



Inhibitory effect of *Psidium guajava* water extract in the development of 2,4-dinitrochlorobenzene-induced atopic dermatitis in NC/Nga mice

Jae Ho Choi^{a,1}, Bong Hwan Park^{a,1}, Hyung Gyun Kim^a, Yong Pil Hwang^{a,b}, Eun Hee Han^a, Sun Woo Jin^a, Jong Kwon Seo^c, Young Chul Chung^c, Hye Gwang Jeong^{a,*}

^a Department of Toxicology, College of Pharmacy, Chungnam National University, 220 Gung-dong, Daejeon 305-764, Republic of Korea

^b Department of Pharmaceutical Engineering, International University of Korea, Jinju 660-759, Republic of Korea

^c Department of Food Science, International University of Korea, Jinju 660-759, Republic of Korea

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ABSTRACT

Atopic dermatitis (AD) is a chronic, relapsing, and inflammatory skin disease associated with eczematous symptoms and IgE hyperproduction. *Psidium guajava* is an important food crop and medicinal plant with anti-oxidant, anti-inflammatory, and anti-allergic activities, supporting its traditional uses. Our previous studies have shown that *P. guajava* extract inhibits Th2 chemokine expression by suppressing the activation of NF- κ B and STAT1 co-stimulated with TNF- α and INF- γ . In this study, we investigated the inhibitory effect of *P. guajava* water extract (PGW) on 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in NC/Nga mice. Treatment of cream containing PGW onto DNCB-induced AD-like skin lesions in NC/Nga mice ameliorated lesion intensity scores, levels of IgE, thymus and activation-regulated chemokine (TARC), TNF- α , and IL-4 in serum and ears. In contrast, PGW increased level of the immunosuppressive cytokine IL-10. Histological analyses demonstrated decreased thickening of the epidermis/dermis as well as dermal infiltration by inflammatory cells. These results suggest that cream containing PGW may be a potential therapeutic modality for AD and adjunctive agent to control pruritus in AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing, and inflammatory skin disease in humans, caused by a complex interrelationship among genetic, psychological, immunologic and skin barrier dysfunction factors (Ikezawa et al., 2010; Niebuhr and Werfel, 2010). AD has been recently increasing in industrialized countries and onset is usually during early infancy and childhood, but can also occur in adulthood (Cooper, 1994; Rudikoff and Lebowitz, 1998).

The earliest event involved in the pathogenesis of AD is considered as a disruption of skin barrier function after scratch. Various chemokines and adhesion molecules are induced by these inflammatory cytokines in the site of injury and mediate infiltration and activation of leukocytes (Kaburagi et al., 2001; Gombert et al., 2005). AD is associated with the paradigm of an allergic T helper (Th) 2-mediated disease, characterized by abnormal IgE production, peripheral eosinophilia, mast cell activation, as well as Th2 and Th1 cytokine upregulation in chronic skin lesions (Wierenga et al., 1991; Neis et al., 2006).

Topical steroids, emollients and oral anti-histamines are used as the first-line therapy for AD, many patients are still worried about

long term use of these agents (Furie et al., 2003; Kobayashi et al., 2010). Therefore, there is a great need for the development of new and effective therapies for AD.

The *Psidium guajava* is grown in tropical and subtropical countries as a food and is also widely used in folk medicines. It is used for the treatment of inflammatory, diabetes, hypertension, wounds, pain, and fever (Gutiérrez et al., 2008). *P. guajava* extracts were shown to shift the Th1/Th2 balance to a Th1-dominant status by attenuating T cell activity (Seo et al., 2005). Our previous studies demonstrated that *P. guajava* ethyl acetate extracts (PGEA) inhibits IgE-mediated allergic responses by blocking Fc ϵ RI signaling (Han et al., 2011a,b). Also, we reported that PGEA inhibits Th2 chemokine expression by suppressing the activation of NF- κ B and STAT1 in Ha-CaT co-stimulated with tumor necrosis factor- α (TNF)- α and interferon- γ (INF- γ) (Han et al., 2011a,b). Although *P. guajava* extracts has been reported in various bioactivity assays, the effect of *P. guajava* extracts as an anti-atopic agent for AD remains unclear. In this study, we investigated the inhibitory effect of *P. guajava* water extracts (PGW) on the development of AD in NC/Nga mice.

2. Materials and methods

2.1. Chemicals

DNCB (2,4-dinitrochlorobenzene) was obtained from Sigma-Aldrich (Milwaukee, WI, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IgE, TARC,

* Corresponding author. Tel.: +82 42 821 5936.

E-mail address: hjeong@cnu.ac.kr (H.G. Jeong).

¹ The first two authors contributed equally to this work.

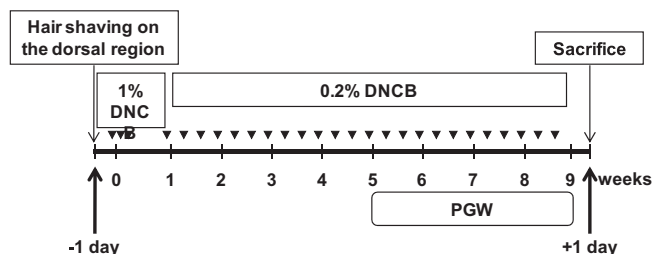


Fig. 1. Schematic diagram of the experimental protocol in mice. Mice were divided into four groups ($n = 5$ per group). To induce AD-like immunologic and skin lesions, DNCB was applied to the dorsal skin and ears. After complete removal of dorsal hairs within an area approximately 8 cm^2 , $200 \mu\text{L}$ of 1% DNCB was applied on the dorsal skin and ears for three consecutive days for sensitization. Four days after sensitization, the dorsal skin and ears were challenged with $200 \mu\text{L}$ of 0.2% DNCB solution 3 times per week for 8 weeks. After inducing AD, cream containing PGW was applied to the dorsal skin and ears of the mice 6 times per week for 4 weeks. Control and DNCB-treated mice were applied to the cream without PGW on dorsal skin and ears. Animals were sacrificed 64 days after the first application of DNCB.

and IL-10 were obtained from R&D Systems (Minneapolis, MN, USA) and BD Biosciences (San Diego, CA, USA). Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Daejeon, Korea). All chemicals and solvents were of the highest grade commercially available.

2.2. Preparation of *P. guajava* water extracts (PGW)

PGW was prepared from the air-dried leaves (130 g) of *P. guajava* with boiling water (250 ml) for 24 h, and used after freeze-drying.

2.3. Animals and treatment

Specific pathogen-free male 6-week-old NC/Nga mice were obtained from SLC, Inc. (Shizuoka, Japan). Animals were acclimatized to the temperature ($22 \pm 2^\circ\text{C}$) and humidity ($55 \pm 5\%$) of controlled rooms with a 12 h light/dark cycle for at least 2 weeks prior to experiments. Animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water. All experimental protocols for animal care were performed according to the rules and regulations of the Animal Ethics Committee, Chungnam National University. The mice were divided into four groups ($n = 5$ per group). To induce AD-like immunologic and skin lesions, DNCB was applied onto the dorsal skin and ears. After complete removal of dorsal hairs within an area approximately 8 cm^2 , $200 \mu\text{L}$ of 1% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) was applied for three consecutive days for sensitization. Four days after sensitization, the dorsal skin and ears were challenged with $200 \mu\text{L}$ of 0.2% DNCB solution 3 times per week for 8 weeks. After inducing AD, cream containing PGW was applied to the dorsal skin and ears of the mice 6 times per week for 4 weeks. Control and DNCB-treated mice were applied to the cream without PGW on dorsal skin and ears. Animals were sacrificed 64 days after the first application of DNCB (Fig. 1). Blood was collected from the vena cava, and the right ear was removed and used for histopathological examination.

2.4. Measurement of ear thickness

Ear thickness was measured with a micrometer (Mitutoyo, Kawasaki, Japan) on the day of sacrifice.

2.5. Histopathological studies

The right ear was removed from each mouse and fixed in 10% formalin solution. It was subsequently embedded in paraffin, sectioned ($4 \mu\text{m}$), and stained with hematoxylin and eosin. Histological changes were examined by light microscopy.

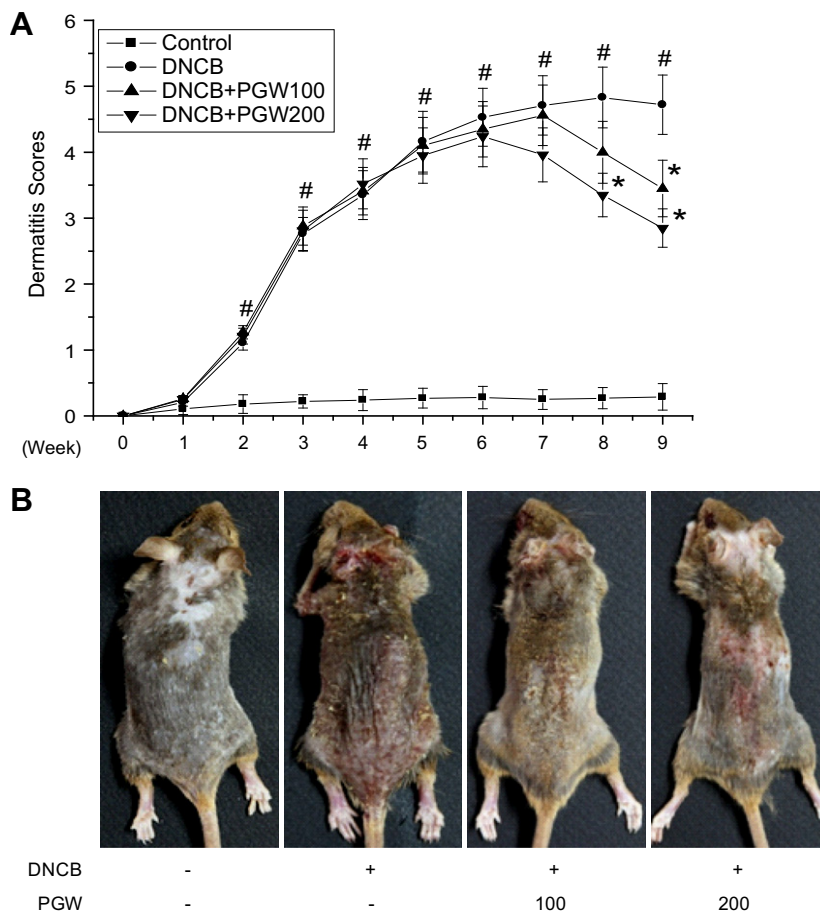


Fig. 2. Effects of PGW on DNCB-induced AD development in NC/Nga mice. (A) The dermatitis score was defined as the sum of scores for seven clinical criteria: erythema, hemorrhage, edema, excoriation, erosion, scarring, and dryness. (B) Example of the clinical severity of AD-like skin lesions. Results shown are representatives of five observations. Results are presented as mean $\pm$ SEM ($n = 5$). # $p < 0.05$, compared to the control group; * $p < 0.05$, compared to the DNCB-treated group.

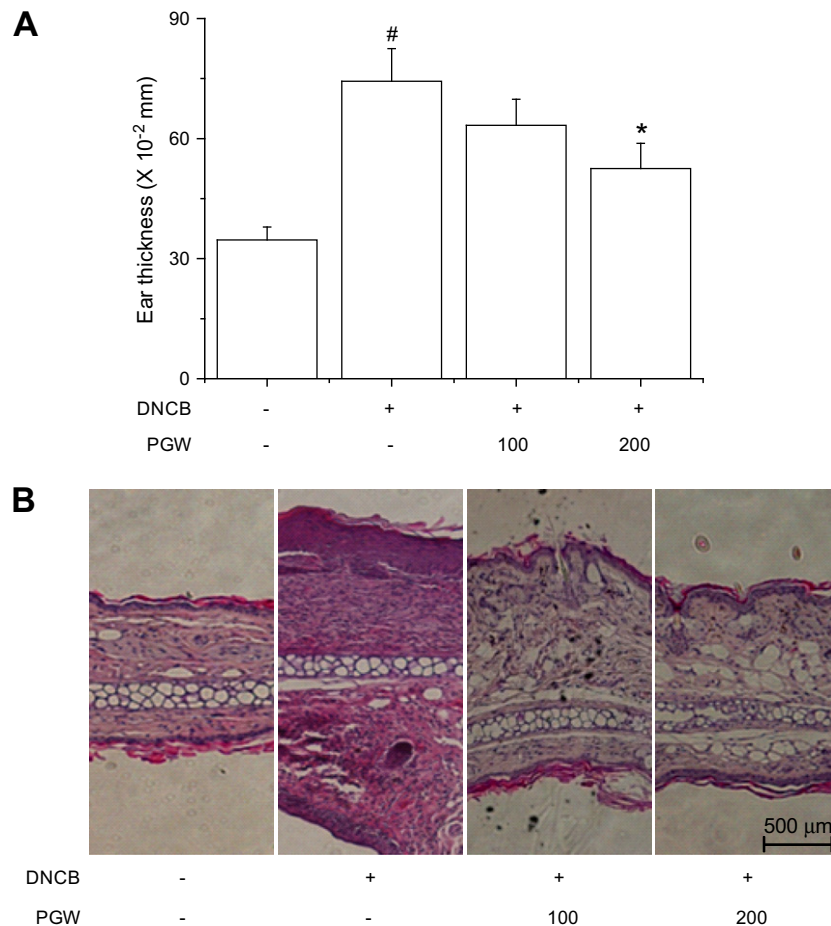


Fig. 3. Histological features of AD-like skin lesions treated with PGW in NC/Nga mice. (A) Ear thickness was measured with a micrometer. (B) Ears were removed and fixed with 10% formaldehyde solution. Thin sections (4 μ m) were cut and stained with hematoxylin and eosin. Magnification, 100 $\times$. Results shown are representatives of five observations. Results are presented as mean $\pm$ SEM ($n = 5$). [#] $p < 0.05$, compared to the control group; ^{*} $p < 0.05$, compared to the DNCB-treated group.

2.6. Evaluation of skin dermatitis severity

The severity of dermatitis in the dorsal skin and ear lesions was evaluated twice a week. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was defined as the dermatitis score (Matsuda et al., 1997).

2.7. Semi-quantitative reverse transcription (RT)-PCR analysis

Total RNA was extracted from left ear lesion using the RNAiso reagent (Takara, Kyoto, Japan), according to the manufacturer's protocol, and stored at -80°C until use. Then, 0.5 μ g RNA was used for reverse transcription and amplified by polymerase chain reaction (PCR) using the access RT-PCR system Takara thermal cycler (Takara, Seoul, Korea). The primer sets used in the PCR amplification were as follows: TNF- α (forward: 5'-CCT GTA GCC CAC GTC GTA GC-3', reverse: 5'-TTG ACC TCA GCG CTG AGT TG-3'); IL-4 (forward: 5'-GAA TGT ACC AGG AGC CAT ATC-3', reverse: 5'-CTC AGT ACT ACG AGT AAT CCA-3'); and GAPDH (forward: 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse: 5'-TCC ACC ACC TGT TGC TGT-3'). The PCR conditions for each target gene were as follows: TNF- α and IL-4: 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s; GAPDH: 28 cycles at 94°C for 60 s, 54°C for 60 s, and 72°C for 60 s. The termination cycle included a prolonged extension at 72°C for 7 min. The PCR products were separated on a 2% agarose gel containing ethidium bromide, visualized, and photographed using a gel documentation system (UVP, Cambridge, UK). Band intensity was quantified by densitometry using Image J software (Image Processing and Analysis in Java, NIH, USA). The relative expression levels of target genes were normalized using GAPDH as an internal control.

2.8. ELISA assay

Cytokines in serum level were measured by sandwich ELISA using the OptEIA Set mouse IgE kit from BD Biosciences, the DuoSet mouse IL-10, and the mouse CCL17/TARC kits from R&D Systems, according to the manufacturer's instructions. Cytokine concentrations were calculated using a linear regression equation obtained from standard absorbance values.

2.9. Statistical analysis

All experiments were repeated at least 3 times. Results are reported as means $\pm$ standard error of the mean (SEM). Statistical significance was determined by a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparisons test. A significant value was defined as $p < 0.05$.

3. Results

3.1. Effect of PGW on DNCB-induced AD-like skin lesions in Nc/Nga mice

The skin conditions were evaluated twice a week for 64 days using the dermatitis severity score. As shown in Fig. 2A, repeated topical application of DNCB increased clinical severity scores in NC/Nga mice. Furthermore, repeated application of DNCB in mice induced dermatitis symptoms of erythema, hemorrhage, edema, scarring, erosion, and ear swelling (Figs. 2B and 3A). However, the application of cream containing PGW inhibited these symptoms of AD in a dose dependant manner.

3.2. Effect of PGW on DNCB-induced AD-like histopathological changes in Nc/Nga mice

Fig. 3B shows the histopathological features of the ear lesions in DNCB-treated NC/Nga mice. On day 64 after the first application of DNCB, it was found that ear lesions showed hyperkeratosis, thickening of the dermis and epidermis, as well as accumulation of inflammatory cells in DNCB-treated mice compared with the

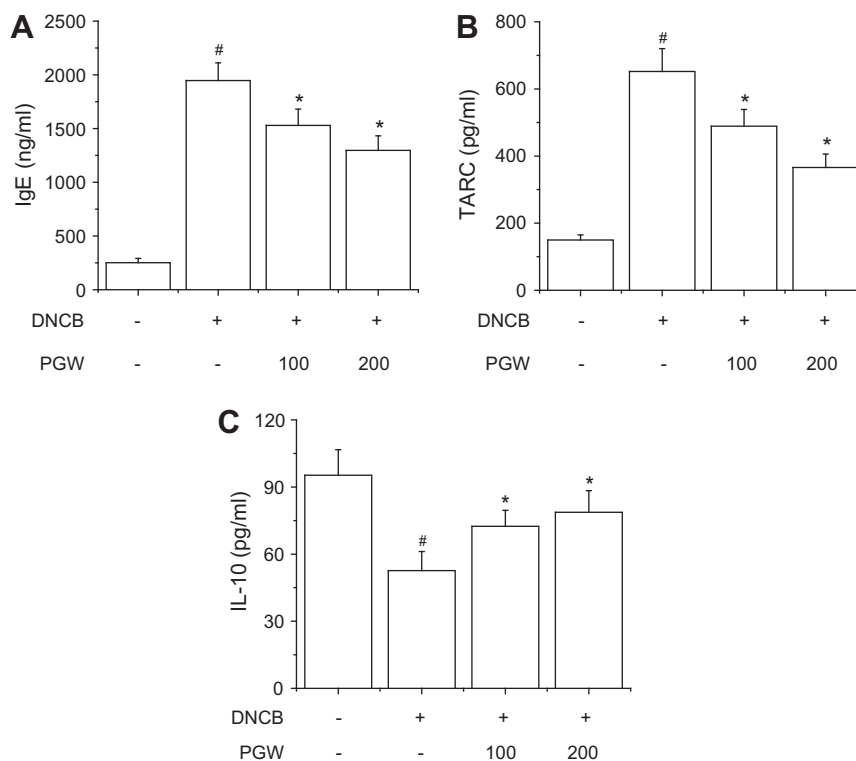


Fig. 4. Effects of PGW on DNCB-induced cytokines level in NC/Nga mice. Serum was collected 24 h after the last PGW treatment. Serum levels of (A) IgE, (B) TARC, and (C) IL-10 were analyzed by ELISA. Results are presented as mean $\pm$ SEM ($n = 5$). [#] $p < 0.05$, compared to the control group; ^{*} $p < 0.05$, compared to the DNCB-treated group.

control mice. These observations were all inhibited by application of cream containing PGW. These results indicate that PGW effectively decreases AD symptoms in NC/Nga mice.

3.3. Effect of PGW on DNCB-induced IgE and TARC release in NC/Nga mice

Mast cells are known as key effector cells in IgE-mediated allergic disorders. Upon activation, mast cells undergo degranulation and release a variety of biologically active substances such as IL-4, IL-5 and IL-13, which play an important role in the development of AD (Amin, 2012; Wesolowski and Paumet, 2011). An increase in serum IgE levels is an important component of AD. TARC is a chemokine involved in lymphocyte migration. It has chemotactic activity specific to type 2 helper T (Th2) cells in which the TARC receptor CCR4 is expressed. TARC is overproduced in the skin of patients with AD (Morita et al., 2010). Repeated application of DNCB increased serum IgE and TARC level in mice. Cream containing PGW reduced the serum levels of IgE and TARC (Fig. 4A and B). The function of IL-10 appears to be the prevention of extensive tissue damage after inflammation and infection (Ouyang et al., 2011; Akdis and Akdis, 2009). T-cell responses to allergens upon allergen-specific immunotherapy, high dose allergen exposure, and chronic viral infections have been shown to be suppressed by IL-10 signals (Akdis and Akdis, 2009; Boyman et al., 2007). As shown in Fig. 4C, DNCB reduced serum IL-10 level compare to control in mice. Cream containing PGW treatment restored the serum level of IL-10.

3.4. Effect of PGW on DNCB-induced cytokine expression in NC/Nga mice

We examined the effect of PGW on the mRNA expression of cytokines in the ears. The Th2 cytokine, IL-4, IL-5, and IL-13, is

expressed in acute stage of AD. The Th1 cytokine, TNF- α and INF- γ , is expressed in chronic stage of AD. Repeated application of DNCB markedly increased mRNA expression of Th1 and Th2 cytokines in mice. PGW inhibited the DNCB-increased Th1 and Th2 cytokines mRNA expression (Fig. 5). These results indicate that PGW treatment downregulated cytokine expression, leading to the inhibition of skin inflammation caused by the infiltration of inflammatory cells.

4. Discussion

AD is an increasingly common pruritic inflammatory skin disorder that affects at least 15% of children (Geha, 2003; Leung and Bieber, 2003). Research on the cause of AD is progressing and new treatments are being developed, but these agents have severe side effects, which limit their clinical applications. Recent anti-AD drugs make the use of natural agents. These agents include licorice, green tea, soybean, acai berry, turmeric and pomegranate (Fowler et al., 2010). Previous study reported that *P. guajava* leaf extracts showed a potent inhibition of histamine release from mast cells and blocked IL-10-mediated *in vitro* induction of T regulatory cells from CD4⁺ splenocytes of C57BL/6 mice (Seo et al., 2005). We also reported that PGEA has anti-allergic and anti-atopic effects. PGEA significantly inhibited IgE-mediated allergic responses by blocking Fc ϵ RI signaling (Han et al., 2011a,b). Also, PGEA inhibits TARC expression by suppressing the activation of NF- κ B and STAT1 in HaCaT co-stimulated with TNF- α and INF- γ (Han et al., 2011a,b). However, the effect of PGW on AD-like skin inflammation remained unclear. Here we examined the inhibitory effect of cream containing PGW on AD-like skin lesions induced by DNCB in NC/Nga mice.

Inducer of AD-like skin lesions was used to DNCB, TNCB, or mite. DNCB is structural similarity of TNCB. DNCB is the extensively studied animal model of AD based on the reproducibility

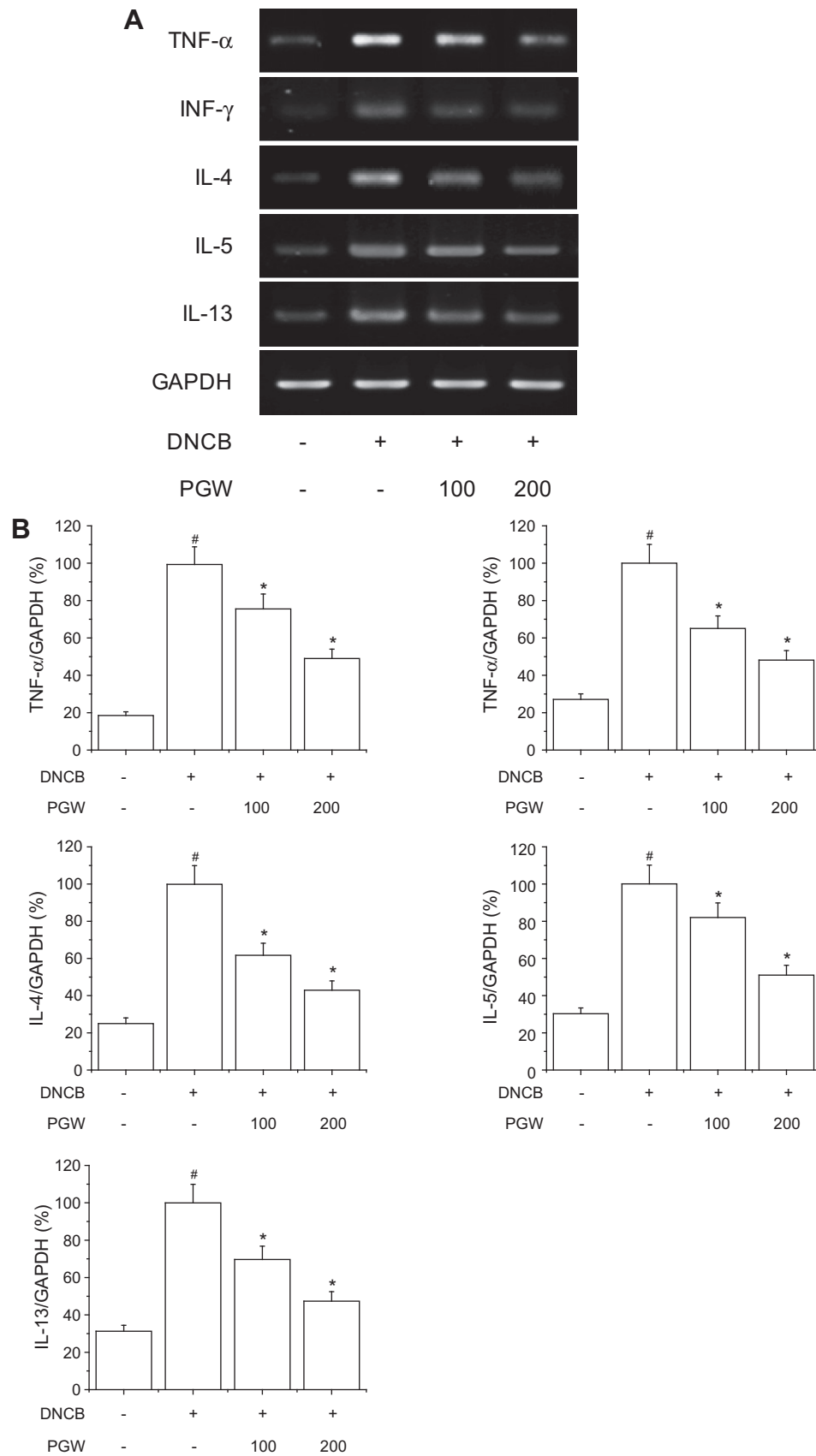


Fig. 5. Effects of PGW on DNCB-induced cytokines mRNA expression in NC/Nga mice. (A) Total RNA was extracted from ear tissue and mRNA levels of TNF- α , INF- γ , IL-4, IL-5, IL-13, and GAPDH were determined by semi-quantitative RT-PCR. Results shown are representatives of five observations. (B) The intensity of PCR bands was measured using the NIH Image J program. Results are presented as mean $\pm$ SEM ($n = 5$). [#] $p < 0.05$, compared to the control group; ^{*} $p < 0.05$, compared to the DNCB-treated group.

and repeatability (Yang et al., 2011; Fujii et al., 2009). Also, the pathogenesis of DNCB-induced contact hypersensitivity is predominantly the result of T cell-mediated immune responses (Zhang et al., 2009). DNCB induce AD, which is characterized by a skin disease with Th2-dominant allergic inflammation. Although Th2 cytokines are dominant in the acute phase, Th1 cytokines are expressed and contribute to the pathogenesis of AD in the chronic phase.

NC/Nga mouse is the most extensively studied animal model of AD based on the reproducibility and repeatability (Kwon et al., 2010). Experimental animal models of AD use DNCB treatment to simulate the clinical features of human AD-like skin symptoms, such as erythema, hemorrhage, and edema (Pokharel et al., 2008). Treatment with cream containing PGW suppressed the DNCB-induced development of AD-like skin symptoms in NC/Nga mice.

AD is an allergic disease that results from dermal inflammation, a hallmark feature of which is a breakdown in the immunologic balance between Th1 and Th2 cells (Oyoshi et al., 2009). Although the etiology and pathology of AD are not fully understood, recent studies suggest that typical symptoms of AD involve increased levels of Th2-mediated cytokines and a deficiency in Th1-derived cytokines. Thus, given the immunologic imbalance in AD, one of the goals of AD therapy is to manipulate the cytokine network to selectively promote the Th1 response or preferentially inhibit the Th2 response. Progression of AD is dependent on the production of the cytokines IL-4, IL-5, and IL-13 by allergen-specific Th2 cells that are generated from naive precursors (Kim et al., 2011).

Elevated IgE level is a hallmark of AD and the expression of IL-4 contributes to this elevation. IL-4 stimulates IgE production in B cells. IgE release from B cells bind to mast cells. Mast cells degranulate and release of various biological mediators in IgE-mediated AD (Kishimoto and Hirano, 1988; Poulsen and Hummelshoj, 2007; Amin, 2012). Several studies have demonstrated that serum IgE and TARC levels are elevated in patients with AD. In this study, PGW suppressed the DNCB-induced serum levels of IgE and TARC levels and mRNA expression of Th1 and Th2 cytokines in the ear lesions.

Moreover, PGW increased serum IL-10 level decreased by DNCB. IL-10 is an important mediator preventing allergic inflammation and AD-like skin lesions might be associated with suppression of regulatory cytokines. Interleukin (IL)-10 play a central role in the maintenance of peripheral homeostasis and the establishment of controlled immune responses in allergic inflammatory tissues. IL-10 induces an allergen-specific T-cell proliferation and suppresses Th1 and Th2 type cytokine secretion. IL-10 production were decreases severe AD as compared to normal controls (Pickard et al., 2007; Niwa, 2000). IL-10 inhibits IL-4 and IL-5 production by Th2 cells and TNF- α and INF- γ production by Th1 cells (Akdis and Blaser, 2001; Moore et al., 1993). These results indicated that IL-10 was an important factor in preventing allergic inflammation and suggested that AD lesions might be associated with suppression of regulatory cytokines expression.

T regulatory (Treg) cells as key regulators of immunologic processes in peripheral tolerance to allergens have reported an important cells in the prevention and treatment of allergic diseases (Akdis and Akdis, 2011; Jutel et al., 2006). Recently, many papers have shown that the T cells constitutively expressing CD4⁺CD25⁺, Treg cells, regulate AD (Stassen et al., 2004; Szegedi et al., 2009). Treg cells directly or indirectly suppress effector cells of allergic inflammation, such as mast cells, basophils, and eosinophils, and contribute to remodeling in AD. The increased level of IL-10 produced by Treg cells can potentially suppress IL-4, IL-5, IL-13, TNF- α , INF- γ , and IgE production. Therefore, Treg cells suppress DNCB-induced AD-like skin disease by PGW. Additional studies with PGW will be needed to clarify for the mechanism studies.

In conclusion, this study demonstrated that topical application of cream containing PGW suppressed the development of AD-like

skin lesions in NC/Nga mice. Downregulation of the Th1 and Th2 responses and upregulation IL-10 level may be the major mechanisms for the therapeutic effect. Clinical studies are required to determine whether PGW reduces serum IgE level and improves skin lesions. Our results indicate that PGW treatment could provide an effective alternative therapy for the management of AD.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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