



Effect of peiminine on DNCB-induced atopic dermatitis by inhibiting inflammatory cytokine expression *in vivo* and *in vitro*

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ABSTRACT

Peiminine (PMN) is the main component derived from *Fritillaria ussuriensis* and is used in traditional medicine in East Asia. The aim of this study was to evaluate the effects of PMN on atopic dermatitis (AD) induced by a dinitrochlorobenzene (DNCB) in Balb/c mice. Inflammatory cytokine expression of PMN was investigated *in vitro*. Eosinophil infiltration and the thickness of DNCB-induced AD mouse skin were measured. The levels of IgE, IL-4, IL-6, IL-13, and TNF- α in the serum were measured by ELISA. The effects of PMN on the transcription level of MAPK and nuclear factor (NF)- κ B were evaluated in mouse skin. In addition, the inhibitory effect of TNF- α , IL-1 β , COX-2 and PGE2 were measured in RAW264.7 cells; TARC was investigated in HaCaT cells; and β -hexosaminidase was examined in RBL-2H3 cells. PMN decreased the number of eosinophils in the dermis as well as mast cells and decreased the thickness of the epidermis and dermis. The PMN High group had a significantly reduced serum level of IgE, IL-4, IL-13 and TNF- α . Moreover, P-ERK and P-P38 were inhibited in the PMN High group compared with the DNCB-treated group. PMN additionally attenuated the expression of inflammatory cytokines in cells, including RAW264.7, HaCaT and RBL-2H3 cells. Our results suggest that PMN could be a potential therapeutic candidate for the treatment of AD.

1. Introduction

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease and is characterized by symptoms of pruritic, erythematous and excoriated papules, which are caused by high levels of histamines and inflammatory cytokines [1,2]. AD is a worldwide health problem, and 5–20% of AD patients throughout the world are children [3]. According to recent reports, severe AD can lead to impaired school performance, sleep disruption, stress and anxiety [4,5].

Most treatments for AD consist of corticosteroids, which are widely used in steroidal treatments and are effective; however, their long-term use could lead to severe side effects [6]. The side effects of corticosteroids in or on the skin are as follows: skin atrophy, flushing, hypertrichosis, capillary dilatation, acne and worsening of bacterial/fungal/viral skin infections [7]. There have been efforts to overcome the limitations of AD treatment, such as immunotherapy, T-cell inhibitors and antimicrobial treatments [8]; however, the pathogenesis and mechanism of AD are still poorly understood. Therefore, the development

of alternative pharmacological agents or compounds remains necessary.

Peiminine (PMN) is the major component of *Fritillaria ussuriensis* (FU, refers to various *Fritillaria* species, including *Fritillaria thunbergii* Miq.) [9,10]. FU has been used to reduce blood pressure or as an expectorant and painkiller in East Asian countries [11]. Moreover, FU has been reported to reduce blood pressure in rats [12] and to have anti-tussive, expectorant and anti-inflammatory effects [13].

Our previous study showed that FU and PMN had inhibitory effects on inflammatory cytokines in HMC-1 cells and on anaphylactic shock *in vivo* [14,15].

The aim of this study was to evaluate the effect of PMN in a dinitrochlorobenzene (DNCB)-induced AD-like skin disease model. We investigated the effect of PMN on the thickness and immune cell infiltration into the dermis and epidermis, cytokine level in mouse serum, and MAPK level in tissue in the AD model. Additionally, we studied the effect of PMN on inflammatory mediators in macrophage and human keratinocyte cell lines as well as on β -hexosaminidase in RBL-2H3 cells.

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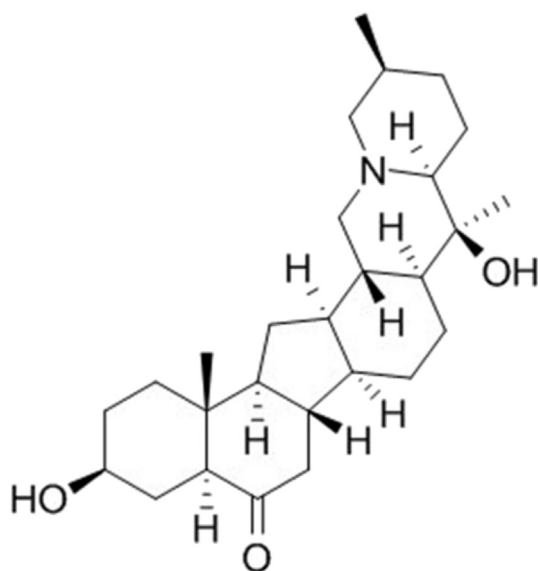


Fig. 1. The chemical structure of peiminine.

2. Materials and methods

2.1. Reagents

PMN (Purity > 98%, #ab143679) was provided by Abcam (Cambridge, UK). The molecular formula of PMN is $C_{27}H_{43}NO_3$ (Fig. 1), and its molecular weight is 429.645 g/mol. PMN was dissolved in DMSO (#d8418), with a final DMSO concentration lower than 0.05%. 2, 4-Dinitrochlorobenzene (DNCB, #237329) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Mouse IgE, IL-4, IL-6, and TNF- α ELISA kits were obtained from BD Biosciences (San Jose, CA). PCR primers were purchased from Genotech (Daejeon, Korea). Western blot antibodies, including phosphorylation-ERK (p-ERK, #4370), ERK (#4695), phosphorylation-P38 (p-P38, #4511), P38 (#9212), phosphorylation-JNK (p-JNK, #4668), JNK (#9258) and NF- κ B (#8242), were acquired from Cell Signaling (Danvers, MA). Peroxidase IgG (#31732) was obtained from Jackson ImmunoResearch (West Grove, PA, USA). Protease inhibitor cocktail (#P8340) and phosphatase inhibitor (#P0044, #P5726) cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM (#LM00-05) cell medium was purchased from Welgene (Daejeon, Korea). Fetal bovine serum (FBS, #16000-044), Penicillin/streptomycin (#15140-122) and Dulbecco's phosphate buffered saline (DPBS) were provided by Gibco (CA, USA).

2.2. Animals

Female BALB/c mice were purchased from the Nara Bio animal center (NARA Biotech, Seoul, Korea). These experiments were approved by the guidelines of the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP (SE)-16-075). The mice were maintained in a controlled environment with a temperature of $23 \pm 3^\circ\text{C}$, a relative

Table 1
Sequences of the primers for RT-PCR.

Name	Primer sequences
TNF- α	Forward: 5'-gca gaa gag gca ctc ccc ca-3' Reverse: 5'-gat cca tgc cgt tgg cca gg-3'
IL-1 β	Forward: 5'-ggc tgt gga gaa gct gtg gc-3' Reverse: 5'-ggg tgg gtg tgc cgt ctt tc-3'
COX-2	Forward: 5'-caa aag ctg gga agc ctt ct-3' Reverse: 5'-cca tcc ttg aaa agg cgc ag-3'
GAPDH	Forward: 5'-aag ttt ggc att gtg gaa gg-3' Reverse: 5'-aca cat tgg ggg tag gaa ca-3'

humidity of 40–60% and a 12 h light/dark cycle. Food and water were provided *ad libitum*. The 7-week-old female BALB/c mice were divided into four groups (n = 8 per group): Normal (Nor) group, DNCB-sensitized (Con) group, PMN 1 mg/kg (PMN Low) group, and PMN 5 mg/kg (PMN High) group.

2.3. Allergic dermatitis model and treatment protocols

For the first induction of AD in the BALB/c mice, the backs of the mice were shaved and challenged with 200 μ L of 0.5% DNCB in acetone/olive oil (3:1) for three days (day 1 - day 3). After 10 days from the first induction, 1% DNCB was painted onto the backs of the mice every three days (day 14, 17, 20, 23, 26 and 29). Concentrations of 1 mg/kg and 5 mg/kg PMN in 200 μ L of saline/olive oil (9:1) were applied to the dorsal skin once daily for 16 days. On day 30, all animals were sacrificed (Fig. 2).

2.4. Analysis of mouse blood

Blood was collected from each experimental group via cardiac puncture and was centrifuged at 2000 rpm and 4°C for 10 min to obtain the serum. The levels of IgE, IL-4, IL-6, IL-13 and TNF- α were measured with an enzyme-linked immunosorbent assay kit (Merck Millipore, #MTH17MAG-47 K-07, USA), following the manufacturer's instructions.

2.5. Histological analysis

The skin tissues of the mice were embedded in 10% neutral buffered formalin (NBF) for 24 h. After fixation, the skins were embedded with paraffin. The paraffin blocks were sectioned at a 5 μ m thickness. To measure the skin thickness and the number of eosinophils, the sections were stained with hematoxylin (sigma, #HHS32) and eosin (sigma, #e4382) (H & E). The number of mast cells were counted following toluidine blue staining. All sections were examined by light microscopy (Olympus, Tokyo, DP73), using 3–8 fields at 100- and 400-fold magnification.

2.6. Western blot

Frozen skins from the mice were homogenized in a HEPES buffer (containing 20 mM HEPES (pH 7.5), 1.5 mM MgCl_2 , 0.2 mM EDTA,

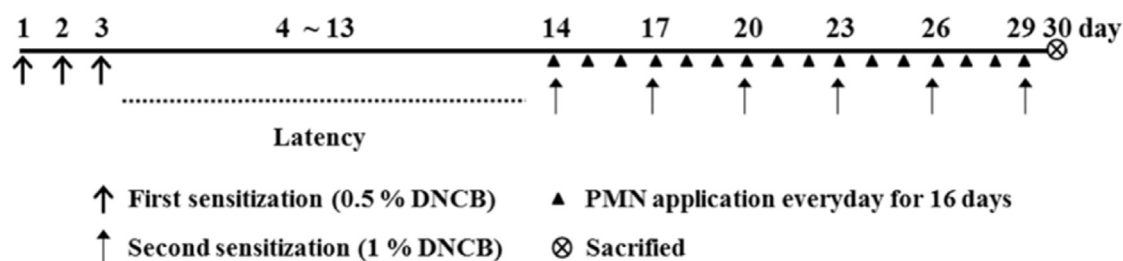


Fig. 2. Schedule for the induction of AD by topical administration of DNCB.

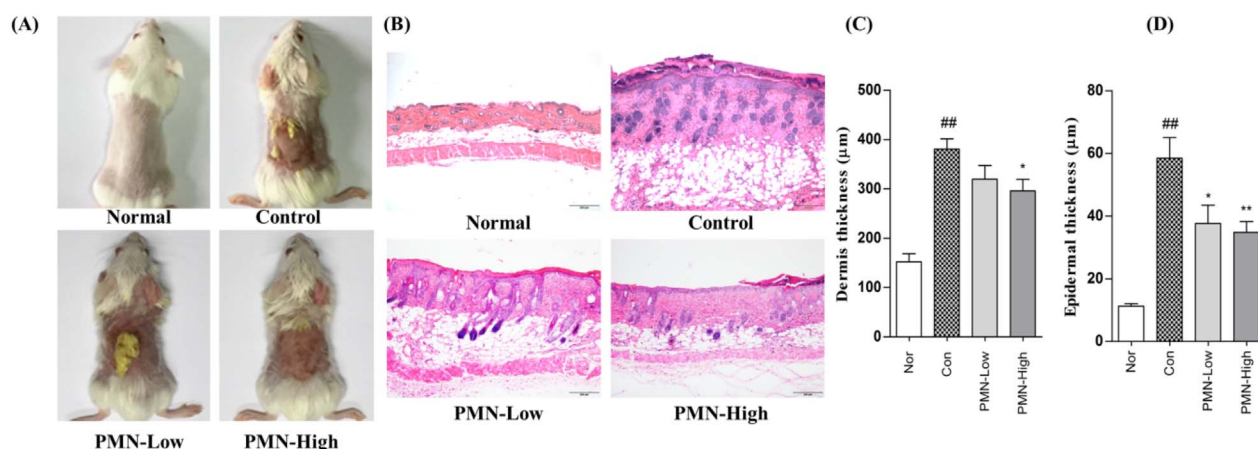


Fig. 3. (A) Image of a DNCB-induced mouse. (B) Representative sections of the epidermis and dermis by H&E staining (magnification $\times 100$, scale bar: 200 μm). (C) Measurement of the dermal thickness. (D) Measurement of the epidermal thickness. The sections were obtained from all mice of four groups ($n = 8$ per group): Normal (Nor) group, DNCB-sensitized (Con) group, PMN 1 mg/kg (PMN Low) group, and PMN 5 mg/kg (PMN High) group. The data are represented as the mean $\pm$ SEM. ## $p < .01$ compared with the normal and * $p < .05$, ** $p < .01$ compared with the control.

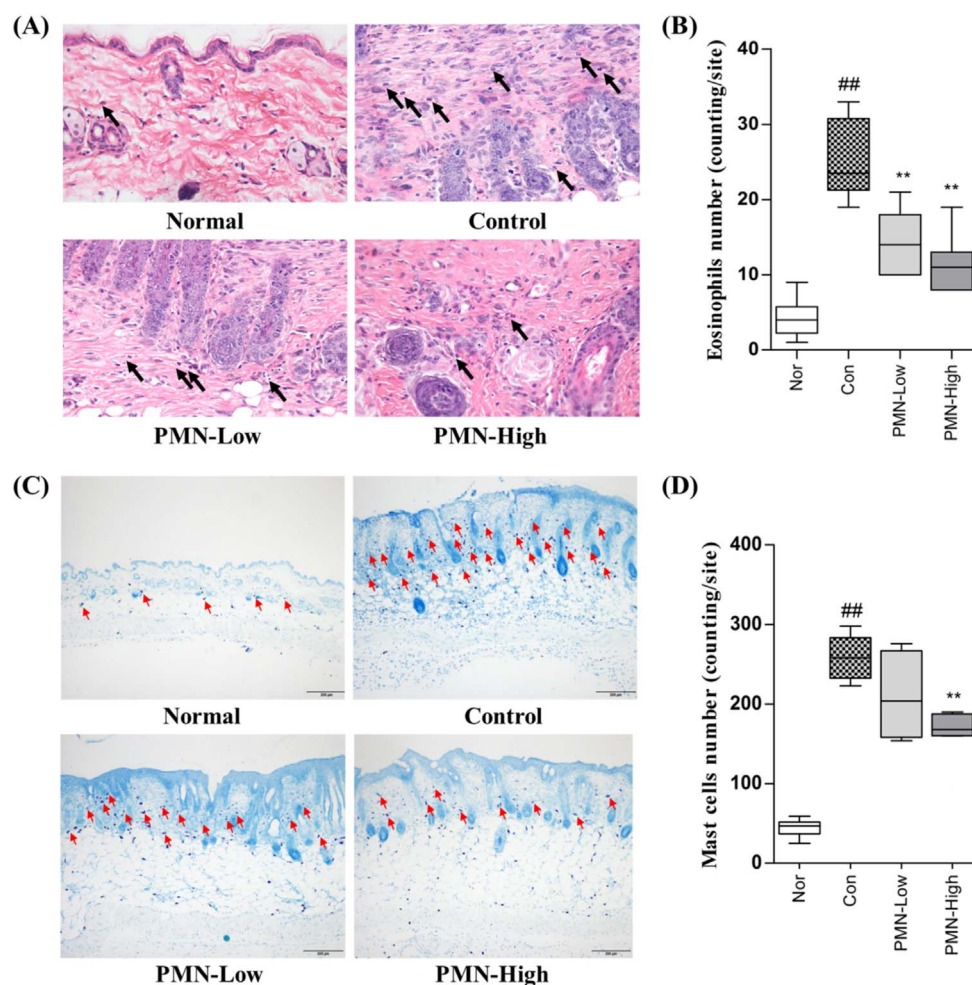


Fig. 4. (A) Infiltration of eosinophils into dermis lesions was examined by H&E staining (magnification $\times 400$). (B) The number of eosinophils that infiltrated into dermis lesions was observed in six high-power fields ($\times 400$) of each sections from all experimental mice. (C) Infiltration of mast cells was examined by toluidine blue staining ($\times 100$). (D) The number of mast cells were observed in three fields ($\times 100$) of each sections from all experimental mice. The data are represented as the mean $\pm$ SEM. ## $p < .01$ compared with the normal and ** $p < .01$ compared with the control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

0.1 M NaCl, 0.2 mM DTT, and 0.5 mM Na_3VO_4). The samples were centrifuged for 10 min at 4 °C, and the supernatants were obtained. The nuclear proteins were lysed and purified using the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce, #78833 Rockford, IL, USA). The proteins were separated by 10% SDS-PAGE gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was kept for 1 h at room temperature and then incubated overnight at 4 °C with a primary antibody (NF- κB 1:500 or MAPK 1:1000,

diluted in TBS-T including 1% albumin). The membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h at room temperature. The antibody-specific proteins were visualized with an enhanced chemiluminescence (ECL) detection reagent and quantified using the ImageJ software.

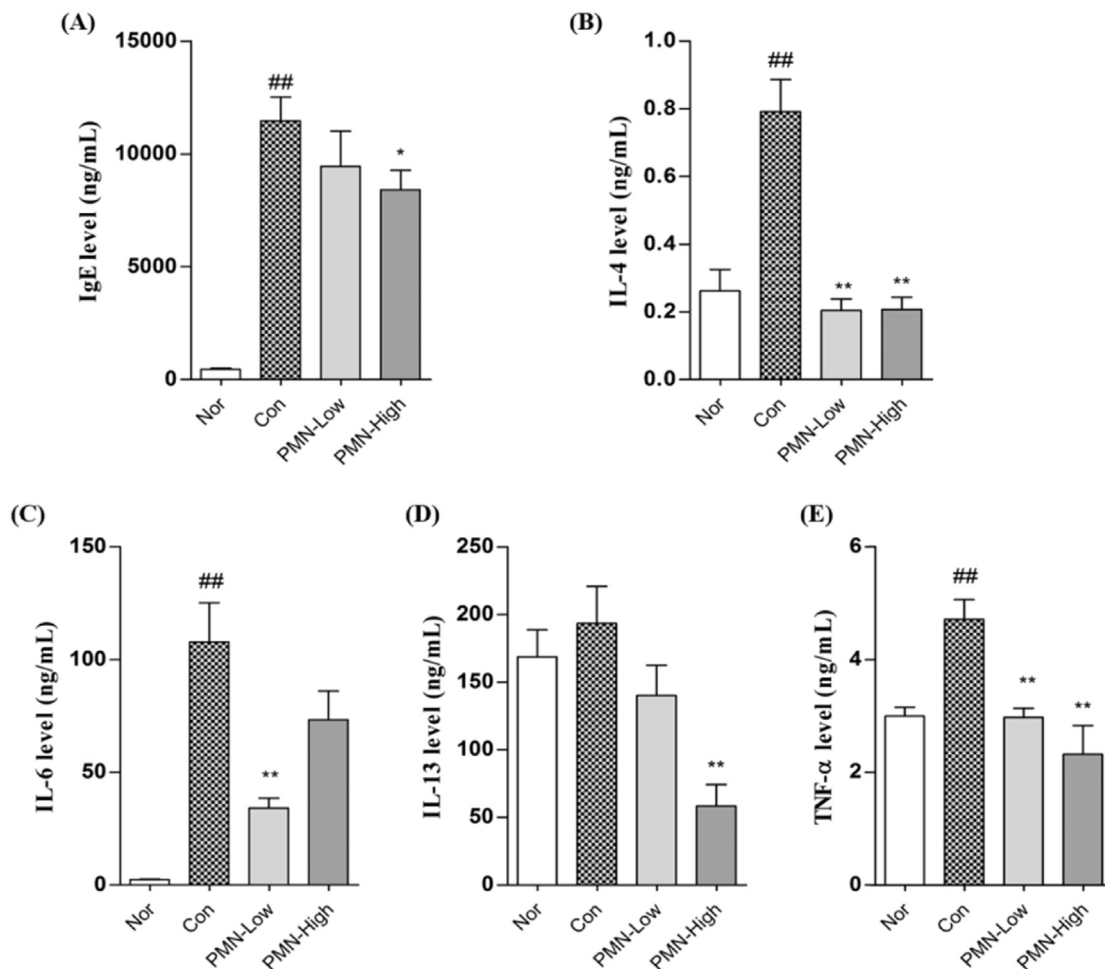


Fig. 5. Effect of PMN on the serum levels of IgE, IL-4, IL-6, IL-13 and TNF- α in DNCB-induced BALB/c mice. The data are represented as the mean $\pm$ SEM from three independent experiments. ## p < .01 compared with the normal and * p < .05, ** p < .01 compared with the control.

2.7. Cell culture and cell viability

The RAW264.7 and RBL-2H3 cell lines were obtained from the Korea Cell Line Bank (Seoul, Korea). The HaCaT cells were purchased from ATCC (Manassas, VA, USA). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated FBS at 37 °C, 5% CO₂, and 95% humidity. Cell viability was tested using the MTS colorimetric assay. The RAW264.7 cells (5×10^4), RBL-2H3 cells (4×10^4) and HaCaT cells (1.5×10^4) were plated into 96-well plates and incubated for 24 h in a 37°C incubator with various GMHGYGT concentrations. MTS solution (2 mg/mL) was added to each well, and the cells were incubated for 2 h at 37°C. The optical density (OD) value of the 96-well culture plates was measured at 490 nm using an ELISA reader. The formazan OD value of the untreated control cells was considered to represent 100% viability.

2.8. Measurement of cytokines in RAW264.7

The RAW264.7 cells were pretreated with PMN concentrations of 10, 25 and 50 μ g/mL for 1 h. Next, the cells were stimulated with LPS (1 μ g/mL) for 8 h, and the supernatants were measured by the enzyme-linked immunosorbent assay (ELISA) method. The levels of IL-6, IL-8 and TNF- α were measured using a commercial cytokine/chemokine ELISA kit (BD Biosciences, San Jose, CA). The levels of prostaglandin E2 (PGE2) were measured using ELISA kits for PGE2 from R&D Systems, Inc. (Minneapolis, MN, USA) with a spectrophotometer, and standard

curve plots were made for each cytokine using the standard solution in the kits.

2.9. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in RAW264.7 cells

The RAW 264.7 cells were pretreated with 10, 25 and 50 μ g/mL of PMN for 1 h and stimulated with LPS for 6 h. The total RNA was extracted using Trizol (TAKARA BIO, Otsu, Japan), and cDNAs were prepared using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The list of TNF- α , IL-1 β and COX-2 primers is shown in Table 1.

2.10. Thymus and activation-regulated chemokine (TARC) assay in HaCaT cells and β -hexosaminidase in RBL-2H3

The HaCaT cells were incubated with PMN for 1 h and then stimulated by TNF- α /IFN- γ for 24 h. The supernatant was centrifuged at 4000 rpm for 5 min. The levels of TARC and IL-4 in the HaCaT cells were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions.

Degranulation in the RBL-2H3 cells was induced with calcium ionophore A23187 (#C7522, Sigma-Aldrich, St. Louis, MO, USA) for 15 min after pretreatment with PMN for 1 h. The supernatant was reacted with 1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (p-NAG, #N9376, Sigma-Aldrich, St. Louis, MO, USA) for 1 h. The reaction was

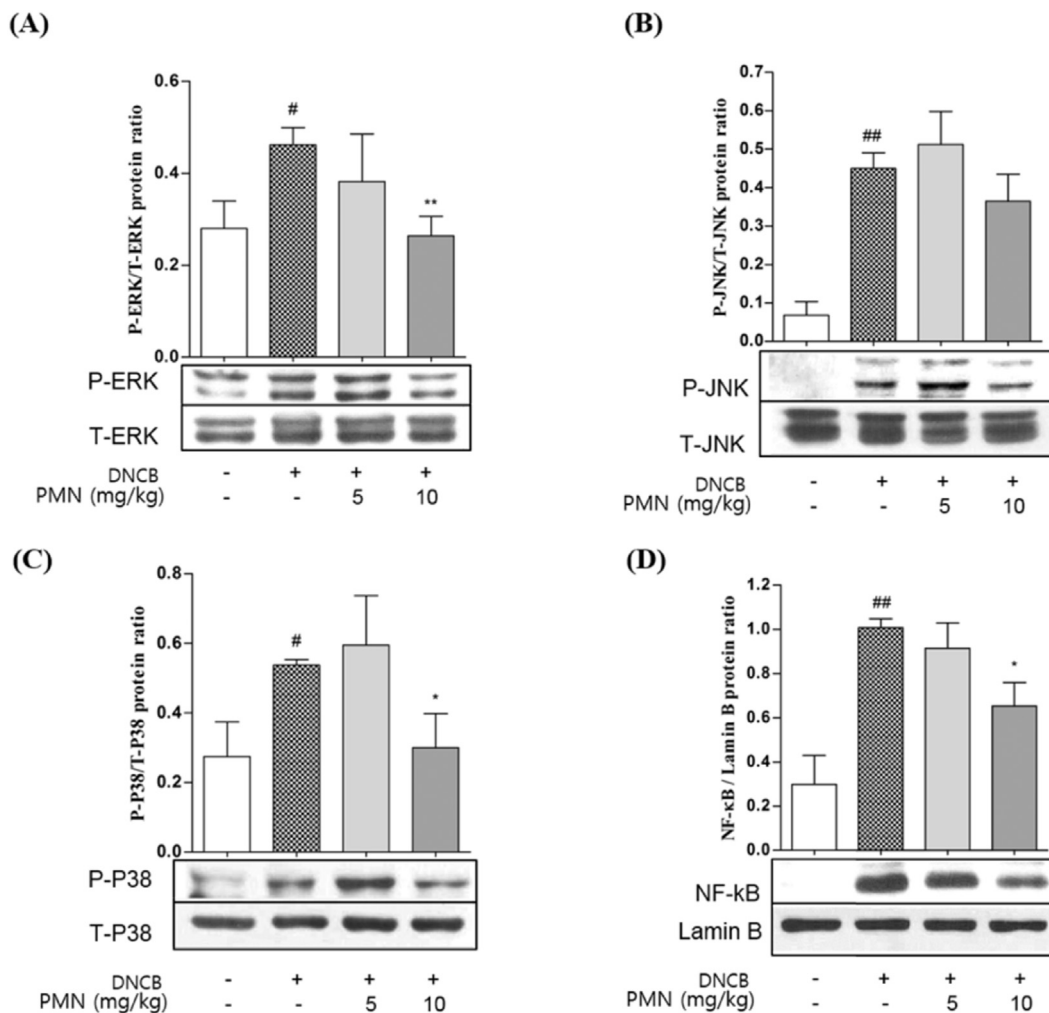


Fig. 6. Effects of PMN on the MAPK and NF- κ B protein expressions in DNCB-induced BALB/c mice. (A) The level of phosphor-ERK1/2, (B) phosphor-JNK, (C) phosphor-P38 expression was examined by Western blot. (D) The level of NF- κ B expression was examined by Western blot. The data are represented as the mean $\pm$ SEM from three independent experiments. # $p < .05$, ## $p < .01$ compared with the normal and ** $p < .01$, * $p < .05$ compared with the DNCB-treated control.

stopped with $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ (pH 10), and the wavelength was measured at 405 nm.

2.11. Statistical analysis

Statistical analysis results are presented as the mean $\pm$ SEM. All data were analyzed using the Graph Pad PRISM Software (Graphpad Software, Inc., CA, USA). One-way ANOVA was used to evaluate the treatment effect, followed by Dunnett's multiple comparison test. Values with a $p < .05$ were considered significant.

3. Results

3.1. Effects of PMN on the DNCB-induced epidermis and dermis thickness

The experimental schedule to establish a DNCB-induced AD mouse model is shown in Fig. 2. We examined the skin thickness changes in the back skins of the mice with DNCB-induced AD. The DNCB-treated group had a thick dermis and epidermis compared with the normal group. The skin thicknesses of the PMN Low and High group were decreased significantly (Fig. 3).

3.2. Effects of PMN on the DNCB-induced immune cell infiltration

The number of eosinophils and mast cells in the dermis of the PMN-

treated group was decreased significantly compared to that of the DNCB-treated group. The number of eosinophils in the PMN Low and High group was reduced to 65% and 45%, respectively, compared to that of the control group (Fig. 4A, B). The number of mast cells in the PMN High group was reduced to 67% compared to that of the control group (Fig. 4C, D).

3.3. Effect of PMN on the DNCB-induced serum cytokine levels

To demonstrate the effect of PMN on the serum cytokine levels in the AD model, the expression of IgE, IL-4, IL-6, IL-13 and TNF- α were measured using ELISA (Fig. 5). The PMN High group had a significantly reduced serum level of IgE, IL-4, IL-13 and TNF- α . The PMN Low group had a significantly reduced serum level of IL-4, IL-6 and TNF- α .

3.4. Effect of PMN on the MAPK and NF- κ B protein levels in the skin tissue of the DNCB-induced mice

To measure the mitogen-activated protein kinases in the skin tissue treated with PMN, Western blots were performed. The results show that P-ERK and P-P38 were inhibited in the PMN High group compared to the DNCB-treated group (Fig. 6). In contrast, P-JNK was reduced in both the Low and High PMN groups, but there was no significant change. We investigated NF- κ B activation in the DNCB-induced mouse model treated with PMN. The results show that the NF- κ B protein level was

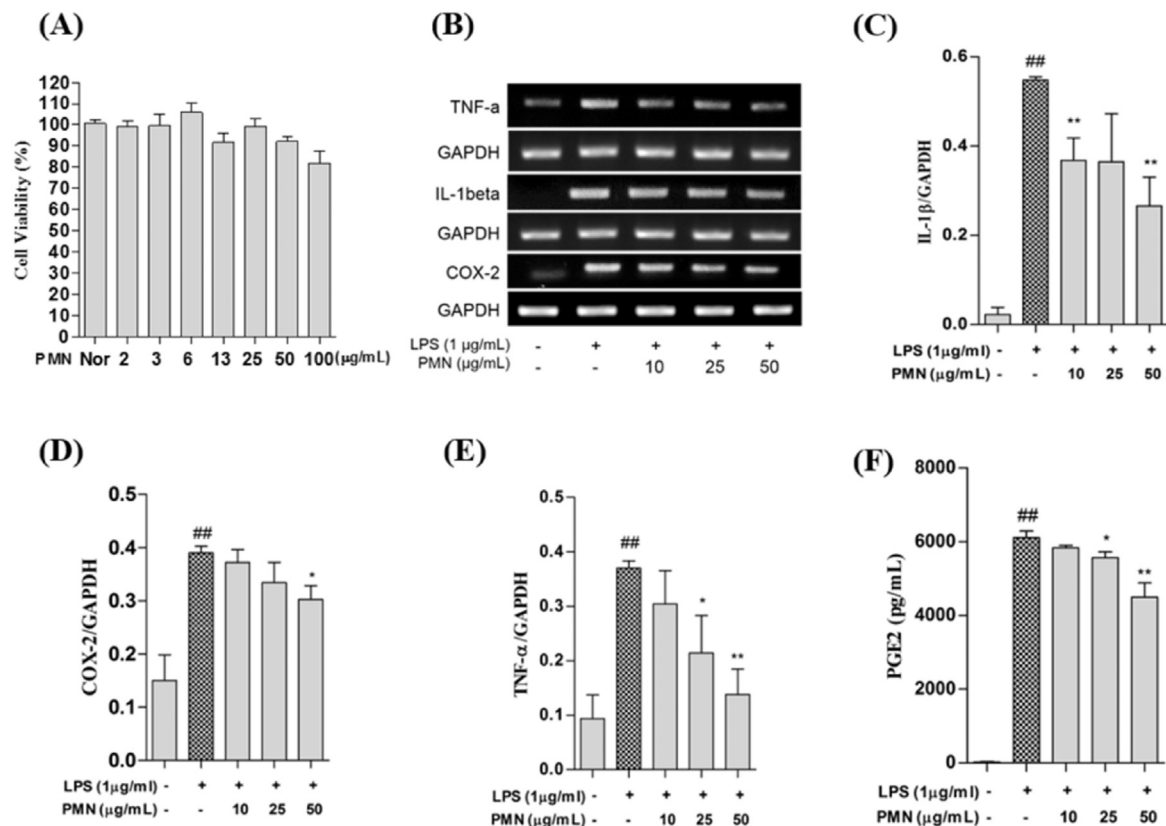


Fig. 7. (A) Cell viability of PMN-treated RAW264.7 cells. The optical density of the culture plates at 490 nm was measured using a spectrophotometer. The value represents the mean $\pm$ SEM from three independent experiments. (B-E) Effect of PMN on the cytokine mRNA expression in RAW264.7 cells. TNF- α , IL-1 β and COX-2 mRNA expressions were measured by RT-PCR. The value represents the mean $\pm$ SEM from three independent experiments. (F) The effects of PMN on PGE2 in RAW264.7. The data are represented as the mean $\pm$ SEM from three independent experiments. ##p < .01 compared with the normal and *p < .05, **p < .01 compared with the LPS-treated control.

significantly reduced in the PMN High group (Fig. 6D).

3.5. Effects of PMN on inflammatory mediators in the RAW264.7 cells

To determine the cell viability, the MTS assay was performed. The RAW264.7 cells were treated with PMN at various concentrations. PMN concentrations from 2 to 50 μ g/mL did not affect the cell viability (Fig. 7A). The expression of TNF- α , IL-1 β and COX-2 mRNAs induced by LPS were inhibited by pretreatment of the cells with PMN (Fig. 7B-E). PMN at 25 and 50 μ g/mL significantly decreased the expression of TNF- α . Pretreatment with PMN at concentrations of 10 and 50 μ g/mL significantly inhibited the LPS-induced gene expression of IL-1 β . PMN at 50 μ g/mL reduced the expression of COX-2. The expression of PGE2 was measured using ELISA. The result shows that PMN reduced the PGE2 level induced by LPS (Fig. 7F). A PMN concentration of 25 and 50 μ g/mL significantly inhibited the PGE2 expression in the RAW264.7 cells.

3.6. Effects of PMN on TARC and IL-4 expression in HaCaT cells and degranulation of RBL-2H3 cells

PMN concentrations of 50 and 100 μ g/mL demonstrated a significantly reduced level of TARC in the HaCaT cells (Fig. 8B). PMN at 100 μ g/mL reduced the expression of IL-4 in the HaCaT cells (Fig. 8C). Moreover, PMN significantly inhibited degranulation of the RBL-2H3 cells (Fig. 8E).

4. Discussion

AD symptoms, such as thickened or cracked skin, are initiated by the interaction of Th 1 and Th 2 cells [16]. Th 2 cells produce cytokines,

such as IL-4 and IL-13 [17], that can increase vascular adhesion, which is molecularly related to the adhesion of eosinophils to allergic dermatitis lesions [18–20]. Th 2 cells induce the proliferation of B cells, which can recruit immunoglobulin (Ig) E [21]. IgE binds to receptors on the mast cell surface, and then, mast cells can release inflammatory mediators and cytokines, such as histamine, IL-6, IL-1 β and TNF- α [22]. This study revealed that PMN inhibited serum IL-6 and TNF- α in the DNCB-induced AD animal model. Moreover, our previous study showed that PMN had an inhibitory effect on PMACI-induced pro-inflammatory mediators, including histamine, IL-6, IL-1 β and TNF- α , in human mast cells [15]. Therefore, PMN inhibits the expression of serum inflammatory cytokines, which can effectively suppress the symptoms of AD, such as thickening of the dermis and epidermis and infiltration of eosinophils and mast cells into the dermis.

MAPK signaling is essential in the pathogenesis of the inflammatory response [23,24]. As mentioned in previous studies, topical p38 MAPK inhibition attenuates severe inflammatory responses in the dermis [25]. In this study, MAPK signaling in the DNCB-induced AD skin tissue was reduced by PMN treatment. This result suggests that PMN has an effect on the DNCB-induced AD response by inhibiting MAPK signaling. MAPK regulates NF- κ B gene expression upstream in the pathway [26]. NF- κ B is a transcription factor that is known to be a controller that regulates the expression of genes in inflammatory responses [27]. In this study, the PMN High group had reduced MAPK and NF- κ B signaling. Thus, it appears that PMN can reduce dermatitis-like skin responses by inhibiting MAPK and NF- κ B signaling.

RAW 264.7 cells murine macrophages that originated from an Abelson-leukemia-virus-infected mouse [28]. Macrophages are innate immune cells that have a central role in inflammation responses [29]. To verify the mechanism of PMN in inflammation, we observed the effect of PMN on inflammatory mediators in RAW 264.7 cells. PGE2 is

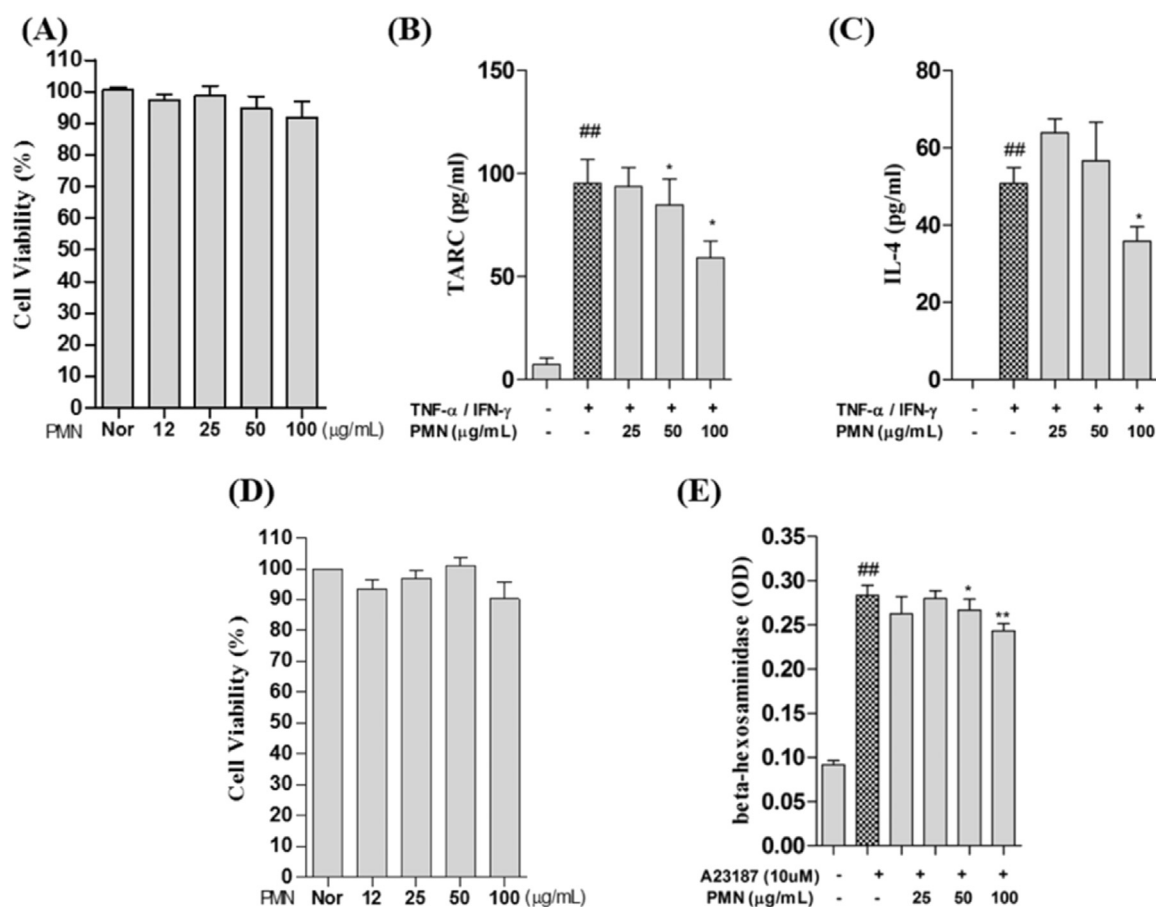


Fig. 8. (A) Cell viability of PMN-treated HaCaT cells. Effect of PMN on (B) TARC and (C) IL-4 expression in HaCaT cells. (D) Cell viability of PMN-treated RBL-2H3 cells. (E) Effect of PMN on β -hexosaminidase in RBL-2H3. The data are represented as the mean $\pm$ SEM from three independent experiments. ##p < .01 compared with the normal and *p < .05, **p < .01 compared with the control.

activated through the inflammatory response and stimulated by an increase in COX-2 expression [30]. These mediators are associated with the induction of mast cells, which can induce inflammatory cytokines, such as TNF- α and IL-1 β [31]. In the case of AD mice or patients, TARC is overexpressed in keratinocytes [32]. TARC upregulates Th2 cytokines, such as IL-4 and IL-13 [33]. TARC in HaCaT cells, which are human keratinocytes, were decreased by PMN. A degranulation marker named β -hexosaminidase can be used to measure the degranulation quantity of mast cells, which contain allergic mediators [34]. PMN reduced β -hexosaminidase release in RBL-2H3 cells.

5. Conclusion

Our results showed that PMN has an inhibitory effect on PGE2 expression and the levels of COX-2, TNF- α and IL-1 β mRNA in a macrophage cell line. This result may be explained as follows: PMN has an inhibitory effect in the AD animal model and *in vitro*.

Authors' contributions

Jeong-Min Lim and Bina Lee designed the study, and Jeong-Min Lim and Ju-Hee Min contributed to the execution of the research. Jeong-Min Lim and Bina Lee wrote the manuscript. Eun-Young Kim, Jae-Hyun Kim and Youngjoo Sohn contributed the statistical analysis. SooYeon Hong carried out the blood sample collection in the field. Jwa-Jin Kim provided some reagents/materials and relative discussion. All authors read and approved the final manuscript.

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Declaration of interests

The authors declare no conflict of interests.

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