



Inhibitory effects of polysaccharide-rich extract of *Phragmites rhizoma* on atopic dermatitis-like skin lesions in NC/Nga mice

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ABSTRACT

Aims: *Phragmites rhizoma* was reported to have anti-oxidative and free radical scavenging activity. It also has been traditionally used to suppress inflammation. In the present study, we aimed to evaluate the topical effects of the polysaccharide-rich extract of *P. rhizoma* (PEP) on atopic dermatitis.

Main methods: We induced AD-like skin lesions by an extract of the house-dust mite *Dermatophagoides farinae* (Dfb) in NC/Nga mice, and then performed macroscopic analysis, immunohistochemical staining and measurement of total serum IgE and cytokine production by ELISA.

Key findings: Topically applied PEP suppressed dermatitis with a decrease in dermatitis score and scratch number. The histological manifestations of atopic skin lesions including thickened epidermis and increased numbers of mast cells, polymorphonuclear leukocytes and nerve fibers were significantly attenuated. The activation of IgE and the levels of cytokines such as IFN- γ IL-4 and IL-10 were also decreased.

Significance: Our results indicated that PEP might have an inhibitory effect on atopic dermatitis-like lesion and be a promising natural resource in the treatment of atopic dermatitis.

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Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disorder characterized by a scaly and itchy rash. It is a major public health problem worldwide, occurring at rates of 10–20% in children and 1–3% in adults (Leung and Bieber, 2003). The prevalence of atopic dermatitis has increased in recent years, but the pathophysiology of this condition is only partly understood. Although the complex interrelationships between genetic, environmental, skin barrier, pharmacological, psychological and immunological factors contribute to the pathogenesis of AD, the immunological basis of the disease is of considerable importance and has been extensively studied (Leung, 2000). In AD, increased concentrations of cytokines secreted by activated Th2 cells, including interleukin-4 (IL-4), IL-5 and IL-13 activate host allergic response, ultimately up-regulate IgE synthesis in B cells (van Reijssen et al., 1992; Thepen et al., 1996; Homey et al., 2006). In

patients with chronic AD, the expression of IFN- γ , produced by Th1 cells has also been reported (Grewe et al., 1994; Werfel et al., 1996). Additionally, atopic dermatitis is characterized serologically by increased eosinophil counts and elevations in free IgE.

NC/Nga mice are a well-described animal model for atopic dermatitis; they exhibit many of the characteristic clinical traits of the disease, including chronic cutaneous xerosis and severe pruritus, as well as increased serum concentrations of IgE (Matsuda et al., 1997; Suto et al., 1999; Hashimoto et al., 2004). Notably, NC/Nga mice do not display these atopic dermatitis-like symptoms in specific-pathogen-free (SPF) conditions, and instead require antigen exposure to develop cutaneous immunologic hypersensitivity reactions.

Phragmites rhizoma has been used to purify polluted water in the wetlands (Lesage et al., 2007; Song et al., 2010). *P. rhizoma* was reported to have anti-oxidative and free radical scavenging activity (Kim et al., 1997). It was noted that *Phragmites communis* polysaccharides obviously increased the activity of catalase, superoxide dismutase, and glutathione peroxidase in the blood of aged mice (Miao et al., 2004). Although the medical use of *P. rhizoma* has been poorly studied, it has been traditionally used to suppress inflammation. Thus, we hypothesized that *P. rhizoma* polysaccharides might be effective against AD. The present study was performed to evaluate the inhibitory effects

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of polysaccharide-rich extract of *P. rhizoma* (PEP) on the development of AD-like skin lesions in NC/Nga mice by measuring symptom severity, scratching behavior, lymph node cell counts and levels of IgE, IFN- γ IL-4, and IL-10. Our finding is that PEP treatment attenuated the atopic dermatitis-like skin lesions in gross and histological examination. The effect of PEP might be associated with suppressing immunological and inflammatory response in atopic dermatitis.

Materials and methods

Preparation of PEP ointment

P. rhizoma was purchased from Woori Oriental Medicine Materials Co. (Seoul, Korea). Polysaccharide, the main bioactive component, was extracted with optimal techniques using hot water decoction (Miao et al., 2004; Zhang et al., 2007). In brief, sliced and dried raw materials of *P. rhizoma* were blanched at 100 °C for 4 h (43.3 g/l water), and then allowed to cool to room temperature. After cooling, the supernatant was applied to a SephadexG-150 (2.7 × 72 cm) column and bound materials were eluted with a linear gradient of NaCl (0.1 mol/l NaCl). The fractions containing carbohydrate were pooled and precipitated three times with ethanol. The resultant polysaccharide extract was freeze-dried to make a PEP powder. Carbohydrate concentration in the fractions was determined using the phenol-sulfuric acid method. Carbohydrate content of the final product was 85.6%, protein and nuclear acid contamination in the extract was negligible. To make an ointment formulation, the powder was mixed with distilled water and wax in 65 °C boiling water bath. The final concentration of PEP was adjusted to 5%.

Animals

Female five week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan) and kept under conventional conditions, including a 12 h light/12 h dark cycle and freely available food and water. The temperature of the colony room was maintained at 22–23 °C and humidity at 55 ± 15%. The mice were anesthetized with ether and the hair on the upper back was shaved with a clipper and a shaver. To induce atopic lesion formation, house-dust mite *Dermatophagoides farinae* (Dfb) ointment (100 mg) was applied topically to the shaved dorsal skin and both surfaces of each ear twice a week for 3 weeks. The Dfb-applied animals were then divided into 3 groups of 8 mice: group I was treated with the vehicle (control), group II was treated with PEP-containing ointment (experimental), and group III was treated with hydrocortisone cream (HCC, positive control). The PEP ointment and hydrocortisone cream were applied topically to exposed mouse back skin every other day for 3 weeks. Another group (non-induction) was not exposed to Dfb ointment for normal control.

All experimental protocols involving the use of animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication No. 85-23, revised 1996), and approved by the Animal Care and Use Committee of the Chung-Ang University of Korea.

Histopathologic analysis

The dorsal (upper back) skin specimens were collected from the experimental mice on the final day of the schedule, fixed in 10% formalin and paraffin embedded. The skin sections (2 μ m) were cut and stained with either hematoxylin–eosin (H&E) staining for evaluation of edema and polymorphonuclear leukocytes (PMN) number, or with toluidine-blue staining for detection of mast cells. In addition, rabbit anti-protein gene product (PGP) 9.5 polyclonal antibodies (Chemicon International, CA, USA) were used to stain nerve fibers.

The histopathologic scores were evaluated in the tissue sections with a microscope at a magnification of 400 $\times$.

Evaluation of the severity of dermatitis and observation of scratching

Skin lesion severity scores and scratching behaviors were evaluated five times per week for three weeks. To compare the skin lesions between mice, the severity of the dermatitis was scored using previously described macroscopic diagnostic criteria for human AD. Briefly, dermatitis severity was evaluated once per week by assessing for four specific criteria: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion. Each component was scored on a three point scale: 0 (none), 1 (mild), 2 (moderate) and 3 (severe), and the sum of the individual scores was used to denote overall dermatitis severity. The frequency of scratching behaviors such as scratching of the nose, ears and the dorsal skin with hind paws was measured in a 1 h period.

Total serum IgE

For measurement of total serum IgE, blood specimens were obtained from the retro-orbital sinus on days 0, 11, and 21. The concentration of total IgE in the serum was quantified using the sandwich ELISA method. Briefly, the total IgE level was determined using 100 μ l of rat anti-mouse IgE at 1 μ g/ml (BD Biosciences, San Jose, CA, USA) in 50 mM sodium carbonate buffer (pH 8.5) as a capture antibody and incubated overnight at 4 °C. The wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween (PBST) and then blocked with 200 μ l of blocking buffer [2% bovine serum albumin (BSA) in PBS, pH 7.2], for 1 h at 37 °C; subsequently, the wells were washed three times with PBST. Serum samples were diluted in blocking buffer (1:20) and incubated with the bound anti-IgE monoclonal antibody overnight at 4 °C. The plate was further washed with PBST three times and then 100 μ l of biotinylated rat anti-mouse IgE (Caltag Laboratories, Burlingame, CA, USA) was added to each well (diluted with blocking buffer, final concentration 2 g/ml), and the plate was incubated at 37 °C for 1 h. The plate was further washed with PBST and incubated with 100 μ l of avidin-conjugated alkaline phosphatase (1:3000) (Sigma, MO, USA) for 1 h and developed with p-nitrophenol phosphate (1 mg/ml) (Sigma) in 0.1 M di-ethanolamine buffer (pH 9.8) for 60 min at 37 °C. The plate was read after 60 min of development after terminating the reaction with 3 M NaOH. Absorbance at 405 nm was read using a microplate reader (Bio-Rad Laboratories, Inc., CA, USA). Purified mouse anti-IgE antibody (BD Biosciences) was used as a standard.

Total lymph node cell counting

To obtain axillary lymph node cell suspensions, mice were sacrificed by cervical dislocation, their lymph nodes were aseptically removed and pressed through a sterile nylon mesh. The cell suspensions were filtered and washed twice in RPMI 1640 medium (Sigma, MO, USA). The total cell count in the resulting lymph node cell suspension was measured.

Cytokine production

The cell suspensions were prepared at a concentration of 5 × 10⁶ cells/ml in RPMI1640 medium containing 10% heat-inactivated FBS, 100 U/ml penicillin (Mediatech, VA, USA) and 100 μ g/ml streptomycin (Mediatech). The lymph node cells were cultured with 10 μ g/ml of Dfb for 48 h at 37 °C under 5% CO₂. After the incubation period, the culture supernatants were collected for measurement of IFN- γ , IL-4 and IL-10 by ELISA using antibody pairs against mouse IFN- γ , IL-4 and IL-10 (BD Biosciences). The experiments were performed 6–8 times to measure the cytokine production.

Statistical analysis

All the data are presented as mean $\pm$ SEM. ANOVA (analysis of variance) and Fischer's exact test were used to determine the statistical significance of the differences.

Results

Effects of PEP ointment on Dfb-induced AD-like skin lesions in NC/Nga mice

To examine the effects of PEP on Dfb-induced AD-like skin lesions in NC/Nga mice, the mice were treated with PEP every other day for three weeks. Many of the classic cutaneous findings of AD – including erythema, bleeding, edema, desquamation, skin dryness, hair loss, abrasions and erosions – were observed in the NC/Nga mice after the AD-like syndrome was induced by Dfb ointments. In both the HCC group and the PEP group, the symptoms associated with AD improved significantly compared with the non-treated control mice (Fig. 1A). Skin lesion scores were in parallel with the results in the

gross examination. The skin lesion severity scores in each group were zero before the initiation of Dfb treatment, and three weeks after the start of treatment, application of PEP ointment or HCC resulted in significant inhibition of the development of skin lesions. Dermatitis score of the control mice was 6.7 ± 0.5 , while the score was 4.7 ± 0.2 ($P < 0.01$) in PEP-treated mice three weeks after the treatment (Fig. 1B).

Histopathologic examination revealed an amelioration of the hypertrophy and hyperkeratosis of the epidermis in both the PEP-treated and HCC-treated mice (Fig. 2A, B). In tissue sections stained with H&E or toluidine blue, the number of PMN and mast cells was significantly reduced in both the PEP- and HCC-treated mice, compared to non-treated control mice (Fig. 2A, C, D). Furthermore, infiltrated nerve fibers in the Dfb-induced AD-like skin lesions were decreased by treatment with PEP or HCC (Fig. 2A, E).

Effects of PEP on scratching behaviors and serum levels of IgE

At the start of the experiment, scratching behaviors were not significantly different between the PEP-treated and the control mice.

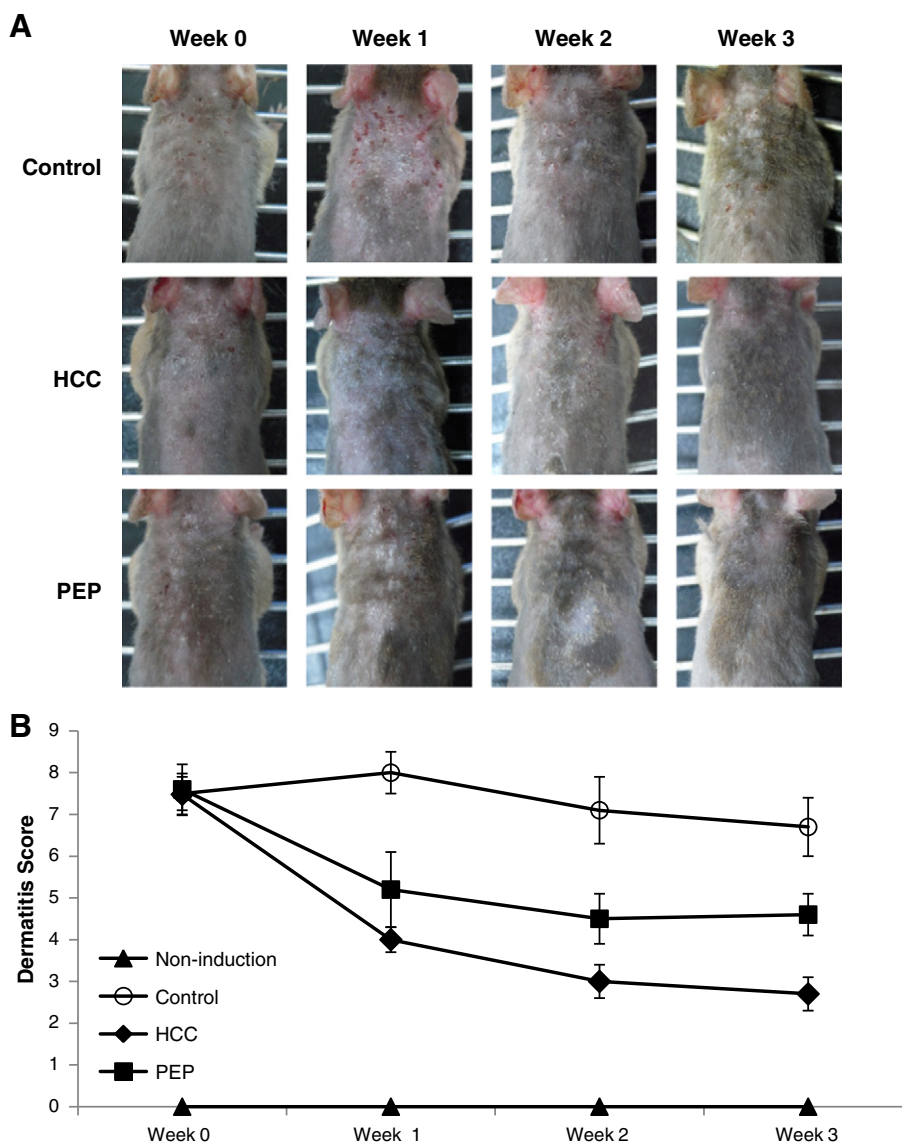


Fig. 1. Effects of PEP on the development of Dfb-induced AD-like skin lesions in NC/Nga mice during experimental periods. (A) Clinical observations indicated that treatment with PEP significantly reduced the severity of AD-like lesions in NC/Nga mice. (B) Dermatitis scores are the sum of individual scores based on the various AD symptoms. Non-induction: not exposed to Dfb ointment for normal control, HCC: hydrocortisone cream, PEP: polysaccharide-rich extract of *Phragmites rhizoma*.

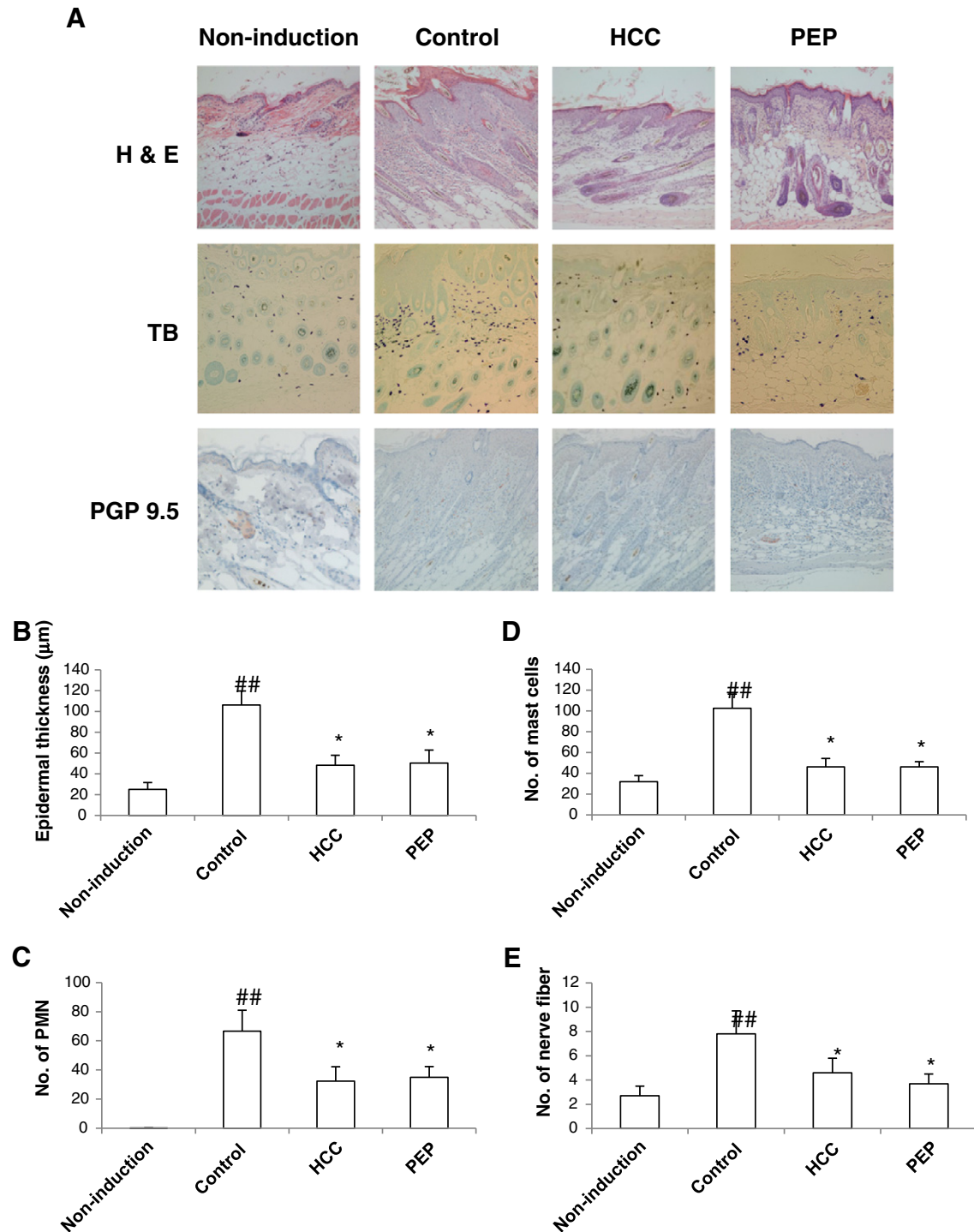


Fig. 2. Effects of PEP on histopathologic changes in the skin lesions in NC/Nga mice. The dorsal skin samples from the mice were collected on the final day of the protocol, and the sections were then stained with H&E staining, toluidine blue (TB) staining for mast cells, and immunohistochemical staining with anti-PGP 9.5 antibodies for nerve fibers. The PMN, mast cells and nerve fibers in the tissue sections were counted with a microscope at a magnification of 400×. Non-induction: normal, HCC: hydrocortisone cream, PEP: polysaccharide-rich extract of *Phragmites rhizoma*. ^{##} $P < 0.01$ vs. Non-induction; ^{*} $P < 0.01$ vs. control.

However, three weeks after the start of treatment, the PEP had resulted in significant suppression of the scratching behaviors compared with that of the control mice. The frequency of scratching behaviors in the PEP-treated group was $31.0 \pm 15.9/1$ h as compared with $381.4 \pm 112.3/1$ h in the control mice ($P < 0.05$) (Fig. 3A). During the three week treatment period, serum IgE levels were measured. PEP significantly decreased serum IgE levels in the treated mice as compared with the non-treated control mice (21.2 ± 2.1 ng/ml vs. 29.1 ± 2.6 ng/ml; $P < 0.01$) three weeks after the treatment (Fig. 3B).

Effects of PEP on lymph node size and levels of cytokines

As shown in Fig. 4A, the axillary lymph nodes of the Dfb-induced control mice were larger than those of the non-induced group. Decreases in the size of the lymph nodes were observed in both the PEP-treated and HCC-treated mice, compared with the control mice. Furthermore, the levels of IFN- γ , IL-4 and IL-10 in the PEP or HCC-treated mice were significantly lower than in the control mice (Fig. 4B–D).

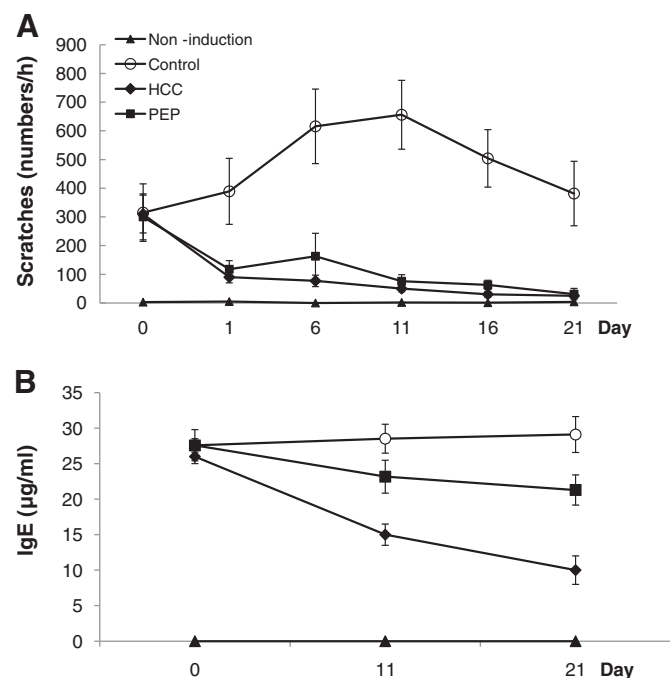


Fig. 3. Effects of PEP on scratching behaviors and serum IgE level. (A) Scratching behaviors were evaluated five times per week for three weeks. The frequency of scratching behaviors was measured in a 1 h period. (B) The concentration of total IgE in the serum was quantified three times over three weeks using the sandwich ELISA method. Non-induction: normal, HCC: hydrocortisone cream, PEP: polysaccharide-rich extract of *Phragmites rhizoma*.

Discussion

In this study, we investigated whether PEP attenuated the severity of AD-like skin lesions in Dfb-induced NC/Nga mice. AD-like skin lesions in NC/Nga mice highly resemble those of human AD, showing various grades of signs and symptoms such as erythema and hemorrhage, followed by edema, superficial erosion, deep excoriation, alopecia and skin dryness. NC/Nga mice with AD-like skin lesions also show immunological dysregulation such as IgE hyperproduction in serum, similar to immunological signs in human AD. Moreover, the anticipated histological manifestations do occur in AD-like skin lesions in NC/Nga mice such as thickened epidermis and increases in mast cells, polymorphonuclear leukocytes and nerve fibers.

The results indicate that topical treatment of PEP to NC/Nga mice produced a resolution of AD-like skin lesions. Itching is one of the major diagnostic criteria of AD, and scratching aggravates skin lesions (Koblenzer, 1999; Wahlgren, 1999). Therefore, effective control of itching and scratching is required in patients with AD. In humans, scratching the skin with the finger nails appears to be an important factor in progression to dermatitis by increasing the intensity of various immunological responses, such as serum IgE elevation and increases in the numbers of mast cells (Hashimoto et al., 2004; Mihara et al., 2004). The treatment of our mice with PEP inhibited scratching behavior and improved skin lesion severity resulting from hyper-scratching. Gross analysis showed severe edema, hemorrhage, acanthosis and excoriation in the control group, whereas PEP treatment prevented these skin changes. In addition, the total symptom severity scores were reduced by PEP treatment. Histologically, PEP treatment decreased hypertrophy, hyperkeratosis and infiltration of inflammatory cells in the skin.

Evidence pertaining to the immunomodulatory activity of polysaccharides derived from bacteria to animal origins is accumulating in recent years. Ginsan, an immunomodulatory polysaccharide from *Panax ginseng* has been shown to have critical effects on various immune cells (Lee et al., 1997; Shin et al., 2002; Song et al., 2002). *Ganoderma*

lucidum polysaccharides were reported to enhance immunity and antioxidant activities (Pan et al., 2013). Pacific oyster-derived polysaccharides enhanced Th1 immunity in vitro and in vivo (Cheng et al., 2012). Previous studies have reported that natural substances from plants or herbal therapy could inhibit the development of AD-like skin lesions in NC/Nga mice by modulating Th1 and/or Th2 cell response (Kotani et al., 2000; Gao et al., 2005; Sung et al., 2011). We thus checked the levels of Th2 cytokines such as IL-4 and IL-10 in cell suspension after PEP treatment, and found that the treatment of PEP significantly reduced IL-4 and IL-10 levels. IL-4 was not detectable in the serum of NC/Nga mice (data not shown) although the topical application of extracts from *Alnus japonica* was reported to reduce serum levels of IL-4 (Choi et al., 2011).

Atopic dermatitis seems to result from various factors including genetic polymorphism, dysregulation of immune cells, innate immune dysfunction and Th1/Th2 cytokine imbalance (Bohm and Bauer, 1997; Sicherer and Leung, 2011). Of these, the immunological hallmark of AD is a Th1/Th2 imbalance leading to IgE over-expression and hyperactivation. Several studies have reported that serum IgE levels are increased in NC/Nga mice with AD-like skin lesions (Matsuda et al., 1997; Kotani et al., 2000; Ohmura et al., 2004). The IgE binds environmental allergens and the allergen–IgE complex activates immune and inflammatory cell, which induces degranulation via intracellular signaling. In general, Th2 cytokines such as IL-4 and IL-10 upregulated IgE production (Lacy et al., 2009; Ko et al., 2011). The Th2-cytokine IL-4 is necessary for IgE synthesis. IL-10 is produced by Th1 and Th2 lymphocytes and increased levels have also been identified in peripheral blood mononuclear cells from atopic dermatitis (Lee et al., 2000).

In the present study, total IgE levels in the groups treated with PEP were significantly lower than in the control group. In general, IgE synthesis by B cells is primarily regulated by cytokines; Th2 cytokines such as IL-4 play a key role in the hyperproduction of IgE, whereas Th1 cytokines, especially IFN- γ , are strong inhibitors of IgE synthesis, Th2 cell proliferation and IL-4 receptor expression on T cells (Leung and Bieber, 2003). The reaction to high molecular weight environmental allergens (e.g. pollen, house dust mites), production of IgE and activation of eosinophil granulocytes result from Th2 dominance. Additionally, IL-4 inhibits the generation of Th1-cells (Bohm and Bauer, 1997). These results strongly suggest that PEP treatment can reduce the serum levels of total IgE by suppressing the Th2 cell response. Consistent with our results, it has also been reported that the elevated serum levels of total IgE correlate with the increased production of Th2 cytokines (Matsuda et al., 1997). However, further study is necessary to elucidate the exact mechanisms of the anti-itching effect of PEP in AD patients.

Conclusion

In the present study, topical treatment of PEP suppressed the aggravation of AD-like dermatitis in NC/Nga mice without any apparent side effects. Our results also suggest that the mechanism of the observed alleviation of the AD-like skin lesions in NC/Nga mice might be due to the suppression of scratching behaviors which are known as one of the most critical exacerbating factors for AD. It is expected that PEP might be an important alternative or complementary therapeutic options for the treatment of patients with AD.

Conflict of interest statement

None.

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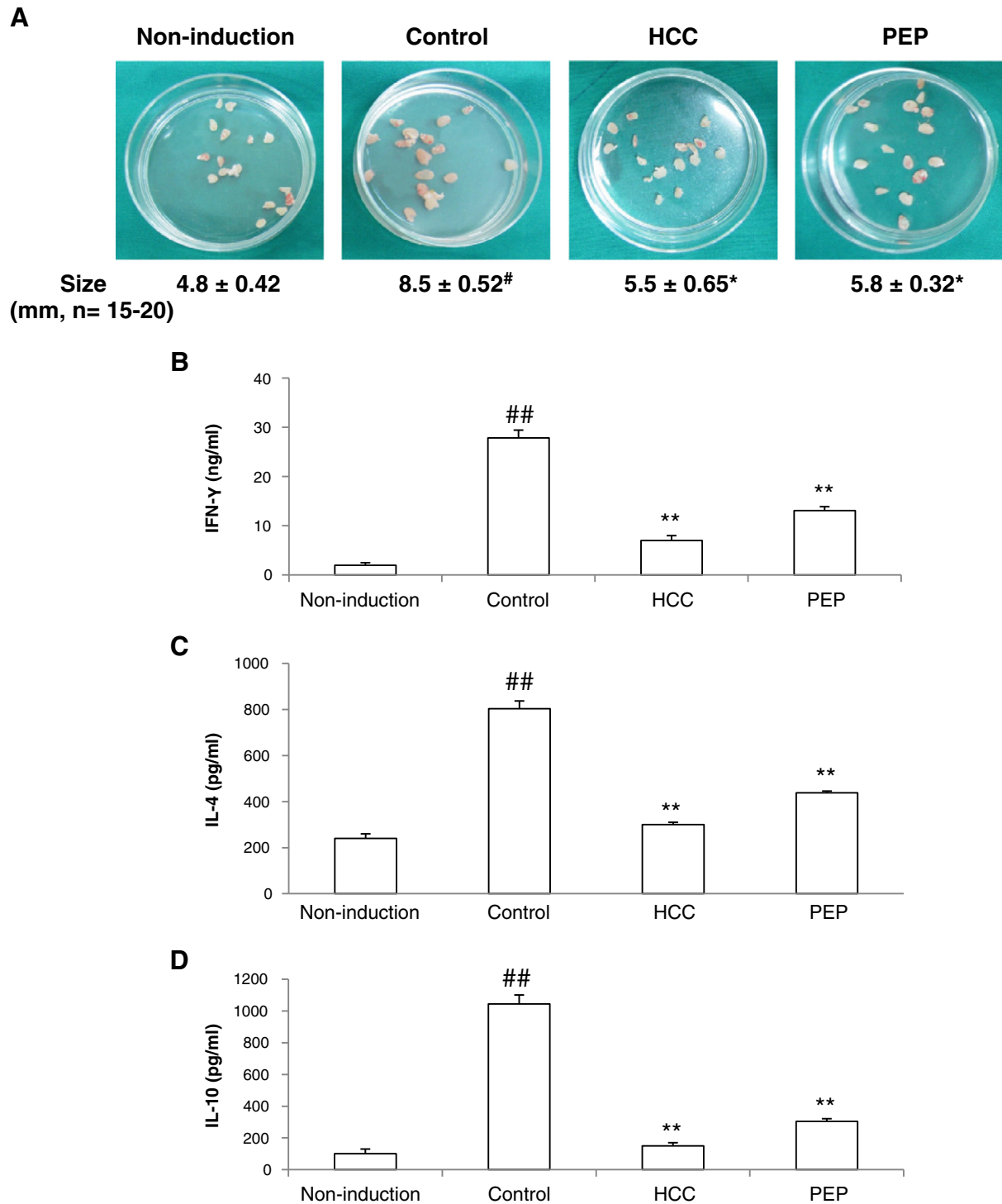


Fig. 4. Effects of PEP on axillary lymph node size (A) and levels of cytokines in cell suspension (B–D). The lymph nodes were aseptically removed and photographed (A). The lymph node cells were cultured with 10 µg/ml of Dfb for 48 h at 37 °C under 5% CO₂. IFN-γ (B), IL-4 (C) and IL-10 (D) were measured by ELISA. Non-induction: normal, HCC: hydrocortisone cream, PEP: polysaccharide-rich extract of *Phragmites rhizoma*. # P < 0.05, ## P < 0.01 vs. Non-induction; * P < 0.05, ** P < 0.01 vs. control.

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