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ORIGINAL ARTICLE

$NaHCO_3$- and NaCl-Type Hot Springs Enhance the Secretion of Inflammatory Cytokine Induced by Polyinosinic-Polycytidylic Acid in HaCaT Cells

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Background: Hot springs have been traditionally used as an alternative treatment for a wide range of diseases, including rheumatoid arthritis, bronchial asthma, diabetes, hypertension, psoriasis and atopic dermatitis. However, the clinical effects and therapeutic mechanisms associated with hot springs remain poorly defined. **Objective:** The purpose of this study was to demonstrate the different effects of hot springs on cellular viability and secretion of inflammatory cytokines on keratinocyte in two geographically representative types of hot springs: $NaHCO_3$-type and NaCl-type, which are the most common types in South Korea. **Methods:** We performed WST-1, BrdU measurements, human inflammatory cytokine arrays and enzyme-linked immunosorbent assay in HaCaT cells stimulated with toll-like receptor 3 by polyinosinic-polycytidylic acid. **Results:** The interaction effects of cell viability and cell proliferation were not significantly different regardless of polyinosinic-polycytidylic acid stimulation and cultured hot springs type. Cytokine array and enzyme-linked immunosorbent assay analysis showed increased expression of inflammatory cytokines such as interleukin-6 and granulocyte-macrophage colony-stimulating factor by polyinosinic-polycytidylic acid stimulation, with expression levels differing according to hot springs hydrochemical composition. Cytokine reduction was not significant. **Conclusion:** The effects and mechanisms of hot springs treatment in keratinocytes were partially elucidated. **(Ann Dermatol 33(5) 440～447, 2021)**

-Keywords-
Cytokines, Hot Springs, Inflammation, Keratinocytes, Poly I-C, Toll-like receptors

INTRODUCTION

Hot springs (HS) are found all over the world and have long been used as an alternative treatment for various diseases. The conditions that can be alleviated by HS therapy include symptoms of neurological and allergic diseases and joint, gynecological, gastrointestinal, metabolic and cutaneous disorders[1-3]. HS in Europe contain certain minerals (e.g., calcium, magnesium, carbonate) and have been reported to modulate inflammatory properties and clinical treatment efficacy[4,5]. Recent research has reported that HS therapy effectively prevents adverse dermatologic events in breast cancer patients after chemotherapy and improves patient quality of life[6]. In Japan, a study compared 2,194 subjects who had the required initial level of

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care and the required level of care over a long period of time and reported that the presence of an HS water supply in the home reduces the need for long-term care[7].
In Japan, a study compared 2,194 subjects who had the required initial level of care and the required level of care over a long period of time and reported that the presence of an HS water supply in the home reduces the need for long-term care.
Evidence-based effects of HS bathing on various systems of the body have also been reported[8]. In particular, the concentrations of extracellular calcium ions found in HS reportedly affect not only the regulators of differentiation in keratinocytes, but also their proliferation and the release of pro-inflammatory mediators[9]. In previous experiments, we have been applied toll-like receptor (TLR) 1 to 6 agonists. Herein we selected polyinosinic-polycytidylic acid (poly I-C), as a TLR3 agonist, which was observed inflammatory responses[10]. Until now, the therapeutic effect or mechanism of various trace components in HS has not been clearly elucidated. In this study, we investigated immune responses in keratinocytes stimulated by TLR3 depending on the two major hydrochemical constituents in South Korean HS.

MATERIALS AND METHODS

NaCl- and $NaHCO_3$-type hot springs

We conducted water quality analysis for two representative hydrochemical types, $NaHCO_3$-type and NaCl-type, in the 404 HS in South Korea. $NaHCO_3$ HS were found at 215 sites (53.22%), mainly in granitic strata, while NaCl HS were found at 78 sites (19.31%), in most other types of geological strata. We selected five $NaHCO_3$ HS (Baegam, Suanbo, Ildong, Yousung, and Dukgu) and four NaCl HS (Dongrae, Haeundae, Mageumsan, and Seokmodo), as shown in (Supplementary Fig. 1). The hydrochemical compositions of the five $NaHCO_3$ HS and four NaCl HS are listed in (Supplementary Table 1, 2), respectively. HS temperature, electrical conductivity, dissolved oxygen and pH were measured at the sampling points. HS fluid samples were collected in plastic bottles for chemical analysis and filter-sterilized through a 0.2 μm Nalgene bottle-top filter in a laboratory.

Cells and reagents

HaCaT cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The WST-1 cell viability kit was purchased from Roche Applied Science. A 5-bromo-2′-bromodeoxyuridine (BrdU) cell proliferation assay kit was purchased from cell. Poly I-C was used as a TLR3-stimulating reagent. This study was approved by the Institutional Review Board of the Uijeongbu St. Mary's Hospital (UC18EESI0007).

Cell culture in hot spring water

Because the osmolalities of HS water were too low to culture keratinocytes, one liter of Dulbecco's modified Eagle medium (DMEM; #12800017; Gibco, Eggenstein, Germany) was dissolved in cell culture media in distilled water. The control group used only DMEM solution without HS water. Osmolalities of HS water and HS water with DMEM powder were measured by freezing-point depression (Osmometer 2020; Advanced Instruments, Norwood, MA, USA) and pH was measured with a pH meter (pHBasicpuls; Sartorius AG, Goettingen, Germany) (Supplementary Table 3). Both analyzers were calibrated according to the manufacturer's instructions. In the Haeundae and Seokmodo HS, total dissolved solids were higher than in other HS; Haeundae HS water was used after two-fold dilution and Seokmodo HS water was used after eight-fold dilution. All media were supplemented with 10% fetal bovine serum and cultured in 5% CO_2 at 37°C.

WST-1 cell viability assay

WST-1 measurement was performed according to the manufacturer's protocol. Briefly, HaCaT cells were seeded at 6×10^3 cells in 200 μl of culture media into a 96-well plate and incubated overnight to obtain 70% confluence. Poly I-C was then added to obtain final concentrations of 20 μg/ml and incubated for 24 or 48 hours. Next, 10 μl of WST-1 was added and the preparation was incubated for another two hours at 37°C in the incubator. The plates were then read at 450 nm with a SpectraMax i3x Micro plate Reader (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed using the micro-plate reader software Softmax Pro (Molecular Devices).

BrdU cell proliferation assay

BrdU measurements were conducted according to the manufacturer's protocol. Briefly, the HaCaT cells were seeded at 6×10^3 cells in 200 μl of culture media into a 96-well plate and incubated overnight to obtain 70% confluence. Poly I-C was then added to obtain final concentrations of 20 μg/ml and incubated for 24 or 48 hours. BrdU was added and incubated for four hours at 37°C. The medium was moved by tapping. Cells were then fixed and denatured for 30 minutes at room temperature. After thorough removal of the fixing solution, 100 μl of detection antibody solution was added to each well, which were incubated for one hour at room temperature. Wells were rinsed three times with 200 μl of washing solution and 100 μl of horseradish peroxidase-conjugated secon-

dary antibody solution was added. The wells were rinsed three times with 200 μl of washing solution and 100 μl of substrate solution was added for 30 minutes. After adding 100 μl of stop solution, the plates were read at 450 nm with a SpectraMax i3x Microplate Reader (Molecular Devices). Data were analyzed using the micro-plate reader software Softmax Pro (Molecular Devices).

Wound-healing assay

HaCaT cells were seeded (1.5×10^5 cells/well) in a 24-well plate and incubated at 37°C overnight. When the cells reached 100% confluence, the monolayer was scratched to create a wound using a 200 μl pipette tip. The monolayer was washed with sterilized phosphate buffer saline and the cells were treated with poly I-C at a final concentration of 20 μg/ml and incubated for 24 hours at 37°C. Images were captured with an Olympus IX-71 microscope (Olympus, Tokyo, Japan) at 0, 24, and 48 hours. The area of the scratch was quantified by three independent observers and the measurements were used as an indication of cell migration. Relative wound-healing ability was calculated using the following formula: Percent wound healing=[(100−area at 0, 24, or 48 hours)/(area at 0 hour)]× 100; an average was calculated from three replicates. Under this setting, untreated control cells exhibited 100% wound-healing ability after 48 hours.

Sample preparation and antibody array for inflammatory cytokine assay

Cell culture supernatants were measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) using Multiskan FC (Thermo, Waltham, MA, USA). The purity of the sample was confirmed on an ultraviolet spectrum. A Human Inflammation G3 Array slide (RayBiotech, Norcross, GA, USA) (Supplementary Table 4) was dried for two hours at room temperature and incubated in 40 μl of blocking solution at room temperature for 30 minutes. After decanting the blocking buffer from each sub-array, 400 μl of diluted sample was added and incubated for two hours at room temperature. After decanting the samples, each array was washed three times with 800 μl of wash buffer I at room temperature for 5 minutes with shaking. A glass chip was placed into the container and wash buffer I was added to submerge the entire chip for 10 minutes, followed by shaking two times and a second advanced washing step with wash buffer II. Biotin-conjugated anti-cytokine antibodies were prepared and incubated for two hours at room temperature with gentle shaking and washed with 150 μl of wash buffer I at room temperature with shaking. Cy3-conjugated streptavidin stock solution was added and incubated for two hours at room temperature with gentle shaking and was washed twice with wash buffer I for 10 minutes at room temperature. After washing, the slide was rinsed with deionized water using a plastic wash bottle and the slide was centrifuged at 1,000 rpm for 3 minutes to remove water.

Data acquisition and analysis

Slide scanning was performed using a GenePix 4100A Scanner (Axon Instrument, Union City, CA, USA). The slides were completely dry before scanning at 10 μm resolution with optimal laser power and photomultiplier tubes. Scanned images were gridded and quantified with GenePix 7.0 Software (Axon Instrument). Numeric data were analyzed using Genowiz 4.0TM (Ocimum Biosolutions, Hyderabad, India). After analyzing, the protein information data were annotated using UniProt DB.

Quantification of inflammatory cytokine by ELISA

The release of inflammatory cytokines from HaCaT cells was quantified using different high-sensitivity human commercially available enzyme-linked immunosorbent assay ELISA kits (Supplementary Table 5) according to the manufacturer's instructions. The plates were read at 450 nm with a SpectraMax i3x microplate reader (Molecular Devices). Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices). Quantification was performed using an optimized standard curve supplied with the ELISA sets.

Statistical analysis

Data are expressed as mean±standard deviation of at least three experiments performed in duplicate. South Korea HS are classified as the two most common types, including nine HS. Cell viability, proliferation, wound healing and ELISA data were analyzed by repeated measures (RM) analysis of variance, in which the culture time, HS type and poly I-C stimulation were the RM factors and the regular culture condition was the fixed effect. Statistical analysis was performed using Statistical Analysis Systems (SAS) for Windows V 9.4 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Hydrochemical contents in $NaHCO_3$ and NaCl HS in South Korea

Supplementary Table 1, 2 show the difference in hydrochemical contents of the two types of HS. NaCl HS exhibited higher electrical conductivity and total dissolved solids and contained more of the cations Na, K, Mg and Ca; anions of Cl, Br, NO_3 and SO_4; and microelements Mn, Fe, Co, Ni, and Ba than did $NaHCO_3$ HS. $NaHCO_3$

HS exhibited higher pH values and contained more anions of F, CO_3 and HCO_3 and microelements Cu, Zn, Mo and U than were found in NaCl HS.

Effect of hot spring type on cell health with or without poly I-C stimulation

Cell viability and proliferation was determined in two groups with or without poly I-C stimulation in cultures made from distilled water or HS. Regardless of poly I-C stimulation, cell viability was higher in HS than in regular culture media and between HS was higher in NaCl HS than in $NaHCO_3$ HS (Fig. 1A, B). There was a significant difference in cell viability according to type of HS, poly I-C stimulation and culture time, but the interaction effect was not significant. Cell proliferation was slightly lower in general culture than in HS at 48 hours but showed similar results regardless of poly I-C stimulation and culture type. There was a significant difference in cell proliferation according to poly I-C stimulation and culture time, but type of HS and interaction effect were not significant (Table 1). A wound-healing assay can be used to demonstrate collective movement and is reported to be suitable for cell types such as the keratinocytes used in this study[11]. Wound healing was determined in two groups with or without poly I-C stimulation in cultures made from distilled water or HS (Fig. 1C). In the control group, wound healing was slightly different in regular culture medium, $NaHCO_3$ HS and NaCl HS at 24 hours and all wound areas in all three groups were restored after 48 hours. In the poly I-C stimulated group, normal culture medium and $NaHCO_3$ HS were significantly higher than those of NaCl HS. There was a significant difference in wound healing according to type of HS, culture time and interaction effect. However, poly I-C stimulation was not significant (Table 1).

Effect of hot spring type on inflammatory cytokine secretion with or without stimulation

Inflammatory cytokine arrays were analyzed using RayBiotech's platform, which can compare the expression of 40 inflammatory cytokines including three positive controls and nine negative controls, duplicated for each cytokine (Supplementary Fig. 2). We screened and compared the secretion of the granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, IL-8, chemokine CXCL10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1 α, MIP-1 β, and chemokine CCL5 (RANTES), all of which were increased in poly I-C stimulated HaCaT cells. Interestingly, expression

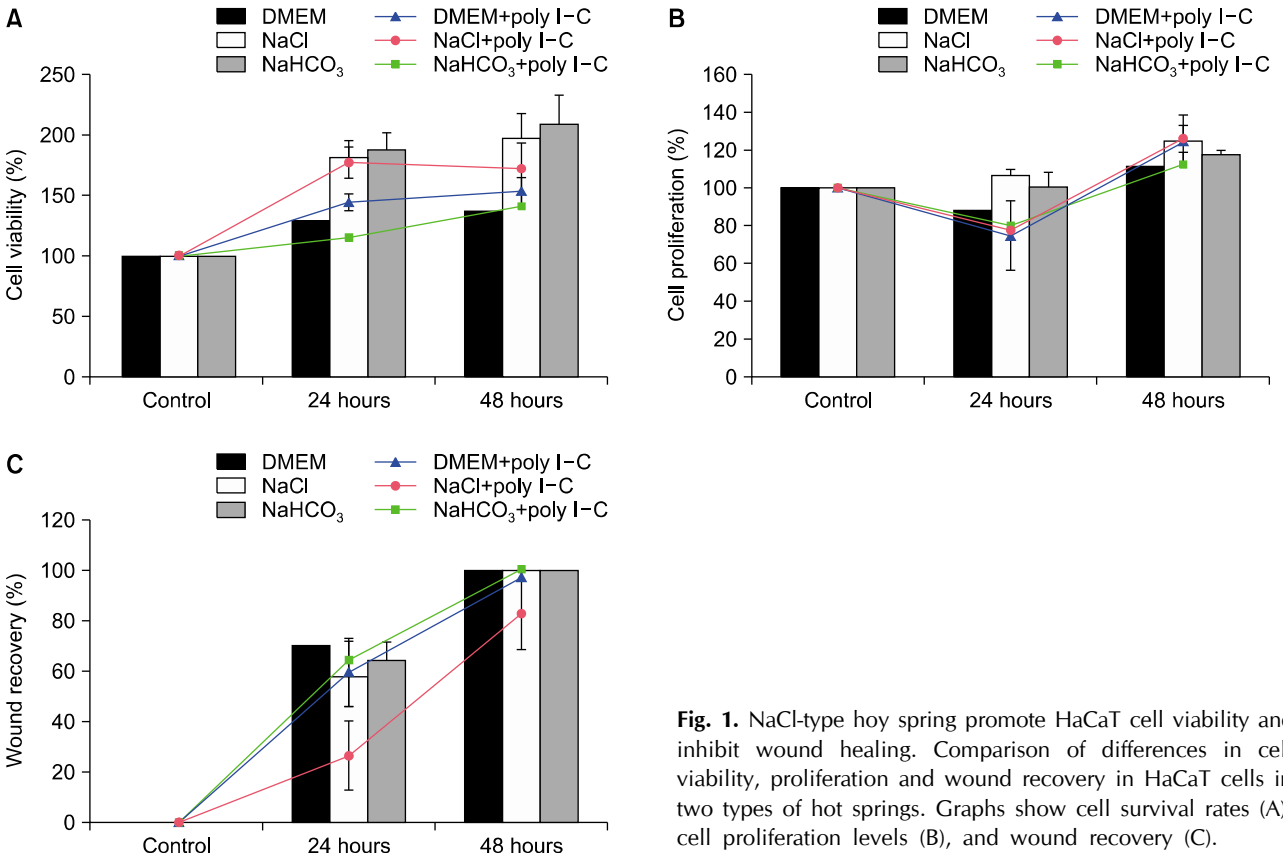

Fig. 1. NaCl-type hoy spring promote HaCaT cell viability and inhibit wound healing. Comparison of differences in cell viability, proliferation and wound recovery in HaCaT cells in two types of hot springs. Graphs show cell survival rates (A), cell proliferation levels (B), and wound recovery (C).

Table 1. Results of repeated measures ANOVA of cell viability, proliferation and wound healing

Anlysis	*p*-value				
	Group	Treatment	Time	Group vs. treatment	Group vs. treatment vs. time
Cell viability	0.0007	0.0163	0.0082	0.2759	0.1194
Cell proliferation	0.2616	0.0436	0.0001	0.3929	0.3104
Wound healing	0.0043	0.0722	0.0001	0.0351	0.4729

Group: DMEM, NaCl- and $NaHCO_3$-type hot springs, Treatment: With/without poly I-C, Time: 0 hour, 24 hours, and 48 hours.

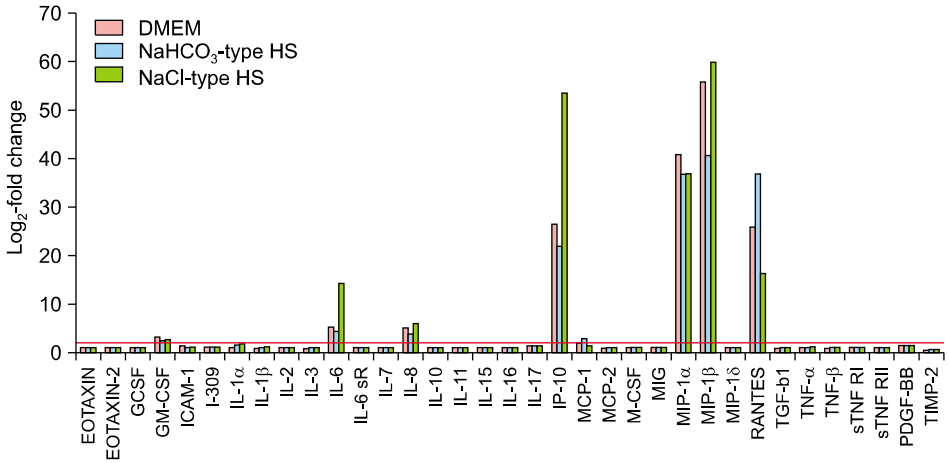

Fig. 2. Hot springs (HS) increased the secretion of several inflammatory cytokines in toll-like receptor (TLR) 3-stimulated HaCaT cells compared with regular culture media. Fold-change in expression of inflammatory cytokines from polyinosinic-polycytidylic acid (poly I-C)-stimulated HaCaT cells, $NaHCO_3$- and NaCl-type HS compared with regular DMEM. Horizontal red line indicates the threshold level. EOTAXIN: eosinophil chemotactic protein, GCSF: granulocyte colony-stimulating factor, GM-CSF: granulocyte-macrophage colony-stimulating factor, ICAM: intercellular adhesion molecule, IL: interleukin, IP: interferon gamma-induced protein, MCP: monocyte chemoattractant protein, M-CSF: macrophage colony-stimulating factor, MIG: monokine induced by interferon-gamma, MIP: macrophage inflammatory protein, RANTES: regulated on activation, normal T cell expressed and secreted, TGF: transforming growth factor, TNF: tumor necrosis factor, PDGF: platelet derived growth factor, TIMP: metalloproteinase inhibitor.

of these cytokines increased by more than two times in the $NaHCO_3$ and NaCl HS compared with the regular media group. Separately, expression of TIMP-2 was lower in the regular culture media, $NaHCO_3$ HS and NaCl HS (Fig. 2).

The expression of GM-CSF, IL-6, IL-8, IP-10, MCP-1, MIP-1 α, MIP-1 β, and RANTES was verified by quantikine ELISA. MIP-1 α, MIP-1 β, and IL-8 presented slightly higher levels in $NaHCO_3$ HS, while GM-CSF, IL-6, IP-10, RANTES, and MCP-1 were higher in NaCl HS compared with regular culture media (Fig. 3). The ELISA analysis revealed that concentrations of GM-CSF, IL-6, IP-10, RANTES, and MCP-1, which were high in NaCl HS, presented significant differences according to type of HS and poly I-C stimulation. However, only IL-8 and MIP-1 β among MIP-1 α, IL-8, and MIP-1 β, which were low in $NaHCO_3$ HS, showed significant differences according to poly I-C stimulation. GM-CSF, IL-6, and IP-10 also showed significant differences regarding their interaction effect.

DISCUSSION

Most HS in South Korea have low mineral content and show the qualities of an alkali environment. The distribution of HS is consistent with that of areas of granite[12]. This study analyzed two representative types ($NaHCO_3$ and NaCl, the two most common HS types in South Korea) of HS as classified by mineral constituents. The release of pro-inflammatory cytokines from HaCaT cells increased in HS media. HaCaT cells are immortalized keratinocytes widely used as an alternative to normal human keratinocytes as they have high differentiation potential in cell cultures[13] and contribute to a variety of cytokines associated with T-cells, including IL and tumor factor necrosis al-

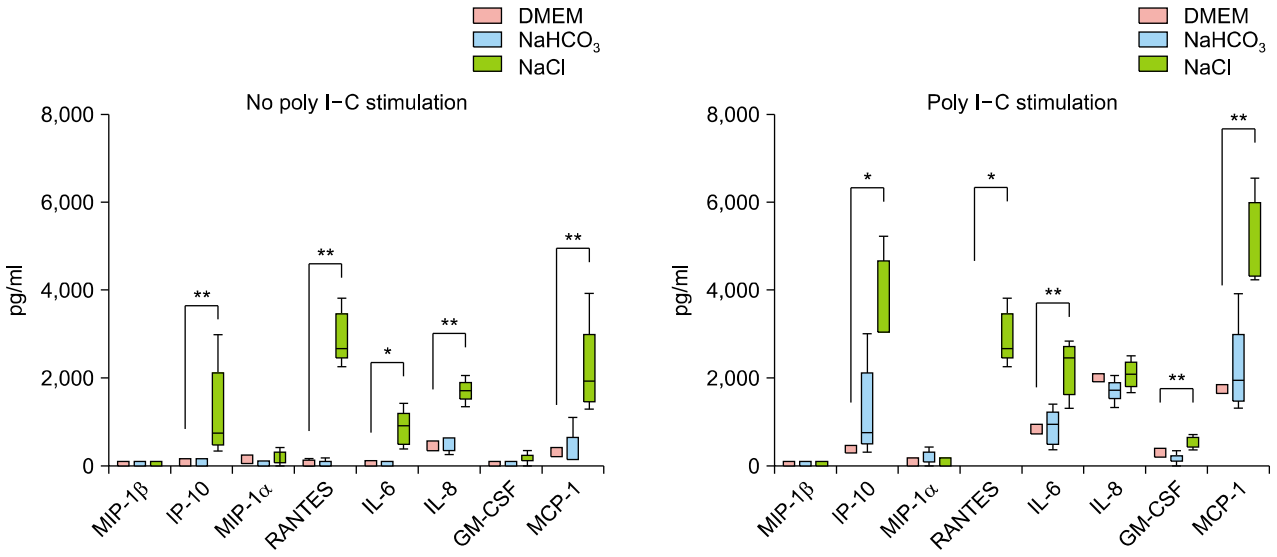

Fig. 3. Hot springs were associated with increased secretion of inflammatory cytokines compared with regular culture media. The results of inflammatory cytokine were verified by ELISA. The left panel shows the change in inflammatory cytokine secretion in the control HaCaT cells. The right panel shows the change in inflammatory cytokine secretion in polyinosinic-polycytidylic acid (poly I C)-stimulated HaCaT cells. MIP: macrophage inflammatory protein, IP: interferon gamma-induced protein, RANTES: regulated on activation, normal T cell expressed and secreted, IL: interleukin, GM-CSF: granulocyte colony-stimulating factor, MCP: monocyte chemoattractant protein. *Significant differences ($p<0.05$), **significant differences ($p<0.01$), n=3.

pha[14]. We first examined the effect of HS on cell viability and cytokine expression in HaCaT cells. Culturing poly I-C stimulated cells in HS did not alter the secretion of anti-inflammatory cytokines IL-10, interferon-alpha, or transforming growth factor beta[15] but it did increase secretion of inflammatory cytokines and chemokines such as IL-6 and GM-CSF. Among the increased cytokines, MIP-1 β showed particularly significant differences according to HS type, while expression of RANTES and MCP-1 showed differences with respect to poly I-C stimulation. IL-6, IP-10 and GM-CSF also presented significant differences in interaction effects. The interaction effect means that there are significant differences between groups by type of HS when poly I-C is treated.

The increase in cytokines in HS media after poly I-C stimulation was considered to have a pro-inflammatory effect. IL-6, IL-8 and GM-CSF are secreted from keratinocytes and play an important role in regulation of the epidermis[14,16]. IL-6 and IL-8 are soluble mediators that affect inflammation, immune responses and hematopoiesis. Their expression induces an alarm signal and activates host defense mechanisms against external stress[17,18]. Keratinocytes play a major role in the production of excess GM-CSF in atopic dermatitis skin; the nature and intensity of inflammatory infiltration found in tissues can therefore be explained by the high secretion of GM-CSF[19]. The immune response of keratinocytes is mediated through secretion of various cytokines and chemokines involved in activation of T-cells and dendritic cells[20,21]. However, these cytokines have not only pro-inflammatory effects, but beneficial effects on wound healing. Recent studies have shown that poly I-C stimulates TLR3 to promote wound healing in human and murine skin[22] and to accelerate abundant HaCaT cell migration through IL-8 secretion[23]. In human corneal epithelial cells, poly I-C induced the secretion of IL-6, IL-8, RANTES, type I interferons and interferon gamma-induced protein 10[24]. Other studies have reported that mineral water baths can regulate proliferation and differentiation of HaCaT cells and murine atopic dermatitis[25,26]. As with the results of previous studies, cell proliferation and viability increased after HS treatment in this study.

We examined the response of keratinocytes in the two most abundant types of HS in South Korea. The immunomodulatory activity of HS is complex due to the hydrologic properties of the many quantitatively variable compounds found in HS of different origins. For a better understanding of the mechanisms of HS-mediated therapeutic effects on various diseases, it is necessary to understand these hydrologic characteristics as well as the regulation of human cytokine secretion. Although this study has several limitations, such as the use of only a portion of keratinocytes without comparing the entire skin layer, it suggests that response to a disease may differ according to hydrochemical contents of HS. In addition, since HS containing certain minerals regulate the inflammatory response in psoriasis murine model[27], therapeutic HS need

to establish appropriate treatments that take into account factors such as Hydrochemical composition and water temperature etc., depending on the disease. Further research is needed to provide a more precise guide on the use of HS as a therapeutic remedy in medicine.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-33-440-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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