



Original article

The prevention of TNF- α /IFN- γ mixture-induced inflammation in human keratinocyte and atopic dermatitis-like skin lesions in Nc/Nga mice by mineral-balanced deep sea water



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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease caused by environmental and chemical allergens. Despite the complexity of its pathogenesis, many investigations have shown that substances having anti-inflammatory activities alleviated the pathology of AD. Here, we evaluated the effects of mineral-balanced deep sea water (DSW) on AD-like skin damage in both *in vitro* and *in vivo*. The results showed that mineral-balanced DSW regressed inflammatory chemokines, such as macrophage-derived chemokine (MDC), thymus- and activation-regulated chemokine (TARC) and regulated on activation, normal T-cell expressed and secreted (RANTES), and cytokines, interleukin (IL)-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA expression in HaCaT immortal human keratinocyte treated with tumor necrosis factor (TNF)- α /interferon (IFN)- γ mixture. Furthermore, increased cyclooxygenase (COX)-2 protein expressions were also reversed, filaggrin gene expression was enhanced and decreased involucrin transcriptions was recovered by mineral-balanced DSW in TNF- α /IFN- γ mixture-treated HaCaT human keratinocyte. Moreover, we revealed that the inhibitory effects of mineral-balanced DSW were mediated with the suppression of signal transducer and activator of transcription (STAT) 1 phosphorylation. In animal experiments, we showed that hardness 2000 of mineral-balanced DSW decreased the serum levels of IgE, IL-4, and histamine, and alleviated the severity score and numbers of scratching in dinitrochlorobenzene (DNCB)-treated Nc/Nga mice. Furthermore, increased epidermal thickness and mast cell infiltration by DNCB treatment were reversed by the application of hardness 2000 mineral-balanced DSW. Taken together, the present investigation indicates that mineral-balanced DSW is a potent substance with anti-atopic dermatitis activity.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with primary occurrence in children [1]. Although the pathogenesis of AD is not fully understood due to the wide variety of causes, many investigations have shown that it is related to allergic inflammation in keratinocyte and disturbance of skin barrier [2]. Therefore, anti-inflammatory agents, such as corticosteroids, are widely used to treat AD patients. The typical characteristics of AD are as follows: Skin redness, increase of skin thickness, itchiness, and skin dryness caused by inflammation responses. Activated keratinocyte by AD developing-agents produces and secretes proinflammatory chemokines, such as macrophage-derived chemokine (MDC/CCL22), thymus- and activation-

regulated chemokine (TARC/CCL17) and regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5), and they trigger infiltration of immune cells, involving monocyte, mast cell and T lymphocytes, to induce skin inflammation [3]. The stimuli also increase the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to AD chronicity and severe inflammation through the penetration of the immune cells in the skin [4]. Moreover, cytokines secreted from Th1 cells trigger inflammation on AD skin lesion through the increased production of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukine (IL)-1 β , and IL-6, in keratinocytes [5,6]. Furthermore, intercellular adhesion molecule-1 (ICAM-1) is overexpressed in AD skin, leading to leukocyte infiltration into the skin [7].

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Many investigations have shown that the inflammatory responses, involving skin inflammation caused by AD, are regulated by janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinases (MAPKs), and nuclear factor-kappaB (NF- κ B) signaling pathways [8–12]. The activation of these signaling pathways by inflammatory responses leads to the synthesis of proinflammatory chemokines and cytokines. In vitro experiments using HaCaT human keratinocyte showed that these signaling pathways were activated by treating TNF- α and/or interferon (IFN)- γ with increased inflammatory chemokines and cytokines expressions [13–16]. Furthermore, increased inflammatory chemokines and cytokines expressions were reversed by anti-inflammatory candidates via the inhibition of STAT, PI3K/Akt, MAPKs, and/or NF- κ B signaling pathway(s) [13–16]. These reports demonstrate the importance of the signaling pathways in regulating inflammation of the skin.

Deep sea water (DSW) has been recognized as natural source of mineral and one of the potent substances that provide various physiological activities, such as anti-diabetes, anti-hypercholesterolemia, anti-obesity, and anti-cancer [17–20]. Moreover, a previous clinical trial has shown that drinking DSW in AD patients was associated with the restoration of mineral imbalance, indicating the relationship between essential mineral balance and skin inflammation in AD and supporting that DSW is an effective essential mineral source [21]. These investigations demonstrate the benefit of DSW on hindering physiological disorders and suggest the importance of mineral on metabolic disease, cancer and inflammation. However, clinical trial in AD patients was done with mineral-imbalanced DSW and the DSW was not directly administrated on AD-damaged skin. Also, biological mechanism of anti-inflammatory effect of DSW on AD skin was not shown in previous studies. Therefore, we investigated the effects of mineral-balanced DSW on atopic-like skin inflammation in Nc/Nga mice and HaCaT immortalized human keratinocytes.

2. Materials and methods

2.1. Materials

Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from GenDEPOT (Barker, TX, USA), and antibodies for cyclooxygenase (COX)-2, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (IkB α), phospho-IkB α (Ser32), c-Jun N-terminal kinase (JNK)1/2, phospho-JNK1/2 (Thr183/Tyr185), extracellular signal-regulated kinase (ERK)1/2, phospho-ERK1/2 (Thr202/Tyr204), NF- κ B, phospho-NF- κ B (Ser536), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), Akt, phospho-Akt (Ser 473), STAT1, phospho-STAT1 (Tyr701), c-Jun, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were bought from Cell Signaling Technology (Beverly, MA, USA). HRP conjugated goat anti-rabbit IgG and bi-cinchoninic acid (BCA) protein assay kits were obtained from Pierce Biotechnology (Rockford, IL, USA). Dinitrochlorobenzene (DNCB) was obtained from Sigma Aldrich (St. Louis, MO, USA) and primers for quantitative RT-PCR were produced from Bioneer (Daejeon, Korea). Polyvinylidene fluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA).

2.2. Preparation of DSW

Mineral-balanced DSW was offered from Seawater Utilization Plant Research Center in the Research Institute of Ships and Ocean Engineering (Goseong, Korea). DSW was taken from a depth of 500-m in the East Sea. It was filtrated via reverse osmosis and electro-dialyzed to remove saline and to extract minerals [18]. Then, the extracted minerals containing Mg and Ca (the ratio of Mg:Ca was 3:1) were dissolved in desalinated DSW (H0) to prepare DSW with hardness 4000 (H4000); it was composed of 835.6 mg/L Mg, 279.9 mg/L Ca,

213.7 mg/L Na, and 81.2 mg/L K. Moreover, we prepared high Mg DSW with hardness 5400 (the ratio of Mg:Ca was 40:1) containing 1298.26 mg/L Mg, 29.93 mg/L Ca, 376.60 mg/L Na, and 423.20 mg/L K. H4000 was serially diluted with H0 to prepare for various hardness of mineral-balanced DSW, and high Mg DSW with hardness 5400 was also diluted with desalinated DSW to prepare for high Mg DSW with hardness 1500 (Mg40). DMEM medium was prepared with H4000, Mg40 and H0 to perform cell experiments and DMEM medium containing artificial Mg and Ca (hardness 1500) was also manufactured. DMEM medium containing artificial Mg and Ca was called Exo (Mg:Ca = 3:1), and MgCl₂ and CaCl₂ were used as sources for Mg and Ca. The hardness was calculated using the following formula: Hardness = [Mg (mg/L) $\times$ 4.1] + [Ca (mg/L) $\times$ 2.5].

2.3. Cell culture and treatment

HaCaT immortalized human keratinocytes were kindly gifted from professor Su-Kyung Kim (Department of Pharmacology, Keimyung University School of Medicine) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (ATCC, USA) and 1% antibiotic-antimycotic solution (Welgene, Korea).

2.4. Quantitative real-time PCR

Total RNA in HaCaT human keratinocyte and Nc/Nga mice skin was extracted using the easy BLUE total RNA Extraction Kit (iNtRON Biotechnology, Sungnam, Korea). Then, cDNA was synthesized with GoScript reverse transcriptase (Promega Corporation, Madison, WI, USA). The reaction for quantitative real-time PCR was performed with the Eco Real-Time PCR System (Illumina, San Diego, CA, USA) by a QGreen™ Sybr Green Master Mix Kit (CellSafe, Suwon, Korea); cDNA was preheated at 95 °C for 10 min, and PCR amplification was followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. The relative expression of the target genes was calculated using the Eco™ Software v3.1.7 using the $\Delta\Delta$ CT method. Used primer sequences are shown in Table 1.

2.5. Western blotting

Whole cells lysates in HaCaT human keratinocyte were prepared with protease inhibitor- and phosphatase inhibitor-contained RIPA lysis buffer [50 mM Tris-HCl (pH7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate, 0.1% SDS and 2 mM ethylenediaminetetraacetic acid]. The protein concentration was determined using the BCA assay, and the equal amount of protein in whole cell lysates was separated via SDS-polyacrylamide gel electrophoresis. Then, total protein was transferred to the PVDF membrane prior to being blocked by 5% skim milk or 1% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBS-T) solution. The primary antibodies for COX-2, IkB α , phospho-IkB α , JNK1/2, phospho-JNK1/2, ERK1/2, phospho-ERK1/2, NF- κ B, phospho-NF- κ B, p38 MAPK, phospho-p38 MAPK, Akt, phospho-Akt, STAT1, phospho-STAT1, c-Jun, and GAPDH were probed onto the PVDF membrane, overnight at 4 °C; then the secondary antibody (which was diluted at 1:5000 in TBS-T solution) was incubated for 1 h at room temperature. All primary antibodies were diluted at 1:3000 with 5% skim milk or 1% BSA in TBS-T solution. To detect the target proteins, chemiluminescent substrate was reacted with HRP conjugated to secondary antibody for 1 min at room temperature. Target protein bands were observed and photographed by Luminescent Image Analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

2.6. Animals and treatment

Animal experiment was approved by the Institutional Animal Care and Use Committee of Dongguk University (IACUC-2017-005); we conducted all experiments using Nc/Nga mice. Male Nc/Nga mice were

Table 1
Sequences of quantitative real-time RT-PCR primers.

Gene		Primer sequence	AnnealingTemp. (°C)	Product Size(bp)
MDC	Forward	5'-GTTGCTCCTCGTCTCTGC-3'	60	150
	Reverse	5'-GGAGTCTGAGGTCCAGTAGAAGTG-3'		
RANTES	Forward	5'-AGTGTGTGCCAACCCAGAGA-3'	60	117
	Reverse	5'-AGCAAGCAGAAACAGGCAAA-3'		
TARC	Forward	5'-GTCTTGAAGCCTCTCACCC-3'	60	147
	Reverse	5'-GGATCTCCCTCACTGTGGCT-3'		
IL-6	Forward	5'-TACCCCAAGGAGAAGATTCC-3'	60	199
	Reverse	5'-GCCATCTTTGGAAGGTTCA-3'		
GM-CSF	Forward	5'-ACTTCCTGTGCAACCCAGATT-3'	59	118
	Reverse	5'-CATCTGGCCGGTCTCACTC-3'		
ICAM-1	Forward	5'-GCTGGTGACATGCAGCACC-3'	60	399
	Reverse	5'-CTCCTCACCAGCACCGTGG-3'		
Filaggrin	Forward	5'-GCAAGGTCAAGTCCAGGAGAA-3'	60	123
	Reverse	5'-CCCTCGGTTTCCACTGTCTC-3'		
Involucrin	Forward	5'-GCAAGGTCAAGTCCAGGAGAA-3'	60	89
	Reverse	5'-CCCTCGGTTTCCACTGTCTC-3'		
GAPDH	Forward	5'-CTGCTCTCTCTGTTTCGACAGT-3'	60	101
	Reverse	5'-CCGTTGACTCGACCTTCAC-3'		

obtained from Orient Bio Inc. (Gapyung, Korea), and cared at $25 \pm 2^\circ\text{C}$ in $50 \pm 5\%$ relative humidity (dark/light cycle: 12 h). Mice were adapted for 1 week and divided into five groups: The normal control, negative control (DNCB-treated only), DNCB + H1000, DNCB + H2000, and positive control (DNCB + 1% hydrocortisone) groups. Three days before administering the experimental treatment, animals were shaved and pre-challenged with 200 μl of 1% DNCB in an acetone and olive oil mixture (3:1) [22]. Then, the mice were treated twice per week with 0.4% DNCB and tap water, DSW or hydrocortisone was applied every day for 8 weeks.

2.7. Scoring of skin severity

The severity scoring of the skin was estimated 1 time per week. The eczema severity index was evaluated by the following SCORAD method; dryness, redness, crusts, traces of scratching, and thickened skin were scored on a 4-point scale, 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The number of itching behavior was counted for 1 h. The severity was blindly scored, and the sum of scores in each individual was taken as the severity score [23].

2.8. Histological analysis

After 8 weeks of treatment, mice were sacrificed and skin tissues were isolated and fixed with 10% neutral formalin solution. The skin tissues were paraffin-embedded prior to section to 4 μm . (Ed note: please confirm phrase) The sections were stained with hematoxylin and eosin to observe skin thickness. To count the infiltrated mast cells in the skin, the sections were stained with toluidine blue. The number of mast cells was counted in three sites that were randomly selected.

2.9. Enzyme-linked immunosorbent assay for serum parameters

Blood was collected and held for 10 min until blood clots were formed. Blood clots were removed with centrifugation at 1000 rpm for 15 min, and Sera were transferred to 1.5-ml microtubes. Sera were used for analysis of the serum parameters, such as histamine, IgE, IL-4, and IFN- γ . An analysis of the serum parameters was conducted using the commercial ELISA kit in accordance with the manufacturer's instruction. The ELISA kits for IgE and IL-4 were purchased from BioLegend (San Diego, CA, USA), ELISA kit for histamine was bought from IBL international GmbH (Hamburg, Germany) and ELISA kit for IFN- γ was obtained from KOMABIOTECH (Seoul, Korea). Quantitation was performed with SpectraMax M2e (Molecular Devices, Sunnyvale, CA, USA).

2.10. Statistical analysis

The data for cell all experiments were statistically analyzed using Student's *t*-test or Mann-Whitney U test followed by Tukey or Kruskal-Wallis test.

3. Results

3.1. Down-regulation of inflammatory chemokines, cytokines and adhesion molecule expression by mineral-balanced DSW in HaCaT keratinocyte

Inflammatory chemokines and cytokines, such as MDC, TARC, RANTES, IL-6 and GM-CSF, expression in keratinocyte are triggered by the inflammatory responses. Adhesion molecule ICAM-1 is also enhanced by the responses. The relationship of AD severity with the levels of MDC, TARC, and RANTES in the serum or skin has previously been reported [24–27]. Moreover, increased GM-CSF expression has been shown in AD keratinocytes, and the induction of IL-6 and ICAM-1 were detected in HaCaT cells by TNF- α -induced inflammatory response [4,7,28]. Therefore, we investigated the effect of mineral-balanced DSW on inflammatory chemokines and cytokines gene expression in HaCaT human keratinocyte treated with TNF- α /IFN- γ mixture. In addition, the effects of mineral-balanced DSW on these expressions were compared with Mg40 and Exo, respectively. The result showed that both mineral-balanced DSW and Mg40 reversed inflammatory chemokines, such as MDC, TARC and RANTES, and cytokines, such as GM-CSF and IL-6 (Fig. 1). Simultaneously, ICAM-1 transcription in HaCaT cells was regressed by mineral-balanced DSW or Mg40 treatment (Fig. 1). Although Exo inhibited MDC, RANTES, and GM-CSF transcription, the expressions of TARC and ICAM-1 were not affected by Exo. These results suggest that prevention is more effective by mineral-balanced DSW and Mg40 compared with Exo.

3.2. Up-regulation of filaggrin and involucrin gene expressions by mineral-balanced DSW in HaCaT keratinocytes

Skin functions as a barrier in animals, protecting the body from external stresses, such as chemical, physical, and biological stress, and from moisture loss. In AD patients, defects in the skin barrier are observed, leading to skin inflammation caused by facilitated pathogenic infection and allergen infiltration. Therefore, we estimated that mineral-balanced DSW, Mg40, and Exo would have an effect on gene expression of filaggrin and involucrin, major components of the stratum corneum (SC). We found that down-regulated involucrin gene expression by TNF- α /IFN- γ mixture was recovered by the administration of

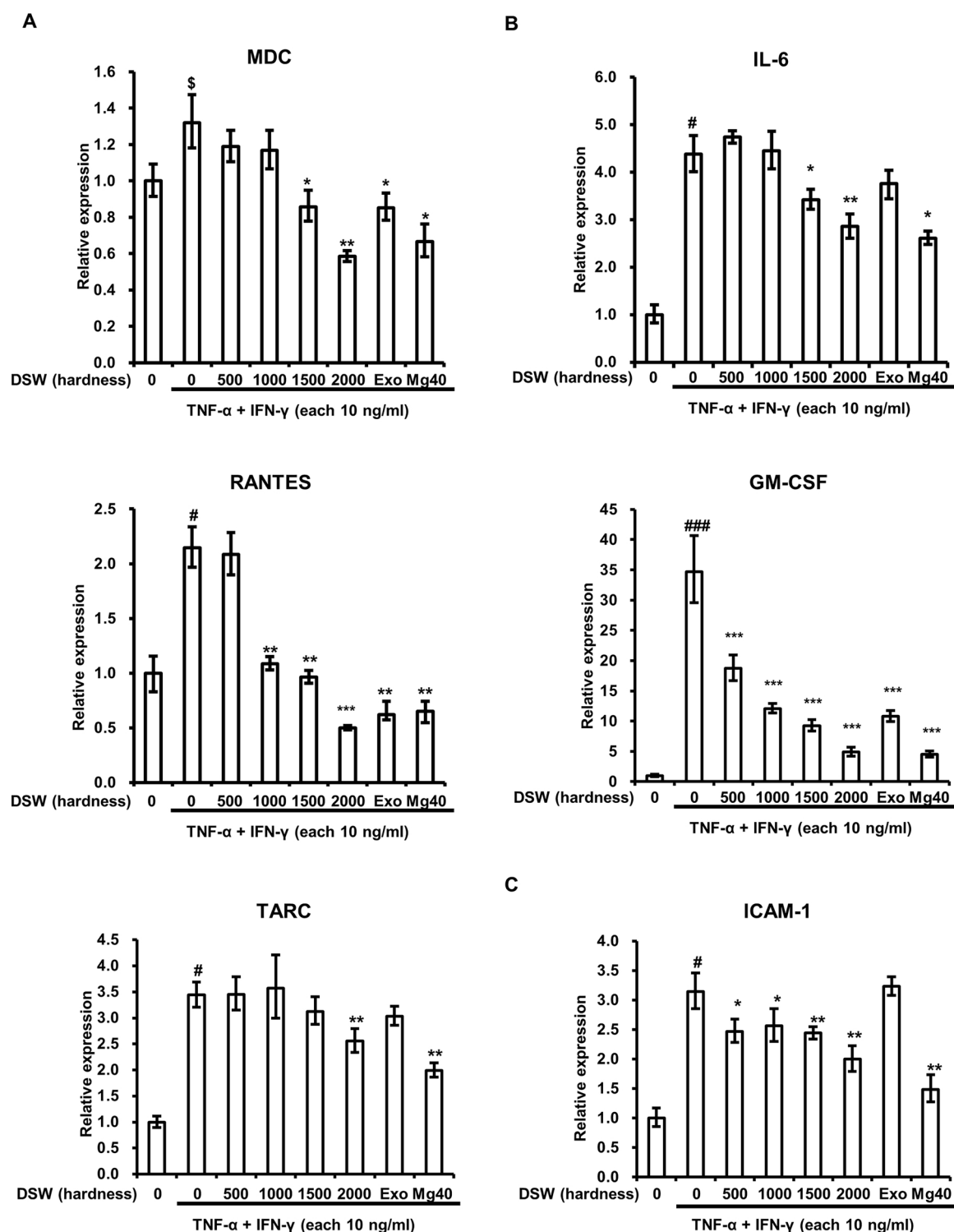


Fig. 1. Effects of mineral-balanced DSW, Mg40, or Exo on gene expressions of inflammatory chemokines and cytokines, as well as ICAM-1 in HaCaT keratinocytes. (A) Suppression of inflammatory chemokines, such as MDC, RANTES and TARC, gene expressions by mineral-balanced DSW, Mg40 or Exo. (B, C) Regulation of inflammatory cytokines, such as IL-6 and GM-CSF, and ICAM-1 gene expressions by mineral-balanced DSW, Mg40 or Exo. (A–C) Cells were simultaneously treated with mineral-balanced DSW, Mg40, or Exo, and TNF- α /IFN- γ mixture (10 ng/ml each other) for 24 h. Relative expression of all genes were in triplicate, verified with quantitative RT-PCR. GAPDH was served as the internal control to normalize expression levels in each sample. All data are represented as the means $\pm$ SD of triplicate experiments. [#], ^{*} and ^{###} indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. hardness 0 treated with TNF- α /IFN- γ mixture, respectively.

mineral-balanced DSW and Exo (Fig. 2). Furthermore, although filaggrin expression was not regressed by TNF- α /IFN- γ mixture treatment, the expression was up-regulated by mineral-balanced DSW and Exo (Fig. 2). Contrastingly, Mg40 increased the transcription of involucrin, but not that of filaggrin (Fig. 2). Interestingly, Exo was dramatically

enhanced involucrin gene expression (Fig. 2). The result shows benefits of mineral-balanced DSW, Mg40, and Exo preventing skin barrier disruption by AD-like inflammation induced by TNF- α /IFN- γ mixture in HaCaT keratinocytes.

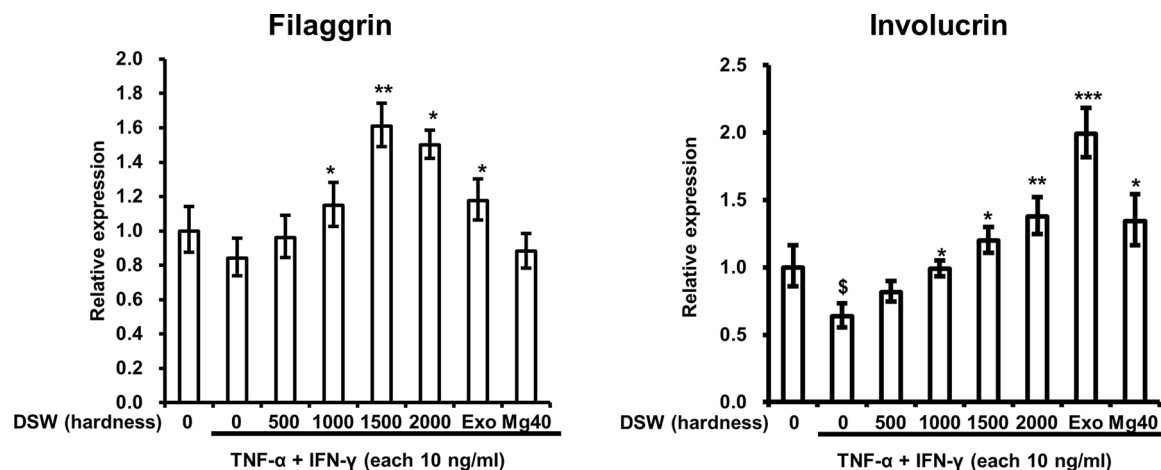


Fig. 2. Effects of mineral-balanced DSW, Mg40, or Exo on gene expression of filaggrin and involucrin in HaCaT keratinocytes. Changes of filaggrin and involucrin gene expressions by mineral-balanced DSW, Mg40, or Exo. Cells were simultaneously treated with mineral-balanced DSW, Mg40, or Exo, and TNF- α /IFN- γ mixture (10 ng/ml each other) for 24 h. Relative expression of all genes were in triplicate, verified with quantitative RT-PCR. GAPDH was served as the internal control to normalize expression levels in each sample. All data are represented as the means $\pm$ SD of triplicate experiments. \$ indicates $P < 0.05$ vs. hardness 0-only. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. hardness 0 treated with TNF- α /IFN- γ mixture, respectively.

3.3. Down-regulation of COX-2 expression by mineral-balanced DSW in HaCaT keratinocyte

High level of COX-2, which is an inducible proinflammatory enzyme that catalyzes PGE₂ biosynthesis, is detected in AD skin [29]. Moreover, the increase of COX-2 and PGE₂ by urban particulate matter should lead to skin barrier dysfunction through the inhibition of filaggrin expression [30]. Therefore, the regulation of COX-2 expression is important for the management of skin inflammation. Here, we showed the effect of mineral-balanced DSW, Mg40, and Exo on COX-2 expression induced by TNF- α /IFN- γ mixture. The results showed that mineral-balanced DSW and Mg40 significantly regressed TNF- α /IFN- γ mixture-enhanced COX-2 expression in HaCaT human keratinocyte; however, Exo did not have the same effect (Fig. 3). The results suggest that mineral-balanced DSW and Mg40 have anti-COX-2 activity in TNF- α /IFN- γ mixture-treated HaCaT keratinocytes.

3.4. Down-regulation of STAT1 and JNK1/2 phosphorylation and c-Jun expression by mineral-balanced DSW in HaCaT keratinocyte

The expression of inflammatory factors, such as COX-2, MDC, RANTES, TARC, IL-6, and GM-CSF, in HaCaT human keratinocyte treated with TNF- α /IFN- γ mixture were primarily regulated by STAT, NF- κ B and/or MAPK signaling pathway(s) [31,32]. Therefore, we studied the effects of mineral-balanced DSW, Mg40, and Exo on the phosphorylation of STAT1, NF- κ B, and MAPK. In addition, the changes of Akt phosphorylation and c-Jun expression by mineral-balanced DSW, Mg40, or Exo treatment were also investigated. The results showed that mineral-balanced DSW, Mg40, and Exo reversed STAT1 and JNK1/2 phosphorylation, but they did not control NF- κ B, I κ B α , ERK1/2, and Akt phosphorylation (Fig. 4A–C). In addition, enhanced c-Jun expression by TNF- α /IFN- γ mixture was down-regulated by mineral-balanced DSW, Mg40, and Exo (Fig. 4A). The results demonstrate that the anti-inflammatory effects of mineral-balanced DSW, Mg40, and Exo are associated with STAT1 and JNK1/2 signaling pathways.

3.5. Prevention of DNCB-induced AD-like skin lesions by mineral-balanced DSW in Nc/Nga mice

We evaluated the effects of mineral-balanced DSW on atopic like skin lesions induced by DNCB in Nc/Nga mice. The results showed that hardness 2000 mineral-balanced DSW (H2000) improved DNCB-induced AD-like skin lesions with lower scratching numbers ($P = 0.032$)

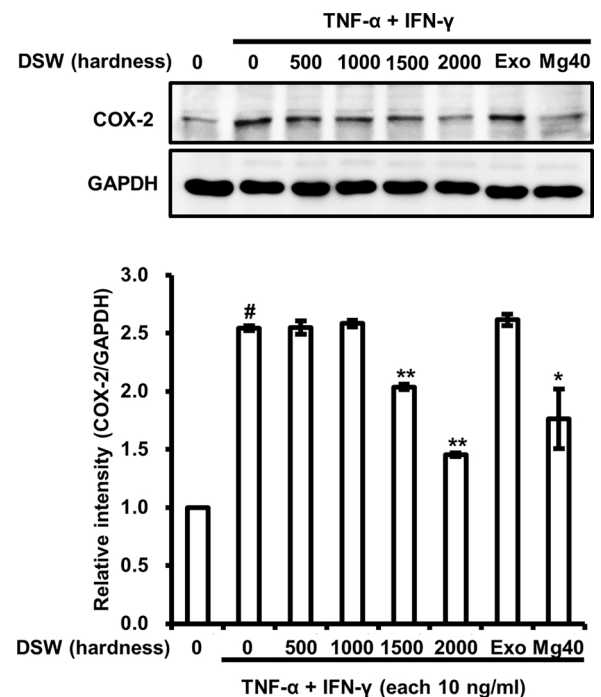


Fig. 3. Effect of mineral-balanced DSW, Mg40, or Exo on COX-2 expression. The induction of COX-2 protein by TNF- α /IFN- γ mixture was suppressed by mineral-balanced DSW, Mg40, and Exo, respectively. Cells were simultaneously treated with mineral-balanced DSW, Mg40, or Exo, and TNF- α /IFN- γ mixture (10 ng/ml each other) for 24 h. Relative intensities were densitometrically analyzed using Scion Image. GAPDH was used as the loading control to normalize expression levels in each sample. The values are represented as the means $\pm$ SD of triplicate experiments. # indicates $P < 0.001$ vs. hardness 0-only. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. hardness 0 treated with TNF- α /IFN- γ mixture, respectively.

and clinical severity ($P = 0.0002$) than the DNCB-only group (Fig. 5). Furthermore, increased skin thickness by DNCB was significantly restored by the application of H2000 ($P = 0.049$) (Fig. 6A and B). Moreover, H2000 reversed the increase of histamine ($P = 0.031$), IgE ($P = 0.024$), and IL-4 ($P = 0.012$) concentration (Fig. 7). However, we could not observe the change of mast cell number increased by DNCB (Fig. 6C). Consequently, these results describe that mineral-balanced DSW may prevent or treat atopic-like skin lesions induced by DNCB.

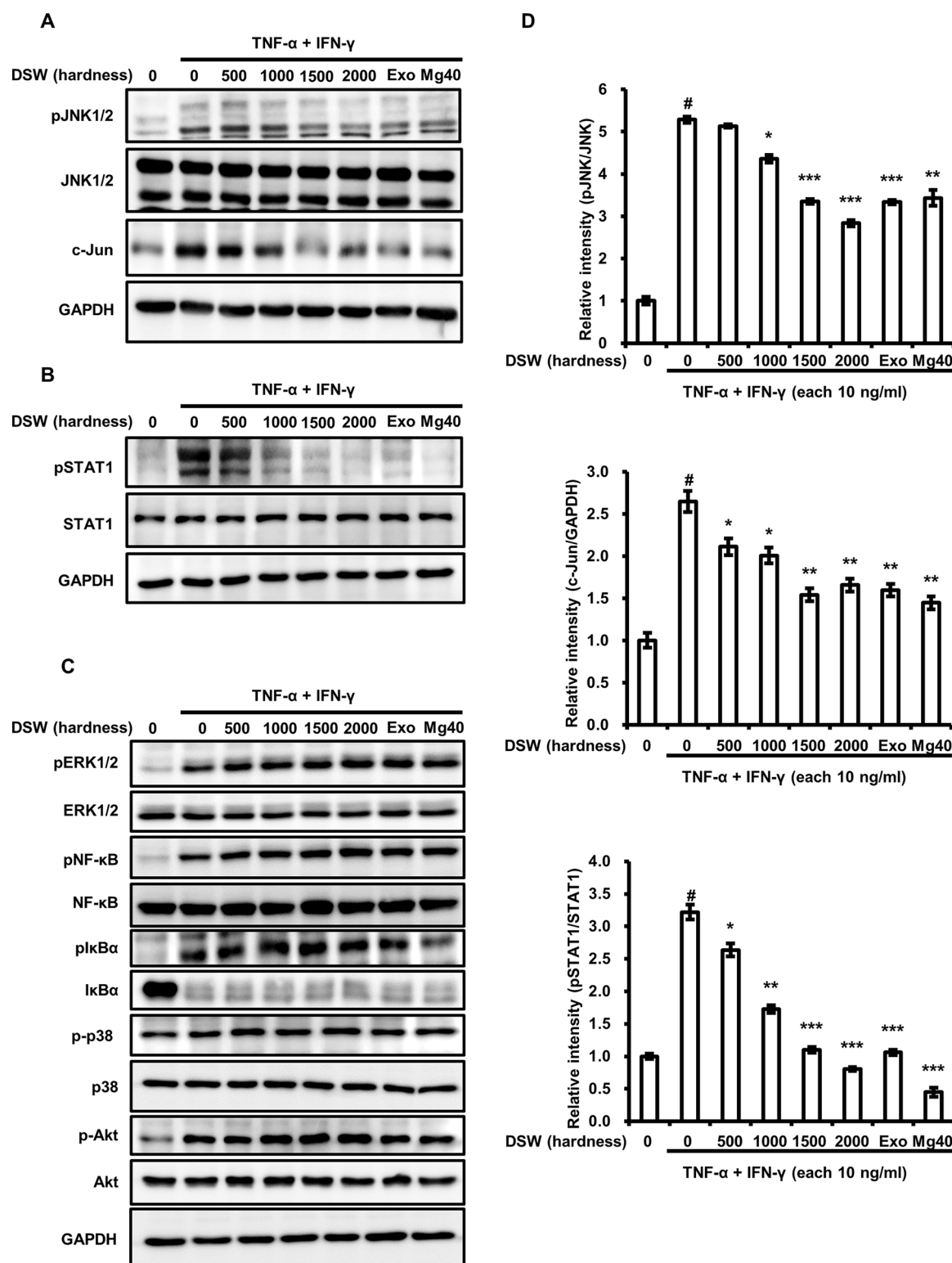


Fig. 4. Effects of mineral-balanced DSW, Mg40, or Exo on inflammatory signaling pathways. (A) The suppression of JNK 1/2 phosphorylation and c-Jun expression by mineral-balanced DSW, Mg40, or Exo. (B) The inhibition of STAT1 phosphorylation by mineral-balanced DSW, Mg40, or Exo. (C) Mineral-balanced DSW, Mg40, and Exo did not affect ERK 1/2, NF- κ B, I κ B α , p38, and Akt phosphorylation. (A–C) Cells were pre-treated with mineral-balanced DSW, Mg40, or Exo for 24 h prior to the treatment of TNF- α /IFN- γ mixture (10 ng/ml each other) for 10 min. GAPDH was used as the loading control. (D) Relative intensities of pJNK1/2, c-Jun and pSTAT1 were densitometrically analyzed using Scion Image. Values of pJNK1/2, c-Jun and pSTAT1 were normalized with JNK1/2, GAPDH and STAT1, respectively. GAPDH was used as the loading control. The values are represented as the means $\pm$ SD of triplicate experiments. # indicates $P < 0.001$ vs. hardness 0-only. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. hardness 0 treated with TNF- α /IFN- γ mixture, respectively.

4. Discussion

To determine whether DSW is a useful candidate for improving and/or treating the skin damage in AD patient, we investigated the effects of

mineral-balanced DSW in atopic-like skin inflammation using HaCaT human keratinocyte and Nc/Nga mice. We found that mineral-balanced DSW and Mg40 lessened the increase of inflammatory chemokines, cytokines, and adhesion molecule, involving MDC, RANTES, TARC,

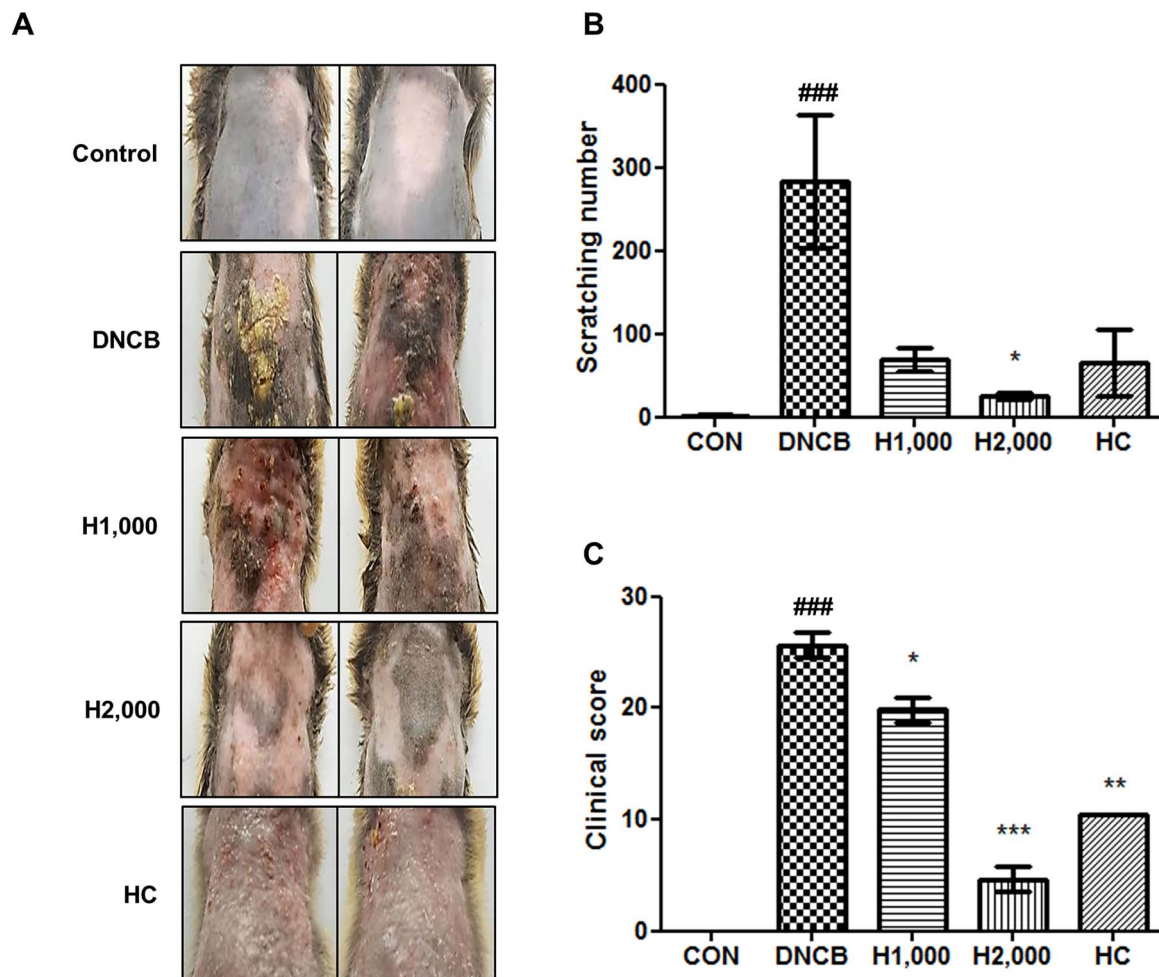


Fig. 5. Effects of mineral-balanced DSW on the development of AD-like skin lesions in Nc/Nga mice induced by DNCB. (A) DNCB-induced AD-like skin damages were prevented in the H2000 group. The mice were divided into five groups (n = 5): Control (treated with vehicle and tap water), DNCB (treated with DNCB and tap water), H1000 (treated with DNCB and hardness 1000 of mineral-balanced DSW), H2000 (treated with DNCB and hardness 2000 of mineral-balanced DSW), and HC (treated with DNCB and 1% hydrocortisone) groups. Mice were administrated with 200 μ l of 0.4% DNCB twice per week, and 200 μ l of tap water, mineral-balanced DSW or hydrocortisone were treated every day. (B, C) Scratching number and clinical severity were blindly monitored once per week for 8 weeks by three researchers. The values are represented as the mean $\pm$ SD of triplicate experiments performed on the 8th week. ^{###} indicates $P < 0.0001$ vs. Con. ^{*}, ^{**} and ^{***} indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. DNCB, respectively (n = 5).

GM-CSF, IL-6, and ICAM-1, by TNF- α /IFN- γ mixture treatment in HaCaT human keratinocytes (Fig. 1A–C). Furthermore, mineral-balanced DSW and Exo restored the expression of involucrin declined by TNF- α /IFN- γ mixture in HaCaT human keratinocytes (Fig. 2). The induction of inflammatory chemokines and cytokines, as well as the reduction of filaggrin and involucrin in the skin are typical responses in AD-like inflammation. Therefore, the results imply that mineral-balanced DSW should alleviate more AD-like inflammation in the skin than Mg40 and Exo.

The increases of COX-2 and IL-6 expressions are associated with the disruption of skin barriers. Lee et al. reported that filaggrin expression in the skin was inversely related to COX-2 expression [30]. Furthermore, IL-6 decreases the synthesis of ceramide, playing the role as a water modulator and a permeability barrier in the SC [33]. In this investigation, we found that enhanced COX-2 protein and IL-6 gene expression by TNF- α /IFN- γ mixture were down-regulated, and filaggrin and involucrin transcription were enhanced by mineral-balanced DSW treatment (Figs. 1B, 2 and 3). Furthermore, mineral-balanced DSW lessened skin thickness in DNCB-treated Nc/Nga mice (Fig. 5A). These results demonstrate that mineral-balanced DSW should prevent and/or recover damage to the skin barrier caused by AD-like lesion through the inhibition of IL-6 gene expression and induction of filaggrin and involucrin transcriptions. In addition, the increase of involucrin by Exo implies that Mg and Ca mixture (the ratio is 3:1) is the most effective on

restoration of skin lesions induced by cytokine-mediated inflammation.

Inflammatory chemokines, such as TARC, MDC, and RANTES, increased in stimulated keratinocyte by inflammatory cytokines trigger the infiltration of leukocyte in AD lesions and T lymphocyte differentiation [34]. Ultimately, the increase of the chemokines in keratinocyte leads to the activation of IgE production, eosinophils, and mast cells, further aggravating AD lesions [35,36]. We found that mineral-balanced DSW suppressed the gene expression of inflammatory chemokines in HaCaT keratinocyte and reduced the serum IgE level in DNCB-treated Nc/Nga mice (Figs. 1 and 7B). These results suggest that the inhibition of IgE production by mineral-balanced DSW in DNCB-treated Nc/Nga mice is associated with the suppression of inflammatory chemokines production in keratinocyte. In addition, IgE is a typical allergic marker. Therefore, these results indirectly demonstrate that mineral-balanced DSW may act as an anti-allergenic substance.

Histamine is known to be an inflammation mediator and as an organic compound that provoke pruritus in AD patients [37]. IL-4 is a critical cytokine in the differentiation of Th2 cells and IgE, contributes to the development of AD, which is increased in AD patients [38]. In this investigation, the numbers of itching behaviors in DNCB-treated Nc/Nga mice were diminished by the administration of mineral-balanced DSW. The reduced numbers of itching behaviors were corresponded with the serum histamine level (Figs. 5B and 7 A). Furthermore, we also ascertained that the serum IL-4 level and skin thickness

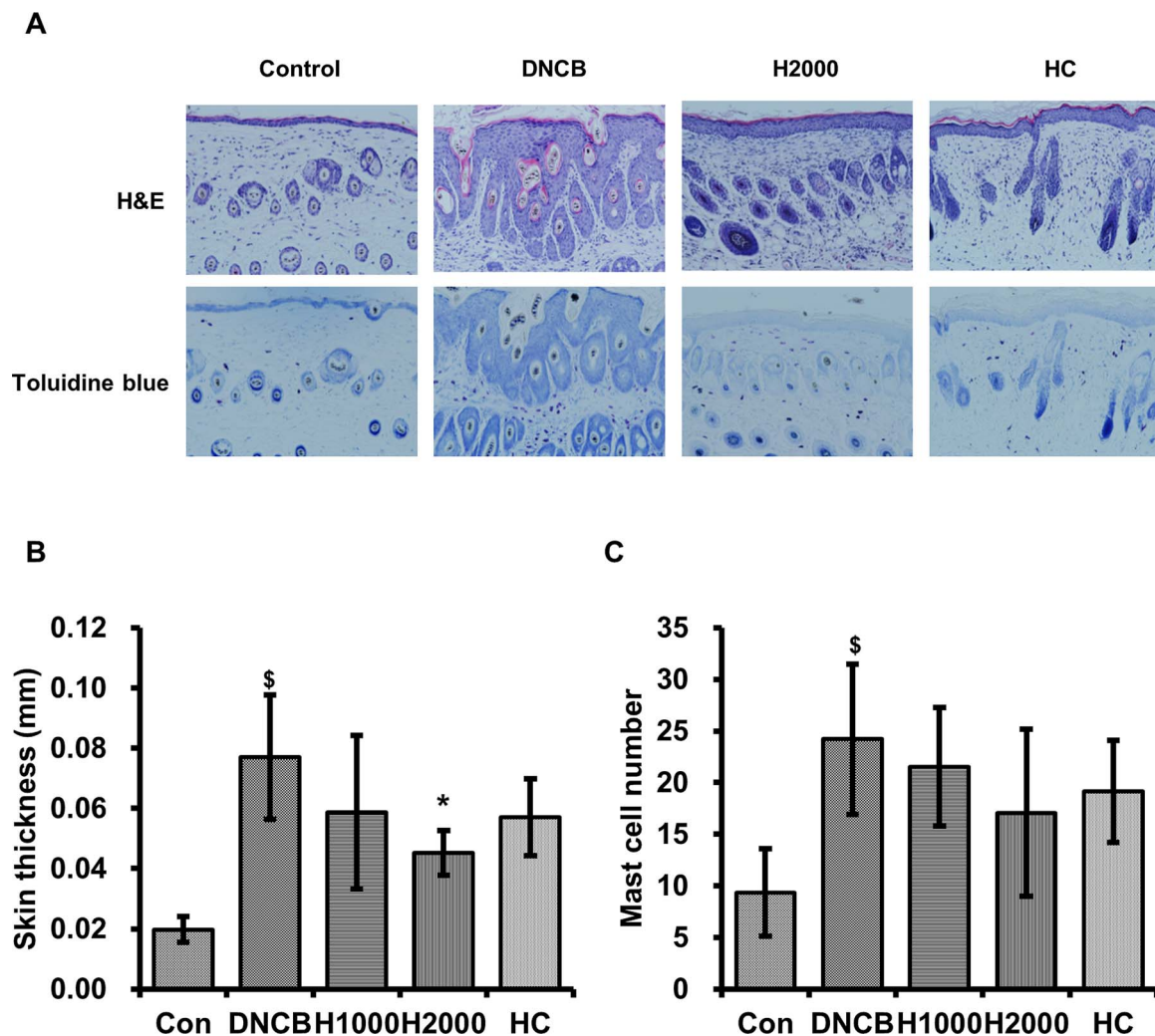


Fig. 6. Effects of mineral-balanced DSW on skin integrity and mast cell infiltration in Nc/Nga mice treated by DNCB. (A) DNCB group showed acanthosis, papillomatosis, papillary dermal chronic inflammation, and increased number of mast cells compared with the control group. Improvement of the pathological findings was shown in the H2000 group. Magnification, $\times 200$. The skin tissue sections were stained using the H&E solution (upper panels) and toluidine blues (lower panels). (B, C) Three regions per animal were randomly selected to assess the skin thickness and to count the number of mast cells, respectively ($n = 5$). The values are represented as the mean $\pm$ SD. ^{\$} indicates $P < 0.05$ vs. Con. ^{*} indicates $P < 0.05$ vs. DNCB.

were reduced by mineral-balanced DSW in DNCB-treated Nc/Nga mice (Figs. 6A and 7C). Skin thickness is closely linked with cutaneous inflammation. Therefore, the results demonstrate that mineral-balanced DSW should lessen histamine-linked pruritus and improve atopic-like skin lesions via the regression of IL-4 production in DNCB-treated Nc/Nga mice.

MAPK and STAT signaling pathways play important roles in the regulation of inflammatory responses in keratinocytes activated by TNF- α /IFN- γ mixture. MAPK and STAT signaling pathways are related to proinflammatory chemokines and cytokines in HaCaT keratinocytes [31,32,39,40]. Here, we showed that the expressions of proinflammatory chemokines and cytokines and phosphorylation of STAT1 and JNK 1/2 were repressed by mineral-balanced DSW, Mg40, or Exo in activated HaCaT keratinocytes by TNF- α /IFN- γ mixture (Figs. 1 and 4A and B). Furthermore, mineral-balanced DSW, Mg40, and Exo reversed c-Jun expression, a subunit of the activator protein 1 (Fig. 4A). However, mineral-balanced DSW did not affect cytokine-induced phosphorylation of NF- κ B, I κ B α , ERK1/2, and Akt (Fig. 4B). The results suggest that STAT1 and JNK 1/2 participate in the inhibition of mineral-balanced DSW, Mg40, and Exo on the expression of proinflammatory chemokines and cytokines in HaCaT keratinocytes but not NF- κ B, I κ B α , ERK1/2, and Akt.

Here, we compared the effects of mineral-balanced DSW in AD-like

inflammation in TNF- α /IFN- γ mixture-treated HaCaT keratinocytes using Mg40 and Exo. Mg40 and Exo also suppressed the inflammatory chemokines and cytokine(s) gene expression and enhanced transcription(s) of filaggrin and/or involucrin, component(s) of SC (Figs. 1 and 2). However, the effects were no more effective than mineral-balanced DSW. Consequently, the results indicate that the ratio of Mg:Ca is important to determine the effectiveness of DSW for the prevention and treatment of AD-like inflammation and that trace mineral in mineral-balanced DSW may affect the inhibition of inflammation in keratinocyte activated by inflammatory cytokines.

Bak et al. also confirmed the benefit of DSW on AD-like skin lesions in Nc/Nga mice [41]. However, the investigation was performed with concentrated DSW containing high concentration of Na (4,070 mg/L), Ca (102,100 mg/L) and Mg (34,800 mg/L) and they did not show the biological mechanism of DSW on skin inflammation. In contrast, our investigation was conducted with mineral-balanced DSW and we revealed the importance of Mg:Ca ratio for the prevention and treatment of AD-like inflammation and also showed the biological mechanism of the anti-inflammatory effect of DSW.

We confirmed the anti-inflammatory effects of DSW in cytokines-treated HaCaT cells and DNCB-treated Nc/Nga mice with various hardness of DSW. The results showed that hardness 1000 and/or 1500 mineral-balanced DSW (H1000 and H1500) also suppressed the

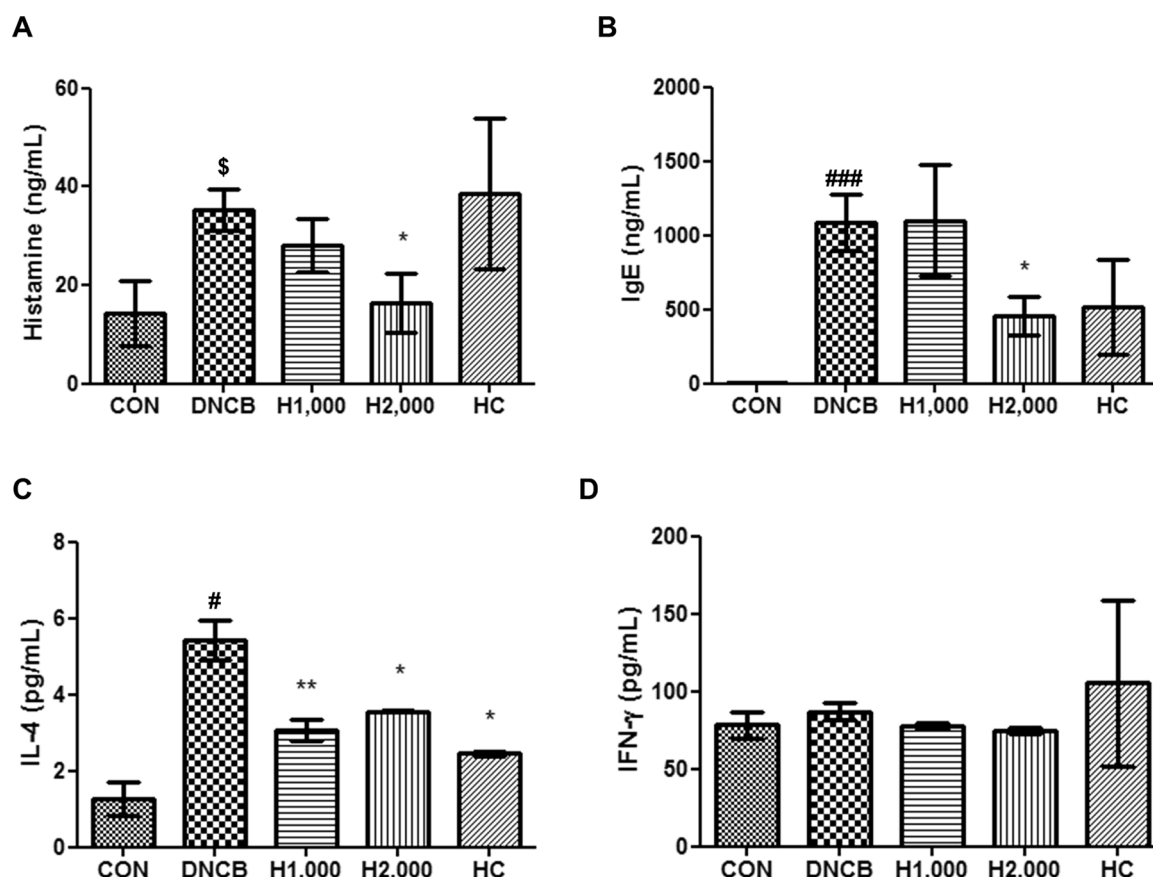


Fig. 7. The effects of mineral-balanced DSW on histamine, IgE, IL-4, and IFN- γ levels in the serum. (A, B) Lower levels of histamine and IgE were shown in the H2000 group compared with the DNCB group. (C) Lower level of IL-4 was shown in the H1000, H2000, and HC groups compared with the DNCB group. (D) Level of IFN- γ did not change in all groups. (A–D) Blood was collected from the hearts of mice, and the levels of serum histamine, IgE, IL-4, and IFN- γ in each group were evaluated using the commercial ELISA kit. The values are represented as the mean $\pm$ SD of triplicate experiments. ^{\$}, [#] and ^{###} indicate $P < 0.05$, $P < 0.001$ and $P < 0.0001$ vs. Con, respectively. ^{*} and ^{**} indicate $P < 0.05$ and $P < 0.01$ vs. DNCB, respectively.

activation of inflammatory response induced by TNF- α /IFN- γ mixture (Figs. 1A–C, 3). Also, filaggrin and involucrin gene expressions were increased by H1000 and H1500 (Fig. 2). However, H1000 and H1500 did not affect TARC transcription. Furthermore, H1000 did not fully improve skin damage and levels of histamine and inflammatory cytokines (Figs. 5–7). These results demonstrate that H2000 is more effective to improve AD-like inflammation.

Taken together, the present investigation demonstrates that mineral-balanced DSW prevents or treats atopic-like skin lesions through the suppression of proinflammatory chemokines and cytokines expressions, as well as the inhibition of histamine and IgE production underlying STAT1 and JNK1/2 signaling pathways. Also, this study describes that restoration of skin health by mineral-balanced DSW is associated with the up-regulation of filaggrin and recovery of involucrin expressions and the inhibition of IL-4 production. Therefore, the results suggest that mineral-balanced DSW may be a potent material for the prevention or treatment of skin inflammation caused by skin disorders, including atopic dermatitis.

Conflict of interest

Not declared.

Acknowledgments

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