

# 7-MEGA™ 500 regulates the expression of COX-2, MMP-3 and type 1 procollagen in UVB-irradiated human keratinocytes and dermal fibroblasts

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**Abstract.** AlaskOmega® Omega 7 500, also known as Omega-7 fatty acid or 7-MEGA™, is a highly concentrated palmitoleic acid (C16:1). Little is known about how 7-MEGA regulates skin inflammation and wrinkle formation in cultured skin cells. The present study aimed to investigate the effects of 7-MEGA on the expression of cyclooxygenase-2 (COX-2), matrix metalloproteinase (MMP)-1/3 and type 1 procollagen, which are markers of skin inflammation and wrinkle formation, in ultraviolet B (UVB)-irradiated human dermal fibroblasts (HDFs) and keratinocytes (HaCaT). No toxicity was observed upon treatment of HDFs and HaCaT cells with 0.5-2.5  $\mu$ l/ml 7-MEGA. The exposure of HaCaT cells to 10 mJ/cm<sup>2</sup> UVB for 6 h resulted in increased protein and/or mRNA expression of COX-2 and MMP-3. Treatment of HaCaT cells with 2.5  $\mu$ l/ml 7-MEGA suppressed the UVB-induced expression of COX-2 and MMP-3 in these cells. In addition, treatment with 2.5  $\mu$ l/ml 7-MEGA attenuated the UVB-induced expression and phosphorylation levels of c-Fos and c-Jun, two components of the activator protein-1 (AP-1) transcription factor, in HaCaT cells. Exposure of HDFs to 60 mJ/cm<sup>2</sup> UVB for 6 h significantly decreased the expression of type 1 procollagen protein, whereas treatment with 2.5  $\mu$ l/ml 7-MEGA partially reversed the effects of UVB on the expression of type 1 procollagen protein. These results demonstrated for the first time that 7-MEGA regulated the expression of COX-2, MMP-3 and type 1 procollagen in UVB-irradiated skin cells. The present study

suggested that 7-MEGA may serve as a novel agent against UVB-induced skin inflammation and damage.

## Introduction

Solar ultraviolet (UV) radiation is harmful to the skin (1). Previous studies have demonstrated that sustained or excessive exposure to UV radiation leads to the development of several skin pathologies, including skin inflammation, aging and cancer (2-4). In addition, UV-induced skin inflammation and damage, such as wrinkles, are closely associated with altered expression of inflammation-related enzymes and proteins, such as cyclooxygenase (COX)-2, matrix metalloproteinases (MMPs) and type 1 collagen (5-7). Thus, any reagent that alters the expression of COX-2, MMPs and type 1 collagen induced by UV radiation in skin cells may be used as a potential anti-inflammatory agent.

COX-2 is an inducible enzyme that is mainly involved in the hyperproduction of prostaglandins (PGs) from arachidonic acid (8). COX-2-derived PGs mediate a number of inflammatory diseases (9), thus supporting the role of COX-2 and PGs in inflammation. Previous studies have suggested that COX-2 expression is significantly increased in multiple types of cells and tissues that have been exposed to internal or external stimuli, such as inflammatory cytokines, mitogenic factors, tumor promoters and UV rays (10-13). In addition, stimuli-induced transcription of COX-2 activates signaling proteins that lead to the activation of transcription factors, such as activator protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), which bind to the COX-2 promoter (14-16). The COX-2 promoter contains multiple cis-acting elements, including AP-1, NF- $\kappa$ B and cAMP response element (CRE), that are crucial for the transcriptional upregulation of COX-2 (15-17). The role of AP-1 and NF- $\kappa$ B in the UVB-induced COX-2 expression in HaCaT human keratinocytes has also been reported (18,19).

Type 1 collagen is a major component of the dermal extracellular matrix (ECM) (20) that accounts for 90% of the dermis (21) and provides hydration, resilience and structural integrity to the skin (22). A reduction in the content of type 1 collagen in the skin, due to decreased expression (synthesis) or

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increased degradation, is considered to be the most common cause of skin damage associated with normal aging or photoaging (6,23,24). UVB exposure results in the downregulation of type 1 procollagen by regulating the expression and/or phosphorylation levels of mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), AP-1 and NF- $\kappa$ B in HaCaT cells and/or human dermal fibroblasts (HDFs) (25,26). MMPs are zinc-containing endopeptidases that can degrade dermal ECM proteins such as type 1 collagen and elastin (7,27). MMP-1 (also known as fibroblast collagenase) and MMP-3 (also known as stromelysin 1) are actively involved in the degradation of type 1 procollagen and other ECM components in the dermis associated with normal aging and photoaging (27,28). The expression of MMP-1 and MMP-3 can be stimulated by UVB, which leads to the production of skin wrinkles (29).

Palmitoleic acid (C16:1), also known as Omega-7 fatty acid, has been reported to exhibit several potential health benefits, such as maintaining the health of the heart, anti-inflammation, anti-aging and accelerated wound healing (30,31). AlaskOmega<sup>®</sup> Omega 7 500 (7-MEGA<sup>™</sup>) is a highly concentrated Omega-7 fatty acid (50% palmitoleic acid). A recent study has demonstrated that 7-MEGA may protect against H<sub>2</sub>O<sub>2</sub>-induced damage in HaCaT human keratinocytes by inhibiting cellular oxidative stress and inflammation (32). At present, little is known about the role of 7-MEGA in the regulation of UVB-induced skin inflammation. The present study aimed to investigate the effects of 7-MEGA on the expression of COX-2, MMP-3 and type 1 procollagen in UVB-irradiated human keratinocytes (HaCaT) and human dermal fibroblasts (HDFs).

## Materials and methods

**Cell culture.** HaCaT cells (American Type Culture Collection), an immortalized human keratinocyte cell line, were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (cat. no. LM 002-04; Welgene, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; cat. no. S001-01; Welgene, Inc.), 2 mM glutamine (cat. no. LS 002-01; Welgene, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (cat. no. LS202-02; Welgene, Inc.). Human dermal fibroblasts (HDFs; ATCC) were cultured in DMEM (cat. no. LM 001-05; Welgene, Inc.) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. HaCaT cells and HDFs were cultured at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>.

**7-MEGA preparation.** 7-MEGA purified concentrates containing >500 mg/g palmitoleic acid were obtained from Organic Technologies. 7-MEGA was diluted 10-fold with 70% ethanol and the resultant solution was stored overnight at room temperature (RT; 25–27°C). Subsequently, the solution was diluted 10-fold with PBS for use in experiments.

**UVB irradiation.** Cells were irradiated in cell culture plates using a Bio-Link BLX-312 UVB lamp (Vilber Lourmat Sté) with an emission wavelength peak at 312 nm. Prior to UVB irradiation, the culture medium was replaced with 1 ml/well PBS. The plate lid was removed, and the cells were irradiated

at 10 or 60 mJ/cm<sup>2</sup> UVB. Subsequently, PBS was replaced with complete culture medium containing either 7-MEGA or vehicle control for the indicated times and concentrations prior to harvesting.

**Cell count analysis.** HaCaT cells and HDFs were seeded into 24-well plates (1x10<sup>5</sup> cells in 500  $\mu$ l/well) and cultured overnight. The cells were treated with 0, 0.5, 1, 2.5 or 5  $\mu$ l/ml 7-MEGA. At each time point, cells were stained with trypan blue, and the viable (unstained) cells were counted under a Nikon Eclipse TS100 phase-contrast microscope (Nikon Corporation). The cell count assay was performed in triplicate, and ~100 cells were counted in each evaluation. The survival rate was expressed as a percentage relative to the untreated control group.

**Preparation of whole cell lysates.** Following the indicated treatments, HaCaT cells and HDFs were washed twice with PBS supplemented with 1 mM Na<sub>3</sub>VO<sub>4</sub> and 1 mM NaF and subsequently exposed to cell lysis buffer [20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA)]. The cells were then harvested and centrifuged for 15 min at 4°C and 12,074 x g. The supernatant was extracted, and protein concentrations were determined by bicinchoninic acid assay at 560 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

**Western blotting.** Proteins (50  $\mu$ g/lane) were separated by SDS-PAGE (10%) and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were washed using TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween-20 (TBST) and blocked with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated overnight with primary antibodies specific for COX-2 (1:2,000; cat. no. 155745; Cayman Chemical Company), type 1 procollagen (1:2,000; cat. no. ab34170; Abcam), phosphorylated (p-) c-Jun (1:1,000; cat. no. 2361; Cell Signaling Technology, Inc.), total (T-) c-Jun (1:1,000; cat. no. 9165; Cell Signaling Technology, Inc.), c-Fos (1:500; cat. no. sc-8047; Santa Cruz Biotechnology, Inc.) or  $\beta$ -actin (1:10,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA) at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies goat anti-rabbit immunoglobulin G (IgG; H+L; 1:2,000; cat. no. 111-035-045; Jackson ImmunoResearch Laboratories, Inc.) and goat anti-mouse IgG (H+L; 1:2,000; cat. no. 115-035-062; Jackson ImmunoResearch Laboratories, Inc.) specific to the primary antibody for 2 h at RT, followed by washing with TBST at RT. The immunoreactivity of the membranes was detected using enhanced chemiluminescence reagents (cat. no. K12045-D50; Advanta, Inc.).  $\beta$ -actin was used as a loading control.

**Reverse-transcription polymerase chain reaction (RT-PCR).** Following the indicated treatments, total RNA was isolated from HaCaT cells using TRIzol<sup>®</sup> (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (3  $\mu$ g) was reverse-transcribed using a 40  $\mu$ l reaction mixture comprising

8  $\mu$ l Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation), 5X buffer, 3  $\mu$ l 10 mM dNTPs, 0.45  $\mu$ l 40 U/ $\mu$ l RNase inhibitor, 0.3  $\mu$ l 200 U/ $\mu$ l M-MLV RT and 3.75  $\mu$ l 20  $\mu$ M oligo dT (Bioneer Corporation). Single-stranded cDNA was amplified using PCR with 4  $\mu$ l 5X Green Go-Taq<sup>®</sup> Flexi reaction buffer, 0.4  $\mu$ M 10 mM dNTPs, 0.1  $\mu$ l 5 U/ $\mu$ l Taq polymerase, 1.2  $\mu$ l 25 mM MgCl<sub>2</sub> (all Promega Corporation) and 0.4  $\mu$ l primer (20 pM/ $\mu$ l). The sequences of primers used in PCR were as follows: COX-2 forward, 5'-CCGGACAGGATTCTATGGAGA-3' and reverse, 5'-CAATCATCAGGCACAGGAGG-3'; MMP-3 forward, 5'-CCTCTGATGCCAGAAATTGA-3' and reverse, 5'-GAAATTGGCCACTCCCTGGGT-3';  $\beta$ -actin forward, 5'-GGTGAAGGTCCGTGTGAACG-3' and reverse, 5'-GGTAGGAACACGGAAGGCCA-3'. The PCR conditions for COX-2 were as follows: 30 cycles of denaturation at 95°C for 30 sec, annealing at 59°C for 30 sec and extension at 72°C for 30 sec. The PCR conditions for MMP-3 were as follows: 30 cycles of denaturation at 95°C for 60 sec, annealing at 60°C for 120 sec and extension at 72°C for 180 sec. The PCR conditions for  $\beta$ -actin were as follows: 25 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 30 sec. PCR products were separated on a 1% agarose gel, and visualized and photographed in the Gel Doc XR+ system (Bio-Rad Laboratories, Inc.).  $\beta$ -actin was used as an internal control to evaluate the relative expression of COX-2 and MMP-3.

**Quantitative PCR (qPCR).** Total cellular RNA was isolated from the treated HaCaT cells using the RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's protocol. Total RNA (3  $\mu$ g) was reverse-transcribed using a 40  $\mu$ l reaction mixture containing 8  $\mu$ l Molony Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega Corporation) 5X buffer, 3  $\mu$ l 10 mM dNTPs, 0.45  $\mu$ l 40 U/ $\mu$ l RNase inhibitor, 0.3  $\mu$ l 200 U/ $\mu$ l M-MLV RT and 3.75  $\mu$ l 20  $\mu$ M oligo dT (Bioneer Corporation). Single-stranded cDNA was amplified using PCR with the primers listed above. The thermocycling conditions were as follows: 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The SYBR Green PCR Master Mix (Takara Bio, Inc.) was used with the LightCycler 96 Machine (Roche Diagnostics GmbH). The reactions were performed in triplicate for each sample. The results were quantified using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (33). The C<sub>q</sub> value for each sample was normalized to the value for  $\beta$ -actin.

**Statistical analysis.** Data were expressed as the mean  $\pm$  SEM. Differences between values were analyzed using one-way ANOVA followed by Dunnett's post hoc test (SPSS software, version 11.5; SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Treatment with 0.5-2.5  $\mu$ l/ml 7-MEGA for 4 and 6 h has low to no cytotoxic effect on HDFs and HaCaT keratinocytes.** The effects of various concentrations and treatment durations of 7-MEGA on the viability of HaCaT cells and HDFs were determined using Trypan blue staining and cell count analysis. Compared with the untreated control group, treatment with 0.5-2.5  $\mu$ l/ml

7-MEGA for 6 h did not significantly affect the survival rate of HaCaT cells; however, treatment with 5  $\mu$ l/ml 7-MEGA for 6 h was cytotoxic to HaCaT cells (Fig. 1A). In addition, treatment with 0.5 or 1  $\mu$ l/ml 7-MEGA for 8 h did not significantly affect the survival of HaCaT cells, whereas treatment with 2.5 or 5  $\mu$ l/ml 7-MEGA for the same duration resulted in a significant decrease in cell survival rate (Fig. 1B). Treatment with 0.5-2.5  $\mu$ l/ml 7-MEGA for 4 and 8 h exhibited no effect on HDF survival; however, treatment with 5  $\mu$ l/ml 7-MEGA for the same durations resulted in a significant decrease in the cell survival rate (Fig. 1C and D). These results suggested that treatment with 0.5-2.5  $\mu$ l/ml 7-MEGA for 4 or 6 h is not toxic to HDFs or HaCaT cells.

**Exposure of HaCaT keratinocytes to 10 mJ/cm<sup>2</sup> UVB for 6 h increases the expression levels of COX-2 and MMP-3.** Since upregulation of COX-2, MMP-1 and MMP-3 is linked to skin inflammation (5,7), the effects of exposure to different doses of UVB irradiation for 6 h on the protein and mRNA expression levels of COX-2 and MMP-3 were investigated in HaCaT cells. Western blotting experiment results demonstrated that compared with the non-irradiated control group, 10 mJ/cm<sup>2</sup> UVB irradiation for 6 h significantly induced the expression of COX-2 protein in HaCaT cells (Fig. 2A and B). Similarly, the results of the RT-PCR analysis revealed increased expression of COX-2 in HaCaT cells irradiated with 10 mJ/cm<sup>2</sup> UVB for 6 h (Fig. 2C). Exposure of HaCaT cells to 10 mJ/cm<sup>2</sup> UVB for 6 h strongly induced MMP-3 mRNA expression in these cells (Fig. 2C), whereas exposure to the tested doses of UVB for 6 h resulted in only a slight increase in MMP-1 mRNA expression (data not shown). Subsequent qPCR analysis demonstrated that peak induction of COX-2 and MMP-3 mRNA expression levels occurred in HaCaT cells irradiated with 10 mJ/cm<sup>2</sup> UVB for 6 h (Fig. 2D). However, there was little or no induction of the expression of MMP-1 mRNA under the same experimental conditions (data not shown). Time course experiments were performed to evaluate the time at which the expression levels of COX-2 and MMP-3 protein and/or mRNA were induced in HaCaT cells irradiated with 10 mJ/cm<sup>2</sup> UVB. Western blotting results demonstrated that whereas COX-2 protein expression was not detected in HaCaT cells exposed to 10 mJ/cm<sup>2</sup> UVB for 0.5 or 1 h, strong COX-2 protein expression was observed in the cells exposed to UVB for 6 h (Fig. 2E and F). In addition, the RT-PCR results demonstrated that the mRNA expression levels of COX-2 and MMP-3 were higher in HaCaT cells exposed to 10 mJ/cm<sup>2</sup> UVB for 3 or 6 h compared with those in the non-irradiated control (Fig. 2G). The peak expression levels of COX-2 and MMP-3 mRNA were observed in HaCaT cells exposed to 10 mJ/cm<sup>2</sup> UVB for 6 h. The protein and mRNA expression levels of the control  $\beta$ -actin remained largely unchanged under these experimental conditions (Fig. 2A, C, E and G). The results of the qPCR confirmed that exposure to 10 mJ/cm<sup>2</sup> UVB irradiation for 6 h maximally induced the expression levels of COX-2 and MMP-3 mRNA in HaCaT cells (Fig. 2H). Based on these results, 10 mJ/cm<sup>2</sup> UVB for 6 h was selected for use in subsequent experiments.

**Treatment with 7-MEGA inhibits UVB-induced expression of COX-2 and MMP-3 in HaCaT keratinocytes.** The effects of 0.5-2.5  $\mu$ l/ml 7-MEGA on the protein and mRNA expression

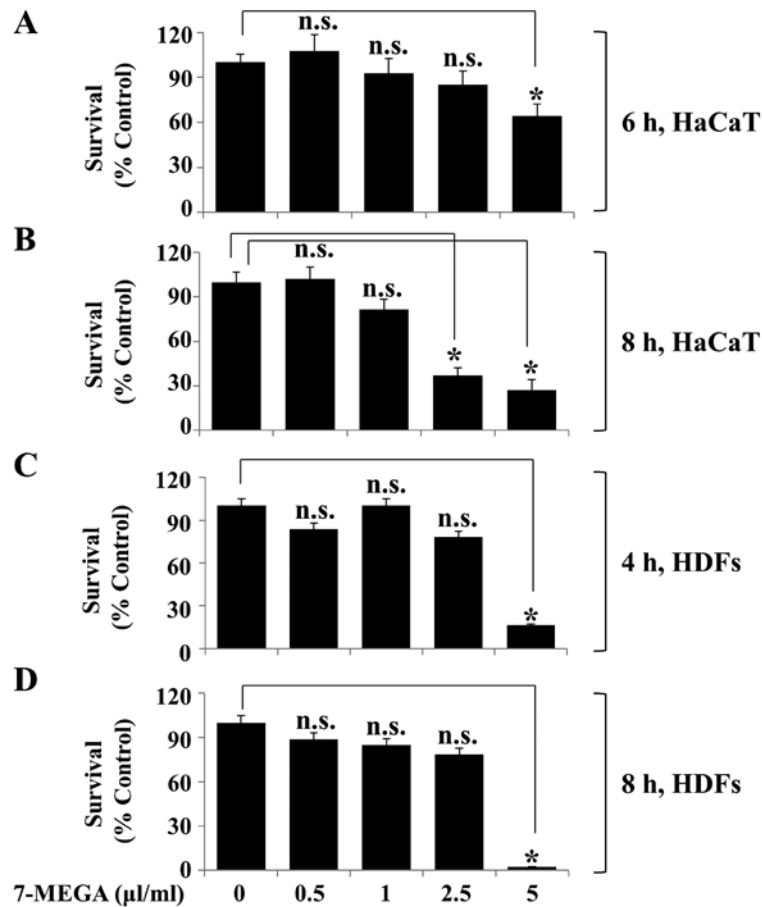


Figure 1. Effects of 7-MEGA on the growth of human keratinocytes and HDFs. (A) HaCaT human keratinocytes were treated with vehicle control or 7-MEGA at the indicated concentrations for 6 or (B) 8 h. (C) HDFs were treated with vehicle control or 7-MEGA at the indicated concentrations for either 4 or (D) 8 h. At each time point, the number of surviving cells was recorded by trypan blue staining analysis. Data are expressed as the mean  $\pm$  SEM of three independent experiments. \* $P < 0.05$  vs. 0  $\mu\text{g/ml}$  7-MEGA. HDFs, human dermal fibroblasts; 7-MEGA, AlaskOmega® Omega 7 500; n.s., not significant.

levels of COX-2 and MMP-3 in HaCaT cells exposed to 10 mJ/cm<sup>2</sup> of UVB for 6 h were evaluated. Treatment with 7-MEGA blocked the UVB-induced COX-2 protein expression in HaCaT cells; 2.5  $\mu\text{l/ml}$  7-MEGA exhibited the strongest effect (Fig. 3A and B). Similarly, the RT-PCR results demonstrated that 7-MEGA treatment inhibited UVB-induced COX-2 mRNA expression in HaCaT cells (Fig. 3C). Treatment with 7-MEGA also suppressed UVB-induced MMP-3 mRNA expression in HaCaT cells.  $\beta$ -actin protein and mRNA expression levels remained constant under these experimental conditions (Fig. 3A and C). The results of the qPCR analysis also demonstrated the ability of 1 and 2.5  $\mu\text{l/ml}$  7-MEGA to significantly suppress UVB-induced COX-2 and MMP-3 mRNA expression levels in HaCaT cells (Fig. 3D). Due to the strong suppressive effects on UVB-induced COX-2 and MMP-3 expressions in HaCaT cells, 2.5  $\mu\text{l/ml}$  7-MEGA was selected as the optimal concentration for subsequent experiments.

*Treatment with 2.5  $\mu\text{l/ml}$  7-MEGA inhibits the UVB-induced increase of c-Fos expression and c-Jun phosphorylation in HaCaT keratinocytes.* UVB-induced expression of COX-2 and MMP-3 in HaCaT cells is affected by AP-1 (18,25,26), which is composed of c-Fos and c-Jun (34). Therefore, the effects of exposure to 10 mJ/cm<sup>2</sup> UVB irradiation on the protein

expression and/or phosphorylation of c-Fos and c-Jun in HaCaT cells were determined in the present study. Compared with the non-irradiated control group, an induction of c-Fos protein expression was observed in HaCaT cells exposed to UVB for 1, 3 or 6 h (Fig. 4A). In addition, compared with the non-irradiated control group, the levels of c-Jun phosphorylation and expression were upregulated in HaCaT cells exposed to UVB at the tested time points (Fig. 4A and B). The peak expression and/or phosphorylation of c-Fos and c-Jun was observed in HaCaT cells exposed to UVB for 3 h (Fig. 4A and B).

The effects of treatment with 2.5  $\mu\text{l/ml}$  7-MEGA on the expression or phosphorylation of c-Fos and c-Jun in HaCaT cells exposed to UVB for 3 h were examined next; treatment with 2.5  $\mu\text{l/ml}$  of 7-MEGA largely blocked the UVB-induced expression of c-Fos protein in HaCaT cells (Fig. 4C). Of note, treatment with 2.5  $\mu\text{l/ml}$  7-MEGA further partly inhibited the UVB-induced phosphorylation of c-Jun without affecting its total protein expression in HaCaT cells. No significant changes were observed in the control  $\beta$ -actin protein expression levels under these experimental conditions (Fig. 4A and C).

*Treatment with 2.5  $\mu\text{l/ml}$  7-MEGA inhibits UVB-induced reduction of type 1 collagen expression in HDFs.* The loss or reduction of type 1 collagen contents is associated with the production of skin wrinkles in photoaging (6,24). To investigate

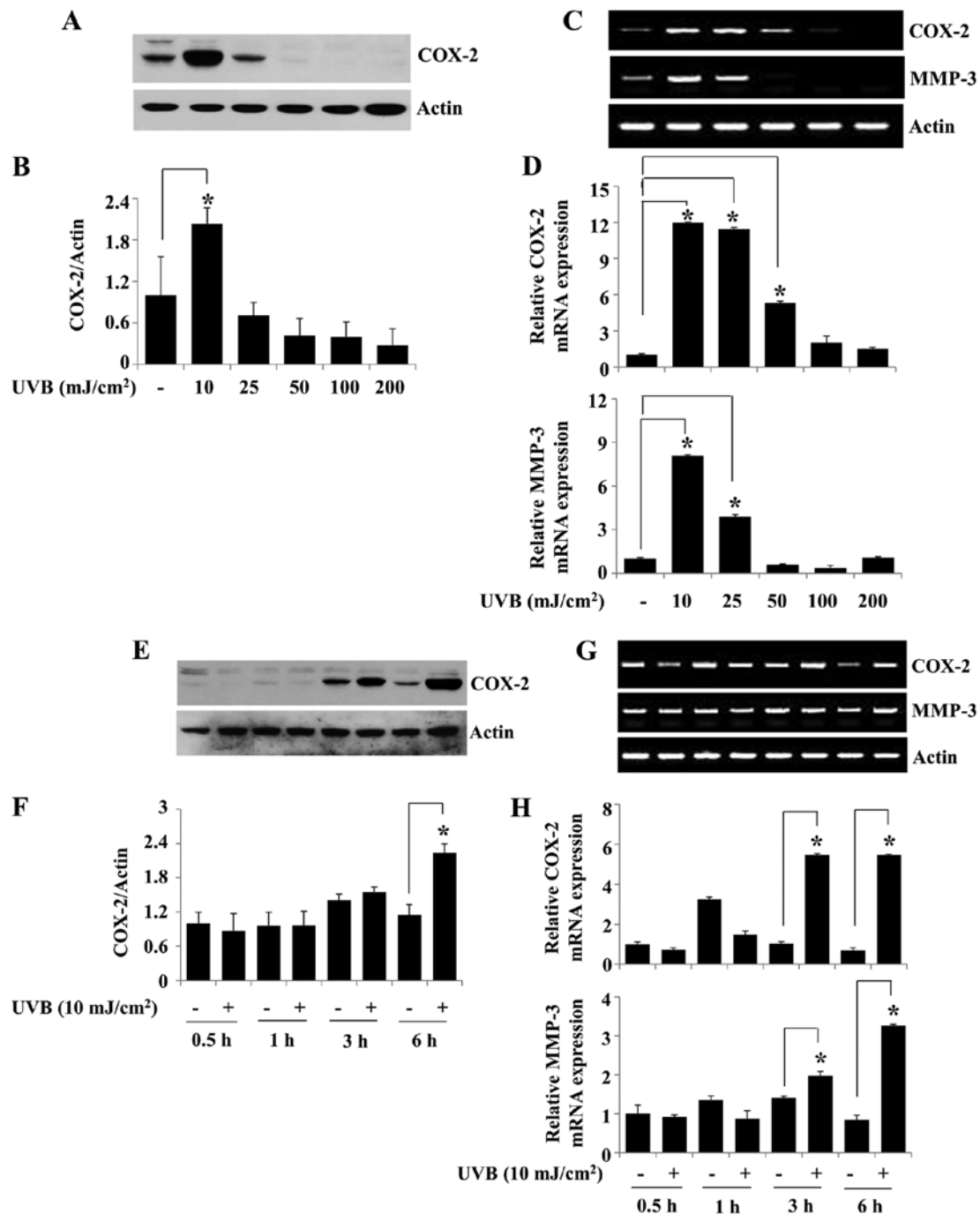


Figure 2. Effects of UVB irradiation on the expression levels of COX-2 and MMP-3 in HaCaT human keratinocytes. (A) HaCaT cells were irradiated with UVB at 0, 10, 25, 50, 100 or 200 mJ/cm<sup>2</sup> for 6 h. Whole cell lysates were extracted from the irradiated cells and analyzed by western blotting to determine the protein expression levels of COX-2 and  $\beta$ -actin. (B) Densitometry analysis results of panel A. (C) RT-PCR and (D) qPCR analysis of the mRNA expression levels of COX-2, MMP-3 and  $\beta$ -actin. (E) HaCaT cells were irradiated with 10 mJ/cm<sup>2</sup> UVB for 0, 0.5, 1, 3 or 6 h. At each time point, whole cell lysates were extracted from the conditioned cells and analyzed by western blotting to determine the protein expression levels of COX-2 and  $\beta$ -actin. (F) Densitometry analysis results of panel E. (G) RT-PCR and (H) qPCR analysis of the mRNA expression levels of COX-2, MMP-3 and  $\beta$ -actin. \*P<0.05 vs. non-irradiated control. COX-2, cyclooxygenase-2; MMP-3, stromelysin-1; UVB, ultraviolet B; RT-PCR, reverse transcription-PCR; qPCR, quantitative PCR.

the role of 7-MEGA on collagen production *in vitro*, the effects of UVB irradiation (60 mJ/cm<sup>2</sup>) and treatment with 7-MEGA (2.5  $\mu$ l/ml) on the protein expression levels of type 1 procollagen were determined in HDFs. In preliminary experiments, we have analyzed the protein expression levels of type 1 procollagen in HDFs after exposure of different doses (30, 60, and 90 mJ/cm<sup>2</sup>) and times (2, 4, and 8 h) of UVB and found that 60 mJ/cm<sup>2</sup> of UVB irradiation for 4 h most strongly decreases the protein

expression levels of type 1 procollagen in these cells compared to no UVB treatment (data not shown). As anticipated, a strong decrease in the expression levels of type 1 procollagen was observed in HDFs exposed to 60 mJ/cm<sup>2</sup> UVB irradiation for 4 h (Fig. 5A and B). However, treatment with 2.5  $\mu$ l/ml 7-MEGA partially blocked the UVB-induced reduction of expression of type 1 procollagen in HDFs. The expression levels of  $\beta$ -actin remained constant under these experimental conditions.

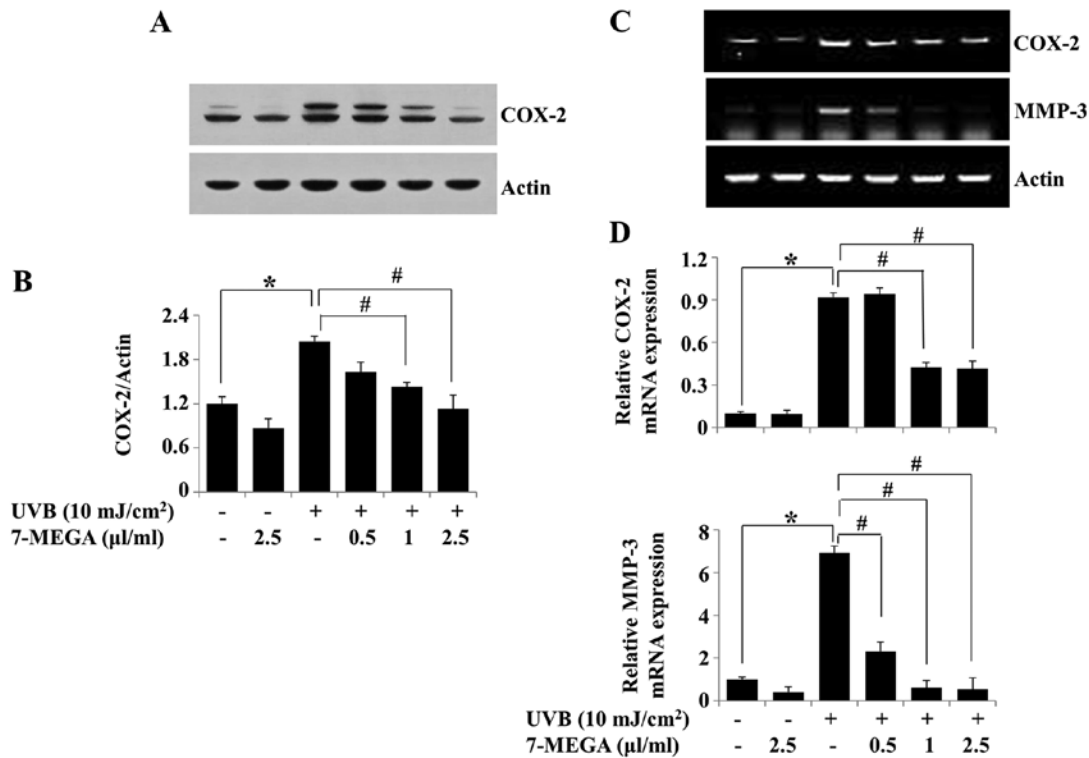


Figure 3. Effects of 7-MEGA on UVB-induced expression of COX-2 and MMP-3 in HaCaT human keratinocytes. HaCaT cells were irradiated with 10 mJ/cm<sup>2</sup> UVB and treated with 0, 0.5, 1, or 2.5 μl/ml 7-MEGA for 6 h. (A) Whole cell lysates were extracted from the cells and analyzed by western blotting to determine COX-2 and β-actin protein expression. (B) Densitometry analysis results of COX-2 expression levels. (C) Total RNA was extracted from the cells and analyzed by reverse transcription-PCR or (D) quantitative PCR to measure the mRNA expression levels of COX-2, MMP-3 and β-actin. \*P<0.05 vs. non-irradiated control; #P<0.05 vs. UVB-irradiated cells without 7-MEGA. 7-MEGA, AlaskOmega® Omega 7 500; COX-2, cyclooxygenase-2; MMP-3, stromelysin-1; UVB, ultraviolet B.

## Discussion

AlaskOmega® Omega 7 500 (7-MEGA) is a highly concentrated Omega-7 fatty acid (30,31). The mechanism of 7-MEGA regulation on UVB-induced skin inflammation, damage and wrinkles is largely unknown. The present study investigated the effects of 7-MEGA on the expression levels of COX-2, MMP-1, MMP-3 and type 1 procollagen, which are markers of skin inflammation, damage and wrinkle formation, in UVB-irradiated HDFs and HaCaT keratinocytes. To the best of our knowledge, this is the first study to demonstrate that 7-MEGA may inhibit not only the UVB-induced upregulation of COX-2 and MMP-3 in HaCaT cells, but also the UVB-induced reduction of type 1 procollagen expression in HDFs. These results suggested that 7-MEGA may exhibit anti-inflammatory, anti-photodamage and anti-wrinkle formation effects in UVB-irradiated skin cells.

A recent study has reported that 7-MEGA exhibits a protective effect on H<sub>2</sub>O<sub>2</sub>-induced damage in HaCaT cells and that the effect is mediated through the inhibition of cellular reactive oxygen species production and downregulation of the expression of inflammatory mediators, such as COX-2, interleukin-1β and tumor necrosis factor-α (32). The results of the present study demonstrated that exposure of HaCaT cells to 10 mJ/cm<sup>2</sup> UVB for 6 h significantly increased the expression of COX-2 at the protein and mRNA levels, whereas treatment with 2.5 μl/ml 7-MEGA significantly suppressed these effects. Since COX-2 and the associated PGs mediate UVB-induced cutaneous

inflammation (5,35), these results indicate that 7-MEGA may exhibit an anti-inflammatory effect on UVB-irradiated human keratinocytes by downregulating COX-2. UVB induces the upregulation of MMP-1 and MMP-3 in skin cells and tissues (27,28,36), which causes UV-mediated skin inflammation and damage (7,37). A recent study has demonstrated the skin regenerative effects of 7-MEGA on H<sub>2</sub>O<sub>2</sub>-treated HaCaT cells through the downregulation of MMP-1 and upregulation of type 1 procollagen (32). RT-PCR and qPCR experiments in the present study revealed that the exposure of HaCaT cells to 10 mJ/cm<sup>2</sup> UVB for 6 h resulted in a strong induction of MMP-3, but not MMP-1 mRNA expression. However, treatment with 7-MEGA inhibited the UVB-induced MMP-3 mRNA upregulation in HaCaT cells. These results further suggested that 7-MEGA may exhibit anti-inflammatory and photoprotective effects on UVB-irradiated HaCaT keratinocytes by downregulating the expression of MMP-3. However, considering that the skin inflammation and damage pathways induced by UV irradiation involve multiple pathways other than the COX-2 (eicosanoids) and MMP-3, it should be noted that the suppressive effects of 7-MEGA on UVB-induced COX-2 and MMP-3 expression levels in HaCaT cells demonstrated in the present study may be limited to the cell lines used. Thus, it is currently not possible to conclude that 7-MEGA has preventive and/or therapeutic effects on UVB-induced inflammation and photodamage in the skin. A number of previous studies have demonstrated the induction of COX-2 or MMP-3 expression in HaCaT cells irradiated with UVB at the intensity of 20, 30 or

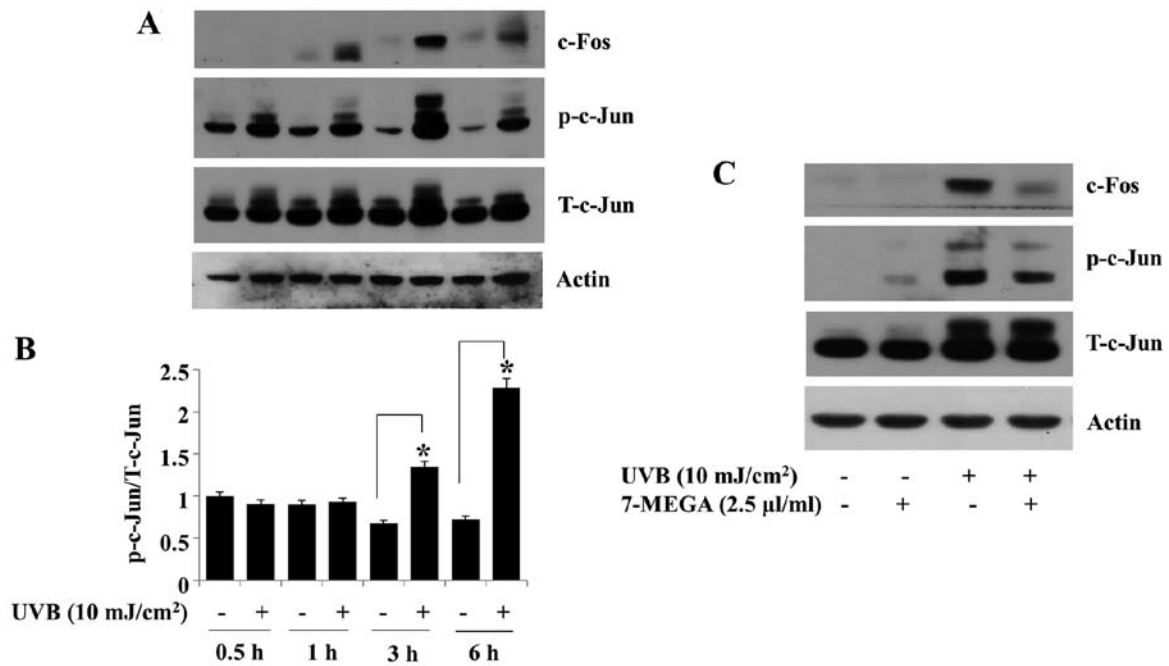


Figure 4. Effects of UVB and 7-MEGA on the expression and phosphorylation of c-Fos and c-Jun in HaCaT human keratinocytes. (A) HaCaT cells were irradiated with 10 mJ/cm<sup>2</sup> UVB for 0, 0.5, 1, 3, or 6 h. At each time point, whole cell lysates were extracted from the conditioned cells and analyzed by western blotting to determine the protein expression and phosphorylation levels of c-Fos, c-Jun and β-actin. (B) Densitometry analysis results of panel A. \*P<0.05 vs. non-irradiated control. (C) HaCaT cells were irradiated with 10 mJ/cm<sup>2</sup> UVB for 3 h either in the absence or presence of 2.5 μl/ml 7-MEGA. Whole cell lysates were extracted from the cells and analyzed by western blotting. 7-MEGA, AlaskOmega® Omega 7 500; UVB, ultraviolet B; p-c-Jun, phosphorylated c-Jun; T-c-Jun, total c-Jun.

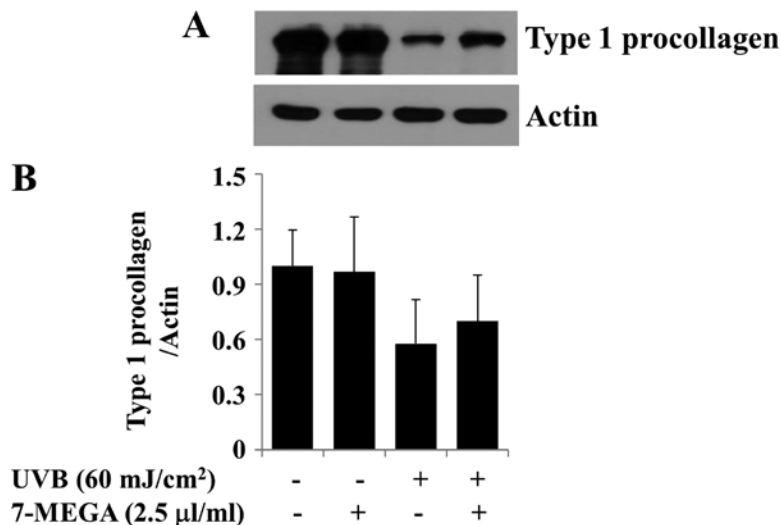


Figure 5. Effects of UVB and 7-MEGA on the expression of type 1 procollagen in HDFs. (A) HDFs were irradiated with 60 mJ/cm<sup>2</sup> UVB for 4 h in the absence or presence of 2.5 μl/ml 7-MEGA. Whole cell lysates were extracted from the cells and analyzed by western blotting. (B) Densitometry analysis results of the expression levels of type 1 procollagen normalized to those of β-actin. 7-MEGA, AlaskOmega® Omega 7 500; UVB, ultraviolet B; HDFs, human dermal fibroblasts.

60 mJ/cm<sup>2</sup> (38-40). In the present study, irradiation with the lowest tested intensity of UVB (10 mJ/cm<sup>2</sup>) for 6 h induced the peak expression levels of COX-2 and MMP-3 in HaCaT cells; this may be due to the use of different passage of HaCaT cells, different duration and devices of UVB-irradiation, different culture conditions (serum-containing or serum-free media) and different cell lysis buffers used in previous studies.

Emerging evidence suggests that the AP-1 transcription factor, composed of c-Fos and c-Jun, is important for the

UVB-induced expression of COX-2, MMP-1 and MMP-3 in HaCaT cells (16,18,25,26). To date, 7-MEGA regulation of AP-1 in UVB-irradiated skin cells has not been reported. Previously, UVB has been demonstrated to induce the activation of AP-1, which is associated with increased c-Fos expression in HaCaT cells (41). Consistent with this result, the present study demonstrated that the exposure of HaCaT cells to 10 mJ/cm<sup>2</sup> UVB for 3 h upregulated c-Fos protein expression. In addition, the expression and phosphorylation

levels of c-Jun were upregulated in the UVB-irradiated HaCaT cells. These results supported the UVB-induced AP-1 activation in HaCaT cells. In addition, the results of the present study demonstrated that 7-MEGA inhibited the UVB-induced c-Fos expression and c-Jun phosphorylation in HaCaT cells. Assuming that the promoters of COX-2 and MMP-3 contain the AP-1 cis-acting element and that 7-MEGA inhibits UVB-induced expression of COX-2 and MMP-3 at their transcript levels in HaCaT cells, the inhibitory effects of 7-MEGA on UVB-induced expression of COX-2 and MMP-3 in HaCaT cells may be attributable to AP-1 inhibition.

UVB-induced reduction of type 1 procollagen expression in HDFs has been previously reported (25,29). Consistent with this result, the present study demonstrated that the expression of type 1 procollagen protein was diminished in HDFs irradiated with 60 mJ/cm<sup>2</sup> UVB for 4 h. However, treatment with 7-MEGA partially blocked the UVB-induced reduction of type 1 procollagen expression in HDFs, thus supporting the anti-photoaging effects of 7-MEGA in culture. Additionally, the family of MMPs expressed and secreted from skin keratinocytes are involved in the degradation of type 1 procollagen and other ECM components in the dermis (27-29). Thus, it may be speculated that 7-MEGA inhibition of UVB-induced MMP-3 expression in HaCaT keratinocytes may mediate the anti-wrinkle effects (enhanced expression of type 1 procollagen on UVB-exposed dermal fibroblasts) of 7-MEGA. Future studies using a co-culture system of HaCaT keratinocytes and HDFs irradiated with UVB in the presence or absence of 7-MEGA are required to clarify this speculation.

The main compounds in 7-MEGA are palmitoleic acid (C16:1 n-7, 53.5%), palmitic acid (C16:0, 25.7%), eicosapentaenoic acid (C20:5 n-3, 0.06%) and myristic acid (C14:0, 0.04%) (32). Treatment of HaCaT cells with 7-MEGA or palmitoleic acid at the same concentration (100 nM) exhibits similar inhibitory effects on H<sub>2</sub>O<sub>2</sub>-induced expression of COX-2 and MMP-1 (32). At present, it is unclear whether 7-MEGA as a whole or its certain constituent(s) exerts the regulatory effects on UVB-induced COX-2, MMP-3 and type 1 procollagen expression in HaCaT cells and HDFs. In the present study, the efficacy of 7-MEGA<sup>TM</sup> 500 and its major compound palmitoleic acid on the UVB-induced COX-2, MMP-3 or type 1 procollagen expression was compared in HaCaT cells and HDFs; however, while treatment with 2.5  $\mu$ l/ml of 7-MEGA is not cytotoxic to UVB-exposed HaCaT cells or HDFs, similar or lower concentrations (0.625, 1.25 and 2.5  $\mu$ l/ml) of palmitoleic acid were largely cytotoxic to these cells (data not shown). Since 0.625, 1.25 and 2.5  $\mu$ l/ml palmitoleic acid are equal to 2.2, 4.4 and 8.8 mM palmitoleic acid and that treatment with high concentrations of fatty acids often lead to lipotoxicity-mediated growth inhibition and cell death in numerous cell types, it is likely that the cytotoxicity of UVB-exposed HaCaT cells or HDFs triggered by palmitoleic acid was due to lipotoxicity. It should be emphasized that palmitoleic acid up to 500  $\mu$ M was not cytotoxic to UVB-exposed HaCaT cells or HDFs, but it was ineffective in counteracting the UVB-induced COX-2, MMP-3 or type 1 procollagen expression in HaCaT cells and HDFs (data not shown). Considering that although 7-MEGA contains 53.5%

palmitoleic acid, it exhibited little cytotoxicity to HaCaT and HDFs, it may be speculated that 7-MEGA as a whole, or other components, in it may serve the function of weakening the cytotoxicity of palmitoleic acid. In the future, the effects of 7-MEGA and each of its constituent compounds (with the exception of palmitoleic acid) on the regulation of COX-2, MMP-3 and type 1 procollagen expression levels in HaCaT cells and HDFs exposed to UVB may be tested and compared. This may clarify the modulatory effects of 7-MEGA and its constituent compounds on UVB-induced expression of COX-2, MMP-3 and type 1 procollagen in skin cells.

The results of the present study demonstrated that 7-MEGA regulated the expression levels of inflammation-, photodamage- and/or wrinkle formation-related proteins and enzymes, such as COX-2, MMP-3 and type 1 procollagen, in 10 or 60 mJ/cm<sup>2</sup> UVB-irradiated HaCaT cells and HDFs. However, although the doses of UVB irradiation administered to HaCaT cells and HDFs in the present study were similar to those used previously in cell culture (42), they are different from those used in *in vivo* studies (200-500 mJ/cm<sup>2</sup>) (43,44). Considering that the HaCaT cells and HDFs used in the present study are cultured cell lines, the damage caused by UVB-irradiation in the human skin is not limited to these cell types and that the effective doses of 7-MEGA may be significantly different in *in vivo* dermal cells, future studies are warranted to evaluate the anti-inflammatory and anti-wrinkle effects of 7-MEGA on animals and their sera. This should be accomplished not only by measuring the expression and secretion of multiple pro-inflammatory, anti-inflammatory and wrinkle formation-related factors, including COX-2, type 1 procollagen, MMP-3 and immunoglobulins, but also using the direct microscopic examination of skin improvement or anti-wrinkle effects in the skin exposed to UVB. This may help establish the anti-inflammatory, anti-photodamaging and anti-wrinkle effects of 7-MEGA.

In conclusion, the results of the present study demonstrated that 7-MEGA regulated the expression levels of COX-2, MMP-3 and type 1 procollagen in UVB-irradiated human keratinocytes and dermal fibroblasts. These results suggested that 7-MEGA may be used as an agent to protect against UVB-induced skin inflammation and potentially wrinkle-formation caused by the dysregulation of the expression of COX-2, MMP-3 and type 1 procollagen.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YKP, AKY and AR performed the experiments. YWR, JYC, YKS, BHK, NYL and BCJ designed the experiments and analysed the data. BCJ wrote the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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