Immunoinflammatory Regulation Effects of Korean Hot Spring Water

Jin-Wou KIM1), Hyung Jin HAHN1), So-Youn WOO2), Seong-Taek YUN3), Jong Tae LEE4), Hong Jig KIM5)

Abstract

Background: The beneficial clinical effects of Korean hot spring spa therapy, as well as their underlying mechanisms are still poorly understood. We performed a series of clinical and laboratory investigations for better understanding of the clinical effects as well as possible mechanisms of their beneficial effects.

Methods: HaCaT cells were prepared and treated with TLR agonist in the presence or absence of HS water for quantification of IL-6, IL-8, GM-CSF, and TNF-α levels. The serum levels of IFN-γ, IL-4, IL-5, and IgE were measured. CD4+ naïve cells were allowed to polarize into Th1, Th2, Th17, and Treg cells, and CD4+ and CFSE+ cells were measured for the degree of proliferation. Total RNA from the lesional skin was transcribed into cDNA using a reverse transcription system, and RT-PCR was performed subsequently. Confocal microscopy and RT-PCR were utilized to evaluate the target skin localization of Th cell subsets and associated inflammatory cytokine milieu.

Results: Treatment with agonists of TLR 1 through 6 induced attenuation of cytokine production in the exposure to HS water. HS water suppressed the proliferation of Th1, Th2, and Th17 cells with anti-CD3 stimulation, while proliferation and differentiation to Treg cells were promoted under HS water treatment. On RT-PCR of the lesional skin, thymic stromal lymphopoietin (TSLP) mRNA decreased dramatically after bathing with HS. IL-33 mRNA decreased markedly in HS water group as compared to control group. Foxp3 mRNA expression, same as in confocal microscopic finding, showed tendency to increase more in HS.

Conclusions: HS water suppressed the proliferation of Th1, Th2, and Th17 cells. In contrast, proliferation and differentiation to Treg cells were promoted under HS water treatment. These results indicate that HS water may affect the distribution of the helper T cells in the immune response, by suppressing the polarization of the Th1, Th2, and Th17 cells. Also, APC induced TNF-α and IL-6 levels were reduced in the presence of HS water. These results showed that TLR-triggered inflammatory responses in APCs might also be modulated under HS water treatment. Overall, our findings suggest that HS spa therapy could be an effective...

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and safe modality for the management of adult AD.

**Keywords:** Korean hot spring water, immunoinflammatory regulation, Toll-like receptor agonists

I  INTRODUCTION

Spa therapy has long been utilized as one of the safe, effective and long-term alternative management options of various dermatologic diseases in Korea. Among them are pruritic, xerotic, immune-inflammatory, infectious, and ulcerative skin diseases. There are about 400 registered spa areas in South Korea, many which are recognized as having specific therapeutic effects, but the beneficial clinical effects of spa therapy as well as their underlying mechanisms are still poorly defined and understood. In Korea, the area of distribution of hot springs (HSs) coincides with areas of granite and is distant from geothermal heat energy related to volcanos. The physicochemical characteristics of South Korean HS is low proportion of high temperature (mean temperature of 32.2°C), low mineral contents (median TDS: total dissolved solids of 695 mg/L), and alkaline pH (mean pH of 8.6) in nature. Natural HS water in South Korea can be classified into five main hydrochemical groups, i.e., Ca(Na)-HCO$_3$ type of low TDS, Na(Ca)-HCO$_3$ type, Na(Ca)-Cl type of high TDS, acidic Ca-HCO$_3$ type, and Ca(Na)-SO$_4$ type. We performed a series of clinical and laboratory studies for better understanding of the clinical effects as well as possible mechanisms of their beneficial effects of several HSs located at different places and having different mineral characteristics.

II  MATERIALS AND METHODS

1.  Hot springs (HSs)

Five HS districts were selected in this study; Haeundae HS (Na-Cl type), located at Busan-si, Sukmodo HS (Na-Cl, Ca-Cl type), at Gangwha-gun, Incheon-si, Suanbo HS (Na-HCO$_3$ type), at Chungju-si, Chungcheongbuk-do, Baekam (Na-HCO$_3$ type) and Dukku (Na-Cl type) HSs, at Uljin-gun, Kyungsangbuk-do.

2.  Hydrochemical analysis of HS water

The temperature, pH, electrical conductivity, and alkalinity of the HS water were measured *in situ*. Collected water samples were filtered with 0.45 m cellulose membranes and stored in polyethylene bottles. Samples for cation analysis were acidified to pH < 2 by adding a few drops of ultra-pure nitric acid. The alkalinity was measured in the field using an acid-neutralizing titration technique and then converted to the equivalent HCO$_3^-$ and CO$_3^{2-}$ concentrations. Water samples were analyzed for Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, SiO$_2$, and total ion concentrations using induced coupled plasma-atomic emission spectrometry (Perkin Elmer OPTIMA 3000XL; Meinhard, Golden, CO, USA) and atomic adsorption spectrometry (Perkin Elmer Analyst100, Meinhard). Anions, such as Cl$^-$, SO$_4^{2-}$, PO$_4^{3-}$, and F$^-$, were analyzed by ion chromatography (Dionex 120: Dionex, Sunnyvale, CA, USA). Quality controls included blank samples and duplicate or triplicate subsamples and standard materials.
3. Diagnosis of atopic dermatitis (AD)
Diagnosis of AD was done by Hanifin & Rajka (1980)\(^4\) criteria.

4. Induction of AD-like skin lesions in mice
All animal procedures were approved by the Ethics of Animal Experimentation Committee of Uijeongbu St. Mary’s Hospital, and conformed to international standards. Four to five week-old female NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan). The mice were housed and bred under conventional conditions at the animal laboratory of the Uijeongbu St. Mary’s Hospital. Dfb ointment was prepared by Biostir Inc. (Kobe, Japan) and one gram of Dfb ointment contained 41.7 mg of protein, 58.5 μg of Der f1 and 22.2 μg of Der f2. In the first induction, the dorsal hair of the mice was clipped using an electric shaver, and the residual hair was depilated using a hair removal cream. Topical application of 100 mg of Dfb ointment on the shaved dorsal skin and surface of both ears was done. In the second induction, barrier disruption was achieved by 150μl of 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and the surface of both ears 3 hours before Dfb ointment application. These procedures were repeated 3 to 4 times a week for up to 2 weeks. For the induction of AD-like skin lesion by hapten sensitization, six-week old female Hos:HRM hairless mice (from Saeron Bio, Korea) were sensitized with cutaneous application of 10μl of 5% oxazolone (Sigma chemical Co., St Louis, USA) solution. After 1 week, daily application of 60μl of 0.1% oxazolone solution for 7 days was done.

5. Bathing
Patients with AD were bathed 3 times a week, for 4 weeks. On each bath, the patients were instructed to avoid scrubbing of the skin and to stay in bath and/or take showers for 30 minutes. After bathing, residual water on the skin was air-dried. Mice were immersed in distilled or HS water baths for 5 minutes, at 37°C to 39°C, daily for 1 to 2 weeks. Specifically designed acrylic bath in thermos-regulated apparatus was used\(^6\).

6. Evaluation of skin lesion
EASI (Eczema Area Severity Index), VAS (Visual Analogue Score), and PGA (Physician’s Global Assessment) were measured to evaluate objective, subjective, and overall clinical symptoms of AD patients\(^5\). Modified SCORAD (SCORing AD) method was used to assess grossly the severity of induced dermatitis in mice\(^6\). Assessment was performed by an investigator who was blind to the grouping of the animals.

7. Measurement of skin barrier parameters
Trans-epidermal Water Loss (TEWL), SC (Stratum Corneum) hydration, skin pH, skin surface temperature were measured with Multiprobe Adaptor Systems (CK electronic, Skin Bioengineering Instrument, Germany) according to the manufacturer’s instructions.

8. Preparation of HS water
Waters were filtered through 0.2 μm filters and stored at 4°C until used for experiments. For the measurement of water osmolarity, Micro-Osmometer 210 (FISKE Associate, Norwood, MA, USA) was used.
9. MTT assay

For the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St.
Louis, MO, USA) assay, cells were plated onto 96-well microtiter plates at a density of 3 x 10⁴/200 μl in fresh medium, and then treated with HS water at a serially diluted concentration.
Cells were cultured for 1, 4, 10, and 24 hours, respectively, to observe a time dependent effect.
After the indicated time, 20 μl of MTT (5 mg/ml in phosphate-buffered saline) was added to
each well, and the plates were returned to the incubator for an additional 4 hours. At the end
of the incubation period, the supernatants were discarded and 200 μl dimethyl sulfoxide was
added to all the wells, in order to dissolve formazan crystals. The plates were subsequently
covered with aluminum foil, gently shaken for 15 minutes, and read at a wavelength of 570 nm.

10. Cell culture

HaCaT cell (human keratinocyte cell line) was cultured in Dulbaco’s Modified Eagle Medium
(DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS,
Gibco- BRL) and 100 U/ml penicillin/streptomycin (Gibco-BRL), at 37℃ in an incubator contain-
ing 5% CO₂.

11. Toll-like receptor (TLR) stimulation

Real-time polymerase chain reaction (RT-PCR) analysis showed that the HaCaT cells
constitutively express mRNA for Toll-like receptors 1 through 9, except for TLR7 and 8. We
stimulated TLR1 through 9 TLR agonists were treated with the following final concentrations
for 24 hours. Tripamitoyl-S-glyceryl-cysteine (Pam3Cys, 1 μl/ml), heat-killed Listeria monocy-
togenes (HKLM, 10⁶ cell/ml), polyriboinosinic polyribocytidylic acid (poly (I: C), 10 μl/ml), li-
popolysaccharide (LPS, 10 μl/ml), flagellin (10 μl/ml), and Pam2CGDPKHPKS (FSL-1, 1 μl/ml)
were from InvivoGen (San Diego, CA, USA). HS waters were added simultaneously or pre-
treated 2 hours before the TLR agonist treatment. Cells were cultured for 1, 4, 10, or 24 hours
in each treatment group.

12. ELISA

Levels of IL-6, IL-8, GM-CSF, and TNF-α (BD OptEIA™, BD Biosciences Pharmingen, San
Diego, CA, USA), IL-1α (Biolegend, San Diego, CA, USA) in HaCaT supernatant treated with
TLR agonist in the presence or absence of HS water were quantified according to the
manufacturer’s protocol. IL-6 and TNF-α in mouse antigen presenting cells (APCs) supernatant
were also measured. The serum levels of IFN-γ, IL-4, IL-5, and IgE were measured using
specific ELISA kits according to the manufacturer’s instructions (Mouse IFN-γ: #430804, ELISA
MAX™ Deluxe Sets; BioLegend, San Diego, CA, USA, Mouse IgE: #432404, ELISA MAX™
Deluxe Sets; BioLegend, IL-4: #555232, Mouse IL-4 ELISA Set; BD Bioscience, San Diego, CA,
USA, IL-5: #555236, Mouse IL-5 ELISA Set; BD Bioscience).

13. Mouse spleen cell isolation

Naïve CD4+ T cells were purified from the mouse spleens, via magnetic isolation (Miltenyi
Biotec GmbH, Gladbach, Germany). Spleens were removed and minced with a Nylon mesh (70
μm pore). After the cells were pelleted, erythrocytes were lysed using hypotonic buffer. The
cells were washed in PBS and incubated with anti-CD4 antibody for 15 minutes, at 4°C. The cells were conducted onto a magnetic separator to isolate the CD4+ cells and collected with positive selection. CD4+ cells were further incubated for 4 hours for the collection of the adherent cells.

14. CD4+ T cell differentiation

CD4+ naive cells were seeded at a density of 2 x 10^5 per well in 96-well plates, and were cultured in DMEM containing 10% FBS. For each of the helper T cell differentiation, the skewing conditions are as followed; IL-12 (25 ng/ml, Biolegend) and anti-CD28 (1 μg/ml, Biolegend) for Th1, IL-4 (25 ng/ml, Biolegend) and anti-CD28 (1 μg/ml, Biolegend) for Th2, IL-6 (25 ng/ml, Biolegend), TGF-β (2 ng/ml, R&D system) and anti-CD28 (1 μg/ml, Biolegend) for Th17, IL-2 (25 ng/ml, Biolegend) and anti-CD28 (1 μg/ml, Biolegend) for regulatory T cell (Treg). All cells stimulated with anti-CD3 antibodies with serial dilutions, in which the initial concentration was 3 μg/ml. Cells were incubated for 3 days in the presence or absence of HIS water.

15. Cell proliferation assay

The CD4+ cells were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE, Cell Trace™ CFSE Cell Proliferation kit, Invitrogen, Paisley, UK). Labeled cells were seeded under each condition for the differentiation of Th1, Th2, Th17, and Treg cells. After 3 days, CD4+ and CFSE+ cells were measured for the degree of proliferation, via a flow cytometry, and were analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA), based on the reduction of CFSE positive cells.

16. Flow cytometry

Antigen presenting cells (APCs) were stimulated with TLR3 agonist, poly (I: C) (10 μg/ml) for 24 hours with or without HIS spring waters, stained with anti-mouse I-A/I-E (Biolegend, clone M5/114.15.2, Rat IgG2b, κ) to analyze the expression of MHC II on the surface of APCs for 20 minutes. Samples were acquired on FACS Calibur system (BD Bioscience, San Jose, CA, USA) and were analyzed using CellQuest software (BD Bioscience). FACS analysis of splenic T cell subsets was as followed; To analyze Th1, spleen cells were stained with PE anti-mouse CD4 (100407; BioLegend) followed by staining with APC anti-mouse IFN-γ (505809; BioLegend). For Th2, cells were stained with PerCP anti-mouse CD4 (100431; BioLegend) followed by intracellular staining with Alexa Fluor 488 anti-mouse IL-4 (504111; BioLegend) and PE anti-mouse IL-9 (514103; BioLegend). For Treg cells, cells were fixed and permeabilized, then stained with Alexa Fluor 488 anti-mouse/rat/human Foxp-3 (320011; BioLegend). Isotype controls were used. Data were acquired on a FACS Calibur system (BD Bioscience, San Jose, CA, USA) and were analyzed using CellQuest software (BD Bioscience).

17. Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) analysis for cytokine production

One day after the final bath, mice were sacrificed and dorsal skin was excised. To study the gene expression of the IFN-γ, IL-4, IL-5, TSLP, IL-33, Foxp3, and IL-17A of the dorsal skin of the mice, total RNA was extracted from the skin using TRIzol (Invitrogen, Carlsbad, CA, USA).
in accordance with the manufacturers instructions. Total RNA was transcribed into cDNA using a reverse transcription system (Promega, Madison, WI, USA). The primers used for the RT-PCR were determined\(^6\), \(^9\). Glyceraldehyde 3-phosphate dehydrogenase genes were used as controls for RNA input. RT-PCR with SYBR green detection was conducted using Maxime PCR premix Kit (Intron Biotechnology, Seongnam, Korea) with an ABI PRISM 7000 sequence detection system (Applied Biosytems, Foster City, CA, USA) with 50ng of cDNA.

18. Histologic examination

The skin lesion samples were fixed in 10% formaldehyde, embedded in paraffin, and stained with standard H&E. For immunohistochemistry, paraformaldehyde-fixed cryosection were incubated overnight at 4°C with antibodies against mouse anti-CD1 (sc-9161; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD4 (sc-70671; Santa Cruz Biotechnology), and anti-CD8 (H-160; Santa Cruz Biotechnology). Immunoreactivity was detected with a peroxidase-conjugated secondary antibody against mouse and rabbit immunoglobulin G (DakoCytomation, Glostrup, Denmark), and counterstained. Non-specific antibody binding was ruled out.

19. Confocal microscopy

Paraffin-embedded tissue was dehydrated and processed for antigen retrieval. Antibodies used are anti-mouse TNF-α PE (12-7321, eBioscience, San Diego, CA, USA), anti-mouse CD25 APC (17-0251, eBioscience, San Diego, CA, USA), anti-mouse IL-17 PE (12-7177, eBioscience, San Diego, CA, USA), anti-mouse Foxp3 PE (12-5773, eBioscience, San Diego, CA, USA), anti-mouse CD4 alexa 488 (100423, Biolegend, San Diego, CA, USA), anti-mouse IL-4 DyLight405 (APC) (NB100-64798v, Novus, Littleton, CO, USA), and DAPI (D1306, invitrogen, Carlsbad, CA, USA)

RESULTS

1. The efficacy and the safety of Haeundae spa therapy for the treatment of adult atopic dermatitis in vivo

Haeundae HS is hydrochemically Na-Cl type. It is a weak alkaline (pH of 7.6 ± 0.1), high electrical conductivity (8,980 ± 1,170 μS/cm), and medium concentration of sea salt content (TDS 3,788 ± 741.9 mg/L). The most common cation present was Na\(^+\), followed by Ca\(^{2+}\), K\(^+\), and Mg\(^{2+}\). The prominent anion was Cl\(^-\), followed by SO\(_4\)\(^{2-}\) and HCO\(_3\)\(^-\). A high concentration of SO\(_4\)\(^{2-}\) (192.9 ± 23.4 mg/L) was observed when compared to the median level of SO\(_4\)\(^{2-}\) (12.3 mg/L) present in the thermal ground water in South Korea. Fifteen adult AD patients (5 with mild to moderate and 10 with severe degree) were enrolled for the study. One month after bathing, EASI (Fig. 1) and VAS (Fig. 2) were significantly decreased. Among the five patients with combined drug therapy, one increased the dosage of the drug, three maintained, and one reduced the dosage. TEWL (Fig. 3), SC hydration, and skin pH showed no significant changes. Of the 15 patients, one patient experienced exacerbation of symptoms and two patients reported a transient prickling sensation during the spa therapy.
Fig. 1  Changes in total EASI score after bathing with Haeundae spring water. $P<0.05$ was noted in weeks 2, 3, and 4 in total and severe groups.

Fig. 2  Changes in VAS score after bathing with Haeundae spring water. $P<0.05$ was noted in weeks 1, 2, 3, and 4 in total and in weeks 2 and 4 in severe group.

Fig. 3  Changes in SCORAD index and TEWL after bathing in the mineral water on Dfb ointment-induced AD-like lesions.
2. Effect of Sukmodo spa spring water on cytokine expression in human keratinocyte HaCaT cells and on differentiation of CD4+ T cells in vitro

Sukmodo HS is hydrochemically Na-Cl, Ca-Cl type. pH is 7.84 and high mineral content (TDS of 24,200 mg/L). The most common cation present was Na+, followed by Ca2+, Mg2+, K+, and Sr2+. The prominent anion was Cl−, followed by SO42−. A high concentrations of SO42− (1,010 mg/L) and SiO2 (65 mg/L) were measured. Initial osmolarity of the Sukmodo HS water was 700 mOsm/kg and the optimal dilution factor of x30 for the cell culture was determined by MTT assay. HaCaT cells were incubated with the HS water at the same time with TLR agonists or cells were preincubated with HS for 2 hours and TLR agonists were applied later. After incubation, levels of proinflammatory cytokines, IL-6, IL-8, GM-CSF, and TNF-α, and IL-1α, in HaCaT supernatant were measured by ELISA assay. TLR1 agonist through TLR6 agonist treatment induced the attenuation of cytokine production in the exposure to HS water in advance or simultaneously with TLR agonist treatment (Fig 4). To evaluate the in vivo suppressive capacity of HS water toward the helper T cell differentiation, CFSE-labeled CD4+ T cells from Balb/c mouse spleen were cultured under conditions, which polarized Th1, Th2, Th17, and Treg with or without HS water. HS water suppressed the proliferation of Th1, Th2, and Th17 cells with anti-CD3 stimulation. In contrast, proliferation and differentiation to Treg cells were promoted under HS water treatment (Figs 5 and 6). With APC isolated from Balb/c mouse spleen and subsequent stimulation of TLR3 with poly (I: C), APC treated with HS water down-regulated the surface level of class II MHC expression and also reduced production of TNF-α and IL-6.

3. Therapeutic effects and immunomodulation of Suanbo, Baekam, and Dukku mineral water therapy in a murine model of AD

Suanbo HS is hydrochemically Na-HCO3 type. pH is 8.39 with low mineral content (TDS 348 mg/L). Sodium bicarbonate is the major component. Compared to other Korean HSs, Suanbo is richer in its proportions of calcium and sulfate. Sixteen NC/Nga mice were sensitized to D. farinae protein containing ointment and AD-like chronic skin lesions were induced. Bathing in the mineral water for 2 weeks alleviated the severity of AD-like skin lesions and markedly reduced dermatitis score and TEWL than distilled water control (p<0.05). Reduced inflammatory infiltrates and normalization of epidermal hyperplasia of the skin also were more apparent in mineral water group. On immunohistochemical staining of the skin, decreased CD1+ and CD8+ cells were marked in mineral water bath group compared to positive control without bath and distilled water bath group. The total serum IgE levels after 2 weeks of AD induction were significantly higher compared to the negative control group. Both mineral and distilled water bath group failed to reduce total serum IgE levels (p<0.05). Mineral water bathing seemed to suppress D. farinae ointment-induced serum IL-4 levels but without significance as compared to distilled water group (p<0.05). Also there were no significant differences in serum IFN-γ and IL-5 levels between two groups after bathing. There was a significant increase in the lesional skin mRNA expression of IFN-γ and IL-4 following AD induction (p<0.05). IL-4 mRNA expression...
Immunoinflammatory Regulation of Korean Hot Spring Water

(a)

(b)

(c)
Fig. 4 (a) to 4 (e) TLR1 agonist through TLR6 agonist treatment induced the attenuation of cytokine production in the exposure to HS water in advance or simultaneously with TLR agonist treatment.

Fig. 5 Effects of bathing in the mineral water on Dfb ointment-induced T cells profile of spleen cell.
was reduced by mineral water bath but was not significant. Both mineral and distilled water groups failed to reduce IFN-ɤ and IL-5 mRNA in the skin. Mineral water bathing seemed to suppress the splenic Th2 cell proliferation without significance, but differentiation into the Treg cells was promoted with mineral water bathing compared to distilled water group (p<0.05).

Baekam HS is hydrochemically Na-HCO₃ type. pH is 8.90 with low mineral content (TDS 64 mg/L). The prominent anion is HCO₃⁻ and SO₄²⁻ and high SiO₂ (87 mg/L). AD-like skin lesions were induced in hairless mice by cutaneous sensitization of oxazolone. After daily bathing for 7 days, confocal microscopic technique and RT-PCR were utilized to evaluate the target skin localization of Th cell subsets and associated inflammatory cytokine milieu. Clinical improvement was observed in both distilled and HS water bath groups (p<0.05). Also, decreased inflammation on H&E staining of the skin was evident in both groups as compared to positive control group without subsequent bathing treatment. On confocal microscopic evaluation of the lesional skin, IL-4 staining showed marked reduction of the fluorescence in HS water group than distilled water group. TNF-α staining also showed similar results. CD4⁺ cells were increased in HS water group than in distilled water group. IL-17 staining, representing TH17 cell, rarely observed in HS water group as compared to distilled water group. Foxp3⁺ staining showed strong fluorescence after AD induction and decreased thereafter. Its fluorescence remained increased in HS water group than distilled water group after 7 days’ bath (Fig 7). On RT-PCR of the lesional skin, thymic stromal lymphopoietin (TSLP) mRNA increased after AD induction, decreased dramatically in both HS and distilled water group. IL-33 mRNA also increased after AD induction, but decreased markedly in HS water group as compared to distilled water group. Foxp3 mRNA expression, same as in confocal microscopic finding, showed tendency to increase more in HS water than distilled water group (p<0.05).

Dukku HS is hydrochemically Na-Cl type. pH is 8.84 with low mineral content (TDS 65 mg/L). High F⁻ (10.3 mg/L) and SiO₂ (34 mg/L) were measured. Hairless mice were sensitized to oxazolone and AD-like skin lesions were induced. After daily bathing for 7 days, confocal microscopic technique was utilized to localize the skin infiltrating CD4⁺CD25⁺Foxp3⁺ Treg cells. Clinical improvement measured by modified SCORAD index did not show any difference between positive control without treatment (p>0.05), HS water and distilled water treatment groups. TEWL showed similar results. Tendency of decreased inflammation on H&E staining
of the skin was evident in both groups as compared to positive control group without bathing. On confocal microscopic evaluation of the lesional skin of HS treatment group, CD4+ cells and CD25+ cells merged on same cells confirmed by DAPI staining, but Foxp3+ staining did not merged into CD4+, CD25+, and DAPI-positive cells and was scattered in the field.

**DISCUSSION**

AD is a chronic, relapsing, inflammatory skin disease with severe itching sensation and associated with genetic diathesis of atopy. The incidence and prevalence of AD are increasing worldwide. In Korea, about 5–20% of prevalence in general population is reported\(^{12}\). AD is basically genetic disease, but due to a multifactorial etiology with various aggravation factors, management of this disease has been unsatisfactory. Many topical and systemic therapeutic modalities, aimed to reduce or to suppress the symptoms of AD, not infrequently result in severe topical as well as systemic complications. Various forms of alternative therapy are preferred among individuals with AD in Korea. Koh et al.\(^{13}\) reported that herbal remedies, health food preparations, spa therapy, and diet changes were the frequent modalities and 82.4% of the studied AD patients wanted to try alterative treatment.

Korea has abundant resources of HS and spa therapy for management of AD is empirically well known among laypeople. Haeundae HS, which is sea salt water, is one of well-known HS effective for the treatment of AD and psoriasis. Our study showed that 15 studied adult AD patients except one experienced clinical improvement after the one month’s spa therapy without serious complications. No significant TEWL changes after the spa therapy might be due to a relatively short period of the treatment and known keratolytic action of the mild alkaline nature of Haeundae HS water. These results suggested that Haundae spa therapy

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*Fig. 7 (a) to (e)  Confocal microscopic images of Foxp3+ staining showing a strong fluorescence*
Table 1  Hydrochemical analysis of Hae-Un-Dae hot spring mineral water

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<td>Li</td>
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<td>Sr</td>
<td>6.4 mg/L.</td>
<td>SO₄²⁻</td>
<td>192.9 mg/L.</td>
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<td>Fe</td>
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<td>Total dissolved solids</td>
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Table 2  Hydrochemical analysis of Sukmodo hot spring mineral water

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<td>Mg^{2+}</td>
<td>254.0 mg/L.</td>
<td>Al</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SiO₂</td>
<td>65.0 mg/L.</td>
<td>F^-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li</td>
<td>5.0 mg/L.</td>
<td>Cl^-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sr</td>
<td>75.0 mg/L.</td>
<td>SO₄²⁻</td>
<td>1,010 mg/L.</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>0.7 mg/L.</td>
<td>Total dissolved solids</td>
<td>24,200 mg/L.</td>
<td></td>
</tr>
</tbody>
</table>
Table 3  Hydrochemical analysis of Suanbo hot spring mineral water

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mg/L)</th>
<th>Ion</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.39</td>
<td>Mn</td>
<td>0.0 mg/L</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.1 mg/L</td>
<td>Cu</td>
<td>0.0 mg/L</td>
</tr>
<tr>
<td>Na⁺</td>
<td>74.9 mg/L</td>
<td>Pb</td>
<td>0.0 mg/L</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>13.0 mg/L</td>
<td>Zn</td>
<td>0.0 mg/L</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.1 mg/L</td>
<td>Al</td>
<td>0.0 mg/L</td>
</tr>
<tr>
<td>SiO₂⁻</td>
<td>35.6 mg/L</td>
<td>F⁻</td>
<td>6.1 mg/L</td>
</tr>
<tr>
<td>Li</td>
<td>0.2 mg/L</td>
<td>Cl⁻</td>
<td>15.9 mg/L</td>
</tr>
<tr>
<td>Sr</td>
<td>0.1 mg/L</td>
<td>SO₄²⁻</td>
<td>32.9 mg/L</td>
</tr>
<tr>
<td>Fe</td>
<td>0.0 mg/L</td>
<td>Total dissolved solids</td>
<td>348 mg/L</td>
</tr>
</tbody>
</table>

Table 4  Hydrochemical analysis of Baekam hot spring mineral water

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mg/L)</th>
<th>Ion</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.90</td>
<td>Mn</td>
<td>0.01 mg/L</td>
</tr>
<tr>
<td>K⁺</td>
<td>0.63 mg/L</td>
<td>Cu</td>
<td>0.03 mg/L</td>
</tr>
<tr>
<td>Na⁺</td>
<td>41.3 mg/L</td>
<td>Pb</td>
<td>0.05 mg/L</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.89 mg/L</td>
<td>Zn</td>
<td>0.02 mg/L</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.02 mg/L</td>
<td>Al</td>
<td>0.05 mg/L</td>
</tr>
<tr>
<td>SiO₂⁻</td>
<td>87 mg/L</td>
<td>F⁻</td>
<td>3.07 mg/L</td>
</tr>
<tr>
<td>Li</td>
<td>0.04 mg/L</td>
<td>Cl⁻</td>
<td>7.12 mg/L</td>
</tr>
<tr>
<td>Sr</td>
<td>0.06 mg/L</td>
<td>SO₄²⁻</td>
<td>32.9 mg/L</td>
</tr>
<tr>
<td>Fe</td>
<td>0.02 mg/L</td>
<td>Total dissolved solids</td>
<td>64 mg/L</td>
</tr>
</tbody>
</table>
could be an effective and safe modality for the management of adult AD. The limitation of the study was small group of the patients, short period of the treatment time, and absence of the control group.

Skin acts as the first line of defense against any foreign materials outside of our body, as a physical barrier. As immune sentinels, keratinocytes can recognize foreign or danger stimuli from the outside via pattern-recognition receptors, such as Toll-like receptors (TLRs), and release innate immune mediators, such as cytokines and chemokines, under the stimulation of keratinocytes. We attempted to determine whether the HS water affected the TLR stimulated production of pro-inflammatory cytokines, including IL-1α, IL-6, IL-8, TNF-α, and GM-CSF, on the HaCaT cells. TLR1 agonist through TLR6 agonist treatment, induced the attenuation of cytokine production in the exposure to HS water in advance or simultaneously with TLR agonist treatment. This finding demonstrated the beneficial anti-inflammatory effect of the HS water. The three main types of CD4⁺ T cells can be found in the skin during inflammatory skin diseases, i.e., Th1, Th2, and Th17. AD is characterized by a predominent expression of Th2 cytokines, IL-4 and IL13, in acute lesion and of Th1 cytokines, IFN-ɤ and IL-12, in chronic lesion. The IL-17 cells were reported to contribute to defend against various fungal or bacterial infections, and possibly induced AD and epidermal changes in psoriasis via secretion of IL-17 and IL-22. Treg cells mediate immunosuppression and tolerogenic responses through contact-dependent or -independent mechanisms. Foxp3⁺ Tregs produce

| Table 5 Hydrochemical analysis of Dukku hot spring mineral water |
|---------------------|----------------|----------------|
| pH                  | 8.84           | Mn             | 0.01 mg/L |
| K⁺                  | 0.47 mg/L      | Cu             | 0.03 mg/L |
| Na⁺                 | 42.3 mg/L      | Pb             | 0.05 mg/L |
| Ca²⁺                | 3.1 mg/L       | Zn             | 0.03 mg/L |
| Mg²⁺                | 0.02 mg/L      | Al             | 0.05 mg/L |
| SiO₂                | 3.4 mg/L       | F⁻             | 10.3 mg/L |
| Li                  | 0.08 mg/L      | Cl⁻            | 4.9 mg/L  |
| Sr                  | 0.03 mg/L      | SO₄²⁻           | 5.82 mg/L |
| Fe                  | 0.02 mg/L      | Total dissolved solids | 65 mg/L |
IL-10 or TGF-β as effector molecules, and the balance between Treg cells and effector T cells is crucial for the maintenance of homeostasis and self-tolerance. It is reported that fine-tuning of the delicate 4-way balance among Th1, Th2, Th17, and Treg cells is required for the control of AD. We attempted to ascertain the potential involvement of HS water in the differentiation of the helper cells. CD4+ naive T cells, prepared from Balb/c mice spleen and cultured under various conditions, polarized to Th1, Th2, Th17, and Treg cells with or without HS water. Along anti-CD3 stimulation, HS water suppressed the proliferation of Th1, Th2, and Th17 cells. In contrast, proliferation and differentiation to Treg cells were promoted under HS water treatment. These results indicate that HS water may affect the distribution of the helper T cells in the immune response, by suppressing the polarization of the Th1, Th2, and Th17 cells. APCs, such as Langerhans cells, have the ability to process antigens in the periphery, transport it to the draining lymph nodes, where they are able to cluster with, and to activate the antigen-specific naive T cells. TLR3 stimulation via poly(I:C) strongly induced MHC II expression on the APCs isolated from Balb/c mouse spleen. By contrast, APCs treated HS water down-regulated the surface level of class II MHC expression, under TLR3 stimulation. Also, APC induced TNF-α and IL-6 under poly(I:C) stimulation was reduced in the presence of HS water. These results showed that TLR-triggered inflammatory responses in APCs might also be modulated under the HS water treatment.

We utilized AD-like skin lesion induced in D. farinae protein-sensitized NC/Nga mice model to investigate the clinical and immunoinflammatory modulation effect of HS water bath. Suanbo HS of NaHCO₃ type with low mineral contents was used. Two weeks’ bathing in the mineral water significantly improved dermatitis both grossly and microscopically accompanied by improved skin barrier parameter compared to distilled water control. In a view that both immunological and skin barrier dysfunction are interrelated and contribute to the pathogenesis of AD, this result seems quite promising. CD1+ cells markedly decreased in HS water group than distilled water control, showing HS water treatment decreased APC population and resultant down regulation of skin immune responses. Serum IgE, IL-4, IFN-γ, IL-5 did not show any significance by HS water treatment. Lesional mRNA expression of IFN-γ, IL-4, and IL-5 also showed no differences. Interestingly, in contrast to the suppressive effect on splenic Th2 cells, differentiation into Treg cells was promoted with the mineral water bathing compared to the distilled water group. These results may suggest that the effect of 2 weeks’ bath with Suanbo HS water is, in view of immunoinflammatory regulation, not too strong enough to suppress the production of inflammatory marker proteins at lesional skin and serum levels, even if it improved clinical and skin barrier function and showed associated reduction of APCs in the skin and increased splenic Treg cell population.

With AD-like skin lesion of ozazolone-sensitized hairless mice, we investigated the clinical and immunoinflammatory modulation effect of bathing with Baekam and Dukku HS of Uljin area. For the purpose of further understanding of localization of helper T cells and cytokine milieu of the target tissue, the skin, we performed confocal microscopic examination. Baekam
HS is of Na-HCO$_3$ type with low mineral contents with HCO$_3^-$ and SO$_4^{2-}$ being prominent anions. Clinical and histopathological improvement was observed in both HS and mineral water bath groups after one week. But on confocal microscopic examination of the lesional skin, marked reduction of IL-4, TNF-$\alpha$, and IL-17 staining and increased fluorescence of the CD4 and Foxp3 in HS water as compared to distilled water control were found. These findings showed possible evidence of reduction of Th17 cells and increased Foxp3+ T cell population of the lesion in HS water bath group. Concomitant findings of decreased expression of TSLP and IL-33 and increased expression of Foxp3 mRNA of the lesional skin were observed in HS water bath group. Dukku HS is of Na-Cl type with low mineral contents with F$^-$ being prominent anion and high SiO$_2$ content. Clinical, histopathological, and skin barrier improvement were observed in both HS and mineral water bath groups after one week. On confocal microscopic examination of the lesional skin, increased CD4$^+$ cells and CD25$^+$ cells merged into the same cells confirmed by DAPI staining, but Foxp3$^+$ staining, scattered in the field, did not. These findings might be due to a poor tissue preparation or the possibilities of initial accumulation of CD4$^+$CD25$^+$Foxp3$^+$ Treg cells and later loss of Foxp3 protein from the cells in the skin lesion by undefined cytokine milieu.

Further studies are needed for better understanding of immune-inflammatory regulatory mechanisms of HS water, specifically based on the individual minerals, their concentrations, and relative ratios.

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Conflict of Interest

No potential conflicts of interest were disclosed.

References


