Agastache rugosa Kuntze Attenuates UVB-Induced Photoaging in Hairless Mice through the Regulation of MAPK/AP-1 and TGF- β /Smad Pathways

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Chronic exposure to ultraviolet (UV) radiation, regarded as a major cause of extrinsic aging or photoaging characterized by wrinkle formation and skin dehydration, exerts adverse effects on skin by causing the overproduction of reactive oxygen species. *Agastache rugosa* Kuntze, known as Korean mint, possesses a wide spectrum of biological properties including anti-oxidation, anti-inflammation, and anti-atherosclerosis. Previous studies have reported that *A. rugosa* protected human keratinocytes against UVB irradiation by restoring the anti-oxidant defense system. However, the anti-photoaging effect of *A. rugosa* extract (ARE) in animal models has not yet been evaluated. ARE was orally administered to hairless mice at doses of 100 or 250 mg/kg/day along with UVB exposure for 12 weeks. ARE histologically improved UVB-induced wrinkle formation, epidermal thickening, erythema, and hyperpigmentation. In addition, ARE recovered skin moisture by improving skin hydration and transepidermal water loss (TEWL). Along with this, ARE increased hyaluronic acid levels by upregulating HA synthase genes. ARE markedly increased the density of collagen and the amounts of hydroxypoline via two pathways. First, ARE significantly downregulated the mRNA expression of matrix metalloproteinases responsible for collagen degradation by inactivating the mitogen-activated protein kinase/activator protein 1 pathway. Second, ARE stimulated the transforming growth factor beta/Smad signaling, consequently raising the mRNA levels of collagen-related genes. In addition, ARE not only increased the mRNA expression of anti-oxidant enzymes but also decreased inflammatory cytokines by blocking the protein expression of nuclear factor kappa B. Collectively, our findings suggest that *A. rugosa* may be a potential preventive and therapeutic agent for photoaging.

Keywords: *Agastache rugosa* Kuntze, collagen, Korean mint, photoaging, skin moisture

Introduction

Skin tissue, located on the outer surface of human body works as a barrier to separate the internal other organs from the external environments, and it is responsible for protecting the human body or internal organs against harmful environmental factors, such as chemical compounds, pathogens, air pollutants, and ultraviolet (UV) radiation [1]. There are two types of skin aging [2]. Intrinsic

aging is the irreversible result of being in senescence over time, whereas extrinsic aging occurs by cumulative and excessive exposure of external stimuli [3]. Intrinsic aging is inevitable as it is predominantly dependent on genetic factors; however, the development of extrinsic aging can be delayed, prevented, and inhibited using functional foods or dietary supplements with anti-aging properties.

Skin consists of two layers as follows: the epidermal layer in which keratinocytes are located and the dermal

layer comprising of fibroblasts and extracellular matrix (ECM) [2]. ECM components including elastic fibers, glycosaminoglycan (GAG), and collagens function to support and maintain the structure of the skin. The architecture of the ECM, conferring elasticity, hydration, and resilience, is closely linked with the features of skin aging. UV, which reaches the surface of the skin and, subsequently, penetrates into the skin, is a harmful damaging external factor causing extrinsic aging or photoaging [4]. Continuous and long-term exposure to UV disrupts the structure of ECM by degrading collagens, remodeling elastic fibers, and aggregating irregular GAG, which in turn, contributes to histological and phenotypic changes consistent with aging including deep wrinkle, skin dehydration, skin thickening, and marked loss of elasticity [2].

Reactive oxygen species (ROS), which is recognized as the main cellular cause of physiological processes of photoaging, damages DNA, induces inflammation, disrupts the anti-oxidant system, and collapses ECM in UVB-exposed skin [5]. In terms of the destruction of ECM, excessive ROS not only degrades collagen, which already supports the structure of ECM, but also inhibits collagen synthesis. With regard to molecular aspects, mitogen-activated protein kinase (MAPK) mediates ROS-induced degradation of collagen by triggering the formation of the activator protein 1 (AP-1) complex in UVB-exposed skin cells [3]. The activation of AP-1, which acts as a transcription factor, upregulates the matrix metalloproteinases (MMPs) or proteins responsible for collagen breakdown. In normal physiological conditions, the transforming growth factor beta (TGF- β)/Smad pathway acts as a key promoter in collagen synthesis; however, the UVB-overproduced ROS inactivates the TGF- β /Smad pathway, consequently preventing the production and formation of collagen [6, 7]. Thus, the signaling pathways related to collagen turnover can act as a molecular target to treat and recover UVB-induced skin damage.

Agastache rugosa Kuntze, commonly known as Korean mint, is predominantly found in Korea, Japan, and China. It has been widely used for food and traditional medicine [8]. *A. rugosa* has been revealed to possess various biological and pharmacological properties including anti-oxidation [9], anti-inflammation [10], and anti-atherosclerosis [11]. Particularly, previous studies have reported that *A. rugosa* alleviated photoaging in UVB-irradiated human keratinocytes with regard to its anti-oxidant effect [1, 4]. However, in the view of collagen turnover, the underlying molecular mechanisms of the photoprotective effect of

A. rugosa on skin have not yet been investigated. In addition, to date, studies investigating its anti-photoaging effect have been only performed with *in vitro* models. Therefore, this study aimed to evaluate the anti-photoaging effect of *A. rugosa* extract (ARE) as elucidated by the collagen turnover-related signaling pathways in UVB-irradiated hairless mice.

Materials and Methods

Preparation of ARE

The ARE used in this study was supplied from Cosmax NBT (Korea). The dried aerial parts of *A. rugosa* were powdered and extracted in water at 95°C for 4 h, followed by filtration. The water in ARE filtrates was evaporated in vacuo using vacuum rotary evaporator (Heidolph Instruments GmbH & Co. KG., Germany) to obtain ARE at a 10% yield (w/w).

Animal Experiment

Five-week-old hairless mice (SKH-1) were purchased from Orient Bio (Korea). The mice were housed under well-controlled environments including a light/dark cycle of 12 h, 25–21°C temperature, and 50–60% relative humidity at Yonsei University Laboratory Animal Research Center (Korea). The mice were randomly assigned to the following four groups (six mice per group): (i) control group (CON); (ii) UVB irradiation group (UVB); (iii) UVB plus 100 mg/kg/day ARE-treated group (UVB+ARE100); and (iv) UVB plus 250 mg/kg/day-treated group (UVB+ARE250). After the 1 week of acclimatization, ARE was orally administered carried out once daily for 12 weeks to the UVB+ARE100 and UVB+ARE250 groups. Meanwhile, the hairless mice in the CON and UVB groups were orally administered saline instead of ARE. During the treatment period, concomitant with oral administration of ARE, the back of each mouse in all groups, except the CON group, was irradiated with UVB using the UV crosslinker CL-1000M (UVP, Upland, USA), three times per week. Starting at 75 mJ/cm², the amount of irradiation was continuously increased by 1 minimal erythema dose (MED) per week, and finally maintained at 3 MED until the end of oral administration. At 12th week, the mice were anesthetized with 325 mg/kg of 2,2,2-tribromoethanol (Sigma-Aldrich, USA) injected intraperitoneally and, then, sacrificed by cardiac puncture. For histological analysis, a small piece of the dorsal section isolated from the mice was fixed with 10% formaldehyde (Junsei Chemical Co., Ltd., Japan). The remaining part of the dorsal skin section was stored at –70°C for the evaluation of molecular mechanism. The animal experiment was reviewed and approved by the Institute of Animal Care and Use Committee (IACUC) of Yonsei University (approval number: IACUC-A-201803-203-02).

Evaluation of Skinfold Thickness

Before anesthetizing the hairless mice, skinfold thickness was

measured using a caliper (Mitutoyo, Japan) according to the method described in our previous study [6].

Measurements of Elasticity, Erythema, and Melanin Accumulation

Before anesthetizing the hairless mice, skin elasticity was evaluated using the Computer MPA580 (CK Electronics GmbH, Germany). Both erythema index and melanin index in skin were measured using the Mexameter MPA580 (CK Electronics GmbH).

Measurement of Hydration and Transepidermal Water Loss (TEWL)

The skin hydration was measured by making contact between the dorsal skin and the probe of the Corneometer 825 (CK Electronics GmbH). TEWL was measured at a temperature of 20–25°C and under 40–60% humidity conditions using the Tewameter TM300 equipped with the Multi Probe Adapter MPA5 (CK Electronics GmbH).

Evaluation of Wrinkles

A skin replica was prepared from the dorsal skin of anesthetized hairless mice using the Replica Kit (Epigem, Korea) according to the manufacturer's protocol. The area, number, depth, and length of wrinkles from the replica were determined with Visioline VL650 (SK Electronics GmbH, Germany).

Histological Analyses

From the piece of dorsal section fixed with 10% formaldehyde (Junsei Chemical Co., Ltd., Japan), paraffin blocks were prepared. The paraffin blocks were mounted and fixed on slides, and, thereafter, subjected to hematoxylin & eosin (H&E) staining to observe the skin epidermal layer, Masson's trichrome (M&T) staining to observe the density of collagen in the ECM, and Verhoeff-van Gieson's (GEV) staining to evaluate elastosis. Images of the stained sections obtained using the inverted microscope (CK40; Olympus, Japan) with the xCope T500 camera (DIXI Science, Korea) were analyzed at 200× magnification.

Measurement of Hyaluronic Acid Level

The skin tissue of the hairless mice was homogenized with the NP40 lysis buffer (Elpis-Biotech, Korea). The concentration of hyaluronic acid (HA) in the homogenate was determined using the hyaluronic acid quantikine ELISA Kit (DHYAL0; R&D Systems, USA) following the manufacturer's protocol. Absorbance was read using the VersaMax tunable microplate reader (Molecular Devices, Inc., USA) at 450 nm.

Measurement of Hydroxyproline Level

The skin tissue of the hairless mice was incubated in 6 N HCl at 95°C for 20 h. After 20 h incubation, the concentrations of hydroxyproline were quantified using the hydroxyproline assay kit (QuickZyme Bioscience, Netherlands), as described by the manufacturer's protocol. At the final step, absorbance was read at

450 nm using the VersaMax tunable microplate reader (Molecular Devices, Inc.).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

From the section of the dorsal skin, total RNA was isolated using the RNAiso Plus (Takara, Japan). The quantity and quality of total mRNA were evaluated using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., USA). An equal amount of mRNA was reverse-transcribed to synthesize cDNA

Table 1. The sequences of forward and reverse primers for target genes.

Gene	Direction	Sequence (5'-3')
HAS1	Forward	CCGGAAGAACTGGCTGCTA
	Reverse	ACTTGGACACGGTAACCAACC
HAS2	Forward	GTTAGTGTCTGGGTTCCGCA
	Reverse	GCTCTTTTGGCTTCGCCACA
HAS3	Forward	TCACATATGGCTCTCATCCTGC
	Reverse	CCTTGGAGAAGCCACTGAGAAT
COL1A1	Forward	GTCCCAATGGTGAGACGTG
	Reverse	GCACGGAACTCCAGCTGAT
COL3A1	Forward	AGCGGCTGAGTTTATGACG
	Reverse	AGCACAGGAGCAGGTGTAGA
COL4A1	Forward	GCCAAAGCCAAACCCATTCC
	Reverse	TGGTACGTGTGGTAACTTCT
COL7A1	Forward	AAGCCGAGATTAAGGGCTGG
	Reverse	CACCAATGGAGCACAGCAG
MMP-2	Forward	CATCTTCATCGCTGCACACC
	Reverse	TCAGCACCTTCTTTGGGCA
MMP-3	Forward	TAGCAGGTTATCTAAAGC
	Reverse	CCAGCTATTGCTCTTCAAT
MMP-9	Forward	CTTACCGGCTAAACCACT
	Reverse	TGTCCCTAACGCCAGTAGA
MMP-13	Forward	AAGCAGTTCCAAAGGCTACAAC
	Reverse	CTGGGTCACACTTCTCTGGTG
Catalase	Forward	GTTTGAGAAGTGCGAAGTGAATG
	Reverse	TTAGGTGGAAAGGCATCGGG
GPx	Forward	GGTGCCCCCACTATTACCC
	Reverse	GAATGTCCGCACCTGAGTGA
IL-1β	Forward	GCCCATCCTCTGTGACTCAT
	Reverse	AGGCCACAGGTATTTGTCTG
IL-6	Forward	AGTTGCCTTCTTGGGACTGA
	Reverse	TCCACGATTTCCAGAGAAC
TNF-α	Forward	CCGATGGGTTGTACCTTGTC
	Reverse	TGGAAGACTCCTCCCAGGTA
β-Actin	Forward	GAAGGAGATTACTGCTCTGGCTC
	Reverse	CTCAGTAACAGTCCGCCTAGAA

with Reverse Transcriptase Premix (Elpis Biotech, Inc., Korea) by incubation 42°C for 55 min and, then, at 70°C for 15 min. PCR was performed with synthesized cDNA, each primer (Bioneer, Korea) for each target gene, and HiPi Premix (Elpis Biotech, Inc., Korea) using the Gene Amp PCR System 2700 (Applied Biosystems, USA). The sequences of reverse and forward primers are shown in Table 1. Pre-denaturation was carried out at 94°C for 5 min. The amplification step consisted of 35-40 cycles composed of denaturation at 94°C for 30 sec, annealing at 55-60°C for 1 min, and extension at 72°C for 1 min. PCR products were stained with Loading Star (DyneBio, Korea), and then separated by 1.5% agarose gel. The bands of the target genes were visualized using the G:BOX EF imaging system (Syngene, UK) and GeneSys program.

Western Blot Analysis

Proteins were extracted from the homogenates of dorsal skin in NP40 lysis buffer (Elpis-Biotech) containing a protease inhibitor cocktail (Sigma-Aldrich, USA). The quantity of the protein was determined using the Bradford assay (Bio-Rad, USA). Equal concentrations of proteins were electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and, thereafter, transferred to nitrocellulose membranes (GE Healthcare, USA) at 110 V for 1 h. To avoid non-specific binding, the membrane was incubated in 5% skim milk (Becton Dickinson, USA) for 30 min at room temperature; subsequently, the membrane was washed three times with Tris-buffered saline containing Tween 20 (TBST) for 10 min each. The blocked membrane was exposed to the primary antibodies against nuclear factor kappa B p65 (NF- κ B p65), phospho (p)-extracellular-signal-regulated kinase (ERK), Smad2/3, Smad7, c-Fos (Santa Cruz Biotechnology Inc., USA), TGF- β , p-c-Jun, c-Jun, ERK, c-Jun N-terminal kinase (JNK), p-JNK, p-38, p-p38, and α -tubulin (Cell Signaling, USA) for 16-24 h at 4°C. Next, it was exposed to horseradish peroxidase-conjugated secondary antibodies (Bethyl Laboratories Inc., USA) for 2 h at 4°C. After washing thrice in TBST for 10 min each, the membrane was developed with enhanced chemiluminescence (ECL) solution (Amersham BioSciences, UK). The intensity of the protein bands was identified using the G:BOX EF imaging system (Syngene, India) and GeneSys program.

Statistical Analysis

All data are expressed as the mean $\pm$ standard deviations (SD), and were analyzed using one-way analysis of variance (ANOVA) on SPSS 25.0 (SPSS, Inc., USA). Statistical differences among groups were determined with Duncan's multiple range test. Statistical significance was considered at $p < 0.05$.

Results

ARE Improved Photoaged Physical Parameters in UVB-Irradiated Hairless Mice

The degree of skin wrinkle formation was evaluated by

measuring wrinkle-related parameters in the dorsal skin of mice. Compared with the CON group, the area, number, depth, and length of wrinkles in the UVB group were remarkably increased by 130.93%, 75.12%, 59.05%, and 61.41%, respectively; however, ARE significantly reduced the values of these factors (Fig. 1A). The results suggests that ARE effectively reduces the UVB-induced development of wrinkles. Repetitive UVB irradiation increased erythema index and melanin index, whereas the values were significantly diminished in the UVB+ARE groups (Figs. 1B and 1C). Histological images showed that the epidermal layer of the UVB group was thicker than that of the CON and UVB+ARE groups (Fig. 1D). Quantitative results showed that the skinfold thickness in the UVB group was increased by 71.96%, whereas it was reduced by 23.72% and 32.37% in the UVB+ARE100 and UVB+ARE250 groups, respectively (Fig. 1E). UVB significantly decreased the elasticity and induced elastosis which refers to the accumulation of irregular aggregates of degraded elastic fibers in the ECM [12], whereas ARE caused the recovery of the UVB-induced loss of elasticity and reduced elastosis, in a dose-dependent manner (Figs. 1F and 1G).

ARE Improved Skin Moisture-Related Factors in UVB-Irradiated Hairless Mice

The effect of UVB and ARE on skin moisture was evaluated by measuring skin hydration, TEWL, and the levels of hyaluronic acid. UVB irradiation reduced skin hydration by 45.37%; these values, however, were increased by ARE treatment in a dose dependent manner (Fig. 2A). While TEWL of the UVB group was increased by UVB irradiation compared to that of the CON, its level was recovered in the UVB+ARE groups (Fig. 2B). Compared to HA level in CON group, UVB reduced HA level by 31.02%, whereas it was increased by 37.43% and 56.19% in the UVB+ARE100 and UVB+ARE250 groups, respectively (Fig. 2C). Hyaluronic acid synthases (HAS) are divided into three different isoforms that are main proteins responsible for synthesis of HA [13]. ARE dose-dependently upregulated the mRNA expression of HAS1, HAS2, and HAS3 which was significantly reduced by UVB irradiation (Fig. 2D).

ARE Stimulated Collagen Synthesis Via TGF- β /Smad Pathway

Photoaging symptoms in skin are closely related with collagen content in the ECM [14]. As compared to the CON group, the decreases in the abundance and density of dermal collagen fibers in the UVB group were

Fig. 1. Effects of ARE on wrinkle-related parameters, erythema index, melanin index, thickness, and elasticity.

The hairless mice were exposed to UVB and orally administered ARE at 100 mg/kg/day or 250 mg/kg/day for 12 weeks. The values of area, number, depth, and length of wrinkles (A) were measured using the skin replica. Erythema index (B) and melanin index (C) were investigated by Mexameter MPA580. After 12 weeks, the dorsal skin was isolated to observe histological changes. Epidermal thickness (D) was observed after staining with H&E. Skinfold thickness (E) was measured using a caliper. Elastic fiber in dermis (F) was stained with Verhoeff-van Gieson's staining. Gross elasticity (Ua/Uf) of the skin (G) was measured using the Cutometer. Results are presented as the mean $\pm$ SD. ^{##} $p < 0.01$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. UVB group.

Fig. 2. Effects of ARE on skin hydration and TEWL.

The hairless mice were exposed to UVB and orally administered ARE at 100 mg/kg/day or 250 mg/kg/day for 12 weeks. Skin hydration (A) and TEWL (B) were measured by the Corneometer and Tewameter, respectively. Hyaluronic acid levels (C) were determined using commercial ELISA kit. The mRNA expression of HAS1, HAS2, and HAS3 (D) was determined using RT-PCR. β -Actin was served as the internal controls. Results are presented as the mean $\pm$ SD. [#] $p < 0.05$, ^{##} $p < 0.01$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. UVB group.

histologically observed; whereas they were ameliorated by ARE treatment (Fig. 3A). The level of hydroxyproline, which is an indicator of matrix collagen level [6], was measured to evaluate whether ARE recovered the UVB-damaged ECM. UVB exposure markedly reduced hydroxyproline levels to less than 32.1% of the CON group; however, ARE dose-dependently increased UVB-reduced hydroxyproline levels to significant degree (Fig. 3B). ARE significantly upregulated the mRNA expression of collagen genes, COL1A1, COL3A1, COL4A1 and COL7A1 which was reduced by UVB (Fig. 3C). TGF- β /Smad pathway is a major cell signaling transduction pathway that regulates the expression of collagen genes [14]. Compared to the

CON group, the expression of TGF- β and Smad2/3 proteins was downregulated by UVB irradiation (Fig. 3D). UVB exposure upregulated the protein expression of Smad7 to 1.13 fold compared to the control, which was nearly restored to normal levels upon ARE treatment. Collectively, ARE not only increased TGF- β and Smad2/3 protein expression but also decreased Smad7 protein level.

ARE Downregulated the Expression of MMPs Via the MAPK/AP-1 Pathway

The MAPK/AP-1 pathway is a key regulator of MMP expression which is involved in the collapse of the structure of the ECM that mostly consists of collagen [2].

Fig. 3. Effects of ARE on hydroxyproline levels and expression of collagen genes, TGF-β and Smads.

After oral administration of ARE in UVB-irradiated hairless mice for 12 weeks, the dorsal skin was isolated. Collagen fibers (A) were stained with M&T staining. Hydroxyproline levels (B) were determined using a commercial ELISA kit. The mRNA expression of COL1A1, COL3A1, COL4A1 and COL7A1 (C) was determined using RT-PCR. The protein expression of TGF-β, Smad2/3, and Smad7 (D) was determined using Western blot analysis. β-Actin and α-tubulin were served as the internal controls. Results are presented as the mean ± SD. [#]*p* < 0.05, ^{##}*p* < 0.01 vs. control group; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. UVB group.

UVB stimulated the phosphorylation of ERK, JNK, and p38, which was significantly suppressed by ARE treatment (Fig. 4A). We observed that with an exception of p-p38 protein expression, the protein levels of p-ERK and p-JNK were reduced compared to those of the CON group in the UVB+ARE250 group (Fig. 4A). The AP-1 complex consisting of c-Jun and c-Fos proteins, works as a transcription factor to regulate MMPs [7]. ARE inhibited the protein levels of p-c-Jun and c-Fos in a dose-dependent manner (Fig. 4B). Subsequently, ARE reduced the mRNA expression of MMP-2, -3, -9, and -13 which was increased by UVB exposure (Fig. 4C).

ARE Upregulated Anti-Oxidant Enzymes and Inhibited Inflammatory Response

ROS abnormally modulate the molecular mechanisms in UVB-irradiated skin cell [3], and causes inflammatory responses by regulating NF-κB or a major mediator of inflammation [5]. In UVB-irradiated dorsal skin, the mRNA expression of catalase and glutathione peroxidase (GPx) was significantly reduced, whereas ARE upregulated these genes (Fig. 5A). UVB treatment increased the protein expression of NF-κB p65 by 137.08%, compared to the CON group; however, its level of protein expression in the UVB+ARE groups was significantly lower than that in the

Fig. 4. Effect of ARE on the expression of MAPK, AP-1, and MMPs.

After oral administration of ARE in UVB-irradiated hairless mice for 12 weeks, the dorsal skin was isolated. The protein expression of p-JNK, JNK, p-ERK, ERK, p-p38, and p38 (A) and p-c-Jun, c-Jun, and c-Foc (B) was determined using Western blot analysis. The mRNA expression of MMP-2, MMP-3, MMP-9, and MMP-13 (C) was determined using RT-PCR. β -Actin and α -tubulin were served as the internal controls. Results are presented as mean $\pm$ SD. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. control group; $^*p < 0.05$, $^{**}p < 0.01$ vs. UVB group.

UVB group (Fig. 5B). NF- κ B which is a mediator of UVB-induced inflammatory responses upregulates the expression of inflammatory cytokines by being translocated into nucleus [7]. ARE significantly reduced the UVB-induced mRNA expression of interleukin (IL)-1 β , IL-6, and tumor necrosis factor alpha (TNF- α) (Fig. 5C), suggesting that ARE downregulated the mRNA expression of inflammatory cytokines by decreasing NF- κ B levels.

Discussion

Overexposure of UV, particularly UVB, elicits adverse biological effects on the skin by inducing abnormal histological and physiological changes, which, in turn, lead to photoaging symptoms, such as wrinkle formation, hyperpigmentation, and skin thickening [6, 15]. There are three types of UV radiation as follows: UVA, UVB, and

Fig. 5. Effect of ARE on the expression of anti-oxidant enzymes, NF-κB, and inflammatory cytokines.

After oral administration of ARE in UVB-irradiated hairless mice for 12 weeks, the dorsal skin was isolated. The mRNA expression of catalase and GPx (A) was determined using RT-PCR. The protein expression of NF-κB p65 (B) was determined using Western blot analysis. The mRNA expression of IL-6, IL-1β, and TNF-α (C) was determined using RT-PCR. β-Actin and α-tubulin were served as the internal controls. Results are presented as mean ± SD. [#]*p* < 0.05, ^{##}*p* < 0.01 vs. control group; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. UVB group.

UVC. With regard to the generation of photoaging, UVB is important as it is not only absorbed into the epidermis and the upper part of dermis but also produces more damage to skin than UVA [4, 15]. Although UVC is more harmful than UVB, it cannot reach on the surface of earth as it is absorbed by the ozone layer [3]. Previous studies have reported that UVB irradiation increased epidermal thickness, and erythema, as well as wrinkle parameters, including area, depth, length, and number in hairless mice [6, 16, 17]. These findings are consistent with the results of the present study of abnormal physiological alterations, such as rough wrinkling, expansion of skin thickness, hyperpigmentation, and erythema in the UVB-exposed dorsal skin (Fig. 1). This indicates that repetitive exposure of UVB to dorsal skin of mice is sufficient to cause the

development and worsening the symptoms of photoaging. However, oral administration of ARE alleviated these symptoms and shifted the condition of damaged skin to a normal physiological state (Fig. 1). Thus, these results suggest that ARE exhibits potential anti-photoaging activity in UVB-irradiated hairless mice.

Another major feature of skin photoaging is skin dehydration; UVB leads to the loss of the ability of the skin to maintain natural moisturizing factors. TEWL is an indicator of skin barrier function as the role of the epidermis is to maintain adequate moisture levels by acting as a skin barrier [18]. In this study, UVB exposure reduced skin hydration and increased TEWL, which were improved by ARE treatment (Figs. 2A and 2B). As the lowering of the water content and an increase in TEWL are accompanied

by a decrease in skin barrier function, the current results suggest that the recovery effect of ARE on skin hydration is attributed to its ability to alleviate the UVB-induced damage to the skin barrier function. Consistently, a mixture of plant oils including *A. rugosa* alleviated the atopic dermatitis-induced skin barrier damage [19]. In addition, as a natural moisturizing factor, HA, which is one of the abundant molecules in the dermis, plays an important role in retaining moisture levels [20]. Thus, the level of HA, which depends on three different HAS isoforms is a representative element of skin moisture [13]. A previous report presented that repetitive UVB irradiation induced loss of HA by reducing the mRNA expression of HAS. Consistently, chronic UVB exposure not only reduced the levels of HA but also decreased the mRNA expression of HAS1, HAS2, and HAS3, which was increased by ARE treatment (Figs. 2C and 2D). These results indicate that ARE possesses a skin moisturizing activity, as evidenced by improved TEWL, skin hydration, and HA levels via the upregulation of HAS.

Collagen is the most abundant protein in ECM; this indicates that ECM function is highly dependent on the roles and structures of collagens [2]. Histologically, a major cause of wrinkle formation in photoaging is the decomposition and fragmentation of collagen as well as the disorganized collagen fragments [21]. M&T staining and hydroxyproline measurement showed that ARE prevented UVB from degrading the collagen content in the ECM (Figs. 2A and 2B). These results suggest that ARE inhibits UVB-induced destruction and decomposition of collagen in UVB-treated dorsal skin. Next, we investigated molecular mechanisms under which UVB and ARE influenced collagen content in the ECM. UVB irradiation prohibits the upregulation of collagen-related genes by inactivating the TGF- β /Smad pathway [7]. Here, ARE significantly increased the mRNA expression of COL1A1, COL3A1, COL4A1 and COL7A1; and stimulated the protein expression of TGF- β and Smad2/3 in UVB-irradiated dorsal section of skin (Figs. 2C and 2D). Smad7 inhibits signaling by competing with Smad2/3 to TGF- β ; thus, increasing the Smad7 protein can lead to TGF- β /Smad pathway suppression [6, 7, 14]. Smad7 is involved in the stimulatory effect of ARE on TGF- β /Smad pathway, as evidenced by the decrease in Smad7 protein expression in the ARE groups, compared to the UVB group (Fig. 2D). These results suggest that ARE rescues the UVB-downregulated collagen gene by activating the TGF- β /Smad pathway. Activating the MAPK/AP-1 signaling cascade is more important than the TGF- β /Smad pathway, as it is a key mechanism not only in the destruction

and collapse of the ECM structure (by degrading the generated collagens) but also in the interference with the new formation of collagen (by decreasing the synthesis of TGF- β) [3, 16]. The UVB-activated MAPK/AP-1 pathway elevates both the mRNA expression and activities of MMPs which not only degrade elastic fibers but also break collagens [2, 6]. Consistent with previous studies that ARE significantly prohibited pro-MMP-2 and pro-MMP-9 in UVB-treated human keratinocytes [1, 4], ARE remarkably decreased the mRNA expression of MMPs. Compared to the UVB group, ARE also downregulated the protein levels of MAPK including, p-ERK, p-JNK, and p-p38; and reduced the levels of components of AP-1, such as c-Fos and p-c-Jun (Fig. 4). These results demonstrate that ARE mitigates the mRNA expression of MMPs by inactivating MAPK/AP-1 pathway. Collectively, ARE can increase the levels of collagen in ECM by improving collagen turnover-related cell signaling transductions in UVB-induced photoaging.

The plant extracts that exhibit anti-oxidant and inflammatory effects have attracted the attention to researchers who have tried to develop therapeutic agents against photoaging [6, 15], as UVB-related extrinsic aging is highly associated with ROS and ROS-triggered inflammatory response. Several polyphenols derived from natural plant extracts have been reported to possess anti-photoaging activities by alleviating oxidative stress and inflammation [22]. Compounds found in ARE, such as caffeic acid, apigenin, kaempferol, and genistein have been reported to reduce UV-induced skin damage [11, 23-26]. For example, caffeic acid downregulated the mRNA expression of MMP-1 and increased the activities of catalase and GPx in UVA-irradiated keratinocytes [23]. Apigenin protected keratinocytes against UVB by inhibiting ROS generation and decreasing NF- κ B [24]. Previously, *A. rugosa* attenuated UVB-induced photoaging by upregulating anti-oxidant enzymes and reducing the production of ROS [1, 4]. In terms of anti-inflammatory effect, the essential oil of *A. rugosa* suppressed nitric oxide (NO) production by inactivating NF- κ B and MAPKs in lipopolysaccharide (LPS)-stimulated RAW264.7 cells [10]. In this study, ARE not only increased the mRNA expression of catalase and GPx but also decreased NF- κ B-induced transcription of inflammatory cytokines in UVB-treated dorsal skin (Fig. 5). These results suggest that the anti-oxidant and anti-inflammatory activities of ARE are partly involved in its anti-photoaging effect.

In conclusion, ARE improved photoaging-related symptoms, such as wrinkling, hyperpigmentation, and

epidermal thickness in UVB-exposed hairless mice. Along with an improvement in skin hydration and TEWL, ARE increased the levels of HA via the upregulation of HAS. At the molecular level, ARE increased collagen levels in the ECM by facilitating TGF- β /Smad signaling and inactivating the MAPK/AP-1 pathway. In addition, the antioxidant and anti-inflammatory effects of ARE were also involved in its anti-photoaging activity by the upregulation of antioxidant enzymes and the downregulation of inflammatory cytokines. Collectively, ARE has an ability to reverse UVB-induced photoaging by attenuating photoaged physical parameters, improving collagen turnover-related pathways, increasing anti-oxidant defense system, and reducing inflammatory response. Supported by clinical trials, ARE can be used as a dietary supplement or an herbal agent for anti-photoaging and skin health.

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Conflict of Interest

The authors declare no conflict of interest.

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