cells were seeded in 6 plates overnight and treated with ZP before PMACI stimulation for another 2 h. Luciferase activity was determined using a Dual-Glo luciferase assay system kit (Promega, Madison, Wisconsin, USA) following the manufacturer's protocols.

Statistical analysis

Results are shown as the mean ± S.D and each experiment was completed at least three times. The statistical results were performed using an independent *t*-test and ANOVA with a Tukey post hoc test. $P < 0.05$ was considered significant.

RESULTS

Effect of ZP on AD symptoms in DNCB-induced AD-like skin lesions

To evaluate the therapeutic effects of ZP on AD symptoms, we used a mouse model of DNCB-induced AD. As shown in Fig. 1A, oral administration of ZP (5 mg/kg and 50 mg/kg) improved DNCB-induced the AD symptoms, such as erythema, edema and dryness. Moreover, we confirmed that the skin severity scores in the ZP treatment group were significantly lower than those in the DNCB-treated group (Fig. 1B). These results demonstrated that ZP possibly may

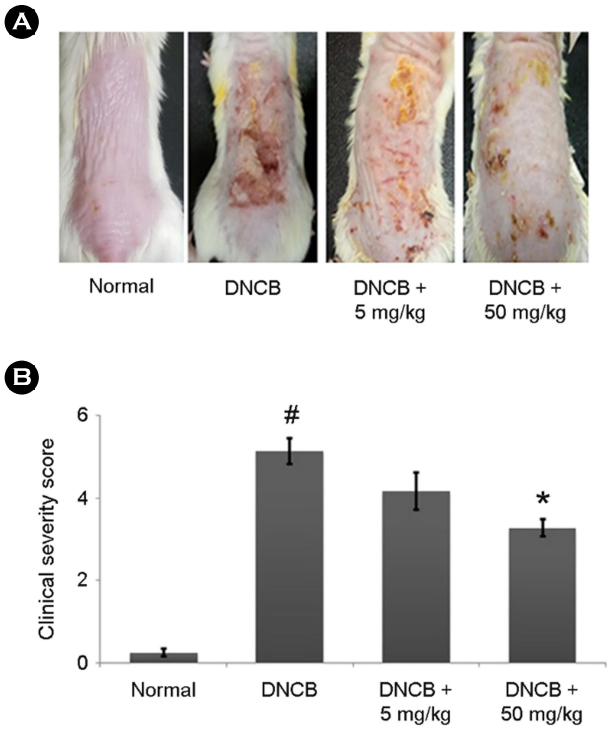

Fig. 1. Effect of ZP on DNCB-induced AD-like skin lesions in mice. (A) Clinical features of AD-like skin lesions. (B) Skin severity score is represented. Values are presented as mean ± SD of independent experiments ($^{\#}P < 0.05$ versus vehicle control group, $^{*}P < 0.05$; versus DNCB- treated group).

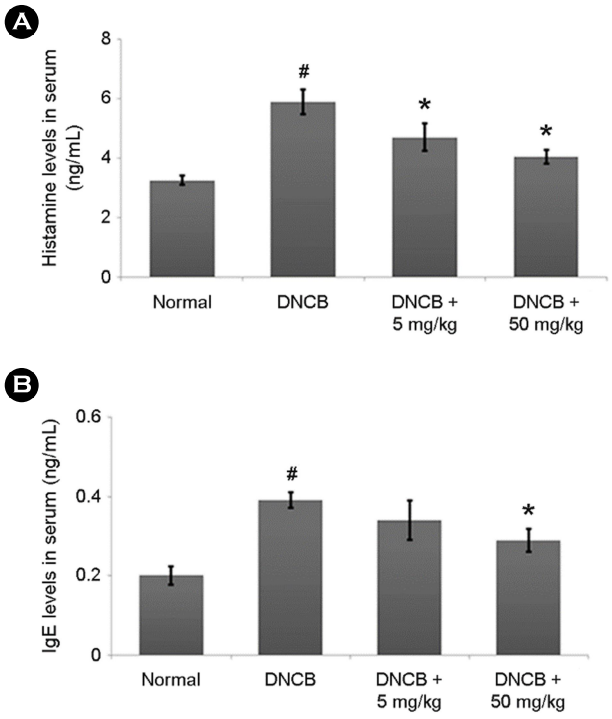

Fig. 2. Effects of ZP on histamine and IgE serum levels in DNCB-induced AD mice. (A and B) Blood samples in DNCB-induced AD mice were collected and then serum level of histamine and IgE was measured using ELISA assay kit assay according to the manufacturer's protocols. Values are presented as mean ± SD of independent experiments ($^{\#}P < 0.05$ versus vehicle control group, $^{*}P < 0.05$; versus DNCB- treated group).

have therapeutic activity by diminishing the clinical symptoms of AD.

Effect of ZP on histamine and IgE serum levels in DNCB-induced AD mice

It has been reported that serum levels of histamine and IgE are increased in AD patients and have been used as diagnostic tools and therapeutic targets (Gomez, 2019). Therefore, we investigated the inhibitory effects of ZP on serum histamine and IgE levels using ELISA. As shown in Fig. 2A and B, application of DNCB to mice resulted in increased levels of histamine and IgE in the serum. However, treatment with ZP reduced the histamine and IgE levels in the serum. The inhibition rates of histamine and IgE by ZP (50 mg/kg) were approximately 31.2% and 25.9%, respectively ($P < 0.05$). From this, we suggested that ZP exerts an anti-atopic effect by suppression of histamine and IgE serum levels in mice.

Effect of ZP on inflammatory cytokine levels in AD-like skin lesions and stimulated HMC-1 cells

Suppression of inflammatory cytokine levels is one of the most widely accepted treatment strategies for allergic inflammation including AD (Furue and Kadono, 2017). Thus, we examined the inhibitory effect of ZP on TNF-α and IL-6 levels in the AD-like skin lesion. At the end of the experiment, dorsal skin lesions were homogenized, and ELISA was performed. The results showed that the levels of TNF-α and IL-6 were significantly increased in skin tissues from DNCB-treated mice compared to that of control. However, administration of ZP decreased the DNCB-induced increased in TNF-α and IL-6 levels. The inhibition rates of TNF-α and IL-6 by ZP (50 mg/kg) were approximately 29.6% and 30.1%, respectively (Fig. 3A).

To investigate the anti-inflammatory activity of ZP, the human mast cell line, HMC-1, was employed in this study.

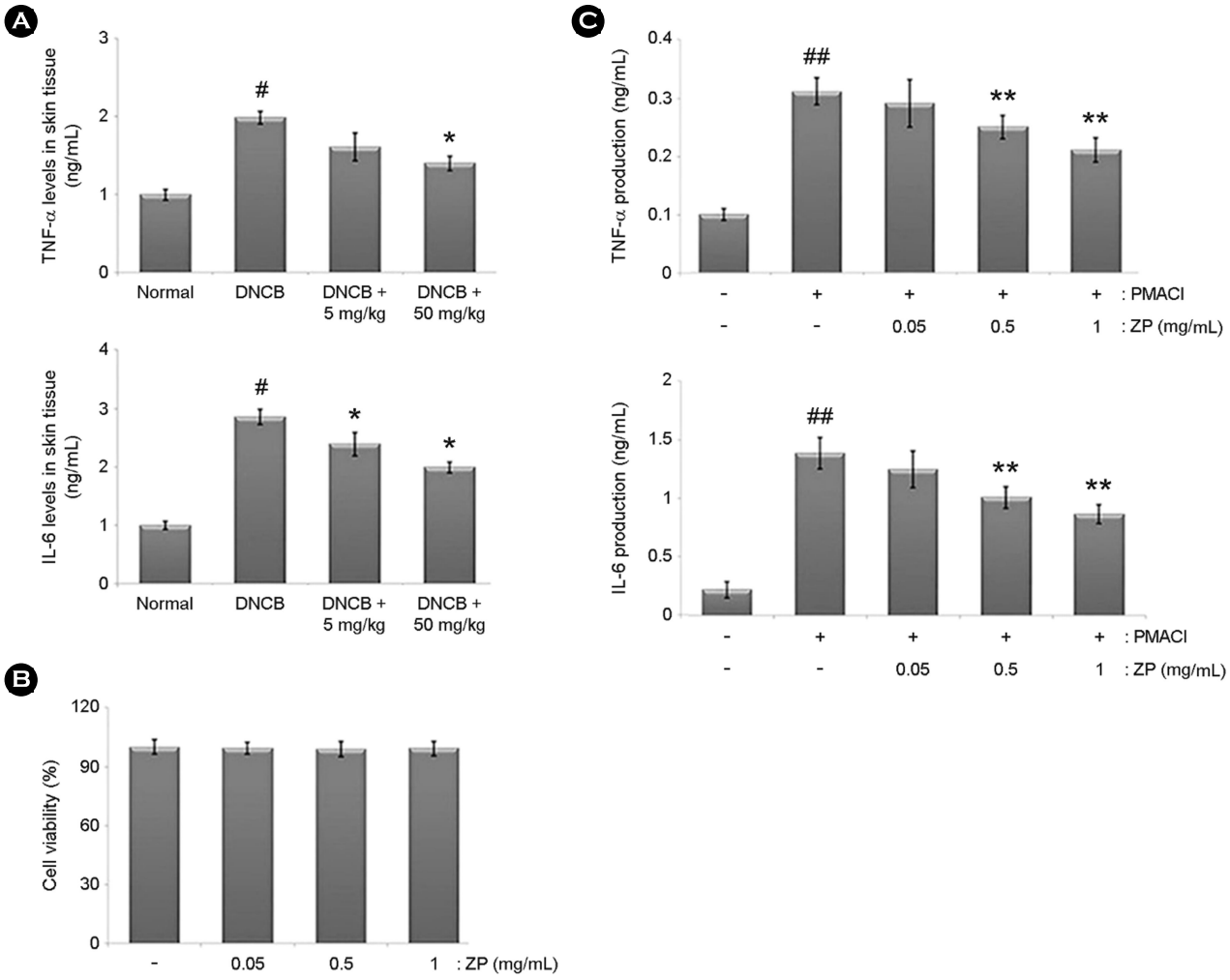

Fig. 3. Effects of ZP on inflammatory cytokines levels in AD-like skin lesion and stimulated HMC-1 cells. (A) At the end of experiment, dorsal skin lesions were cut out and homogenized. The level of TNF-α and IL-6 in the indicated groups was measured *via* ELISA assay according to the manufacturer's protocols. (B) Cells were pretreated with ZP (0.05, 0.5 and 1 mg/ mL) for 12 h. Cell viability was analyzed by MTT assay. (C) Cells were pretreated with or without ZP (0.05, 0.5 and 1 mg/ mL) for 2 h prior to stimulation with PMA (50 nM) plus A23187 (1 μg/mL) for 8 h. The levels of TNF-α and IL-6 in the indicated groups were measured *via* ELISA method. The results are presented as mean ± SD ($^{\#}P < 0.05$ vs. vehicle control mice group, $^{*}P < 0.05$ vs. DNCB- treated mice group, $^{\#\#}P < 0.05$ vs. control, $^{**}P < 0.05$ vs PMACI alone).

First, the cytotoxic effects of ZP were evaluated after treatment with various concentrations of ZP for 12 h using an MTT assay. No cell cytotoxicity by ZP was observed (Fig. 3B). Next, we evaluated the inflammatory effects of ZP on TNF-α and IL-6 production in PMACI-stimulated HMC-1 cells. Cells were pretreated with or without ZP (0.05, 0.5 and 1 mg/ mL) for 2 h prior to stimulation with PMACI for 8 h. As shown in Fig. 3C, PMACI alone markedly induced the secretion o TNF-α and IL-6 compared with the untreated control. However, pretreatment with ZP (0.05, 0.5 and 1 mg/ mL) suppressed TNF-α, and IL-6 production in PMACI-stimulated HMC-1cells in a dose-dependent manner. The maximal inhibition rates of TNF-α and IL-6 secretion by ZP (1 mg/mL) were approximately 32.2% ($P < 0.05$) and 37.4% ($P < 0.05$), respectively. These results demonstrated that ZP exerts anti-inflammatory effects *via* the inhibition of inflammatory cytokine levels.

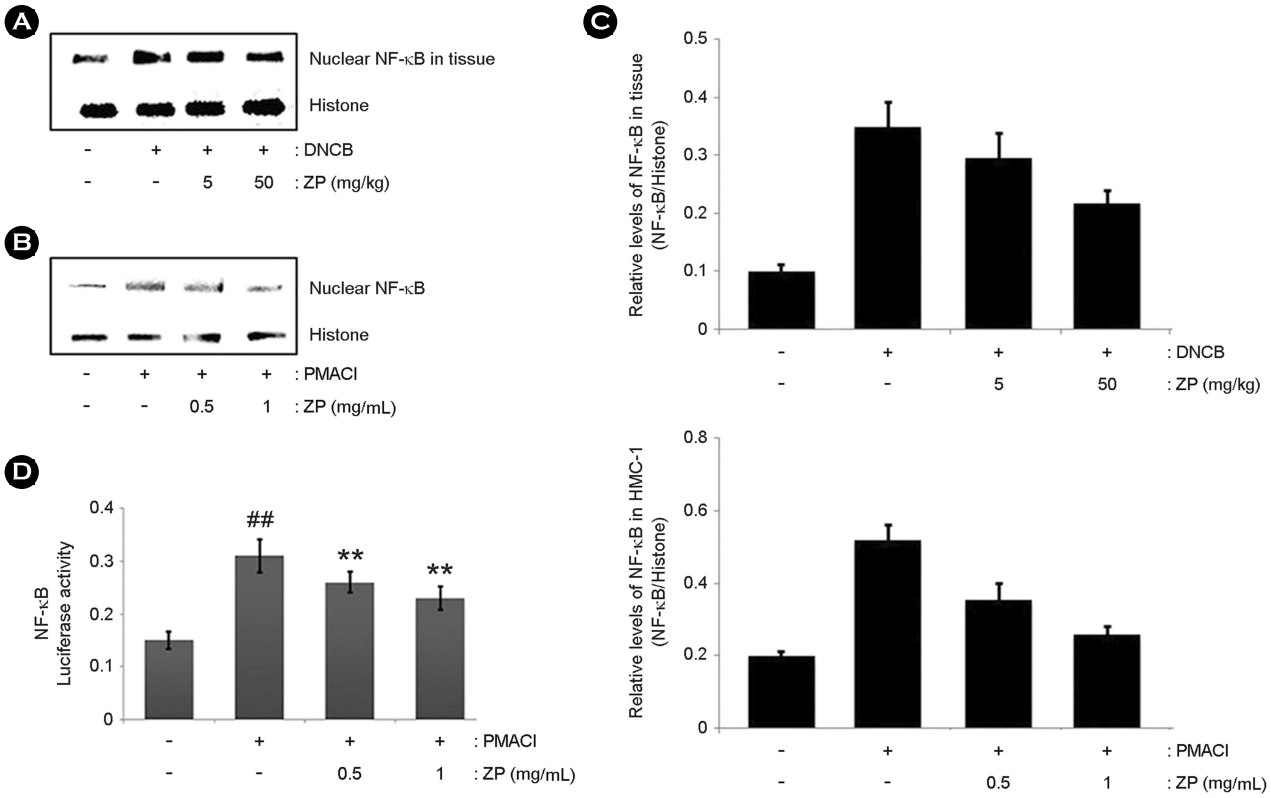

Fig. 4. Effect of ZP on NF-κB activation in AD-like skin lesion stimulated HMC-1 cells. (A) Nuclear extracts from skin tissue were prepared and the NF-κB levels in nucleus measured *via* western blot analysis. (B) Cells were pretreated with or without ZP for 2 h prior to stimulation with PMA (50 nM) plus A23187 (1 μg/mL) for 2 h. The level of NF-κB in nucleus was evaluated for RelA/p65 *via* Western blot analysis. (C) The relative levels of nucleus NF-κB (in AD-like skin lesion and mast cells) were represented. (D) Luciferase activity was determined using a luciferase assay system kit following the manufacturer's instructions. All results are expressed as the means ± SD ($^{\#}P < 0.05$ vs. vehicle control mice group, $^{*}P < 0.05$ vs. DNCB- treated mice group, $^{\#\#}P < 0.05$ vs. control, $^{**}P < 0.05$ vs PMACI alone).

Effect of ZP on NF-κB activation in in AD-like skin lesions and stimulated HMC-1 cells

As the NF-κB activation is associated with the inflammatory response, we theorized that the anti-inflammatory mechanism of ZP may be mediated *via* suppression of NF-κB activation. As activation of NF-κB requires the translocation of the NF-κB into nucleus (Nakamura et al., 2002), we evaluated the effects of ZP on the nuclear pool of NF-κB in AD-like skin lesion. As illustrated in Fig. 4A, we confirmed that the enhanced NF-κB activity in DNCB-induced AD-like skin lesions was down-regulated by treatment with ZP in a dose-dependent manner. Additionally, to determine the molecular mechanism of ZP, we evaluated the regulatory effects of ZP on PMACI-induced NF-κB activation. In PMACI-stimulated cells, the levels of NF-κB in the nucleus were increased, but ZP inhibited these enhanced nuclear levels of NF-κB in a dose-dependent manner (Fig. 4B). The relative levels of nucleus NF-κB (in skin tissue and cells) were represented in Fig. 4C. Moreover, we evaluated the effect of ZP on the promoter activity of NF-κB using luciferase reporter assay. As illustrated in Fig. 4D, ZP reduced the NF-κB-driven luciferase activity in PMACI-stimulated HMC-1 cells. From this, we demonstrated that the anti-inflammatory mechanism of ZP can attributed to the inhibition the activation of NF-κB in AD-like skin lesion and mast cells.

DISCUSSION

Recently, traditional medicines have received revived interest in terms of complementary and alternative therapies

in various diseases. Although ZP has been widely used as a traditional medicine, the precise mechanisms of the effects of ZP on allergic inflammation including AD has yet to be thoroughly elucidated. The findings of this study revealed that ZP reduced the clinical symptoms along with IgE and histamine serum levels in a DNCB-induced AD model. Additionally, we demonstrated that the anti-inflammatory effect and mechanism of ZP can attributed to the attenuation of inflammatory cytokine expression and NF-κB activation in AD-like skin lesion and stimulated HMC-1 cells. This result indicated an important molecular mechanism by which ZP ameliorates the allergic inflammatory reaction.

AD is a chronic inflammatory skin disease associated with pruritic, swelling and skin redness and affects the quality of life of patients (David et al., 2017). The clinical features of AD are infiltration of immune cells, epidermal hyperplasia, elevated serum histamine and IgE levels and increased inflammatory cytokines (Waldman et al., 2018). It has been reported that the pathogenesis of AD is thought to result from a multifactorial interaction of genetic, immune, and environmental factors. Generally, certain drugs including steroid, antihistamine and immunosuppressant are available for the treatment of AD. However, it cannot be administered long-term because of their deleterious side effects (Das and Panda, 2017). Thus, natural products have gained attention for relief of AD (Shiohara et al., 2004). Firstly, we tested the anti-atopic effects of ZP against DNCB-induced experimental AD progression in this study. Various biomarkers are employed to measure the severity of clinical symptoms of experimental AD. In particular, serum IgE and histamine levels are considered one of the crucial markers of AD as patients with it exhibit significantly increased serum IgE and histamine levels compared with healthy patients (Furue et al., 2017). In present study, we found that topical treatment of ZP significantly reduced AD symptoms such as erythema, edema and dryness in mice. Additionally, we observed that ZP suppressed DNCB-induced IgE and histamine levels in the serum. These results demonstrated that ZP possibly may have therapeutic activity by diminishing the clinical symptoms of AD along with serum IgE and histamine levels.

Several researchers have shown that inflammatory cytokines are related to the development of AD. Inflammatory cytokines highly express in activated mast cells and AD skin lesion, which induce allergic inflammation (Lim et al., 2018). It has also been reported that TNF-α and IL-6 levels are elevated in patients with AD and plays an integral role in AD pathogenesis (Fedenko et al., 2011). These results indicate that new biological therapies for AD should focus on suppression the inflammatory cytokines. Additionally, we examined the regulatory effect of ZP on intracellular signaling molecules involved in PMACI signaling pathways in HMC-1. Activated mast cells release an array of cytokines and chemokines with the potential to cause skin inflammation (Voisin and Chiu., 2018). These reports suggest that down-regulation of inflammatory cytokine from mast cells is necessary to successfully modulate AD. In this study, we showed that the levels of TNF-α and IL-6 were increased in AD-like skin lesions compared to those in controls and that ZP reduced these increased TNF-α and IL-6 levels in AD-like skin lesions. In addition, we demonstrated that ZP attenuated the release of TNF-α and IL-6 in PMACI-simulated HMC-1 cells. The inhibition rates of TNF-α and IL-6 by ZP (1 mg/mL) were approximately 32.2% and 37.4%, respectively. These results suggest that ZP exerts an anti-inflammatory effect by suppressing of TNF-α and IL-6 release from mast cells.

NF-κB regulates the transcription of numerous genes involved in allergic inflammation (Gilmore and Garbati, 2011). In inactive state (under normal condition), complexes of NF-κB/IκB is sequestered in the cytoplasm. During the inflammatory process, IκB kinase (IKK) complex phosphorylate and degrade the IκB protein (Huber et al., 2002). As a result, free NF-κB is translocate into the nucleus where it can combine the promoter of target genes and activate various inflammatory factors. Based on these results, suppression of NF-κB activation was identified as an anti-inflammatory strategy. Therefore, to identify the anti-inflammatory mechanism of ZP, we assessed whether ZP could suppress the activation of NF-κB. The results demonstrate that ZP inhibited NF-κB translocation into the nucleus in AD-like skin lesion and stimulated HMC-1 cells. We hypothesized that ZP exerts anti-atopic effects *via* the inhibition of NF-κB activation. Although ZP attenuated NF-κB activation, the effect of ZP on another pathway including MAPK-signaling was not

elucidated. Therefore, further studies are necessary to clarify the role of ZP on the other pathway in mast-cell mediated skin inflammation.

In conclusion, the present study demonstrated ZP might relieve AD clinical symptoms as well as IgE and histamine serum levels in a DNCB-induced AD model. Additionally, we demonstrated that the anti-atopic activity of ZP can attributed to the inhibition the expression of inflammatory cytokine and activation of NF-κB in AD-like skin lesion and stimulated mast cells. These results provide experimental evidence that ZP may be useful candidate for treating inflammatory skin diseases including AD.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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