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Korean red ginseng water extract alleviates atopic dermatitis-like inflammatory responses by negative regulation of mitogen-activated protein kinase signaling pathway *in vivo*

Ju-Hyoung Park[a], Eun-Kyung Ahn[b], Hye-Jin Ko[b], Jae Yeon Lee[b], Seung-Mi Hwang[c], SeonMi Ko[c], Joa Sub Oh[a,*]

[a] College of Pharmacy, Dankook University, Dandae-ro 119, Dongnam, Cheonan, Chungnam, 31116, Republic of Korea
[b] Bio-Center, Gyeonggido Business and Science Accelerator, Gwanggyo-ro 147, Yeoungtong, Suwon, Gyeonggi, 16229, Republic of Korea
[c] Institute of Jinan Red Ginseng, 41 Hongsamhanbang-ro, Jinan-eup, Jinan-gun, Jeonbuk, 55442, Republic of Korea

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Korean red ginseng is a Korean traditional medicine. In this study, we estimated the effects of Korean red ginseng water extract (RGE) in the 1-chloro-2,4-dinitrobenzene (DNCB)-induced BALB/c mouse model which develops AD-like lesions. After RGE administration (100, 200, and 400 mg/kg) to DNCB-induced mice there were improvements in the dermatitis score and skin pH, a decrease in trans-epidermal water loss, and improved skin hydration. RGE also significantly inhibited eosinophil infiltration, increased filaggrin protein levels, and decreased serum IgE levels, epidermal thickness, mast cell infiltration, and ceramidase release. Compared with that in DNCB-induced mice, RGE effectively decreased the mRNA expression levels of interleukin-6 (IL-6), thymic stromal lymphopoietin (TSLP), and tumor necrosis factor-α (TNF-α), as well as the protein level of thymus and activation-regulated chemokine (TARC). These inhibitory RGE effects are mediated by inhibiting the phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. Furthermore, we confirmed that RGE suppresses interferon-γ (IFN-γ) and TNF-α-induced expression of macrophage-derived chemokine (MDC) and TARC genes in human keratinocyte (HaCaT) cells. Taken together, these results demonstrate that RGE may exert anti-atopic related to responses by suppression the expression of inflammatory mediators, cytokines, and chemokines *via* downregulation of MAPK signaling pathways, suggesting that RGE may be an effective therapeutic approach for prevention of AD-like disease.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder characterized by dryness, pruritus, and erythema eczema, and is often accompanied by other atopic diseases such as allergic rhinitis and asthma. AD is caused by complex interactions between multiple factors, including immunological, genetic, pharmacological and physiological, and environmental factors that together can induce an abnormal immune response that results in epidermal barrier dysfunction [1–4]. As a primary immunological abnormality, immunoglobulin E (IgE) sensitization to specific allergens increases the levels of IgE and the activities of T helper 2 (Th2) cells, dendritic cells, and eosinophils. These inflammatory reactions are well-known to induce skin barrier dysfunction [5–7]. Damage to the skin barrier leads to a rapid response by the innate immune system followed by an adaptive immune response in the skin that enhances the penetration of external antigens and reduces the moisture content of the skin [8,9]. In general, the clinical features of AD include a collapse of the skin barrier due to severe itching, inflammation, increases in serum IgE levels, infiltration of eosinophils into the inflammatory site, and development of Th2 cells [10,11]. Other factors that can affect the skin barrier function in AD-like diseases are reduced expression of filaggrin and decrease in ceramide levels. Filaggrin is a protein that is found in the epidermis and plays a critical role in the formation of the skin barrier and is involved in regulating the degree of hydration of the cornified layer, as well as its pH. A reduced expression of filaggrin leads to weakening of skin barrier function, increasing water loss and reducing the intercellular adhesion force. There is also a reduction in ceramide levels in the cornified layer which leads to dry

* Corresponding author at: Dankook University, Dandae-ro 119, Dongnam, Cheonan, Chungnam, 31116, Republic of Korea.
E-mail address: jsoh@dankook.ac.kr (J.S. Oh).

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Table 1
Primers for PCR amplification.

Primer	Forward sequence	Reverse sequence
TSLP	5'-CGA CAG CAT GGT TCT TCT CA-3'	5'-CGA TTT GCT CGA ACT TAG CC -3'
IL-6	5'-AAC CTT CCA AAG ATG GCT GAA -3'	5'-CAG GAA CTG GAT CAG GAC TTT-3'
TNF-α	5'-CTG AGA CAA TGA ACG CTA CA-3'	5'-TTC TTC CAC ATC TAT GCC AC-3'
MDC	5'-GCA TGG CTC GCC TAC AGA CT-3'	5'-CGA GGG AGG GAG GCA GAG GA-3'
TARC	5'-ATG GCC CCA CTG AAG ATG CT-3'	5'-TGA ACA CCA ACG GTG GAG GT-3'
GAPDH	5'-GAG GTA AAC TCA GGA GAG TG-3'	5'-GTA GAC TCC ACG ACA TAC TC-3'

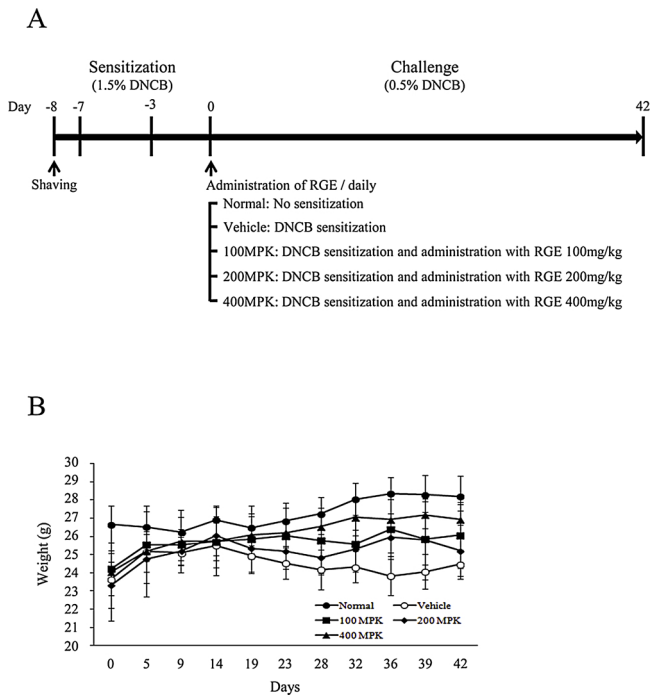


Fig. 1. Schematic diagram of the experimental protocol. (A) During the experiment, the mice were fed with food and water. In order to induce AD, the dorsal skin was sensitized with 200 μl of 1.5% DNCB dissolved in a mixture of acetone/corn oil (3:1) and then 150 μl of 1% DNCB was applied 3 days a week for 6 weeks. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE (100 MP K, 200 MP K, and 400 MP K). The mice were orally administered DW (normal and vehicle group), and RGE (100, 200, and 400 MP K) for 6 weeks. (B) The body weight was measured twice a week.

skin [12–14]. IgE causes allergic skin reaction by stimulating mast cells to release cytokines, which result in the infiltration of other immune cells, such as macrophages, into the skin which leads to the further release of cytokines and an inflammatory response [15–17]. Macrophage-derived chemokine (MDC), and thymus activation-regulated chemokine (TARC) are typical Th2 chemokines that can induce Th2 cell migration and invasion. It has recently been shown that both TARC and MDC are present at high levels in the serum of AD patients and are closely related to AD-like diseases [18,19]. Thymic stromal lymphopoietin (TSLP) which is known to be an important cytokine in AD can also cause a Th2 inflammatory response. TSLP-mediated skin inflammation can induce an AD-like disease which has both innate and adaptive immune response components. TSLP is released from various cells such as epithelial cell, stromal cell, and fibroblast through stimulation by allergens and the TSLP pathway is a promising target for immunological mechanism of AD-like disease [20,21].

Previous studies have shown that herbal medicines may be beneficial for the treatment of AD [22]. Ginseng has long been used as a traditional medicine in Asian countries. Korean red ginseng is a traditional medicine that has been widely used to treat diseases such as cancer, Alzheimer's disease, vascular diseases, and also has antiallergic effects [23–25]. However, the effect of Korean red ginseng from Jinan to suppress AD-like disease has not been investigated and the mechanisms behind its immune-pharmacological effects are poorly elucidated.

The aim of this study was to verify the effects and immunological mechanisms of Korean red ginseng water extract (RGE) treatment in an AD-like disease model established in BALB/c mice using 1-chloro-2,4-dinitrobenzene (DNCB).

2. Materials and methods

2.1. Preparation of RGE

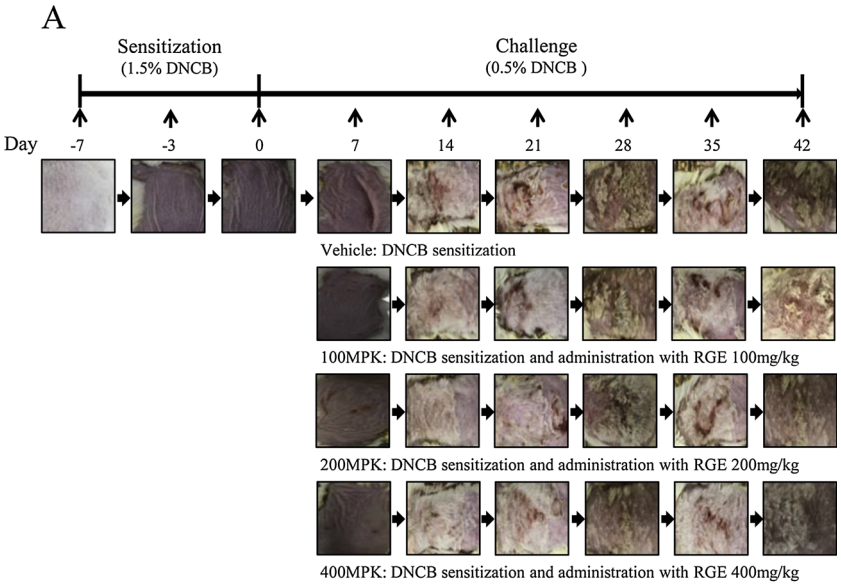
Four-year old Korean red ginseng was purchased from a commercial supplier (Gansando agriculture cooperation, Jinan, Republic of Korea). RGE was prepared by the Jinan red ginseng institute using the following method. Fifty liters of distilled water (DW) was added to 5 g of crushed red ginseng. The soluble components were then extracted using a reflux condenser. The extract was then filtered through filter paper (pore size 8 μm, Whatman) and condensed under a vacuum. RGE from Jinan was converted into a powder using a spray-dryer with the addition of 40% maltodextrin as a carrier. The ginsenoside content was calculated by comparison with a standard solution. RGE from Jinan contained Rg1, Rb1 and Rg3 at a combined concentration of was 6.42 mg/g (Rg1-1.07 mg/g, Rb1-5.06 mg/g and Rg3-0.29 mg/g).

2.2. Animals

Male BALB/c mice, 5 weeks of age, were purchased from Orient, Inc. (Seoul, Republic of Korea). During experiment, the mice were housed in polycarbonate cage and acclimated to standard conditions of a 12 h light/dark cycle, 20 ± 2 °C, and relative humidity between 35% and 55% in a specific pathogen-free environment. According to AAALAC-International guidelines, all experimental procedures were approved by the Institutional Animal Care and Ethical Use Committee of the Gyeonggi Institute of Science & Technology Promotion (Approval No. 2017-08-0009).

2.3. Induction of AD-like skin lesions and RGE treatment

The mice all groups were anesthetized with 2% isoflurane (HanaPharm, Seoul, Republic of Korea) and the back hair of the mice was removed by shaving with a commercially available shaving cream (Veet, Oxy Reckitt Benckiser Ltd., France) to induce AD-like skin lesion.



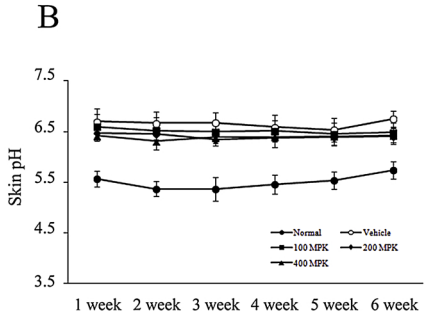


Fig. 2. The effect of RGE on clinical assessment in dorsal skin lesions in DNCB-induced BALB/c mice. (A) To evaluate the effect of RGE on DNCB-induced AD-like disease in BALB/c mice, images of the dorsal skin lesions were used to perform the clinical assessment of skin once a week. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE (100 MP K, 200 MP K, and 400 MP K) for 6 weeks. (B, C) The dermatitis score was determined once a week. Skin pH of the dorsal skin was also analyzed once a week, using a pH meter.

Table 2
The effect of RGE on the dermatitis score in DNCB-induced BALB/c mice.

Group	1 week	2 week	3 week	4 week	5 week	6 week
Normal	0	0	0	0	0	0
Vehicle	5.7 ± 0.2	7.3 ± 0.5	8.5 ± 1.2	8.4 ± 1.6	7.9 ± 1.6	8.5 ± 1.7
100 MP K	5.5 ± 0.7	6.0 ± 0.7*	6.4 ± 0.7*	5.8 ± 1.0*	6.2 ± 1.1	6.5 ± 0.8*
200 MP K	5.5 ± 0.6	5.7 ± 0.4*	6.3 ± 0.5*	6.2 ± 1.8	6.5 ± 0.5	6.8 ± 1.0
400 MP K	5.0 ± 0.6*	5.5 ± 0.4*	6.2 ± 0.5*	5.7 ± 0.7*	6.2 ± 0.9	6.4 ± 0.5*

The dermatitis score of the dorsal skin was evaluated once a week. All data were represented as mean ± SD; * $P < 0.05$ compared with the DNCB-induced group.

Table 3
The effect of RGE on the TEWL in DNCB-induced BALB/c mice.

Group	1 week	2 week	3 week	4 week	5 week	6 week
Normal	2.12 ± 0.29	2.08 ± 0.19	2.14 ± 0.30	2.18 ± 0.33	2.16 ± 0.11	2.20 ± 0.78
Vehicle	35.54 ± 2.48	40.17 ± 2.89	42.65 ± 5.67	40.78 ± 7.00	41.07 ± 7.36	42.93 ± 5.92
100 MP K	33.16 ± 2.97	37.71 ± 5.23	36.76 ± 5.00*	36.67 ± 6.03	35.80 ± 5.25	37.16 ± 3.90
200 MP K	32.88 ± 3.01	36.26 ± 0.92*	35.55 ± 9.88	34.53 ± 7.83	32.01 ± 0.94*	32.63 ± 5.08*
400 MP K	33.10 ± 4.64	36.25 ± 9.89	35.41 ± 3.74*	33.84 ± 3.10*	35.78 ± 3.35*	33.05 ± 5.12*

TEWL was analyzed with skin analyzer in dorsal skin once a week. All data were represented as mean ± SD; * $P < 0.05$ compared with the DNCB-induced group.

On the first day, the dorsal skin was sensitized with 200 μl of 1.5% DNCB dissolved in a mixture of acetone/corn oil (3:1) and then 150 μl of 1% DNCB was applied 3 days a week for 6 weeks. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE 100 mg/kg (MPK), 200 MP K, and 400 MP K. RGE, dissolved in DW, was administered orally daily for a further 6 weeks and the DNCB group was administered DW without RGE. During the experiment, the mice had free access to food and water. Body weight was measured using a top-loading balance twice a week.

2.4. Measurement of serum IgE levels, eosinophils, TARC, and ceramidase

At the end of the experiment, the mice in all groups were anesthetized with 2% isoflurane (HanaPharm, Seoul, Republic of Korea) in

Table 4
The effect of RGE on the skin surface hydration in DNCB-induced BALB/c mice.

Group	1 week	2 week	3 week	4 week	5 week	6 week
Normal	91.20 ± 4.49	90.80 ± 2.28	92.40 ± 4.51	90.20 ± 4.15	91.40 ± 2.30	90.20 ± 32.50
Vehicle	35.30 ± 9.19	33.26 ± 7.23	32.26 ± 4.71	33.60 ± 5.34	31.80 ± 8.12	32.75 ± 3.45
100 MP K	33.50 ± 7.58	33.88 ± 2.22	36.06 ± 3.40	36.93 ± 7.88	35.79 ± 3.41	36.96 ± 3.49*
200 MP K	37.33 ± 9.64	34.13 ± 5.56	38.10 ± 4.34*	38.42 ± 7.11	39.78 ± 5.44*	40.73 ± 5.17*
400 MP K	36.22 ± 8.78	35.29 ± 4.71	39.03 ± 7.00*	37.64 ± 7.42	40.19 ± 4.22*	40.80 ± 5.67*

Skin surface hydration was analyzed with skin analyzer in dorsal skin once a week. All data were represented as mean ± SD; *$P < 0.05$ compared with the DNCB-induced group.

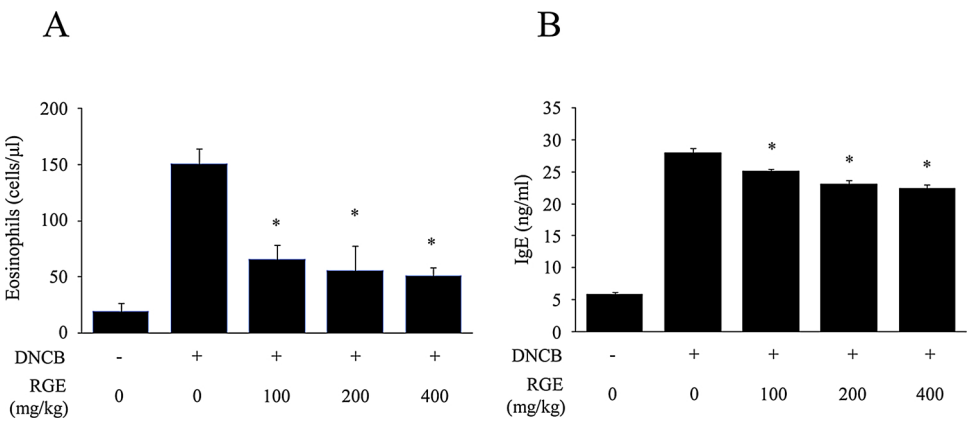


Fig. 3. The effect of RGE on the expression of IgE and eosinophils in DNCB-induced BALB/c mice. Blood sample was collected from the mice and serum was separated at the end of the experiment. (A) The number of eosinophil in whole blood was counted randomly. (B) The level of serum IgE was analyzed with ELISA. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE (100 MP K, 200 MP K, and 400 MP K) for 6 weeks. All data are presented as mean ± SD; *$P < 0.05$ compared with the DNCB-induced group.

order to examine the physiological change that had occurred. Blood from the abdominal vena cava was collected and the number of eosinophils in whole blood was counting using an automated hematology cell counter (Celltac alpha MEK-6253, Nihon-Koden, Tokyo). Blood was centrifuged at 890 *g* for 15 min and separated serum was measured to analyze the level of IgE. The dorsal skin tissue was collected and homogenized with RIPA buffer containing a protease inhibitor cocktail. The lysate was centrifuged at 16,600 *g* for 20 min and the supernatant was measured to analyze the level of TARC, and ceramidase. The levels of total serum IgE, TARC, and ceramidase were determined using enzyme-linked immunosorbent assay (ELISA) Quantitation Kits (BD Biosciences, San Jose, CA, USA). The absorbance was determined at 450 nm using a microplate reader.

2.5. Western blotting

At the end of the experiment, the dorsal skin tissue from all groups was collected and homogenized with the lysis buffer (PROPREP protein extraction solution; iNtRoN Biotechnology, Seoul, Republic of Korea). The lysate was centrifuged at 16,600 *g* for 15 min, and the total protein concentration in the supernatant was measured by the Bradford method. Protein sample (20 μg) was mixed with a loading dye, separated on 10% sodium dodecyl sulfate polyacrylamide gels, and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany). The membrane was blocked in 5% bovine serum albumin (BSA) for 1 h and incubated with primary antibodies against filaggrin (Enzo, Farmingdale, NY, USA), phospho-ERK1/2, phospho-JNK, phospho-p38 (Cell Signaling Technology, Beverly, MA, USA), and β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which were used at a 1:1000 dilution, with gently shaking at 4 °C overnight. The membrane was washed in Tris-buffered saline with 0.1% Tween 20 (TBST) five times and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a 1:2000 dilution, with gently shaking at room temperature for 1 h. The band of protein was enhanced by chemiluminescence reagent and visualized using a ChemiDoc XRS system (Bio-Rad, Richmond, CA, USA).

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was extracted from human keratinocyte (HaCaT) cells and mouse dorsal skin tissue. HaCaT cells were seeded in 6-well plates (1×10^6 cells/well) and treated with RGE (250–1000 μg/ml) for 1 h, followed by stimulation with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α; 10 ng/ml each) for 24 h. The dorsal skin tissue from all groups was collected and homogenized. The total RNA was extracted from the tissue and single stranded cDNA was prepared using random hexamers and the SuperScript®III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Inc.). The amplification of cDNA was performed in a thermal cycler (MG96 G, LongGene Scientific Instruments, Hangzhou, China) using AccuPower® Pfu PCR premix (Bioneer Corporation, Daejeon, Republic of Korea). For amplification, PCR cycling condition was as follows: 5 min at 95 °C followed by 27 cycles of 30 s at 95 °C, 40 s at between 55 and 60 °C, 1 min at 72 °C, and then 10 min at 72 °C for final extension. The primer sequences for PCR amplification are shown in Table 1.

2.7. Histological analysis

At the end of the experiment, the dorsal skin tissues were collected and fixed in 10% neutral buffered formalin and paraffin embedded. To evaluate epidermal thickness, the paraffin embedded tissue was cut into 5-μm sections, stained with hematoxylin and eosin (H&E) and observed

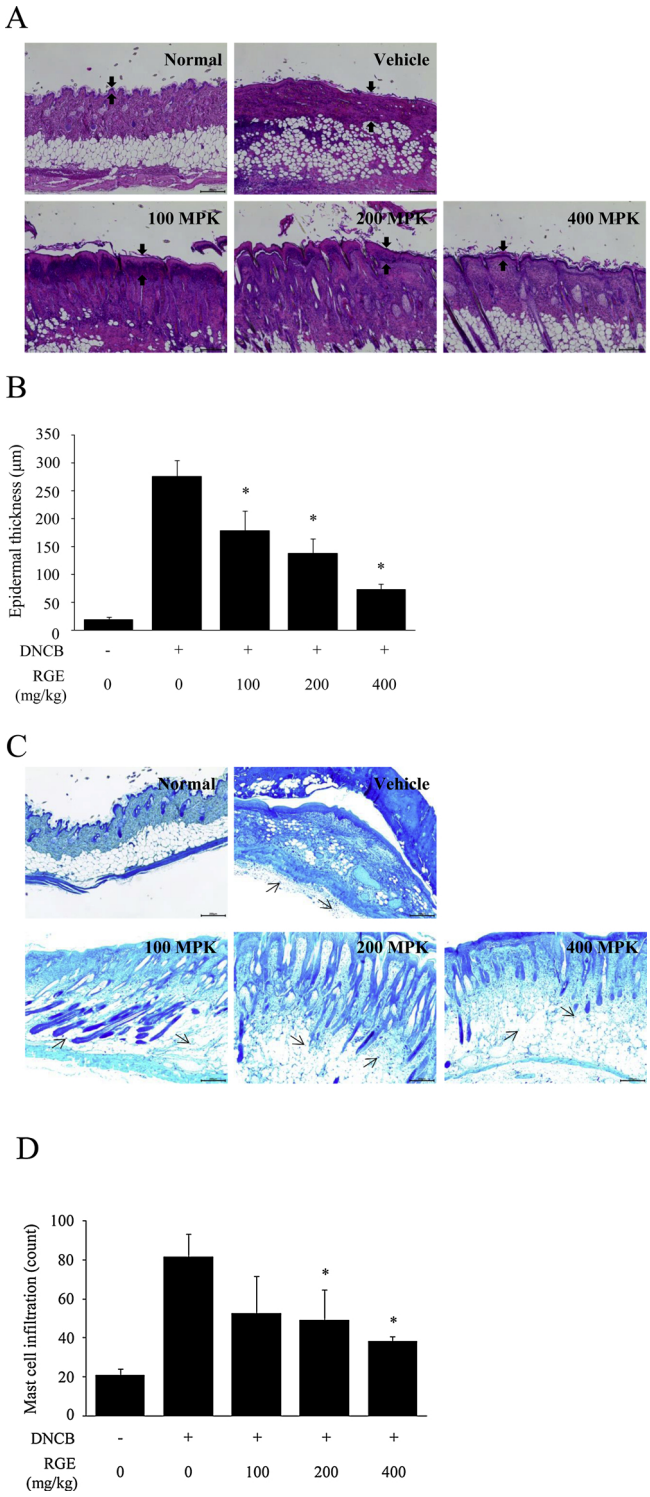


Fig. 4. The effect of RGE on histological analysis of dorsal skin lesions in DNCB-induced BALB/c mice. (A) Dorsal skin tissue was fixed with 10% neutral buffered formalin and paraffin embedded. The paraffin blocks were cut into 5 μm section and stained with H&E and observed using light microscopy at ×400 magnification. (B) Epidermal thickness was measured randomly in dorsal tissue section. (C) Dorsal skin tissue was fixed with 10% neutral buffered formalin and paraffin embedded. The paraffin blocks were cut into 5-μm sections and stained with toluidine blue staining and observed using light microscopy at ×400 magnification. (D) The degree of mast cell infiltration was assessed randomly in dorsal tissue section. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE (100 MP K, 200 MP K, and 400 MP K) for 6 weeks. All data are presented as mean ± SD; *$P < 0.05$ compared with the DNCB-induced group.

using light microscopy at ×400 magnification. To detect mast cell infiltration, each section was also stained with 0.5% toluidine blue to determine the number of mast cells by counting three sections randomly at ×400 magnification.

2.8. Assessment of skin lesions and dermatitis score

During the experiment, the mice in all groups were anesthetized with 2% isoflurane (HanaPharm, Seoul, Republic of Korea) and images of the dorsal skin were obtained using a camera once a week. Additionally, the dermatitis score of the dorsal skin also was evaluated once a week. Based on four symptoms (erythema, dryness, edema and erosion), the total dermatitis score was assessed by calculating the sum of the four symptom scores, ranging between 0 and 3: 0 (none), 1 (mild), 2 (moderate), and 3 (severe). Measurements of skin pH, transepidermal water loss (TEWL), and skin surface hydration were evaluated once a week.

2.9. Cell viability assay

To determine cell viability on the effect of RGE, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. HaCaT cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 12 h. HaCaT cells were treated with RGE (31.25–1000 μg/ml) for 1 h, followed by stimulation with 1 μg/ml lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, MO, USA) for 24 h. After incubation, the medium was removed and 100 μl MTT solution (Duchefa Biochemie, Haarlem, The Netherlands) was added and incubated for 2 h. MTT solution was removed and 100 μl dimethyl sulfoxide (DMSO; Duchefa Biochemie, Haarlem, The Netherlands) was added to each well. The absorbance at a wavelength of 540 nm was measured using microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.10. Statistical analysis

Statistical analysis was determined using one-way analysis of variance, followed by the Dunnett's test. All data are presented as mean ± standard deviation (SD). *P value* < 0.05 was considered statistically significant.

3. Results

3.1. Effect of RGE on AD-like symptoms in the DNCB-induced AD mouse model

We investigated the effect of RGE in an AD-like disease model established in BALB/c mice. In this model, BALB/c mice were treated with DNCB for six weeks and after the presence of AD-like lesion was confirmed the mice were then separated into treatment groups. The mice were orally administered DW (normal mice and DNCB mice vehicle groups) or RGE (100, 200, and 400 MP K) for six weeks (Fig. 1A). RGE did not have any effect on body weight, and furthermore there were no animal deaths (Fig. 1B) confirming that RGE is not toxic.

Pictures of the dorsal skin lesions were used to determine the dermatitis score and this score was then used to evaluate the effects of RGE on the DNCB-induced AD-like disease in BALB/c mice (Fig. 2A). The dermatitis score covered four dermatitis symptoms namely, erythema, edema, dryness, and erosion. In the DNCB treatment group, the dermatitis score was significantly increased compared with the normal group. Importantly, RGE treatment reduced the extent of the AD-like skin lesions and the dermatitis score was reduced compared with the DNCB group (Table 2). Skin barrier function parameters such as skin pH, TEWL, and skin hydration were also measured. Skin pH and TEWL were also higher in the DNCB group compared with the normal group, and RGE treatment reduced both the TEWL and skin pH levels

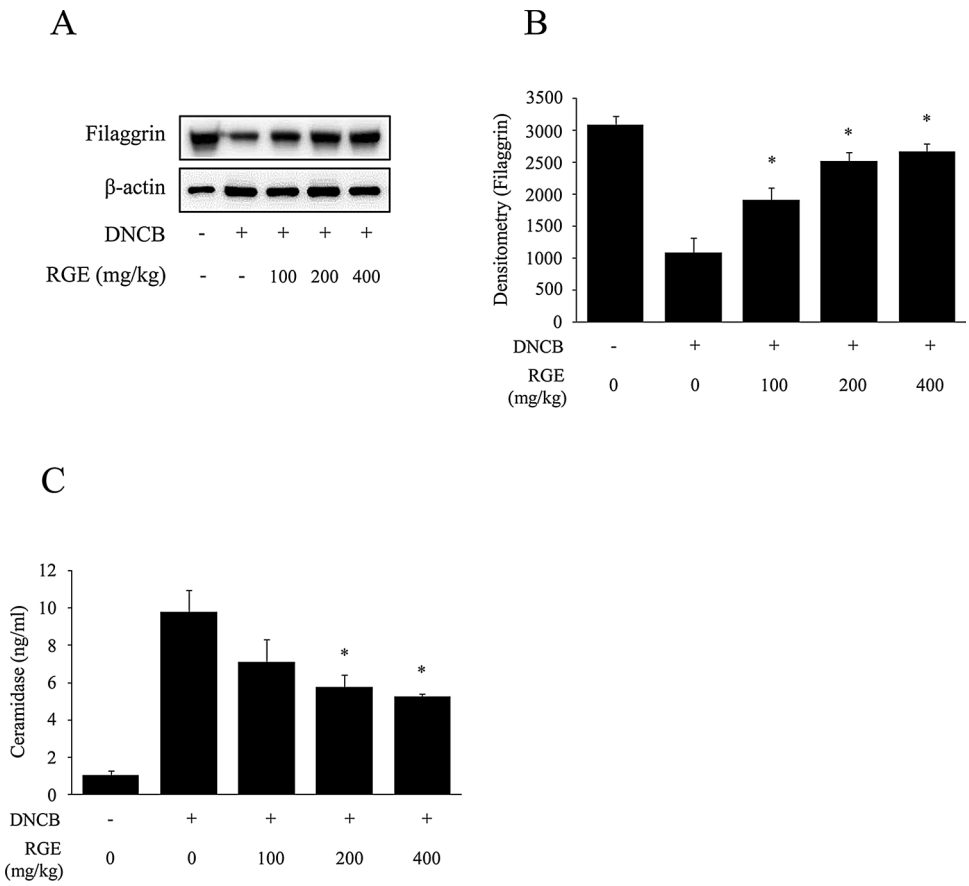


Fig. 5. The effect of RGE on the expression of filaggrin and ceramidase in dorsal skin lesions in DNCB-induced BALB/c mice. (A) At the end of the experiment, the tissue was collected, homogenized, and lysed in lysis buffer. The lysates were centrifuged at 16,600 *g* for 15 min and the expression of filaggrin was analyzed by western blotting. (B) The relative level of filaggrin was calculated using an image analyzer. (C) The level of ceramidase was analyzed with ELISA. The mice were randomly divided into five groups: normal, DNCB, DNCB + RGE (100 MP K, 200 MP K, and 400 MP K) for 6 weeks. All data are presented as mean ± SD; $^{*}P < 0.05$ compared with the DNCB-induced group.

compared with the DNCB group (Fig. 2B and Table 3). Skin hydration was also lower in the DNCB group compared with the normal group. Importantly, RGE treatment increased the level of skin hydration compared with the DNCB group (Table 4). Since the presence of eosinophils and the overproduction of serum IgE are typical characteristics of AD-like disease, we investigated the effects of RGE on the number of eosinophils in whole blood and IgE levels in serum. As expected, the number of eosinophils and the serum IgE levels were increased in the DNCB group, whereas these were both significantly decreased by RGE treatment (Fig. 3).

3.2. Effects of RGE on AD-like skin lesion histology

We performed H&E staining in DNCB-induced dorsal skin to determine whether RGE decreases epidermal thickness in AD-like skin lesions. We observed that the thickness of the epidermis was increased in the DNCB group and that RGE treatment significantly reduced the thickness of the epidermis in a dose dependent-manner (Fig. 4A and B). In addition, we performed toluidine blue staining of dorsal skin to assess effects on mast cell infiltration. We observed clear mast cell infiltration in the dorsal skin of mice in the DNCB group, and that RGE treatment decreased mast cell infiltration in these skin lesions (Fig. 4C and D).

Filaggrin and ceramide are thought to play a role in skin barrier function to maintain skin hydration and a low pH. A deficiency in both filaggrin and ceramide is a major cause of AD [26,27]. Mice in the DNCB group had decreased filaggrin protein levels in the skin lesions compared with the normal group. RGE treatment increased the protein levels of filaggrin in a dose-dependent manner (Fig. 5A and B). In addition, the levels of ceramidase (a glycosphingolipid ceramide deacylase) an enzyme which cleaves fatty acids from ceramide, were sharply increased in the DNCB group. Treatment with 200 and 400 MP K of RGE significantly decreased the levels of ceramidase (Fig. 5C).

3.3. Effects of RGE on proinflammatory cytokines and chemokines in AD-like skin lesions

In order to investigate the effects of RGE on proinflammatory cytokines and chemokines, we measured TSLP, IL-6, and TNF-α at the mRNA level in DNCB-induced skin lesions. The expressions of TSLP, IL-6, and TNF-α mRNAs were increased in the DNCB group compared with that in the normal group. RGE treatment decreased the mRNA expression levels of TSLP, IL-6, and TNF-α in the skin lesions compared with that in the DNCB group (Fig. 6A–E). We also measured the protein levels of TARC in these DNCB-induced skin lesions. RGE treatment effectively decreased TARC protein levels in a dose-dependent manner (Fig. 6F). Taken together, these data suggest that RGE treatment suppresses AD-like disease through an inhibition of the expression of Th2-related inflammatory cytokines and chemokines in AD-like skin lesions.

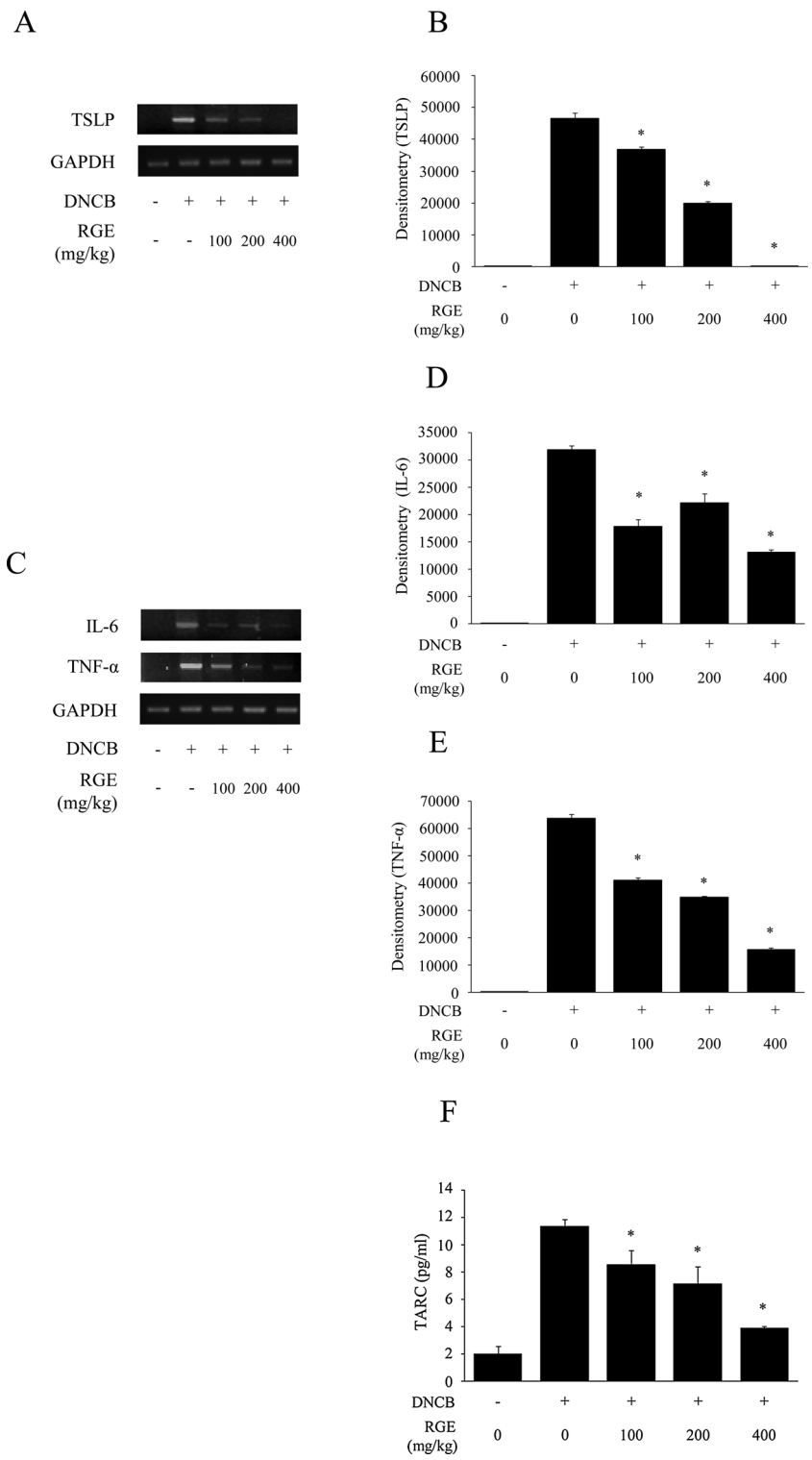


Fig. 6. Effects of RGE on proinflammatory cytokines and chemokines in DNCB-induced BALB/c mice. At the end of the experiment, the tissue was collected and homogenized and the total RNA was extracted. The expression of (A, B) TSLP, (C, D, E) IL-6, TNF-α, and (f) TARC was determined by RT-PCR, ELISA and densitometry protocol. All data are presented as mean ± SD; $^{*}P < 0.05$ compared with the DNCB-induced group.

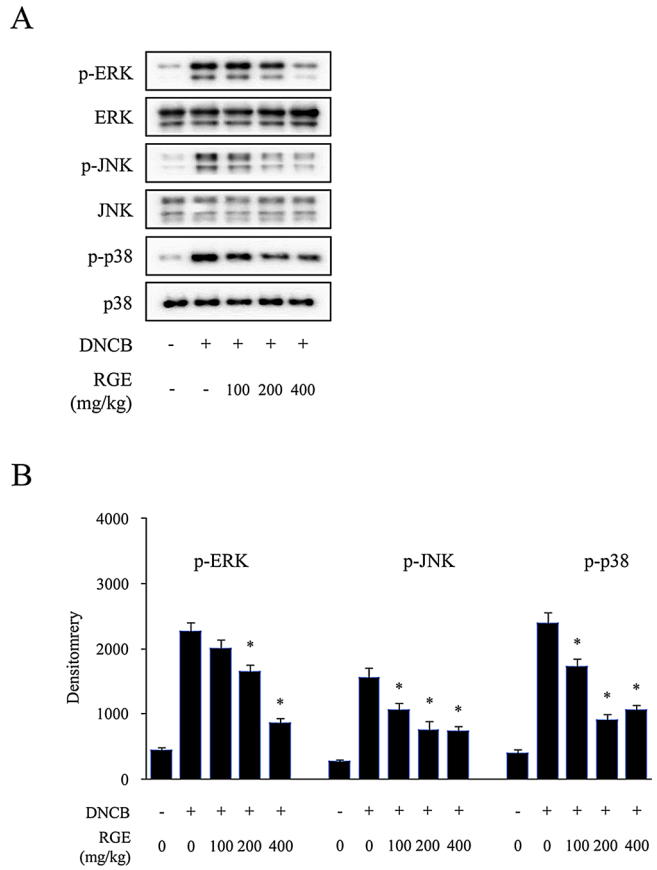


Fig. 7. Effect of RGE on the phosphorylation of MAPKs in dorsal skin lesions in DNCB-induced BALB/c mice. (A) At the end of the experiment, the tissue was collected, homogenized, and lysed in ice-cold lysis buffer. The lysates were centrifuged at 16,600 *g* for 15 min and the expression of total protein was measured. (B) The activities of p-ERK, p-JNK, and p-p38 were determined by western blotting and densitometry protocol. All data are presented as mean ± SD; $^{*}P < 0.05$ compared with the DNCB-induced group.

3.4. Effect of RGE on the phosphorylation of mitogen-activated protein kinase (MAPK) in AD-like skin lesions

The MAPK signaling pathways have previously been implicated in the pathogenesis of allergic inflammatory skin diseases [28]. Accordingly, we assessed whether RGE regulates the MAPK signaling pathways. As shown in Fig. 7, the phosphorylation levels of MAPKs (ERK1/2, JNK, and p38) were increased in the skin of mice in the DNCB group compared with that in normal mice. Importantly, RGE treatment dose-dependently suppressed the phosphorylation levels of ERK1/2. The phosphorylation levels of JNK were also decreased by treatment with 400 MP K RGE, whereas the phosphorylation levels of p38 were significantly decreased by all concentrations of RGE (100, 200, and 400 MP K). These data suggest that the inhibitory effect of RGE on DNCB-induced AD-like inflammatory responses may involve the negative regulation of MAPK signaling pathways.

3.5. Effects of RGE on proinflammatory chemokines in HaCaT cells

Finally, to explain more these results we confirmed RGE effect on the expression of proinflammatory chemokines in IFN-γ and TNF-α-induced HaCaT cells. We performed a preliminary experiment to assess the potential cytotoxicity of RGE treatment (31.25–1000 μg/mL) and found that it did have a significant cytotoxic effect in HaCaT cells (Fig. 8A). Expression levels of MDC and TARC, the chemokines associated with AD, showed significant reductions by treatment of RGE in IFN-γ and TNF-α-induced HaCaT cells (Fig. 8B–D). This *in vitro* inhibitory effect of RGE on TARC expression was similar to the result of *in vivo* potency.

4. Discussion

The skin is the outermost organ that maintains body moisture and performs the necessary barrier function to protect against internal intrusion of external factors. AD is a skin disorder caused by a complex interaction of hereditary factors, skin barrier abnormalities, and environmental factors [29,30]. The major causes of AD are damage to the skin barrier function and the associated immune responses, an abnormality in barrier function such as an increase in both TEWL and skin surface pH, and a decrease in the hydration in the stratum corneum [3,11]. Elevated levels of IgE are a marker of AD-like disease and IgE is released in response to a number of allergens. IgE-mediated responses then lead to the symptoms of allergic inflammation. Eosinophils also play an important immune regulatory role to release cytokines and are associated with numerous allergic diseases such as AD [31,32]. As shown in Fig. 3, RGE significantly reduced the number of eosinophils in whole blood as well as the serum IgE levels compared with that in the DNCB group.

Mast cells play an important role in inflammation by releasing inflammatory mediator cytokines [33]. In this study, using histological analysis with H&E and toluidine blue staining, we demonstrated clear epidermal thickening and infiltration of mast cells in mice with AD-like disease. Treatment of mice with AD-like disease with RGE markedly reduced epidermal thickening and the degree of mast cell infiltration compared with the untreated AD-like skin lesions (Fig. 4).

Filaggrin is considered to be an important genetic factor in AD. Decreased expression of filaggrin leads to decreased hydration in the stratum corneum [27,34]. Ceramides are water-retaining molecules that surround keratinocytes; various types of ceramides exist. Ceramide levels are reduced in AD and this decline in ceramide levels is thought to be due to the excessive degradation of ceramide by ceramidase [35,36]. In keeping with this, we observed that there were decreased filaggrin levels and increased ceramidase levels in the DNCB-induced mice. Importantly, RGE treatment significantly increased filaggrin levels and reduced ceramidase levels compared to the DNCB group, demonstrating that RGE might be able to maintain hydration of the skin barrier (Fig. 5).

Th2 cytokines cause allergic reactions and the cytokines produced by the Th2 immune response directly affect skin barrier function. The production of cytokines caused by allergens is therefore a critical process in the development of skin disease. The Th1/2 pathway has been proposed to play an important pathogenic role in the development of AD. IL-4 and IL-6 are both crucial factors in a Th2 response whereas Th1-mediated immunity is characterized by the release of inflammatory and regulatory cytokines such as TNF-α [37–39]. As shown in Fig. 6, the production of IL-6 and TNF- α is increased in the DNCB-induced mice, and importantly the levels of these cytokines were inhibited by

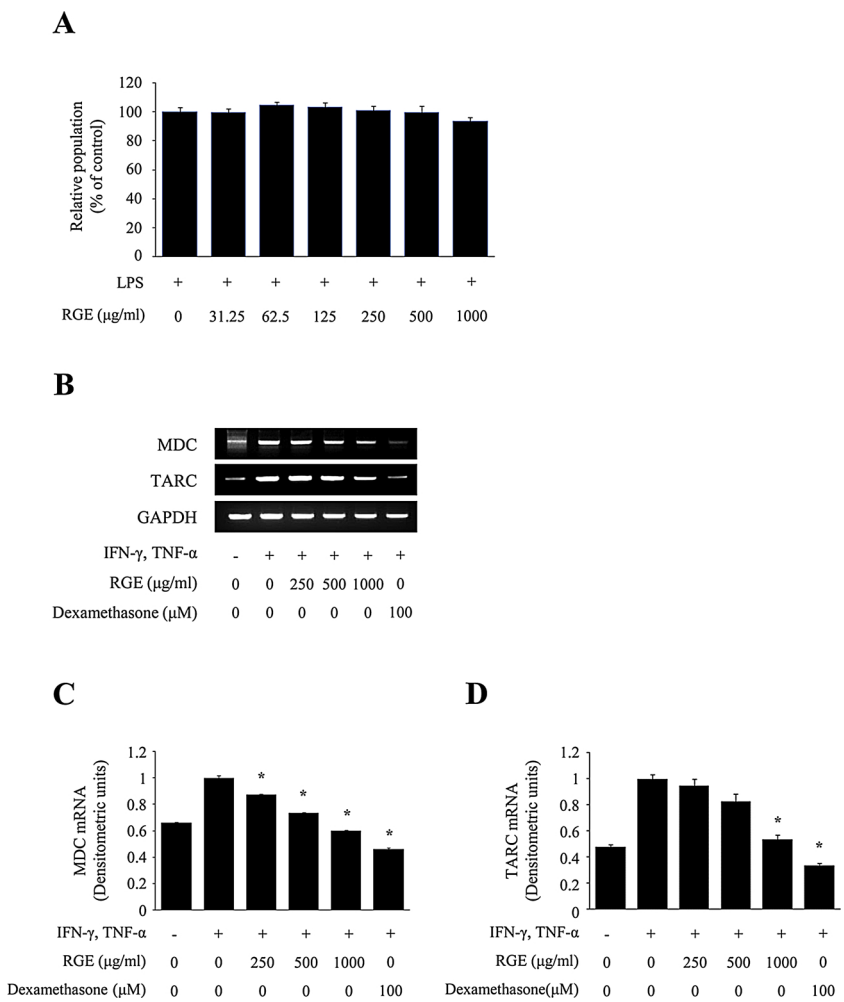


Fig. 8. Effect of RGE on cell viability and chemokines in HaCaT cells. (A) HaCaT cells were treated with RGE (31.25–1000 μg/ml) for 1 h and stimulated with LPS (1 μg/ml) for 24 h. Cell viability of RGE was determined using MTT assay. (B–D) HaCaT cells were treated with RGE (250–1000 μg/ml) for 1 h and stimulated with IFN-γ and TNF-α (10 ng/ml) for 24 h. The expression of MDC and TARC in HaCaT cells was determined by RT-PCR and densitometry protocol. Dexamethasone was used as a positive control. All data are presented as mean ± SD; $^{*}P < 0.05$ compared with control group.

RGE. Th2 cytokines have been shown to inhibit the recovery of skin barrier damage and inhibit ceramide synthesis and filaggrin expression [26,27]. Furthermore, both TARC and TSLP are important in Th2 inflammatory response. Th2 inflammatory response and the infiltration of eosinophils increase the levels of TARC. TSLP is known to induce myeloid dendritic cells and the Th2 inflammatory response [21,40,41]. MDC involved in the infiltration of Th2 type cells is produced in allergic reaction such as inflammation and observed in skin lesions [42,43]. In this study, TARC, MDC and TSLP levels were significantly reduced by RGE treatment of DNCB-induced AD mice and IFN-γ and TNF-α-induced HaCaT cells (Figs. 6 and 8).

MAPK pathways are important signaling pathways in inflammatory responses. It is well-known that as a result of the inflammatory response there is increased phosphorylation of ERK1/2, JNK, and p38 in the DNCB-induced mice [44,45]. In this study, we showed that the DNCB-induced phosphorylation of ERK1/2, JNK, and p38 was especially reduced by RGE (Fig. 7).

In conclusion, the present study demonstrated that RGE attenuates the inflammatory responses in a DNCB-induced AD-like disease in BALB/c mice. RGE caused a decrease in the dermatitis score, skin pH, and TEWL and an increase in skin hydration by suppressing the number of eosinophils and serum IgE levels. Furthermore, RGE improved epidermal thickness, decreased mast cell infiltration and ceramide levels, and increased filaggrin levels. Further, it reduced the expression levels of TSLP, TARC, and Th-2 mediated cytokines by inhibiting the phosphorylation of p38 MAPK in AD-like skin lesions. These findings suggest that RGE alleviates the AD-like inflammatory responses by negatively regulating the MAPK signaling pathways.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2019.109066.

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Supplement Fig. 1 Park et al

Group	CREA	TP	ALB	ALP	GOT	GPT
Normal	0.51 ± 0.05	3.89 ± 0.11	1.42 ± 0.05	1.70 ± 0.80	33.81 ± 2.24	16.03 ± 3.53
Control	0.52 ± 0.02	4.38 ± 0.18	1.26 ± 0.05	1.72 ± 1.62	36.70 ± 3.55	15.96 ± 4.06
100 MPK	0.52 ± 0.03	4.37 ± 0.16	1.23 ± 0.08	2.22 ± 1.89	32.85 ± 3.98	15.26 ± 1.24
200 MPK	0.48 ± 0.02	4.21 ± 0.13	1.24 ± 0.07	1.48 ± 0.42	36.10 ± 3.40	13.42 ± 3.39
400 MPK	0.55 ± 0.14	4.31 ± 0.24	1.34 ± 0.08	2.66 ± 1.45	34.57 ± 2.40	12.55 ± 1.13

Group	T-BIL	BUN	IP	LDH	D-BIL
Normal	0.01 ± 0.01	24.45 ± 2.46	10.69 ± 0.82	326.96 ± 32.36	0.06 ± 0.01
Control	0.03 ± 0.01	26.64 ± 0.73	9.28 ± 1.80	285.47 ± 33.90	0.05 ± 0.00
100 MPK	0.02 ± 0.01	25.78 ± 2.26	8.24 ± 0.53	370.12 ± 35.42	0.04 ± 0.01
200 MPK	0.02 ± 0.01	24.10 ± .316	9.54 ± 0.11	317.00 ± 55.08	0.06 ± 0.01
400 MPK	0.02 ± 0.01	24.06 ± 3.98	11.11 ± 3.19	337.87 ± 36.82	0.05 ± 0.01

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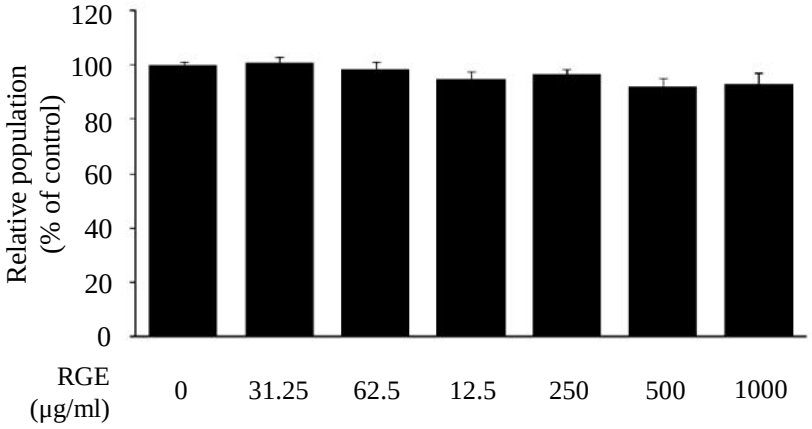

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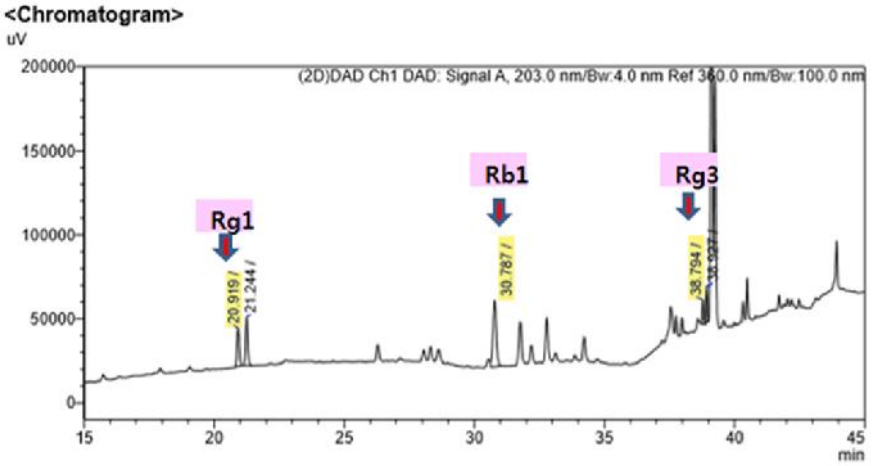

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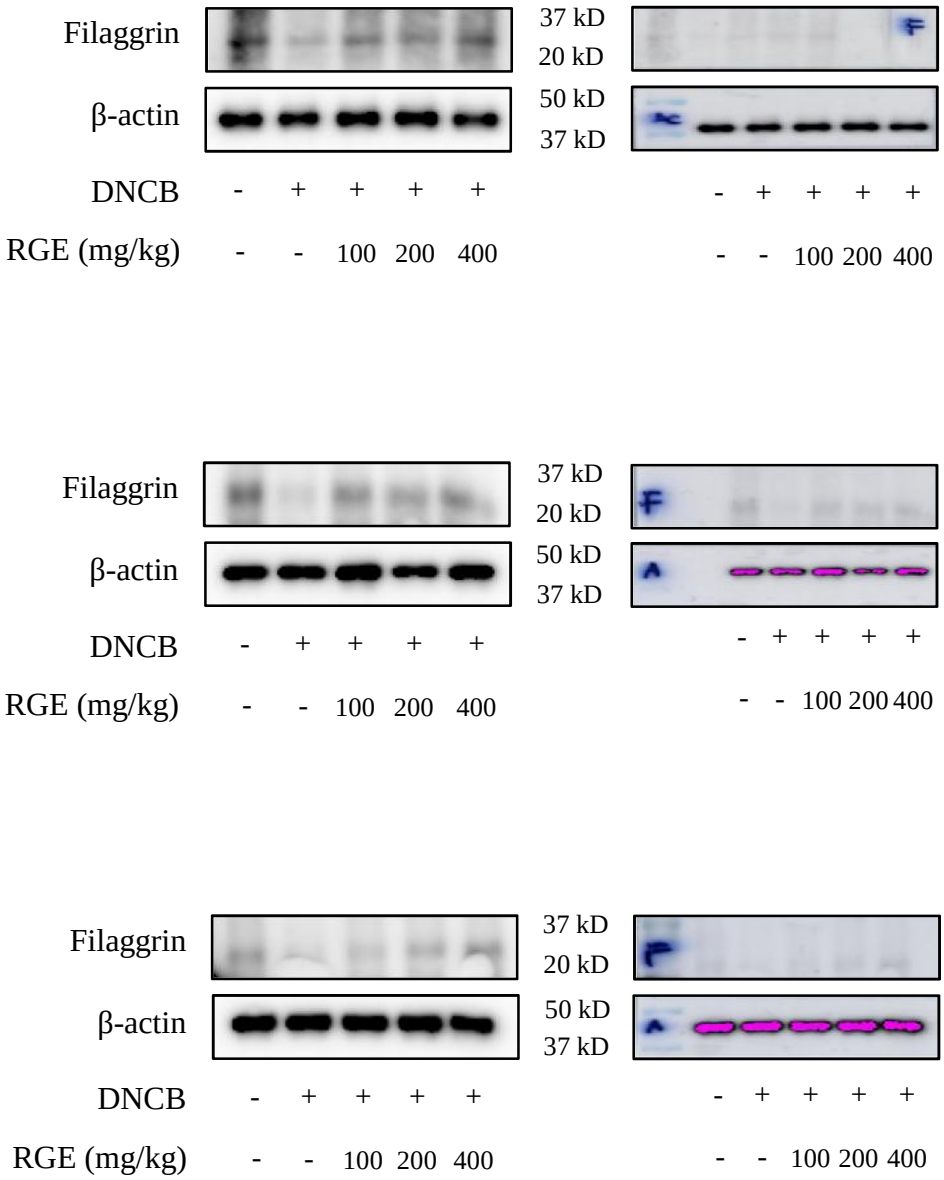

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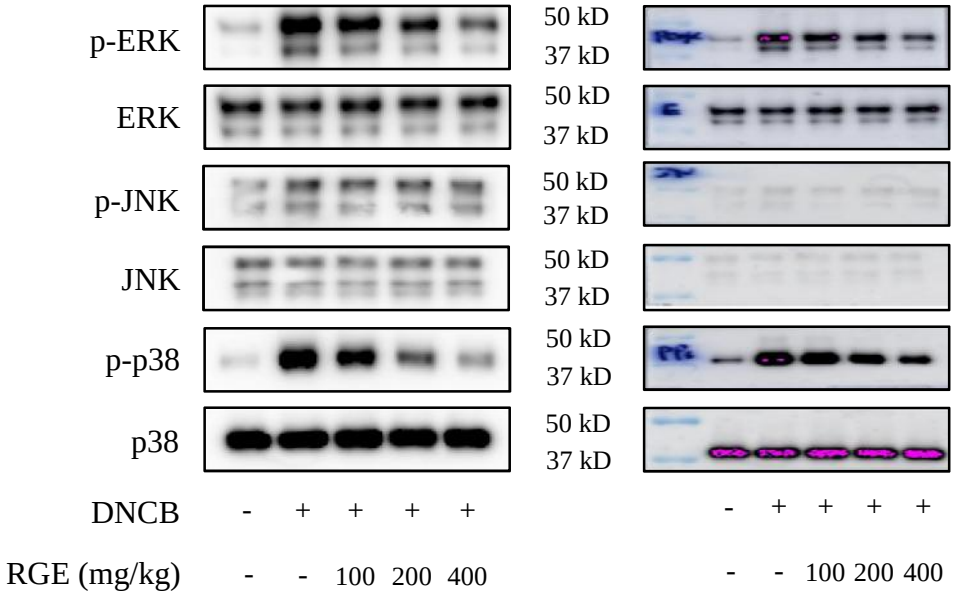

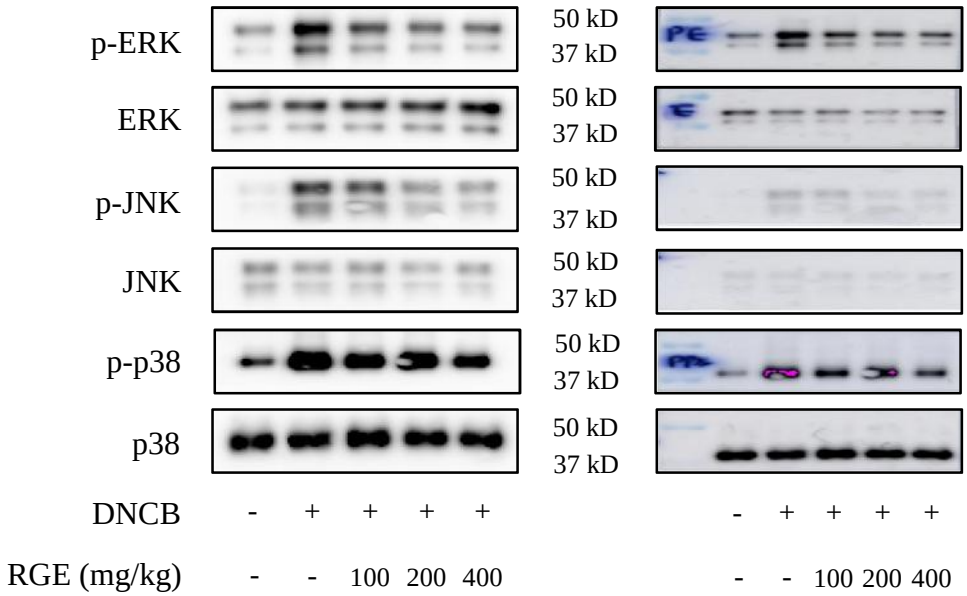
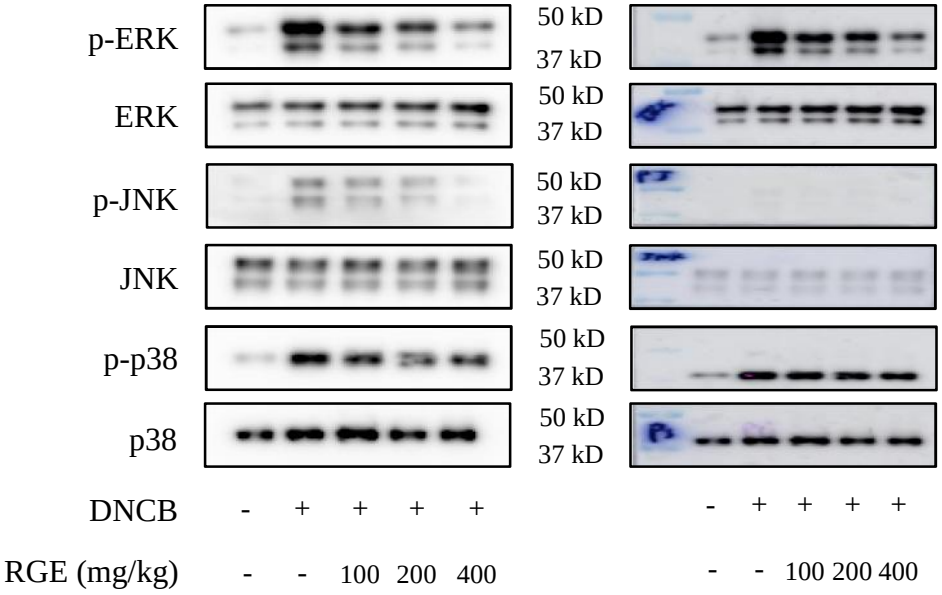
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