Identification of a molecular-level moisturizing effect on the entire skin layer of a mixture composed of seaweed extract from Jeju Island

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Abstract

This study was conducted to develop a new ingredient with moisturizing and water-loss prevention functions at the molecular-level to fundamentally prevent and solve the problem of the lack of water in the skin. We prepared a new moisturizing ingredient with a seaweed extract containing *Gelidium Cartilagineum, Hizikia Fusiforme, Codium Fragile, Ecklonia Cava,* and *Sargassum Fulvellum* from Jeju Island (MIZCHO MARINE FOREST).

We investigated the molecular-level moisturizing effect of the seaweed extract for the entire skin layer in dermal fibroblasts and keratinocytes. The moisturizing activity was determined by the expression levels of Aquaporins (AQPs) and hyaluronic acid synthases (HASs). In addition, Strengthening the skin barrier activity was determined by the expression level of the thigh junction protein (Claudin, Zo-1).

The seaweed extract induced the upregulation of different types of AQP mRNA expression by the skin layer. The results were also confirmed in the 3D full thickness skin model. Likewise, the mRNA levels for HAS1, 2 and 3 were increased by the seaweeds extract; HAS1 was increased more in epidermal cells and HAS2 and 3 more in the dermal cells. The hyaluronic acid (HA) synthesis level in the skin cells was increased shown by the expression level of HAS. Claudin, Zo-1 mRNA and protein expression were also increased by the seaweed extract.

The seaweed extract from the seaweeds found in the sea of Jeju Island has a moisturizing effect by promoting AQPs and HAS differently for each skin layer. Moreover, it promoted the expression of tight junction proteins *in vitro*. Therefore, the new seaweed extract was shown to have potential as a powerful moisturizing ingredient.

Keywords: Moisturizing; Hydration; Seaweeds ; Aquaporins; Tight junction

Introduction.

Water is absolutely essential for the normal functioning of the skin, and the loss of water from the skin must be carefully regulated. Otherwise, various skin problems such as dry skin, itchiness, and damage to skin tissue may occur and reduced skin elasticity and wrinkles; thus, moisturizing is the most basic and essential function in cosmetics. Traditionally, skin care strategies for moisturizing have mainly utilized methods that prevent moisture evaporation from the skin by providing water or moisturizing ingredients directly to the skin or by forming a film on the skin. However, in recent years, substances have been revealed that act as a moisturizing agent at the molecular level. For example, glycerol is present as a natural endogenous wetting agent in the stratum corneum (SC). Hyaluronan, which has been considered a predominantly dermal component, is found in the epidermis and is important for maintaining a normal SC structure and epidermal barrier function. The presence of aquaporin-3, a water transport protein in the epidermis, and the presence of tight junction structures at the junction between the granular layer and the SC are also important findings [1]. Rather than simply supplying water to the skin from the outside, it will be possible to achieve stronger skin hydration through a molecular-level moisturizing approach.

This study was conducted to develop a new ingredient with moisturizing and water-loss prevention functions at the molecular-level to fundamentally prevent and solve the problem of the lack of water in the skin.

Materials and Methods.

Preparation of the seaweed extract

We prepared a new moisturizing ingredient with seaweed extract containing *Gelidium Cartilagineum, Hizikia Fusiforme, Codium Fragile, Ecklonia Cava,* and *Sargassum Fulvellum* (Table1). The seaweeds were collected from the Jeju sea area. The dried *Gelidium Cartilagineum* was added to a 1% acetic acid (w/w) solution that was 60 times the dry weight and extracted at 90-100 °C for 1 hour. The extract was concentrated to 1/10 or less of the primary extract at 60-70 °C, and 50 kg of purified water were added to re-concentrate it. In this manner, re-concentration using purified water was performed three times. After recovering the last concentrate, purified water was added so that 1/2 of the weight of the primary extraction solvent (1% acetic acid) was added and then refrigerated for 12 to 24

hours. The refrigerated concentrate was centrifuged at 12,000 rpm using a centrifuge, and the separated supernatant was collected to obtain a hydrolyzed *Gelidium Cartilagineum* extract. It was confirmed through NMR analysis that this hydrolyzed *Gelidium Cartilagineum* extract contained agarobiose, a functional substance not found in the hot-water *Gelidium Cartilagineum* extract when compared with the hot-water *Gelidium Cartilagineum* extract (data not shown). Finally, a mixture of the hydrolyzed agar extract and the extracts a, b, c, and d were mixed in a 1:1 ratio to prepare the new seaweed extract.

Table1. Composition of the seaweeds extract.

NO.	INCI name	%
1	*Gelidium Cartilagineum* Extract	50
2	*Hizikia Fusiforme* Extract	12.5
3	*Codium Fragile* Extract	12.5
4	*Ecklonia Cava* Extract	12.5
5	*Sargassum Fulvellum* Extract	12.5

Cell cultures and reagents

Keratinocyte (HaCaT) cells and human primary fibroblast cells (HDFn, normal human dermal fibroblasts juvenile foreskin, C-12300, PromoCell, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium high glucose (DMEM high glucose, SH30243.01, Hy-80 clone, Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS, SH30084.03, Hyclone, Logan, UT, USA) and 1% antibiotic- antimycotic agents (Anti-anti, 15240-062, Gibco, Grand Island, NY, USA). Cell cultures were maintained at sub confluence in a 95% air, 5% CO2 humidified atmosphere at 37 °C.

Cell viability assay (MTT assay)

Cells were seeded at $5x10^3$ cells/ well on a 96-well plate and cultured for 24 h under cell culture conditions. Then, we discarded the medium, washed the cells with a solution of phosphate buffered saline (PBS, 21-040- CV; Corning, USA), placed the cells in new serum-free media, treated the cells with concentrations of 0.0001, 0.001, 0.005. 0.01, 0.05, 0.1, 0.5, and 1% of the samples, and incubated the cells for 24 and 48 h. Then, we added 100 μL of MTT solution (0.5%, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H- tetrazolium bromide) to each well and incubated the cells for another 2 hours. After removing the culture media, we added 100 μL of 100% dimethyl sulfoxide (DMSO), shook the cells for 10 minutes, and

measured the absorbance at 590 nm with a microplate reader (EPOCH2 microplate reader, EPOCH2NSC, BioTek, USA).

Quantitative real-time polymerase chain reaction (qRT PCR)

RNA extraction was performed on the cells using an RNA extraction kit (TaKaRa Mini-BEST Universal RNA extraction kit, 9767A, Takara Bio, Inc., Otsu, Japan), and the samples were purified following the manufacturer's instructions. cDNA was synthesized from the isolated RNA (1µg) using a T.R. reagent kit (PrimeScriptTM RT reagent Kit with gDNA Eraser, RR047A, Takara Bio, Inc., Otsu, Japan). Using a SYRB Green Realtime PCR Master Mix (Power SYBRTM Green PCR Master Mix, 4367659, Applied Biosystems^{TM}, Thermo Fischer Scientific) and QuantStudio^{TM} 3 Real-Time PCR instrument, (A28132, Thermo Fisher Scientific), the gene expression levels were standardized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh). The primers used in this study are presented in Table 2. The experiments were performed in duplicate for three independent repeats. Melting curve analysis was performed for each primer set

Table 2. Primer sequences for the quantitative PCR

Gene		Sequences
AQP1	Forward	AGGCTACAAAGCAGAGATCGAC
	Reverse	CACCCTCTAAATGGCTTCATTC
AQP3	Forward	TGCAATCTGGCACTTCGC
	Reverse	GCCAGCACACACACGATAA
AQP9	Forward	GTATTGGTAGAAACAGGAGTC
	Reverse	GGACAATCAAGATGAACGTG
HAS1	Forward	GTGCGGGTACTGGACGA
	Reverse	GACCGCTGATGCAGGATACA
HAS2	Forward	GCAGTGTAAGATATTGGATGGC
	Reverse	CCCATAAATTCTTGATTGTACCAATCTTC
HAS3	Forward	TGTCCAGATCCTCAACAAGTACGA
	Reverse	CTGGAGGAGGCTGTTGC
ZO1	Forward	AGAGCACAGCAATGGAGGAA
	Reverse	GACGTTTCCCCACTCTGAAA
CLAUDIN	Forward	GCACATCCAGTGCAAAGTCT
	Reverse	CATACACTTCATGCCAACGG
GAPDH	Forward	ACCCACTCCTCCACCTTTGA
	Reverse	CTGTTGCTGTAGCCAAATTGCT

Western blot analysis.

Cells were seeded at $1x10^6$ cells/ well on a 6-well plate and cultured for 24 h under cell culture conditions. Then, we discarded the medium, washed the cells with a solution of phosphate buffered saline (PBS, 21-040- CV; Corning, USA), placed the cells in new serum-

free media, treated the cells with concentrations of 0.1% of the samples, and incubated the cells for 48 h. The cells were resuspended in 1X SDS loading buffer (50 mM Tris-Cl, pH 6.8, 2% sodium dodecyl sulfate) and incubated at 95 °C for 10 min. The cell lysate was loaded onto a SuperSep Ace 5–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Wako, Tokyo, Japan) and separated in running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) for 50 min at 180 V. The separated proteins were transferred onto a nitrocellulose membrane using an Atto Western Blotting System (Atto Corp., Tokyo, Japan) with EZ Fast Blot (Atto Corp.) for 15 min at 0.25 A per mini gel. The membrane was blocked with 5% skim milk at 37 °C for 20 min and then washed five times with wash buffer at room temperature for 5 min with shaking. Afterwards, the membrane was incubated with primary antibody at 37 °C for 2 h. The primary antibodies were as follows: anti-AQP1, anti-AQP3, anti-AQP9, anti-HAS1 (LSBIO, USA), anti-HAS2 (LSBIO, USA), and anti-HAS3 (LSBIO, USA). After washing three times with wash buffer at room temperature for 20 min each, the membrane was incubated with a horseradish peroxidase-conjugated anti-rabbit IgG 2nd antibody at 37 °C for 1 h. The membrane was then washed three times with wash buffer at room temperature (20 min each). The protein bands were detected using EzWestLumi plus (Atto Corp., Tokyo, Japan) and analyzed using Luminograph II (Atto Corp., Tokyo, Japan).

Immunocytochemistry.

Cells were cultured on coverslips and treated with 0.1% sample for 24 h. The primary antibodies were as follows: anti-HA (Invitrogen, USA), anti-Zo-1 (Invitrogen, USA) and anti-Claudin (Bethyl Laboratories, Montgomery, Texas, USA). The primary antibody was incubated for 2 h followed by addition of Cy3-conjugated (red signal) FITC- conjugated (green signal) secondary antibody and further incubated for 1 h. The slides were mounted with Vectashield/DAPI. Staining was examined under an a SP8 X Confocal Laser Scanning Microscope (Leica, Mannheim, Germany).

3D reconstructed human skin model

3D reconstructed human full skin model (Keraskin-FT™) and Keraskin-FT™ culture media were purchased from Biosolution Co., Ltd. (Seoul, Korea). Keraskin-FT™ was cultured on a six-well plate at 37 °C in a humidified atmosphere containing 5% CO2 overnight. Then, we discarded the medium, washed the cells with a solution of PBS, placed the cells in new media, treated the cells with concentrations of 0.1% of the samples, and incubated the cells for 72 h.

Histological analysis

For histological examination, all 3D reconstructed human full skin resamples were cut into 10 mm-widths and fixed in 4% phosphate-buffered formalin (PFA) for 24 h, as described previously (Hwang et al., 2020). Fixed samples were paraffin-embedded and cut into 5-μm sections using a microtome (Leica, RM2235), followed by immunohistochemistry.

Data analysis

Data are expressed as the mean ± SD. Data were analyzed using the analysis of variance (ANOVA) or student's t-test. Dunnett's test was used for post hoc analysis to determine the difference from the negative control. P-values of 0.05 or less were considered significant.

Results.

Effects of the seaweed extract on skin cell viability

To measure the cell viability of the seaweed extract on HaCaT and HDFn, the cells were treated with various concentrations (0.0001, 0.001, 0.005. 0.01, 0.05, 0.1, 0.5, and 1%) of the seaweed extract. As a result, the seaweed extract did not significantly reduce the viability of human epidermal cells and fibroblasts at concentrations of 0.1% or less (Fig. 1). Mild cytotoxicity was observed above 0.5%. Therefore, it is safe to use seaweed extract at a concentration of 0.1% or less.

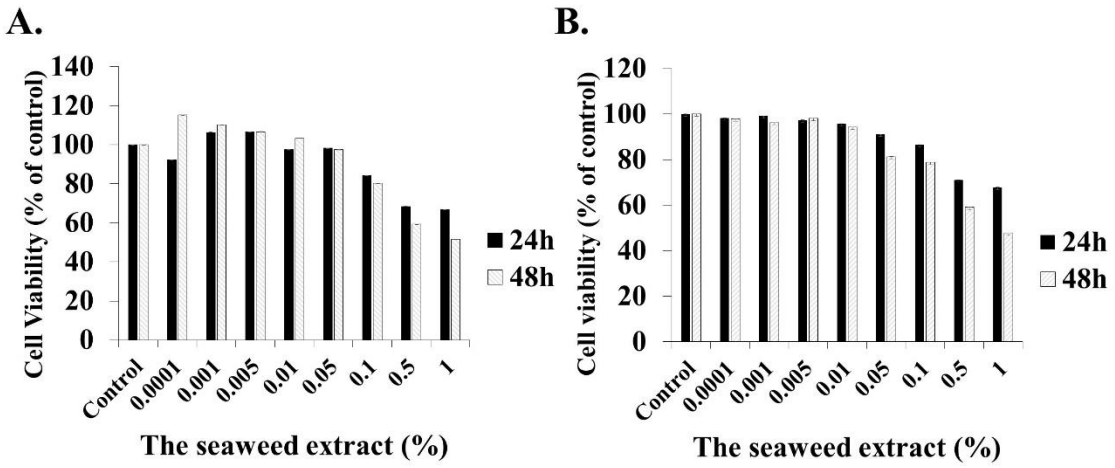

Figure 1. Effects of the seaweed extract on skin cell viability.

The seaweed extract did not significantly reduce the viability of HaCaT (A) and HDFn (B) at concentrations below 0.1%.

Effect of the seaweed extract to regulate the expression of different AQPs for each skin layer

To evaluate whether the new ingredient regulates the expression level of aquaporins in the epidermal and dermal layers of the skin, qRT PCR and western blot were performed after treating the HaCaT and HDFn with the seaweed extract. The seaweed extract induced the upregulation of mRNA expression for different types of AQPs by the skin layer (Fig. 2). When cells were treated with 0.1% of the seaweed extract for 24 h, the gene expression of the AQPs in HaCaT, the cells that make up the epidermis, increased by 1.36 times for AQP1, 1.39 times for AQP3, and 1.12 times for AQP9 compared to the control group (Fig. 2A). Additionally, under the same conditions, the expression levels of the AQP genes in HDFn, the dermal fibroblasts, were increased by 1.21, 1.57 and 1.25 times for AQP1, AQP3, and AQP9, respectively, compared to the control group (Fig. 2C). When skin cells were treated with 0.1% of the seaweed extract for 48 hours, it was confirmed that the expression level of the AQP proteins in the epidermal cells increased by 1.86 for AQP3 compared to the control group (Fig. 2B). In addition, it was confirmed that the expression level of the AQP proteins in the dermal cells increased by 1.05, 1.14 and 1.31 times for AQP1, AQP3, and AQP9, respectively, compared to the control group (Fig. 2D). In conclusion, in the HaCaT cells, the increase of the AQP3 mRNA and protein expression levels by the seaweed extract was greater than that of AQP1 and AQP9. On the other hand, in the HDFn cells, the mRNA and protein levels of AQP9 were more increased compared to those of AQP1 and AQP3.

The results were also confirmed in the 3D full thickness skin model. We used Keraskin-FT^{TM}, a full-thickness skin model, to measure whether the seaweed extract increases the expression of the different types of AQPs for each skin layer in artificial skin that is closer to actual skin than the cells used in the in vitro tests. When 0.1% of the seaweed extract was applied to the 3D artificial skin for 48 hours, the AQP3 protein content was increased in the Epidermis layer compared to the Dermis layer. In addition, AQP1 and AQP9 were more increased in the Dermis layer than in the Epidermis layer.

From the above results, the seaweed extract can contribute to moisturizing the entire skin layer by activating different AQPs for each skin layer.

Effect of seaweed extract on Hyaluronic acid synthesis

To measure the effect of the seaweed extract on hyaluronic acid synthesis, we performed

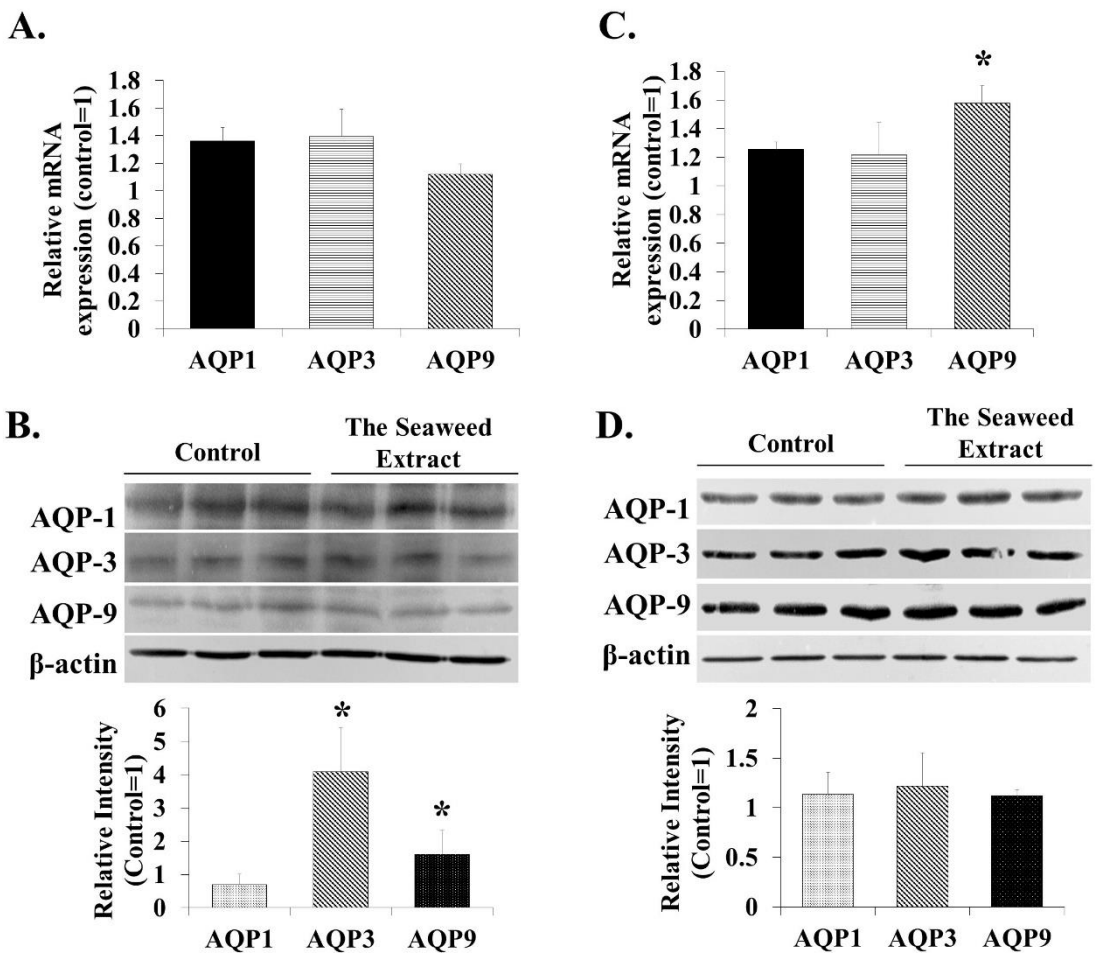

Figure 2. Effects of the seaweed extract on the expression of AQPs in HaCaT and HDFn.

The seaweed extract (0.1%, 24 h) increased the expression of AQP3 in HaCaT (A, B) and AQP9 (C) in HDFn. Data are shown as the mean±S.D. of three experiments. * <0.05 compared to control.

qRT PCR to confirm the gene expression of hyaluronic acid synthase (HAS) and immunocytochemistry to confirm hyaluronic acid production. When HaCaT was treated with 0.1% of the seaweed extract for 24 hours, the HAS1, HAS2 and HAS3 gene expression levels were increased by 1.51, 1.09 and 1.11 times, respectively, compared to the control group (Fig. 4A). Similarly, in the HDFn cells, the expression levels of the hyaluronic acid synthase HAS genes in the cells were increased by 1.3, 1.68 and 2.1 times for HAS1, HAS2 and HAS3, respectively, compared to the control group (Fig. 4B). From this result, it can be seen that 0.1% of the seaweed extract tends to increase the HAS1 gene expression in the

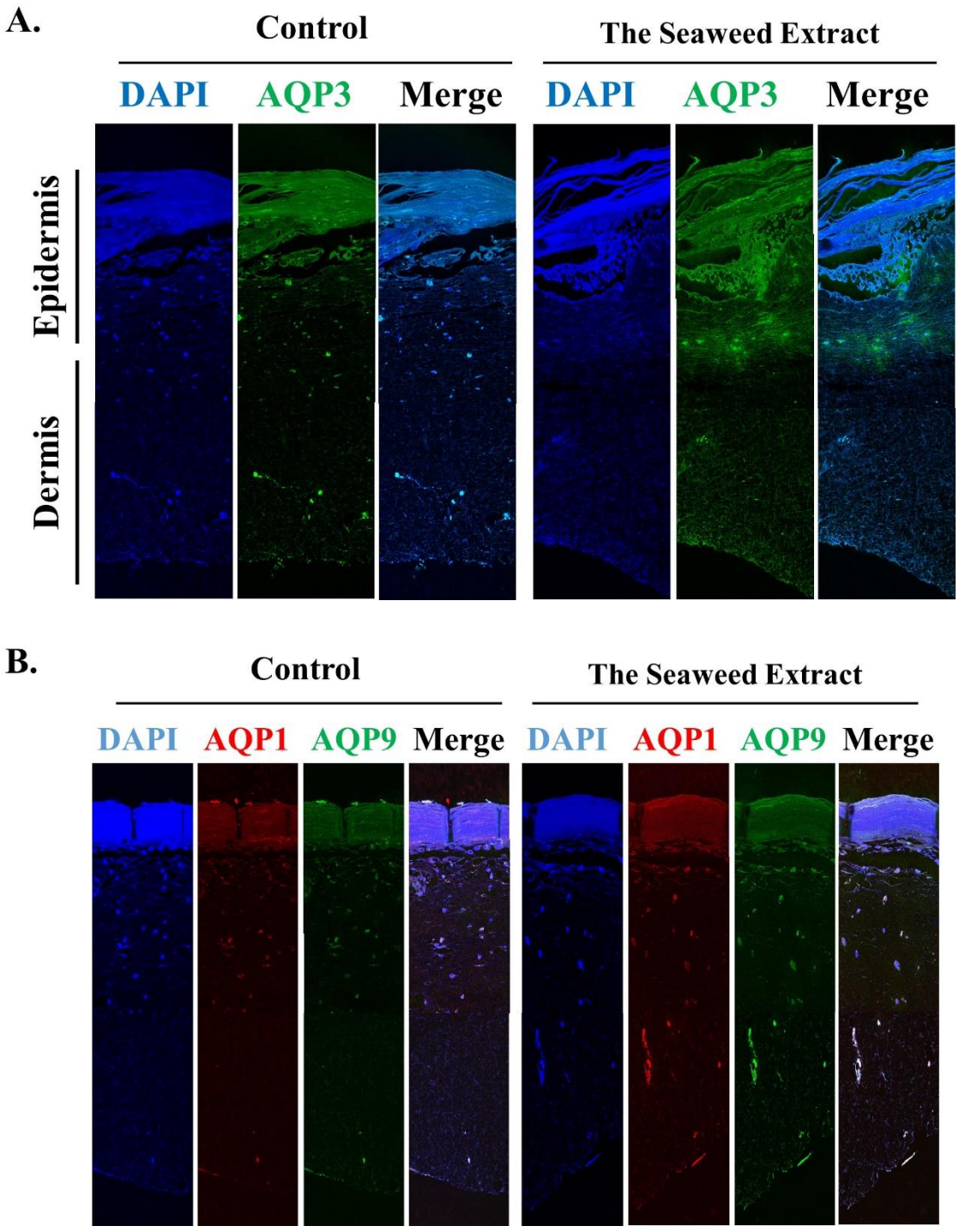

Figure 3. Effects of the seaweed extract on the expression of AQPs in the 3D skin model.

The seaweed extract increased the expression of AQP3 (A), and AQP1 and AQP9 (B).

epidermis and HAS2 and 3 expression in the dermis. In conclusion, the seaweed extract activates different types of hyaluronic acid synthases for each skin layer. It was confirmed through ICH that the amount of hyaluronic acid (HA) synthesis in HDFn actually increased along with the increase in the HAS expression level (Fig. 4C).

Effect of the seaweed extract on tight junction protein expression

We evaluated the effect of the seaweed extract on tight junctions which prevent the evaporation of skin moisture. We performed qRT PCR to investigate the effect on the gene expression of Zo-1 and Claudin which are proteins constituting the tight junctions. When HaCaT cells were treated with 0.1 and 1% of the seaweed extract for 6 and 24 hours, the expression levels of the Zo-1 and Claudin genes were increased (Fig. 5A). In addition, when the HaCaT cells were treated with 0.1% of the seaweed extract for 24 hours, it was confirmed through ICH that the production of the Zo-1 and Claudin proteins in the cells was increased (Fig. 5B).

Discussion.

Proper skin hydration is important for maintaining healthy skin, and moisturizers are one of the most important components of basic skin care. Among several skin molecular-level moisturizing strategies to retain water and prevent water loss, we focused on aquaporins. AQP3 is a member of the family of aquaglyceroporins. Aquaglyceroporins are membrane proteins that form water channels across the cell membrane. They facilitate the transport of water and solutes like glycerol or urea. AQP3 is expressed from the basal layer up to one layer of cells below the SC [2]. AQP3 is known to have a very important role in maintaining skin moisture, such as water circulation and moisturizing. When the seaweed extract was applied to the skin cells and artificial skin, it was confirmed that the expression level of AQP3 was increased in the epidermal cells and epidermal layer (Fig. 2A, 2B, 3A).

Aquaporin-1 (AQP1), a water channel protein controlling the water contents of cells and tissues, exerts pleiotropic effects on various biological activities, including inflammation, angiogenesis, and extracellular matrix remodeling, by regulating cell behaviors and tissue water balance [3]. In the case of AQP1, no significant increase or decrease in expression was observed by the seaweed extract (Fig2, 3).

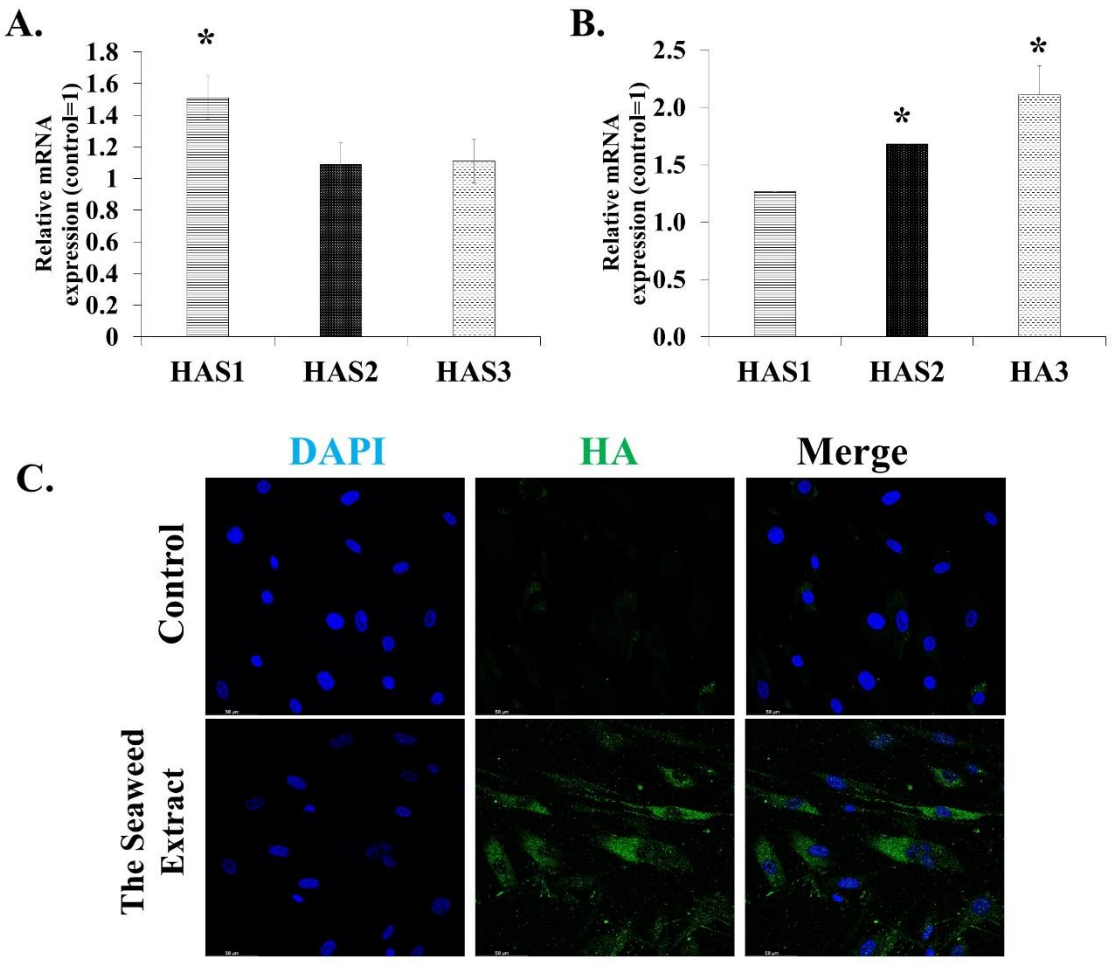

Figure 4. Effects of the seaweed extract on expression of HAS and HA.

The seaweed extract increased the gene expression of HAS1 in HaCaT (A) and HAS2 and 3 in HDFn (B). Additionally, the seaweed extract increased the synthesis of HA in HDFn (C). Data are shown as the mean±S.D. of three experiments. * <0.05 compared to control.

AQP9 is known to be strongly expressed mainly in the Epidermis [4]. However, it was confirmed that the expression of AQP9 in the dermal cells was increased when our new moisturizing ingredient was applied (Fig. 2C, 2D, 3B). The exact physiological function of HDFn and the increased AQP9 in the dermal layer of the artificial skin will be elucidated through further study.

A skin molecule moisturizing substance better known than AQP3 is hyaluronic acid (HA). HA is known to be mainly expressed in the dermis, and its existence was recently confirmed

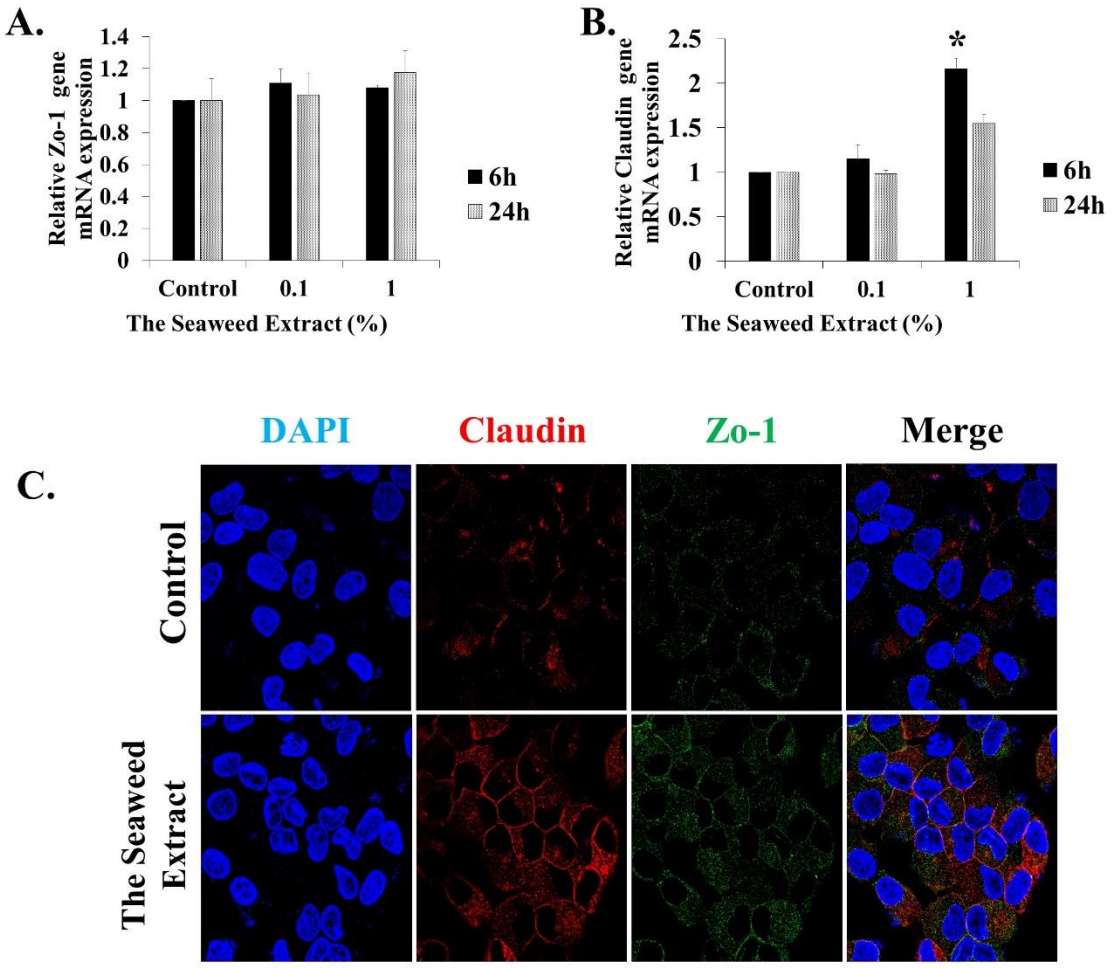

Figure 5. Effects of the seaweed extract on expression of tight junction molecules.

The seaweed extract increased the gene expression of Zo-1 an Claudin (A, b) and the protein expression of Zo-1 an Claudin (C). Data are shown as the mean±S.D. of three experiments. * <0.05 compared to control.

in the SC [1, 5]. Therefore, it can be said that HA is generated throughout the skin layer and has a role in supplying moisture to the skin. In this study, we confirmed that the seaweed extract increases the expression level of the enzyme that promotes hyaluronic acid synthesis and synthesis of HA in HDFn and the effect of activating hyaluronic acid synthase which could be involved in hyaluronic acid synthesis in HaCaT (Fig 4). Through this, it can be seen

that the seaweed extract has the effect of activating different hyaluronic acid synthases in all layers of the dermis and epidermis while accelerating the synthesis of hyaluronic acid according to the characteristics of each skin layer.

A moisturizing strategy that increases the amount of moisture and moisturizing substances in the skin is important, but a strategy to prevent moisture loss in the skin is also important. We focused on the tight junctions which have a major role in skin water loss. The barrier for preventing water loss is not distributed over the entire SC but is located at the junction between the SC and SG which can be referred to as the corneo-epidermal junction. Recent studies have shown that tight junction structures exist in the human epidermis and are also required for regulating skin permeability. TJs consist of proteins such as claudin, occludin, Zo-1, Zo-2, and Zo-3. TJs have an important role in skin permeability, and studies have reported that mice lacking Cludin-1 die from severe body dehydration [6, 7, 8]. In this study, we confirmed that the seaweed extract increased the gene expression and protein production of Zo-1 and Claudin among proteins constituting the TJs (Fig. 5). Through this, it was confirmed that the seaweed extract is a powerful new moisturizing ingredient that can not only increase the moisturizing power of the skin but also suppress water loss.

The ability of the skin to hold water is primarily related to the stratum corneum (SC) which has the role of being a barrier against water loss. Therefore, most moisturizing strategies target the SC. However, we wanted to confirm that our new moisturizing ingredient has a strong molecular-level moisturizing effect in all layers including the dermis, epidermis and stratum corneum of the skin. Our new moisturizing functional ingredient, the seaweed extract, activates different AQPs and hyaluronic acid synthases in the dermal and epidermal layers to increase the moisturizing ability of the skin. In addition, it is a powerful and effective molecular-level moisturizing material that prevents skin water loss by strengthening the TJs.

Conclusion.

The seaweed extract from the seaweeds found in the sea of Jeju Island has a moisturizing effect by promoting AQPs and HAS differently for each skin layer. These factors contribute to moisturizing at the molecular-level. Moreover, it promoted expression of tight junction proteins *in vitro*. Therefore, the new seaweed extract was shown to have potential as a powerful moisturizing ingredient.

Acknowledgments.

Conflict of Interest Statement. NONE.

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