A Thesis for the Degree of Master of Science

A New Combination of Soybean and Haematococcus Extract Potently Alleviates Ultraviolet B-induced Photoaging

대두와 헤마토코쿠스 추출물 특정 비율 복합물의 자외선에 의한 광노화 개선 효능 규명

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ABSTRACT

Soybean has been constantly consumed in Asian countries. It contains high amounts of isoflavones. Soybean-derived isoflavones are known for many advantages such as anti-carcinogenic activity and estrogenic-like effect. In particular, soy isoflavones have been studied on their beneficial effects in improving UV-induced skin damage such as wrinkle formation and inflammation by binding with specific targets which result in regulating signal transduction. *Haematococcus pluvialis* (*H. pluvialis,* Haematococcus) is a carotenoid pigment containing the largest amount of natural astaxanthin. The astaxanthin is a non-provitamin A carotenoid, but it involves in retinoic acid receptor (RAR) or retinoid signaling which are associated with inhibiting activator protein (AP)-1 dependent transcription. Based on the previous studies, I hypothesized that soy extract (SE) and haematococcus extract (HE) can effectively improve UVB-induced photo-aging through specific signaling pathways

by regulating targets. And I investigated an effect of the mixture in different ratios on UVB-induced wrinkles in hairless mice and human dermal fibroblasts (HDFs). The 1:2 ratio of SE and HE mixture (SHM) showed the most promising benefit compared to the other different ratios *in vivo*. Based on *in vivo* study I used primary HDFs for mechanisms study. SHM has an inhibitory effect on wrinkle formation through down-regulating matrix metalloproteinase (MMP)-1 protein and mRNA expression. SHM also inhibits transactivation of AP-1 which has important role in regulating MMP expression. In addition, SHM influences UVB-induced mitogen activated protein kinases (MAPKs) which regulate AP-1 transactivation. These result suggest that SHM could be a potential agent against UVB-induced skin wrinkles.

Keywords: Soybean; Isoflavone; Haematococcus; astaxanthin; Skin wrinkle; MMP-1; AP-1; MAPKs

Student ID: 2014-20706

CONTENTS

Ⅰ. INTRODUCTION

Life expectancy has been improving for decades, accompanied by desire to improve their appearance and reverse the signs of aging [1]. Because skin represents typical phenotype of aging, it is important to maintain skin health for life satisfaction [2]. Skin is the largest and most massive organ. A crucial role of the skin is to protective barrier from external environment [3]. And it is a suitable model allowing the study of intrinsic and extrinsic factors about complex phenomenon of aging [4]. Intrinsic aging occurs naturally by senescence and the symptoms contain dry and finely wrinkled skin [5]. On the other hand, extrinsic skin aging is related to environmental factors like smoking, ultraviolet (UV) exposure [6] and air pollution [7]. It is obvious that extrinsic aging caused by UV exposure from sunlight, photo-aging, contribute to ~ 90 % of overall skin changes [8]. UV light can also cause direct damage to

DNA leading to upregulation of proinflammatory cytokines such as nuclear factor kappa B (NF-kB) [9]. Clinically, photo-aging phenotype includes sagging and deeply wrinkled skin [10].

Sun light is divided into ultraviolet (UV), infra-red (IR), and visible light depending on wavelengths. UV comprises UVA (320~400nm), UVB (280~320nm), and UVC (200~280nm). Since UVC is blocked by ozone layer, UVA and UVB are an actual cause of skin aging [11]. UVB contains more energy than UVA, leading to skin damage such as sunburn, suntanning, immune suppression, photoaging, and photo-carcinogenesis [12]. Chronic exposure to UV can lead to photo-aging by changing the composition of dense collagen-rich extracellular matrix (ECM). ECM is composed of connective tissue and basement membrane proteins such as elastin, glycosaminoglycans and interstitial collagen [13]. In human skin, UV radiation can elevate the levels of various matrix metalloproteinases (MMPs) such as MMP-1,

MMP-3, MMP-9, but not MMP-2 [14, 15]. Among them, MMP-1, fibroblast collagenase, is the key enzyme breaking down dermal components in ECM [16, 17]. Once the elevated levels of MMP-1 initiates degradation of fibrillary type Ⅰ and Ⅲ collagen, further process is followed by MMP-3 and MMP-9 [18]. Therefore, MMP-1 plays an important role in UVB-induced wrinkle formation through ECM degradation. The UV-induced MMP-1 overexpression is followed by mitogen-activated protein kinases (MAPKs) signaling pathway through various factors such as cytokines and growth factor receptors [15]. Activator protein (AP)-1 is a transcription factor which is one of the main effector of MAPK pathway in regulating MMPs expression. AP-1 makes heterodimers between Jun, Fos or activating transcription factor (ATF) proteins [19]. Because the chronic exposure of UVB could occur photo-aging, it is required to search the efficient method to inhibit the MMP-1 expression by inhibiting the transcriptional factor through

specific signaling pathways.

Soy foods have been consistently consumed in Asian countries not only their nutritional benefits but also disease preventive effects [20]. Soybean is well known as a high-quality protein source containing several key bioactive compounds [21]. Among other ingredients, isoflavones are associated with beneficiary effects contributing to the health. They have an anti-carcinogenic activity and estrogen-like effect based on their diphenolic structure [22, 23]. In our previous study, soy-derived isoflavonoids have been studied on the effects in improving UV-induced skin damage by binding with specific targets. Coumestrol which is one of the soybean isoflavonoids had significant effects in preventing UVB-induced skin wrinkle formation by regulating Ras/MEK/ERK and Akt/p70S6K pathway through targeting FLT3 kinase [24]. And biochanin A, one of the isoflavones found in soy, inhibited solar UV-induced cyclooxygenase (COX)-2 expression by directly targeting

MLK3 kinase which results in regulation of MKK4/JNK/c-Jun and MKK3/6/p38/MSK signaling pathways [25]. Furthermore, soybean isoflavones, especially in aglycone form like genistein and daidzein, are considered to have beneficial effects on the skin through the stimulation of fibroblast proliferation, reduction of collagen degradation and other various mechanisms [26]. Based on the previous studies, I hypothesized that soy isoflavones can effectively improve UVB-induced photo-aging through targeting specific proteins.

H. pluvialis, a green alga, is the richest biological source of natural astaxanthin. Astaxanthin is a carotenoid pigment which presents in lots of marine lives such as salmon, shrimp, lobster, and fish eggs [27]. Astaxanthin accumulation in *H. pluvialis* was observed only in encysted cells as a defense mechanism when they are under unfavorable growth conditions such as elevated temperature, nitrogen and phosphate limitation, pH and salinity [28, 29]. And since animal cannot synthesize

the astaxanthin, it should be provided from the diet [27]. Carotenoids have carried biological actions by being converted to retinoids [30]. Most carotenoids such as β-carotene which can be converted into vitamin A have many beneficial effects on human health via direct binding to retinoic acid receptor (RAR) [31]. On the other hand, astxanthin is a non-provitamin A carotenoid which have been considered not to have any retinol converting activity [27]. However, astaxanthin is a non-provitamin A carotenoid, some studies revealed that the astaxanthin involve in RAR or retinoid signaling [30]. In the previous study, non-provitamin A carotenoids such as astaxanthin stimulated RARs like retinoic acid resulting in production of hyaluronan, one of the important components of ECM [32]. It still remains obscure that the astaxanthin could be turned into β-carotene or retinol *in vivo* model [33]. And RARα, -β, and –γ are activated by retinoids and they are associated with inhibiting AP-1-dependent transcription. In addition, RARα

prevents Jun/AP-1 from binding to DNA leading to direct inhibition of the interaction between RARs and c-Jun [34]. But the mechanism is not fully understood how non-provitamin A has the biological activities.

Although soybean extract (SE) and haematococcus extract (HE) have been fully studied on their active compounds and beneficial effects respectively, anti-wrinkle effects of combined SE and HE have not be reported yet. Therefore, it is probably reasonable anticipation that the addition of combined SE and HE to UV-exposed human dermal fibroblasts (HDFs) will prevent UV-stimulated up-regulation of MMP-1 through specific targets and signaling pathways.

In this study, we reported that a protective effect of the mixture of SE and HE on UVB-induced skin aging in hairless mice and HDFs. These finding shows that a new combination of SE and HE could be used as a potential therapeutic anti-aging food.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and reagents.

Dulbecco's modified eagle medium (DMEM) was purchased from Welgene (Gyeongsan, Korea). Fetal bovine serum (FBS) was bought from Sigma-Aldrich (St.Louis, MO) . The MMP-1 antibody was obtained from R&D systems Inc. (Minneapolis, MN). Antibodies against phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204), total ERK1/2, total c-Jun N-terminal kinase 1/2 (JNK1/2), and total p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated JNK1/2 (Thr183/Tyr185), phosphorylated-p38 (Thr180/Tyr182), phosphorylated $p90^{RSK}$ (Thyr359/Ser363), total $p90^{RSK}$ and a tissue inhibitor of metalloproteinases (TIMP)-1 were purchased from Cell

signaling (Cell signaling, MA) 3-[4,5-dimethylatiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) powder was purchased from USB Co. (Cleveland, OH). Penicillin-Streptomycin Solution was purchased from Mediatech, Inc. (Manassas, VA). Protein assay reagent kits were obtained from Bio-Rad Laboratories (Hercules, CA).

2. Sample preparation.

SE was provided by Skyherb Co., Ltd. (Seoul, Korea). Black soybean, *Glycine Max (L.) Merr*, come to soybean meal through vibrating screen cold pressing. Soybean meal was extracted with ethanol and concentrated under reduced pressure. SE was gained after purification, elution, spray drying process. The contents of total isoflavones in SE were 40.4 9% and the contents of genistein, daidzein, genistin, and daidzin were 19.7 5%, 19.52 %, 0.15 %, and 0.83 % respectively. It was dissolved in 0.5 % sodium carboxymethylcellulose

for animal treatment and in dimethylsulfoxide for cell treatment. HE was provided by fuji chemical industry Co. (Toyama, Japan). Microalge HE (AstaREAL®L10) and all other component were mixed by high-shear mixer. AstaREAL®EL25 was gained through encapsulation, granulation, and sieving process. The total astaxanthin content was 2.5-2.7 % and out of total carotenoids were 2.6-2.9 % in AstaREAL®EL25. HE was dissolved in 0.5 % sodium carboxymethylcellulose for animal treatment and in dimethylsulfoxide for cell treatment.

3. Animals and treatments.

Five-week-old female albino hairless mice (Skh-1) were obtained from Orient Bio (Seongnam, Korea). Animals were acclimated for 1 week prior to study and had free access to food and water. All experimental protocols were approved by the Institutional Animal Care and Use Committee (No. 2015-0057) of the Biomedical Research

Institute, Seoul National University Hospital (Association for Assessment and Accreditation of Laboratory Animal Care accredited facility). 6-8 mice were allocated into seven groups.

Sample and vehicle (0.5 % sodium carboxymethylcellulose) were orally administered for 9 weeks. Body weight and food intake were monitored on a weekly basis. A UVB-induced photoaging animal study experiment was performed, as described previously. A UVB irradiation device that included TL20W/12RS UV lamps (Philips, Eindhoven, the Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310–315 nm) served as the UV source. Initially, I measured the minimal UVB dose on dorsal skin of mice as minimal edema dose (MEdD) comparable with minimal erythema dose in human skin. In contrast to human, mouse showed peak responses to UVB mainly as edema, increased thickness of dorsal skin at 48 hours post-UVB irradiation. The irradiation dose was increased weekly by 0.5

MEdD (1 MEdD = 100 mJ/cm^2) up to 2 MEdD and then maintained at 2 MEdD. UVB irradiation was stopped after 9 weeks (Fig. 1A).

Each dose of sample and mixture ratio: SE (11.67 mg/kg of SE), HE (53.33 mg/kg of HE), SHL (11.67 mg/kg of SE and 13.33 mg/kg of HE, about 1:1 ratio of SE and HE), SHM (11.67 mg/kg of SE and 26.67 mg/kg of HE, about 1:2 ratio of SE and HE) and SHH (11.67 mg/kg of SE and 53.33 mg/kg of HE, about 1:4 ratio of SE and HE).

4. Cell Culture and treatments.

Primary HDFs were isolated from the outgrowth of foreskin obtained 12 year-old healthy volunteers from Dr. Chung JH laboratory (Seoul National University Hospital, Korea) under the Institutional Review Board at Seoul National University Hospital (No. H-1101-116-353) and Seoul National University (No. E1408/001-002). HDFs were cultured in DMEM with 10 % (v/v) FBS and 1 % (v/v)

penicillin/streptomycin at 37 °C and 5 % CO_2. UVB irradiation was performed on serum-starved monolayer cultures. HDFs were treated with SHM, which was dissolved in 50% ethanol. HDFs were exposed to UVB at a dose of 0.02 J/cm^2 using UVB source (Bio-Link crosslinker, VilberLourmat, Cedex 1, France) set spectral peak at 312 nm.

5. Cell viability

The cell cytotoxicity was measured using the MTT assay. HDF were cultured in the 96 well plates at a density of 2×10^3 cells/well and incubated in DMEM-10% FBS containing penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were starved in serum-free DMEM for 24 h. The cells and each sample were incubated for 22 h at 37 °C, followed by treatment with MTT solution for 2 h. The medium

was removed and formazan crystals were dissolved by the addition of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was then measure using a microplate reader (Molecular Devices, CA) and SHM treated and non-treated cells were compared.

6. Determination of wrinkle formation.

To determine the severity of wrinkles, each hairless mouse was anesthetized and the UVB-exposed dorsal skin (wrinkle formation area) was photographed. Skin wrinkle replica was made with a silicon rubber (Silflo Dental Impression Materials, Potters Bar, UK) from the backs of unstrained mice. Skin replica was photographed using a coupling charge system video camera. Wrinkle was analyzed by Skin-Visiometer SV 600 software (CK Electronic GmbH, Köln, Germany). The visiometer is a computerized instrument which creates a skin microrelief map from the

replica using a light transmission method.

7. Hematoxylin and eosin staining.

To investigate epidermal thickness, Hematoxylin and eosin staining was performed. Mouse skin samples were fixed with 10 % neutral-buffered formalin, and embedded in paraffin. Serial sections (4 μm) were mounted onto slides. After deparaffinizing, skin sections were re-hydrated and stained with Hematoxylin solution for 5 minutes. And then, slides were washed and stained in counterstain in eosin Y solution for 30 seconds. Next, the slides were dehydrated through 95% alcohol and washed in absolute alcohol, 5 minutes each. Lastly, they were incubated in xylene overnight to clear of any water and then dry them. Skin sections were examined at 400× magnification using an Olympus AX70 light microscope (Tokyo, Japan).

8. Masson's trichrome staining.

To evaluate collagen in the dermis, Masson's trichrome staining was performed. Mouse skin samples were fixed, embedded, and deparaffinized as described above. After deparaffinizing, skin sections were stained with Hematoxylin for 5 min. And then, slides were washed and stained in biebrich scarlet and acid fuchsin. Next, the slides were placed in phosphomolybdic-phosphotungstic acid for 10 min and aniline blue for 5 min to stain collagen. Slides were then washed and incubated in 1 % acetic acid for 15 min. Lastly, they were dehydrated and washed. Skin sections were examined at 400× magnification using an Olympus AX70 light microscope (Tokyo, Japan).

9. Western blot and zymography.

Primary HDFs were cultured for 48 h, and then the cells were incubated in serum-free DMEM for 24 h. After that, the cells were

treated with or without various concentrations of SHM (2.5, 5, 10 μg/ml) for 1 h, followed by UVB (0.02 J/cm^2) irradiation. The media was harvested on ice, and then centrifuged at 18,620 g for 10 min. The protein concentration was measured using a protein assay reagent kits as described by the manufacturer. The cells were lysed with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1 % Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 % glycerol and protease inhibitor cocktail tablet] on ice for 30 min, scraped and then centrifuged at 18,620 g for 10 min. The proteins were separated electrophoretically using a 10 % SDS-polyacrylamide gel and transferred onto an Immobilon P membrane (MERK Millipore). The membrane was blocked in 5 % fat-free milk for 1 h, and then incubated with the specific primary antibody at 4 °C overnight. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, London, UK) after

hybridization with the HRP-conjugated secondary antibody (Life technologies, Waltham, MA).

Zymography was used to determine the activity of secreted MMP-2. Zymography was performed in 12 % polyacrylamide gel in the presence of gelatin (0.1 % w/v). The protein samples were mixed with loading buffer [10 % SDS, 25 % glycerol, 0.25 M Tris (pH 6.8) and 0.1 % bromophenol blue], and then run on 12 % SDS-PAGE gel without denaturation. Afterward, the gel was washed with renaturating buffer (Life technologies, Waltham, MA) for 1 h at room temperature and incubated for 24 h at 37 °C in developing buffer (Life technologies, Waltham, MA). After enzyme reaction, the gel was stained with 0.5 % Coomassie brilliant blue in 10 % acetic acid.

10. Real-time quantitative PCR.

Primary HDFs were treated with SHM for 24 h and harvested in

RNAiso Plus (Takara Bio Inc., Shiga, Japan). RNA was quantified using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). After RT with oligo-dT primers using a PrimeScript™ 1st strand cDNA synthesis Kit (Takara Bio Inc.), Real-time quantitative RT-PCR was performed using IQ SYBR (Bio-Rad Laboratories) and 2 μl of cDNA in triplicate with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. Before PCR amplification, the primers were denatured at 95 °C for 3 min. Amplification was made up of 44 cycles at 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 30. PCR was performed by CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was probed by the following primer: MMP-1 forward (5'-ATT CTA CTG ATA TCG GGG CTT TGA-3'); MMP-1 reverse (5'-ATG TCC TTG GGG TAT CCG TGT AG-3'); GAPDH forward (5'-ATT GTT GCC ATC AAT GAC CC-3'); GAPDH reverse (5'-AGT AGA GGC AGG GAT GAT

GT-3').

11. Luciferase reporter gene assay.

The lentiviral expression vectors, including pGF-AP1-mCMV-EF1-Puro (System Biosciences, CA), and packaging vectors, including pMD2.0G and psPAX, were purchased from Addgene Inc (Cambridge, MA). pGF-MMP-1-mCMV-EF1-puro vector was gently provided provided by Dr, Sung-Keun Jung (Korea Food Research Institute, Sung-Nam, Korea). MMP-1 promoter are coloned to pGF vector [35]. pGF-AP1-mCMV-EF1-Puro vectors and the packaging vectors (pMD2.0G and psPAX) were transfected into HEK293T cells using jetPEI following the manufacturer's instructions. The transfection medium was changed into 24 h after transfection and the cells were then cultured for 36 h. The viral particles were harvested by filtration using a 0.45 mm

syringe filter, then combined with 8 μg/mL polybrenes (EMD, Darmstadt, Germany) and infected into 60 % confluent HDFs overnight. The cell cultured medium was replaced with fresh complete growth medium for 24 h before the cells were selected for using puromycin (Sigma-Aldrich) over 36 h. The selected cells were then used for further experiments. HDFs were cultured for 48 h and then starved to serum-free DMEM for 24 h. After starvation, HDFs were treated with or without various concentrations of SHM for 1 h, followed by 0.02 J/cm^2 UVB irradiation. Cell extracts were prepared for reporter lysis buffer (Promega). HDFs for MMP-1 and AP-1 were lysated after 36 h. The extracts were used for luciferase assay. MMP-1 and AP-1 activity in HDFs were determined by using a luciferase assay kit (Promega), as described by the manufacturers.

12. Statistical analysis.

Statistical analyses were performed using one-way ANOVA followed by Duncan and p values of less than 0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Oral administration of SHM decreased UVB-induced skin wrinkles in hairless mice.

To investigate anti-wrinkle effects of SHM, hairless mice were exposed to UVB on their dorsal skins during 9 weeks (Fig. 1A). I measured wrinkle area, length and depth by analyzing skin replica. Consequently, UVB-induced wrinkles are reduced in the mixture of SE and HE (Fig. 1B and 1C). I found more significant decrease of wrinkles in SHM and SHH than that of only SE or HE through determination of the percentage of wrinkle area (Fig. 1D). All groups reduced wrinkle length against only UVB-irradiated group (Fig. 1E), and I found a similar result in wrinkle depth except for HE (Fig. 1F). Overall, these results indicate that the intake of SHM and SHH effectively decreased UVB-induced wrinkle formation.

Figure 1

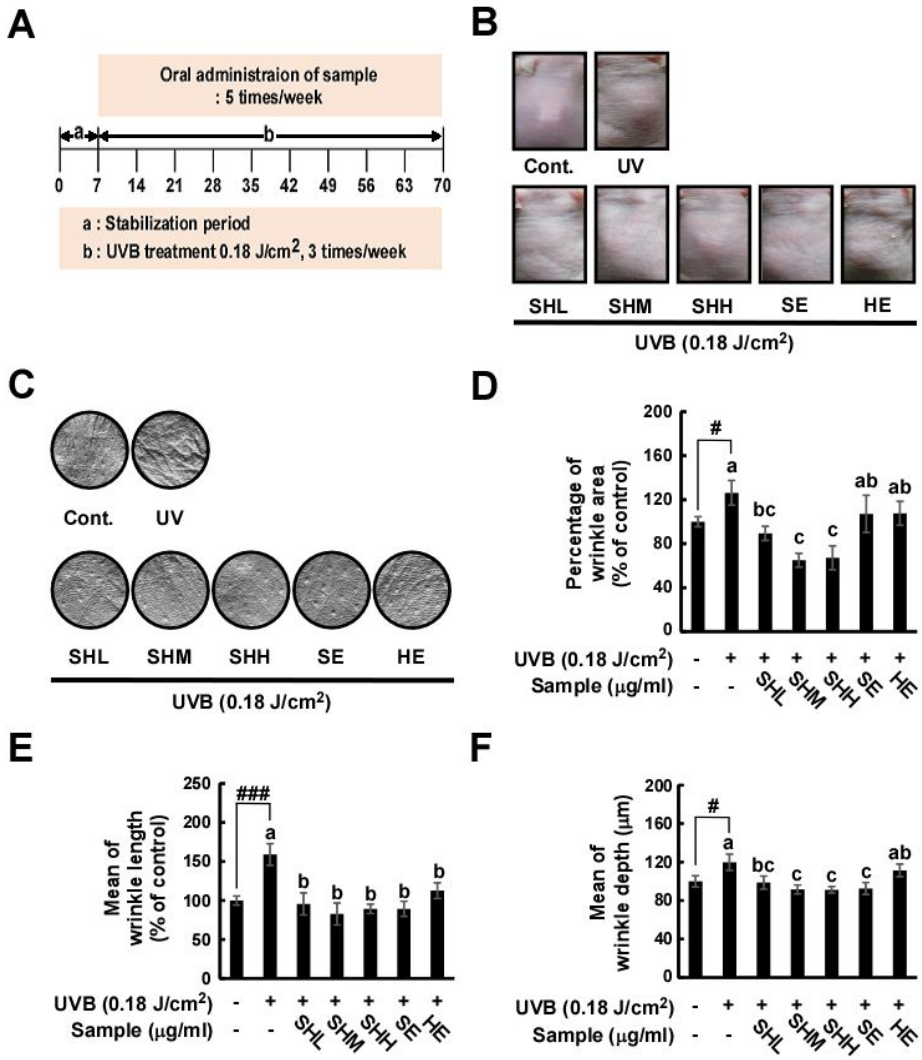
A
Oral administraion of sample
: 5 times/week
a
b
0 7 14 21 28 35 42 49 56 63 70
a : Stabilization period
b : UVB treatment 0.18 J/cm2, 3 times/week
B
Cont.
UV
SHL
SHM
SHH
SE
HE
UVB (0.18 J/cm2)
C
Cont.
UV
SHL
SHM
SHH
SE
HE
UVB (0.18 J/cm2)
D
Percentage of wrinkle area (% of control)
UVB (0.18 J/cm2)
Sample (μg/ml)
E
Mean of wrinkle length (% of control)
UVB (0.18 J/cm2)
Sample (μg/ml)
F
Mean of wrinkle depth (μm)
UVB (0.18 J/cm2)
Sample (μg/ml)

Figure 1. Inhibition effects of soybean and haematococcus extracts mixture medium (SHM) on UVB-induced wrinkle formation in hairless mice.

A, A schematic diagram of experiment. Each group consists of 6-8 mice. *B* and *C,* The dorsal skins of hairless mice were exposed to UVB for 9 weeks and replica of them. *D*, *E*, and *F*, The percentage of skin wrinkle (D), the mean of winkle length (E) and depth (F) were analyzed by Skin-Visiometer software after 9 weeks of UVB treatment. Data represent the means ± SEM (n=6-8). The vehicle was 0.5% sodium carboxymethylcellulose. Means with letters (a-c) within a graph are significantly different from each other at $p < 0.05$.

2. SHM prevented UVB-induced increase of epidermal thickness and collagen degradation in hairless mice.

When the skin was acutely irradiated to UVB, it occurs epidermal hyperplasia [36] and collagen degradation [37]. To identify the effect of UVB exposure on epidermal thickness and collagen degradation, I stained tissues of mouse skin in the way of Hematoxylin and eosin staining and Masson's trichrome staining respectively. Epidermal thickness was effectively reduced in sample groups (Fig. 2A). I showed more significant decrease of epidermal thickness in SHM than that of only SE or HE (Fig. 2C). Collagen fiber levels were recovered in sample supplement groups than that of only UVB-irradiated group (Fig. 2B). Overall, these results indicate that the intake of SHM reduced UVB-induced increase of epidermal thickness and prevented UVB-induced collagen degradation.

Figure 2

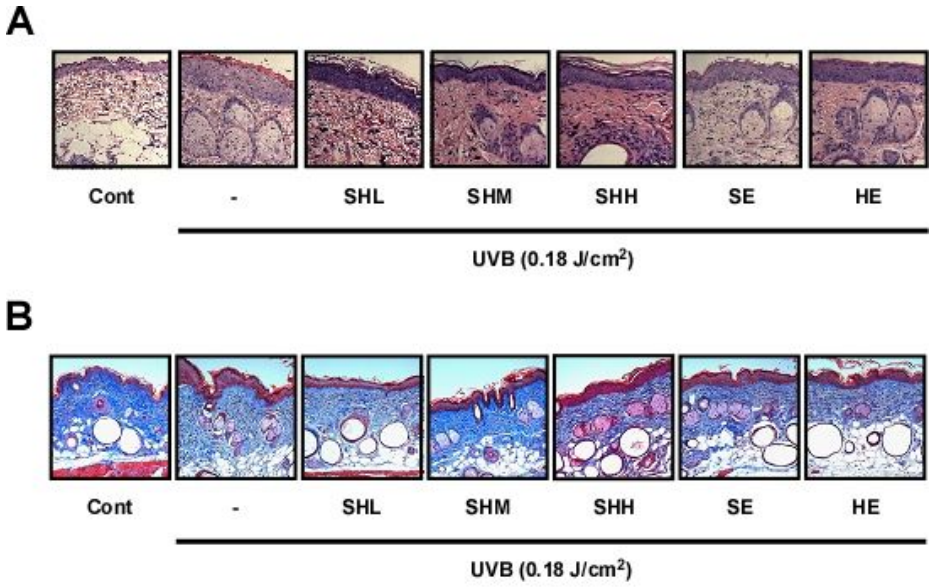
A
Cont
-
SHL
SHM
SHH
SE
HE
UVB (0.18 J/cm2)
B
Cont
-
SHL
SHM
SHH
SE
HE
UVB (0.18 J/cm2)
C
Epidermal thickness (μm)
300
250
200
150
100
50
0
###
a
b
c
c
b
b
UVB (0.18 J/cm2) - + + + + + +
Sample (μg/ml) - - SHL (1:1) SHM (1:2) SHH (1:4) SE HE

Figure 2. Effects of SHM on UVB-induced skin inflammation and collagen degradation in hairless mice.

A and *B*, Dorsal skin sections were stained with hematoxylin & eosin (H&E). The epidermal thickness data were quantified using the image J software analysis as described in materials and methods (Open source Java-written program, NIH, USA). Means with letters (a-c) within a graph are significantly different from each other at $p < 0.05$. Data represent the means ± SEM (n=5). *C*, Masson's trichrome staining for the visualization of collagen fibers as described in materials and methods. Collagen fibers appear blue.

3. SHM significantly decreased UVB-induced MMP-1 overexpression in protein and gene level in cultured primary human dermal fibroblasts.

In animal study, SHM significantly decreased UVB-induced skin wrinkles. To elucidate mechanisms underlying the anti-wrinkle effects of SHM, *in vitro* study was performed. SHM reduced MMP-1 protein expression in a concentration-dependent manner, compared with that of the only UVB-irradiated cells (Fig. 3A), and SHM effectively suppressed UVB-induced MMP-1 mRNA level and transactivation (Fig 3B and 3C). These inhibitory effects of SHM were appeared within the concentration which did not affect cell (Fig 3D). Overall, these results indicate that SHM effectively decreased both UVB-induced MMP-1 transcription.

Figure 3

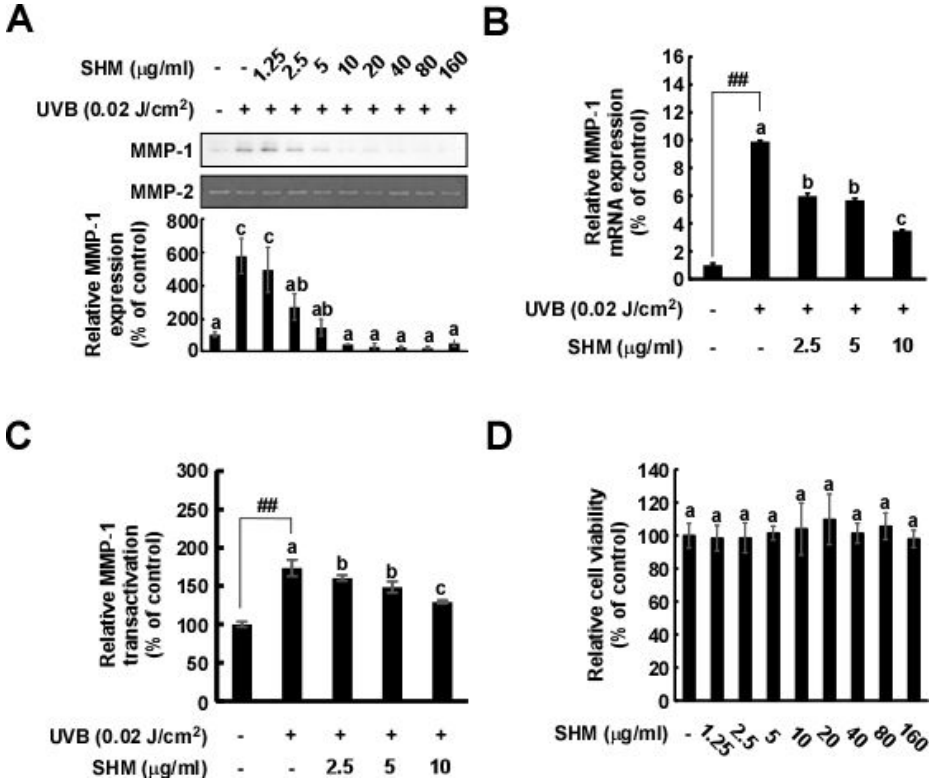
A
SHM (μg/ml) - - 1.25 2.5 5 10 20 40 80 160
UVB (0.02 J/cm²) - + + + + + + + + +
MMP-1
MMP-2
Relative MMP-1 expression (% of control)
800
600
400
200
0
a c c ab ab a a a a a
B
Relative MMP-1 mRNA expression (% of control)
16
14
12
10
8
6
4
2
0
##
a b b c
UVB (0.02 J/cm²) - + + + +
SHM (μg/ml) - - 2.5 5 10
C
Relative MMP-1 transactivation (% of control)
300
250
200
150
100
50
0
##
a b b c
UVB (0.02 J/cm²) - + + + +
SHM (μg/ml) - - 2.5 5 10
D
Relative cell viability (% of control)
140
120
100
80
60
40
20
0
a a a a a a a a a
SHM (μg/ml) - 1.25 2.5 5 10 20 40 80 160

Figure 3. Effects of SHM on UVB-induced MMP-1 transcription in cultured primary human dermal fibroblasts.

A, Expression of MMP-1 was determined by Western blot. MMP-2 was used as a loading control. Cells were pretreated with SHM at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB for 48 h at 37 ℃. The MMP-1 expression data were quantified using the image J software analysis. Data (n=3) represent the means ± SD. *B*, MMP-1 mRNA levels for the SHM group were analyzed by real-time quantitative PCR. Cells were pretreated with SHM at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB for 48 h at 37 ℃. Data (n=3) represent the means ± SD. *C*, MMP-1 transactivation ability of SHM, measured using a luciferase reporter gene assay as described in the Materials and Methods. Cells were pretreated with SHM at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB

for 24 h at 37 ℃. Data (n=3) represent the means ± SD. *D*, Cell viability after SHM treatment. Viability was measured using the MTT Assay as described in the Materials and Methods. Cells were pretreated with SHM at the indicated concentrations for 48 h at 37 ℃. Data (n=4) represent the means ± SD. Means with letters (a-c) within a graph are significantly different from each other at $p < 0.05$.

4. SHM significantly decreased UVB-induced AP-1 transactivation and influenced UVB-induced cellular signal transduction in cultured primary human dermal fibroblasts, Akt and TIMP-1 independently.

MMP-1 transcription is regulated by activator protein (AP) -1, which is the transcription factor activated by UVB irradiation [38]. Therefore I examined an inhibitory effect of SHM on AP-1 transactivation. SHM effectively suppressed UVB-induced AP-1 transactivation (Fig 4A). Base on the previous study, MAPK signaling pathway plays a crucial role in adjusting MMP-1 expression [38]. To identify the possible target in reducing MMP-1 expression, I evaluated how SHM have influences on MAPKs signal transduction. SHM attenuated UVB-induced phosphorylation of major MAPK families via AP-1 transactivation (Fig 4B). On the other hand, SHM have no significant inhibitory effect on Akt signal transduction (Fig 4C).

A TIMP-1 is known for a natural inhibitor of the MMPs which are involved in degradation of the extracellular matrix [25]. SHM have no inhibitory effect on TIMP-1 protein expression (Fig 4D). Overall, these results indicate that SHM have an anti-wrinkle effects through regulating MAPKs without TIMP-1 inhibitory effect.

Figure 4

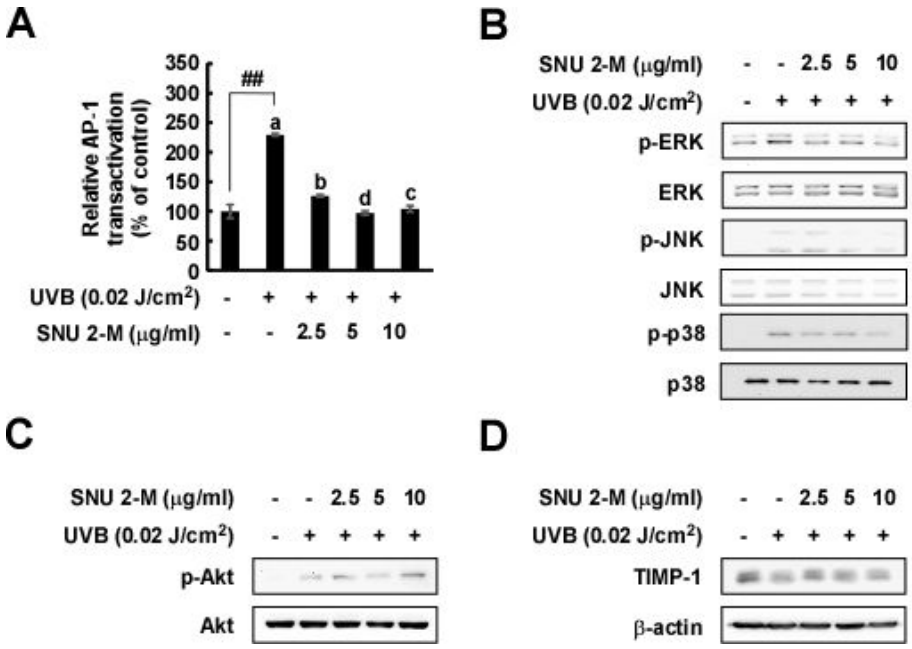
A
Relative AP-1 transactivation (% of control)
350
300
250
200
150
100
50
0
##
a
b
d
c
UVB (0.02 J/cm2) - + + + +
SNU 2-M (μg/ml) - - 2.5 5 10
B
SNU 2-M (μg/ml) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
p-ERK
ERK
p-JNK
JNK
p-p38
p38
C
SNU 2-M (μg/ml) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
p-Akt
Akt
D
SNU 2-M (μg/ml) - - 2.5 5 10
UVB (0.02 J/cm2) - + + + +
TIMP-1
β-actin

Figure 4. Effects of SHM on UVB-induced AP-1 protein expression and signaling pathways in cultured primary HDFs, TIMP-1 independently.

A, AP-1 transactivation ability of SHM was measured using a luciferase reporter gene assay as described in the Materials and Methods. Cells were pretreated with SHM at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB for 36 h at 37 ℃. Data (n=3) represent the means ± SD. *B*, Phosphorylated and total protein levels were conducted by Western blot assay using specific antibodies described in the Materials and Methods. Cells were pretreated with SHM at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm^2 UVB for 30min at 37 ℃.

Ⅳ. DISCUSSION

Skin aging which is fasten by chronic exposure to sun is called photo-aging. [39, 40]. Among the solar UV rays, UVB (280-320 nm) accelerates various physiological changes in the cells. Chronic UVB exposure occurs wrinkle formation, pigmentary changes, and increases of laxity [41]. In the epidermal and dermal cells, AP-1 complex are formed to regulate the transcription of MMPs [42]. The MMPs are members of an enzyme family that require a zinc ion in their active site for catalytic activity [43]. The active MMPs are generated by proteolytic cleavage of an inactive form which is called zymogen. And TIMPs, the inhibitor of MMPs, regulate these activation process [3]. MMPs contribute to the balanced and regulated degradation of ECM proteins [42], they play an important role in UVB-induced wrinkle by breakdown of collagen in dermis. Among them, MMP-1 cleaves α chains of native triple helical type Ⅰ and Ⅲ collagens after glycine (Gly) in a particular

sequence of collagen molecule [2, 44]. Therefore, the inhibition of UVB-induced MMP-1 overexpression represents a promising strategy to prevent photo-aging.

Albino hairless mice (Skh-1) is one of the suitable mouse model for chronic UVB exposure experiment. According to chronically UVB-exposed Skh-1 mice can develop wrinkles, which appear as prominent horizontal creases on the dorsal skin [45]. Although *in vivo* model has a limit that they are not humans, it is need to be verified through clinical study that SHM have an actual effects to human in reducing wrinkle formation after *in vivo* study.

To identify underlying mechanisms against UVB-induced wrinkle formation of SHM, *in vitro* study was performed. A chronic exposure of UVB provokes MMP-1 overexpression, leading to the breakdown of collagen. In the wrinkle formation, the composition of ECM containing connective tissue and basement membrane proteins is

very important. Dermal fibroblasts produce and secrete MMP-1, which plays a key role in dermal remodeling [46]. Therefore, HDFs were used to figure out mechanisms associated with anti-wrinkle effect of SHM.

Until recently, little attention has been paid to less extreme type of sun exposure because it does not cause any short term clinical impact while an extreme type has been widely studied. To have a physiological relevance, in this study, Skh-1 hairless mice were exposed to UVB at a dose of 180 mJ/cm^2 and HDFs were exposed to UVB at a dose of 0.002 J/cm^2. It means that these UVB dose indicate the exposure by sun about 2, and half hours respectively in mid-April in Newyork. [47].

In this study, an oral administration of SHM reduced UVB-induced wrinkle formation and prevented UVB-induced collagen degradation in hairless mice. In addition I revealed that SHM has an anti-wrinkle effect by suppressing UVB-induced MMP-1 protein expression and MMP-1 gene transcription in HDFs. And these

regulations of MMP-1 is derived from reduction of UVB-induced AP-1, which is the major transcription factor of MMP-1, activity and MAPKs signaling pathway. To identify more accurate mechanisms of these anti-wrinkle effects, I have to figure out the particular target in regulating MAPKs signal transduction in the further study.

In conclusion, oral administration of SHM protects against UVB-induced skin wrinkle in hairless mice. SHM has also an inhibitory effect on wrinkle formation through MMP-1 regulation which results from inhibition of AP-1 activity. Also SHM markedly influences MAPKs signaling pathway. Therefore, SHM has been suggested to be a therapeutic agent for the treatment of UVB-induced wrinkles.

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Ⅵ. 국문 초록

피부 주름은 지속적인 자외선 노출에 의한 피부 손상 중 가장 대표적인 증상이며, 자외선에 의해 콜라겐의 합성이 감소하고 세포외 기질 단백질 분해 효소인 matrix metalloproteinase(MMPs)의 발현 증가에 의해 피부 진피층에 존재하는 콜라겐이 붕괴되면서 주름이 생성된다. 따라서 콜라겐 붕괴를 억제하는 것은 자외선에 의한 주름을 예방하는 좋은 전략이 될 수 있다. 본 연구는 아이소플라본 함량이 높으며 예로부터 아시아 국가들에서 식품으로서 널리 소비되어 온 대두의 추출물과 카로티노이드 계열 색소인 아스타잔틴의 풍부한 급원으로 알려져 있는 헤마토코쿠스 플루비알리스 추출물을 복합하여 자외선에 의한 피부주름 예방효능을 확인하고 그 작용기전을 규명하였다. 상기 복합물은 자외선에 의해 과발현된 MMP-1의 단백질 발현을 농도의존적으로 억제하였으며, 이는 MMP-1의 전사 과정이 저해됨으로써 조절되는 것을 확

인하였다. 또한, 본 복합물이 MMP-1의 전사에 주요한 역할을 하는 전사인자인 Activator protein-1 (AP-1) 역시 자외선에 의해 과발현되었을 때, 효과적으로 억제하였다. 작용 기전을 밝히기 위해 MMP-1 발현에 관여하는 MAPKs(Mitogen-activated protein kinases) 신호전달 체계를 알아본 결과, 자외선에 의해 유도된 ERK, JNK, p38의 인산화를 감소시키는 것을 확인하였다. 상기 신호전달 기전을 통해 최종적으로는 MMP-1 발현을 조절함으로써 콜라겐의 붕괴를 억제하여 피부 주름 예방 효과를 지닌다.

본 연구 결과를 통해 대두와 헤마토코쿠스 추출물 복합물은 자외선으로 인해 유도된 피부 노화를 억제함으로써 피부 건강에 도움을 주는 소재로써 식품 또는 화장품으로의 개발이 가능하다.