Alleviation of Ultraviolet B-Induced Photoaging by 7-MEGA™ 500 in Hairless Mouse Skin

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Abstract

The purpose of this study was to investigate the effect of 7-MEGA™ 500 on the improvement of skin aging in an UVB-induced photo-aging model of hairless mice. The dorsal skin of hairless mice was exposed to UVB three times a week for 12 weeks to induce skin wrinkle. After inducing the wrinkle, 7-MEGA™ 500 was orally administered once a day for 4 weeks. Skin thickness, skin barrier function, and wrinkle indicators were improved by treatment with 7-MEGA™ 500. Both gene and protein expression levels of MMP-3 and c-Jun in skin were significantly decreased by 7-MEGA™ 500. Therefore, the intake of 7-MEGA™ 500 is thought to have a positive effect on the improvement of skin aging, although further studies are needed.

Key words: 7-MEGA™ 500, Photoaging, Hairless mice, c-Jun, MMP-3

INTRODUCTION

Ultraviolet (UV) is abundant in the environment. It is the most important risk factor for skin cancer. It not only increases epidermal thickness, but also increases wrinkle formation (1). UV irradiation also induces the synthesis of matrix metalloproteinases (MMPs) and decrease of collagen synthesis (2).

Collagen degradation is closely related to the presence of MMPs that are mainly secreted by epidermal keratinocytes and dermal fibroblasts. MMPs are usually expressed at low levels in unstimulated cells or normal skin tissues. However, they can be induced by various extracellular stimuli, including cytokines, growth factors, and UV radiation. Up-regulation of MMPs can also be induced by even a minimal dose of UV (3). After chronical exposure to UV-irradiation, mouse skin shows epidermal hyperplasia, skin wrinkles, and significant increase of several MMPs, including stromelysin-1 (MMP-3), metalloelastase (MMP-12), and collagenase (MMP-1) (4).

It has been shown that omega polyunsaturated fatty acids (PUFAs) possess anti-oxidative (5), anti-inflammatory (6), neuroprotective (7), and chemopreventive (8) effects. Omega-6 and -9 have been linked to obesity prevention (9) and anti-inflammation (10). Recent studies have shown that PUFAs can defend a wide range of diseases characterized by increased MMPs activity (11). They can also suppress UV-induced expression of proinflammatory cytokines and MMPs in skin cells *in vitro* or skin tissues *in vivo* (1,4). Moreover, it has been reported tht palmitoleic acid (omega-7) and gamma-linolenic acid (omega-6) can affect skin regeneration and repair (12). However, there have been few reports on omega-7 compared to other omega fatty acids. Omega-7, also known as palmitoleic acid (16:1, Cis-9-hexadecenoic acid), is a monounsaturated fatty acid that is found in fish and plants such as macadamias, cold water fish, and sea buckthorn berries (13).

It has been previously shown that omega-3 and omega-6

act as inhibitors of MMPs (11), and 7-MEGA[TM] 500 (more than 50% of palmitoleic acid containing fish oil, omega-7) can show the effects of anti-oxidant and anti-inflammation *in vitro* (14). However, *in vivo* information on its effects on skin has been insufficient. Therefore, the aim of this study was to investigate the effect of 7-MEGA[TM] 500 by observing expression levels of MMP-3 and c-Jun on skin of mouse.

MATERIALS AND METHODS

Preparation of 7-MEGA[TM] 500. 7-MEGA[TM] 500 was obtained from Organic Technologies (OH, USA). Pollock was collected from Alaskan Bering Sea and 7-MEGA[TM] 500 containing palmitoleic acid (> 500 mg/g) was prepared (Table 1). 7-MEGA[TM] 500 was administered by volume of 10 mL/kg after dissolving a defined concentration of each group in 30% EtOH.

Animals. Male HR-1 hairless mice (5-week old, 18-20 g) were purchased from Orient Bio (Seongnam, Korea). After acclimation for one week, they were randomly assigned to five groups (Table 2). The animal room was maintained at a temperature of $22 \pm 3^{\circ}C$ with relative humidity of $50 \pm 10\%$ and 12-hr light/12-hr dark cycle per day. They were provided free access to feed (Purina, Korea) and water *ad libitum* during the experiment period. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Keimyung University, South Korea (permit number: KM-2017-005).

Design of skin photoaging model. The dorsal skin of hairless mice was exposed to UVB three times a week. The irradiation dose was increased weekly by 1 MED (1 MED = 130 mJ/cm^2) to 4 MED. It was then maintained at

4 MED until 12 weeks. After skin photoaging induction (1-8 weeks), the test material or vehicle was orally administered (10 mL/kg, once a day) for 4 weeks.

Determination of wrinkle grade. To determine the severity of wrinkling, each hairless mouse was anesthetized and the UVB exposed dorsal skin (wrinkle formation area) was photographed. The severity of wrinkling was measured using Bissett's visual wrinkle scale. Skin impressions (replicas) were prepared by applying Repliflo Cartridge Kit (CuDerm Corp., Dallas, TX, USA) to dorsal skin of each mousee. Replicas were analyzed using a skin visioline VL650 (CK Electronics GmbH, Cologne, Germany).

Measurement of skin barrier function. Transepidermal water loss (TEWL) and stratum corneum (SC) water content were assessed under standardized conditions (external temperature $23 \pm 3^{\circ}C$ and $50 \pm 10\%$ RH) using a Tewameter (Courage-Khazaka Electronic GmbH, Cologne, Germany) and a Corneometer (Courage-Khazaka Electronic GmbH) apparatus, respectively.

Measurement of skin thickness. Skin thickness was measured using a digimatic micrometer (Mitutoyo Co. Ltd., Tokyo, Japan) once a week. The dorsal skin of each mouse was pulled up from the neck to the bottom of the body by hand and the skin fold thickness was measured between the neck and hips.

Histological observation. Dorsal skin from autopsy was fixed in 10% formalin for 24 hr and embedded in paraffin with common process. Embedded tissue was cut into 4 μm-thick sections and stained with hematoxylin and eosin (H&E). Changes of skin tissue such as epidermal thickness and inflammatory cell infiltration were observed under an optical microscope.

RNA isolation and RT-PCR. Total RNA was isolated from dorsal skin of mouse using TRIzol reagent (Life Technologies Inc., Rockville, MD, USA) according to the manufacturer's protocol. Total RNA was used to synthesize cDNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification of cDNA (4 μL) was performed with PCR premix (SolGent, Daejeon, Korea) and primer pairs (Bionics, Seoul, Korea;

Table 1. The main ingredients of 7-MEGA[TM] 500

Molecular formula	Name	mg/g	%
C14:0	Myristic acid	4.4 ± 5.0	0.04
C16:0	Palmitic acid	257.3 ± 27.1	25.7
C16:1n-7	Palmitoleic acid	535.6 ± 10.9	53.5
C20:5	Eicosapentaenoic acid (EPA)	5.6 ± 6.4	0.06

Table 2. Experimental groups

Groups	Induction of skin photoaging	Test compound	No. of mice
Normal Control (NC)	−	Distilled water (DW)	7
Vehicle Control (VC)	+	30% EtOH	7
Experimental 1 (E1)	+	7-MEGA[TM] 500 (200 mg/kg)	7
Experimental 2 (E2)	+	7-MEGA[TM] 500 (100 mg/kg)	7
Experimental 3 (E3)	+	7-MEGA[TM] 500 (50 mg/kg)	7

Table 3. Primer sequences for RT-PCR

		Primer sequences[1]	Amplicon size (bp)[2]
GAPDH	Sense	5'-*AACTTTGGCATTGTGGAAGG*-3'	223
	Antisense	5'-*ACACATTGGGGGTAGGAACA*-3'	
c-Jun	Sense	5'-*TCCCCTATCGACATGGAGTC*-3'	146
	Antisense	5'-*TTTTGCGCTTTCAAGGTTTT*-3'	
MMP-3	Sense	5'-*CAGGTGTGGTGTTCCTGATG*-3'	317
	Antisense	5'-*GCCTTGGCTGAGTGGTAGAG*-3'	

[1]Primer sequences (Bionics, Seoul, Korea).
[2]bp, basepair.

Table 3). Before PCR amplification, the PCR mixture was denatured at 95°C for 2 min. Amplification consisted of 35 cycles of denaturation at 95°C for 20 sec, annealing at 57°C for 40 sec, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were separated by 1% agarose gel electrophoresis and visualized with 6X loading dye and UV illumination.

Western blot analysis. Mouse dorsal skin sections were homogenized in radioimmunoprecipitation assay buffer (Sigma, USA) containing 1% protease inhibitor cocktail and phosphatase inhibitor cocktail. The homogenate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and protein concentration was estimated by the Bradford protein assay. A 30 µL aliquot of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resulting separated proteins were transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBS-T (Tris-Buffered Saline plus 0.05% Tween 20). The following primary antibodies were used for western blotting: c-Jun, MMP-3, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Amersham, UK). Band intensities were measured using ImageJ software
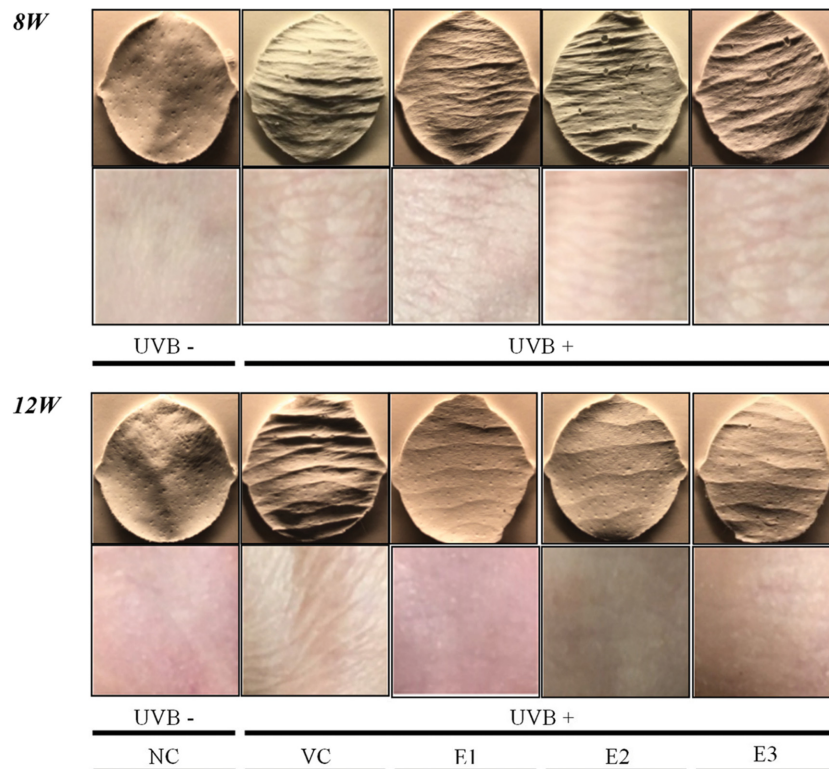

Fig. 1. Replica production and visual wrinkle patterns of skin. 8w: Before oral administration, 12w: Four weeks after oral administration. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg).

(National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. SPSS 17.0 software was used for all statistical analyses. Skin thickness data a and mRNA or protein expression levels were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test. Skin wrinkles and skin barrier function data were analyzed by two-way ANOVA followed by Duncan's multiple range test when appropriate. Data are expressed as mean ± standard error (SE). Value of $p < 0.05$ was considered significant for all comparisons made.

RESULTS

Determination of wrinkle grade. This *in vivo* study demonstrated that oral administeration of 7-MEGA™ 500 alleviated the photoaging effect of UVB-radiation on skin. To investigate the effect of 7-MEGA™ 500 on UVB-induced wrinkle formation, we induced skin photoaging by repeatedly exposing the skin of hairless mice to UVB for 8 weeks. The 7-MEGA™ 500 was then orally administered once a day for 4 weeks. Visual analysis and replica

were made at 8 weeks (before oral administration) and 12 weeks (before autopsy). The prepared replica was analyzed with a wrinkle analyzer (VisioLine, VL650, Courage-Khazaka Electronic GmbH, Cologne, Germany). We found that UVB-irradiated skin showed wrinkles. However, 7-MEGA™ 500 blocked wrinkle formation (Fig. 1, Table 4). The experimental group showed a dose-dependent recovery pattern. At 12 weeks, wrinkles were weakening and skin surface was soft with elasticity. Wrinkles from in the test substance group were significantly reduced compared to those in the vehicle control (VC) group.

Measurement of skin barrier function. Results of moisture content and TEWL after treatment with 7-MEGA™ 500 are shown in Table 5. In both UVB irradiated groups, TEWL gradually increased while water content gradually decreased (1 to 8 weeks). From the first week after treatment with the test substance, moisture content of the skin increased dose-dependently in all groups while TEWL tended to decrease. These results strongly suggest that 7-MEGA™ 500 can help protect against or restore UVB irradiation-induced skin barrier

Table 4. Evaluation of wrinkles through replica analysis of hairless mouse before autopsy

		NC	VC	E1	E2	E3
Wrinkle area (mm^2)	8w	19.0 ± 10.5**	50.0 ± 7.5	41.5 ± 5.8	54.0 ± 31.1	57.8 ± 42.1
	12w	17.8 ± 5.9**	76.6 ± 26.2	19.9 ± 9.9**	33.2 ± 10.9**	35.2 ± 43.6*
No. of wrinkles	8w	52.0 ± 14.2	89.0 ± 40.1	95.0 ± 18.4	101.5 ± 23.3	91.0 ± 7.1
	12w	46.7 ± 25.4*	83.0 ± 28.2	52.0±2.8*	68.5 ± 6.4	67.5 ± 12.0
Total length (mm)	8w	38.5 ± 3.2**	97.5 ± 8.0	92.6 ± 24.2	92.5 ± 29.92	81.2 ± 24.1
	12w	35.5 ± 7.4**	94.9 ± 37.3	30.2 ± 10.3**	38.6 ± 4.1**	49.18 ± 8.06*
Mean length (mm)	8w	0.6 ± 0.1*	0.9 ± 0.3	1.1 ± 0.2	0.9 ± 0.1	0.9 ± 0.2
	12w	0.4 ± 0.1**	1.1 ± 0.1	0.5 ± 0.1**	0.7 ± 0.2**	0.8 ± 0.2**
Mean depth (μm)	8w	90.6 ± 2.8**	116.3 ± 13.1	103.7 ± 8.6	103.6 ± 13.4	102.4 ± 7.0
	12w	84.2 ± 7.1*	120.7 ± 29.0	69.7 ± 14.5*	90.1 ± 5.1	91.7 ± 4.1

8w: Just before oral administration, 12w: Four weeks after oral administration. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg). Values are means ± SE (n = 7). *$p < 0.05$, **$p < 0.01$ as compared to the VC group by ANOVA and Duncan's multiple range test.

Table 5. Changes of trans-epidermal water loss (TEWL) and skin water content (WC) by time and group

		NC	VC	E1	E2	E3
8w	TEWL	4.5 ± 0.6**	29.1 ± 1.3	27.3 ± 1.2*	26.9 ± 1.5*	28.7 ± 1.3
	WC	78.2 ± 2.3**	25.9 ± 2.1	29.4 ± 2.7*	29.7 ± 3.1*	32.0 ± 5.6*
9w	TEWL	5.6 ± 0.5**	31.5 ± 1.4	19.9 ± 1.0**	26.3 ± 1.0**	28.1 ± 1.3**
	WC	77.3 ± 2.1**	24.7 ± 2.2	70.1 ± 2.6**	39.2 ± 3.4**	41.1 ± 4.2**
10w	TEWL	5.6 ± 0.4**	34.8 ± 1.8	18.9 ± 1.2**	25.0 ± 2.2**	27.4 ± 0.6**
	WC	76.2 ± 3.0**	22.8 ± 1.9	75.6 ± 3.0**	49.6 ± 1.0**	47.0 ± 1.5**
11w	TEWL	6.2 ± 0.7**	39.6 ± 1.6	17.6 ± 1.0**	23.6 ± 2.5**	26.1 ± 0.8**
	WC	76.1 ± 2.3**	20.1 ± 3.1	77.1 ± 2.7**	56.3 ± 1.7**	48.5 ± 0.7**
12w	TEWL	6.2 ± 0.6**	40.2 ± 2.0	14.8 ± 1.1**	20.1 ± 2.0**	23.6 ± 0.9**
	WC	74.1 ± 2.4**	16.1 ± 2.2	82.4 ± 1.1**	60.6 ± 1.4**	50.9 ± 1.3**

Unit: TEWL (g/h/m^2), WC (AU). 8w: Just before oral administration, 9w to 12w: From one week to four weeks after oral administration. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg). Values are means ± SE (n = 7). *$p < 0.05$, **$p < 0.01$ as compared to the VC group by ANOVA and Duncan's multiple range test.
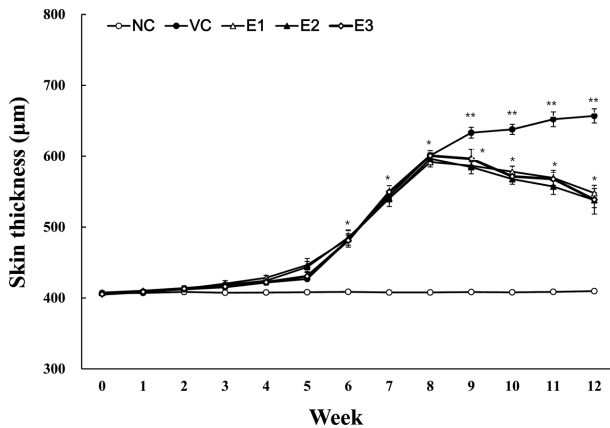
Fig. 2. Effects of the 7-MEGA™ 500 on skin thickness in chronic UVB-irradiated hairless mice. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg). Values represent the mean $\pm$ SE (n = 7). *Significantly different from NC group ($p <$ 0.05). **Significantly different from other groups ($p <$ 0.05).

dysfunction.

Measurement of skin thickness. Skin thickness of hairless mice gradually increased after 8 weeks of UVB irradiation. However, skin thickness significantly decreased from the first week after oral administration of 7-MEGA™ 500. Such decrease was proportional to the duration of administration. These results suggest that 7-MEGA™ 500 can improve skin thickness thickened by ultraviolet light (Fig. 2).

Histological observation. To investigate the effect of 7-MEGA™ 500 on UV-induced photoaging in hairless mice, the thickness of epidermis was observed by H & E staining. Epidermal thickness was measured with a ruler at 400 magnification of microscopy. The epidermal thickness in the VC group (31.5 ± 0.5 μm) was significantly increased compared to that in the NC group (11 ± 0.2 μm) ($p < 0.05$). However, 4 weeks of intake of 7-MEGA™ 500 significantly reduced the thickness of the epidermis. Such decrease was dependent on the dose of the concentration of 7-MEGA™ 500 (E1 < E2 < E3, $15 \pm 0.3 < 21 \pm 0.1 < 23 \pm 0.2$ μm, Fig. 3). These results suggest that 7-MEGA™ 500 treatment can significantly improve the epidermal thickness of hairless mice thickened by UV irradiation.

MMP-3 and c-Jun expression. MMPs and c-Jun can be used as photo-aging markers. Our results revealed that 12 weeks of UVB irradiation significantly increased mRNA and protein levels of MMP-3 and c-Jun in dorsal skin. However, 4 weeks of 7-MEGA™ 500 treatment significantly reduced MMP-1 and c-Jun expression levels in the dose-dependent manner (Fig. 4).
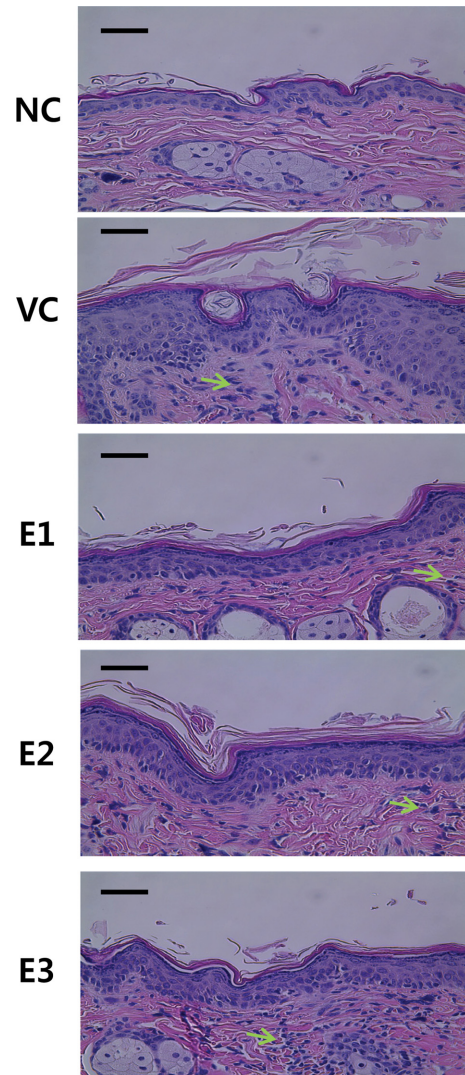

Fig. 3. Histopathological evaluation of 7-MEGA™ 500 treatment on skin thickness in UVB-irradiated hairless mice. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg). Arrow: infiltration of inflammatory cells. bar = 20 μm.

DISCUSSION

UV irradiation is known as a skin photoaging factor. It causes wrinkle, roughness, relaxation, and pigmentation of skin (2). Especially, UVB irradiation causes photo-aging determined by expression of c-Jun and MMPs that can degrade the extracellular matrix (ECM). Developing c-Jun and MMPs inhibitor can be a promising strategy for photo-aging therapy (15). This study suggests that 7-MEGA™ 500 might be useful as a functional ingredient by observing changes in expression levels of c-Jun and MMP-3 on skin.

Effect of 7-MEGA™ 500 on UVB-induced photo-aging was evaluated by determining changes in clinical sign and biomolecular markers. In photo-aging symptoms, wrinkle
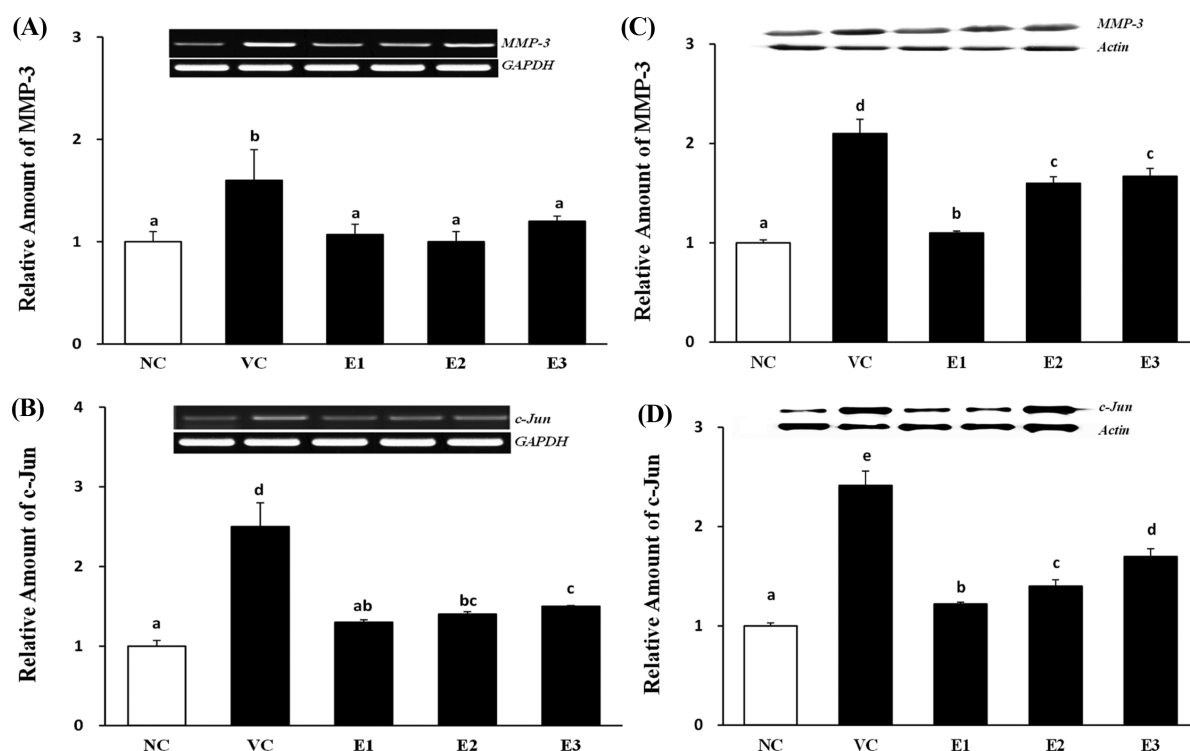
Fig. 4. Effect of the 7-MEGA™ 500 on the expression of MMP-3 and c-Jun induced by UVB in hairless mice skin. (a) MMP-3 mRNA expression level. (b) c-Jun mRNA expression level. (c) MMP-3 protein level. (d) c-Jun protein level. NC: Normal control, VC: Vehicle control, E1: 7-MEGA™ 500 (200 mg/kg), E2: 7-MEGA™ 500 (100 mg/kg), E3: 7-MEGA™ 500 (50 mg/kg). Data are expressed as the mean ± SE (n = 7). Values with different letters are significantly different from each other ($p < 0.05$).

is exacerbated by increased TEWL. In this study, TEWL and wrinkle were also increased in UVB-induced photo-aging model. However, 7-MEGA™ 500 treatment decreased both TEWL and wrinkles compared to VC. Water content in the group treated with 7-MEGA™ 500 was increased compared to that in the VC group. Fine lines are geneally made by alteration in the surface of the skin or the epidermis whereas deep wrinkles are formed by changes in the dermis (16). Treatment with 7-MEGA™ 500 visibly reduced formation of both fine lines and deep wrinkles in hairless mice.

UVB can induce photo-aging by damaging skin and inducing epidermal hyperplasia (16). Thus, measuring the thickness of irritated skin is a reliable indicator of photo-aging (17,18). Previous studies have reported that topical application of polyunsaturated fatty acids can attenuate UV-induced epidermal and dermal thickness in hairless mice (19), consistent with results of this study. Results of H&E staining showed that epidermis thickness and inflammatory cell infiltration were significantly decreased in 7-MEGA™ 500 treated groups, similarly to those in the NC group. Another previous study (20) evaluated the effect of cis-palmitoleic acid supplementation on inflammatory activity and the expression of genes HNF4γ, HNF4α and IL6 in the colonic mucosa of patients with ulcerative colitis (UC). Cis-palmitoleic acid as co-adjuvant therapy for 8 weeks seemed

to decrease the inflammatory activity through the increased expression of HNF4α and HNF4γ in patients with UC.

In this study, high levels of mRNA expression of c-Jun and MMP-3 were observed in the photo-aging model induced by UVB. c-Jun and MMP-3 mRNA expression levels were significantly lower in E1, E2, and E3 groups treated with 7-MEGA™ 500 than those in the VC group. Protein expression levels of c-Jun and MMP-3 using western blot showed the same results. MMPs expression is generally low in unstimulated skin cells or normal skin tissue. However, it is significantly increased concomitant with symptoms such as epidermal hyperplasia and skin wrinkles in hairless mice chronically exposed to ultraviolet (21). In addition, c-Jun is a nuclear protein that is not expressed or expressed very low in normal skin tissue. However, it is rapidly increased by UV stimulation (22). Previous studies have shown that overexpression of c-Jun can reduce the expression of type I collagen (23).

Results of this study suggest that 7-MEGA™ 500 could be effective in preventing and treating photo-aging by altering various indices related to photo-aging induced by UVB. However, further studies are needed to understand which components of 7-MEGA™ 500 are directly responsible for the improvement of photo-aging. In conclusion, results of this study indicate that 7-MEGA™ 500 could

help recover photo-aging induced by UVB. Thus, 7-MEGA™ 500 might be useful as a functional raw material to improve skin photo-aging.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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