



Effects of 7-MEGATM 500 on Oxidative Stress, Inflammation, and Skin Regeneration in H₂O₂-Treated Skin Cells

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Abstract

Environmental stimuli can lead to the excessive accumulation of reactive oxygen species (ROS), which is one of the risk factors for premature skin aging. Here, we investigated the protective effects of 7-MEGATM 500 (50% palmitoleic acid, 7-MEGA) against oxidative stress-induced cellular damage and its underlying therapeutic mechanisms in the HaCaT human skin keratinocyte cell line (HaCaT cells). Our results showed that treatment with 7-MEGA prior to hydrogen peroxide (H₂O₂)-induced damage significantly increased the viability of HaCaT cells. 7-MEGA effectively attenuated generation of H₂O₂-induced reactive oxygen species (ROS), and inhibited H₂O₂-induced inflammatory factors, such as prostaglandin E₂ (PGE₂), tumor necrosis factor- α (TNF- α), and interleukin-1 β (IL-1 β). In addition, cells treated with 7-MEGA exhibited significantly decreased expression of matrix metalloproteinase-1 (MMP-1) and increased expression of procollagen type 1 (PCOL1) and Elastin against oxidative stress by H₂O₂. Interestingly, these protective activities of 7-MEGA were similar in scope and of a higher magnitude than those seen with 98.5% palmitoleic acid (PA) obtained from Sigma when given at the same concentration (100 nL/mL). According to our data, 7-MEGA is able to protect HaCaT cells from H₂O₂-induced damage through inhibiting cellular oxidative stress and inflammation. Moreover, 7-MEGA may affect skin elasticity maintenance and improve skin wrinkles. These findings indicate that 7-MEGA may be useful as a food supplement for skin health.

Key words: 7-MEGA, Palmitoleic acid, Anti-oxidation, Anti-inflammation, Skin regeneration

INTRODUCTION

Skin is the primary barrier that serves to protect our body from various chemical and physical external stimuli. The skin consists of the epidermis, dermis, and subcutaneous tissue, and the epidermis is mostly composed of keratinocytes. These keratinocytes are susceptible to external

stimuli, such as UV radiation, environmental toxins, and heat (1). In particular, reactive oxygen species (ROS) produced by external stimuli induce processes related to skin aging, by decreasing skin regeneration and increasing wrinkle formation (2,3).

Omega fatty acids are essential fatty acids, meaning they are produced only in small quantities in our bodies so

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Abbreviation: 7-MEGA, 7-MEGATM 500; COX-2, cyclooxygenase-2; DPPH, 2,2-diphenyl-1-picrylhydrazyl; H₂O₂, hydrogen peroxide; IL-1 β , interleukin-1 β ; MMP-1, matrix metalloproteinase-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PA, palmitoleic acid; PCOL1, pro-collagen type 1; PGE₂, prostaglandin E₂; ROS, reactive oxygen species; TNF- α , tumor necrosis factor- α .

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they must be ingested primarily through foods. The representative omega fatty acids are omega-3, omega-6, omega-7 and omega-9. Among these, omega-3, which is abundant in fish and soybean oils, has been shown to possess anti-oxidative (4), anti-inflammatory (5), neuroprotective (6), and chemopreventive (7) effects. Omega-6 and -9 have been linked to obesity prevention (8) and anti-inflammation (9).

However, there has been little reported on omega-7 compared with 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 fishes and plants, such as macadamias, cold water fish, and sea buckthorn berries (10). Previous research has shown that omega-7 can improve cardiovascular function (11) and increase insulin sensitivity (12). However, the effects of omega-7 on the health of skin have not yet been elucidated. Thus, we investigated the physiological activity of 7-MEGATM 500 (purity 50% of omega-7, 7-MEGA) in human keratinocytes, which reside in the main outermost layer of the skin. As expected, we determined that 7-MEGA exhibited anti-oxidation and anti-inflammatory effects and improved skin cell regeneration, which suggests it may be useful as a functional food supplement for promoting skin health.

MATERIALS AND METHODS

Preparation of 7-MEGATM 500. 7-MEGATM 500 concentrates were made by Organic Technologies in Eastern Ohio, USA. Pollock was recovered from the Alaskan Bering Sea, and was subsequently processed, purified, and concentrated to produce 7-MEGATM 500 containing more than 500 mg/g of palmitoleic acid (Table 1). 7-MEGA was dissolved in 99.5% ethanol and stocked at -80°C. Palmitoleic acid (purity 98.5% of omega-7, PA) (Sigma-Aldrich, St. Louis, MO, USA) was also dissolved under the same conditions. Every aliquot was used not more than two times.

Cell culture. The human keratinocyte cell line HaCaT (ATCC, Rockville, MD, USA) was maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, Logan, UT, USA), containing 10% FBS (Gibco, CA, USA) and 1% antibiotics (penicillin [100 U/mL], streptomycin [100 µg/mL]) (Sigma-Aldrich) in a 37°C, 5% CO₂ incubator.

Table 1. 7-MEGA main compounds

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

tor. The medium was changed every 2-3 days. Cells were subcultured in a 100-mm culture dish (Nunc, Rochester, NY, USA) 24 hr before treatment.

Free radical scavenging activity assay. The free radical scavenging activity of garlic extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined using the method described by Blois (13). Ascorbic acid (vitamin C, Vc) was used as a positive control. Vc and PA were dissolved in 99.5% ethanol to 1 mg/mL. 7-MEGA concentrates were used at the same volume as PA in 99.5% ethanol. Then, 10 µL of 7-MEGA concentrate was mixed with 90 µL of DPPH solution (126 µg/mL). After incubation for 10 min at room temperature in the dark, the absorbance at 517 nm was measured using a plate reader (BioTek, Winooski, VT, USA). The free radical scavenging activity of the sample was calculated by the following formula:

$$\text{DPPH free radical scavenging activity (\%)} = \frac{(\text{Control group OD} - \text{Sample addition group OD})}{\text{Control group OD}} \times 100.$$

Cell viability assay. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich). HaCaT cells were plated at 2×10^4 cells/well on a 48-well plate (Nunc). After 24 hr incubation, cells were treated with either 7-MEGA (10-100 nL/mL) or PA (100 nL/mL) for 24 hr. We then investigated whether 1 hr of pretreatment with 7-MEGA (10-100 nL/mL) or PA (100 nL/mL) affected the cell viability of HaCaT cells treated with 1 mM H₂O₂ for 24 hr. After the incubation period, 10 µL of the MTT solution (500 µg/mL) was added to each well and cells were incubated for 2 hr in a 37°C, 5% CO₂ incubator. The absorbance was determined at 540 nm using a microplate reader (BioTek).

Oxidative stress assay. The level of intracellular ROS was quantified by fluorescence using dichlorofluorescein diacetate (DCF-DA; Invitrogen, Carlsbad, CA, USA). HaCaT cells were first plated in a 48-well plate. We then investigated whether 1 hr of pretreatment with 7-MEGA (10-100 nL/mL) or PA (100 nL/mL) affected ROS generation in HaCaT cells treated with 1 mM H₂O₂ for 5 min. After washing with PBS, cells were stained with 10 µM DCF-DA in PBS for 20 min in the dark at 37°C. Fluorescence was recorded with an excitation wavelength of 525 nm.

Superoxide dismutase and glutathione assays. Superoxide dismutase (SOD) was measured using a SOD Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). HaCaT cells were seeded in a 100-mm culture dish and cultured in a 37°C, 5% CO₂ incubator for 24 hr. Then,

cells were treated with various concentrations of 7-MEGA and 1 mM H₂O₂ in a 37°C, 5% CO₂ incubator for 24 hr. After that, lysis buffer was added to the cells, which were subsequently homogenized and centrifuged. Then, 10 µL of sample was mixed with 200 µL radical detector and 20 µL xanthine oxidase at room temperature. After 30 min, absorbance was measured at 450 nm using a microplate reader (BioTek). Glutathione (GSH) was measured using a GSH assay kit (Cayman Chemical). HaCaT cells were seeded in a 100-mm culture dish and maintained in a 37°C, 5% CO₂ incubator for 24 hr. After that, cells were treated with various concentrations of 7-MEGA and 1 mM H₂O₂ in a 37°C, 5% CO₂ incubator for 24 hr. After that, lysis buffer was added to the cells, which were homogenized and centrifuged. Then, 50 µL of sample was mixed with 150 µL reaction buffer (which included MES buffer, cofactor mixture, enzyme mix, and DTNB mixture buffer in the dark and at room temperature. After 30 min, absorbance was measured at 410 nm using a microplate reader (BioTek).

Western blotting. After the cells were treated in the same manner as the method for measuring cell viability, total protein from HaCaT cell lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 12% gels. Protein bands were then transferred to PVDF membranes (BioRad, Hercules, CA, USA), which were blocked with 5% skim milk in PBS then incubated with a 1:1000 v/v dilution of primary antibodies against COX-2, PGE₂, PCOL1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Cell Signaling, Danvers, MA, USA), MMP-1 and Elastin (Abcam, Cambridge, MA, USA) in PBS with 1% skim milk overnight at 4°C. The blots were then incubated with peroxidase-conjugated goat anti-rabbit IgG (PGE₂, β-actin, MMP-1, Elastin) and were then incubated with peroxidase-conjugated goat anti-mouse IgG (COX-2, PCOL1) (1:10,000 v/v, Millipore, CA, USA) for 1 hr. The immunoreactions were visualized with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, CA, USA) on a Chemilmager analyzer system (Alpha Innotech, San Leandro, CA, USA).

Inflammatory cytokine analysis. After the cells were treated in the same manner as the method for measuring cell viability, the concentrations of IL-1β and TNF-α in samples of supernatant were determined with ELISA kits (Abcam). In a 96-well plate, 100 µL samples were plated in cell culture medium at room temperature for 150 min. Then, 100 µL biotinylated IL-1β and TNF-α detection antibodies were added and cells were incubated at room temperature for 1 hr. Then, 100 µL HRP-streptavidin solution was added and cells were further incubated at room temperature for 45 min. Next, 100 µL TMB 1-Step Substrate reagent was added and cells were incubated at room temperature for an additional 30 min. The plate was washed

between each step. For the final step, 50 µL stop solution was added to the wells without washing and absorbance was measured at 450 nm using a microplate reader (BioTek).

Statistical analysis. All experiments were performed at least in triplicate, and data were expressed as means ± SEMs. Statistical significance was determined using Student's *t*-test and ANOVA to assess differences between two groups. Differences with *p*-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity and DPPH radical scavenging action 7-MEGA in HaCaT cells. To assess the cytotoxicity of 7-MEGA in HaCaT cells and potential anti-oxidant effects of 7-MEGA, MTT and DPPH free radical scavenging activity assays were conducted. HaCaT cells were treated with 7-MEGA at a concentration of 1~100 nL/mL for 24 hr. There were no visible cytotoxic effects of 7-MEGA until a concentration of 100 nL/mL was used, when compared with untreated control cells (Fig. 1A). At a concentration of 1 mg/mL, 7-MEGA showed DPPH free radical scavenging activity at 71 ± 1.3% of that of Vitamin C, and about 30% higher than that of PA (Fig. 1B). Thus, 7-MEGA showed

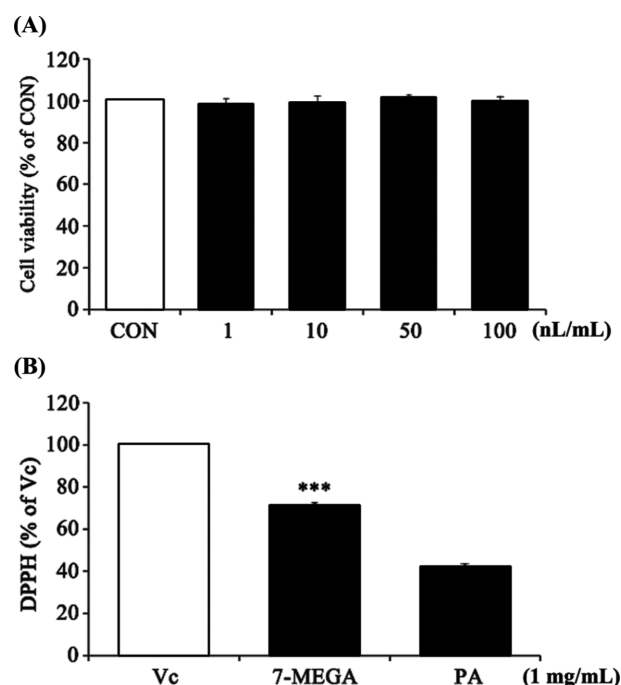


Fig. 1. Cytotoxicity and DPPH radical scavenging ability of 7-MEGA in HaCaT cells. (A) Viability of HaCaT cells after treatment with increasing concentrations of 7-MEGA (1~100 nL/mL) for 24 hr. (B) DPPH radical scavenging ability of 7-MEGA. Data are expressed as the mean ± SEM of three independent experiments, ****p* < 0.001 vs. PA.

no toxicity until a concentration of 100 nL/mL in HaCaT cells, and showed potential as an anti-oxidant material.

Effect of 7-MEGA on cell viability in HaCaT cells under oxidative stress. To quantify the oxidative stress effects of H₂O₂, HaCaT cells were treated with H₂O₂ at various concentrations (0.1~1.5 mM) for 24 hr (Fig. 2A). Cell viability decreased 60% after 24 hr of treatment with 1.0 mM H₂O₂. Thus, 1.0 mM H₂O₂ was used in all subsequent experiments. Pretreatment with 7-MEGA for 1 hr prior to incubation with 1 mM H₂O₂ for 24 hr resulted in significantly increased viability compared with cells incubated with H₂O₂ alone. Especially, compared to using the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA showed a higher cell survival rate. This result shows that 7-MEGA had a higher protective effect against cell damage from oxidative stress when compared to PA (Fig. 2B).

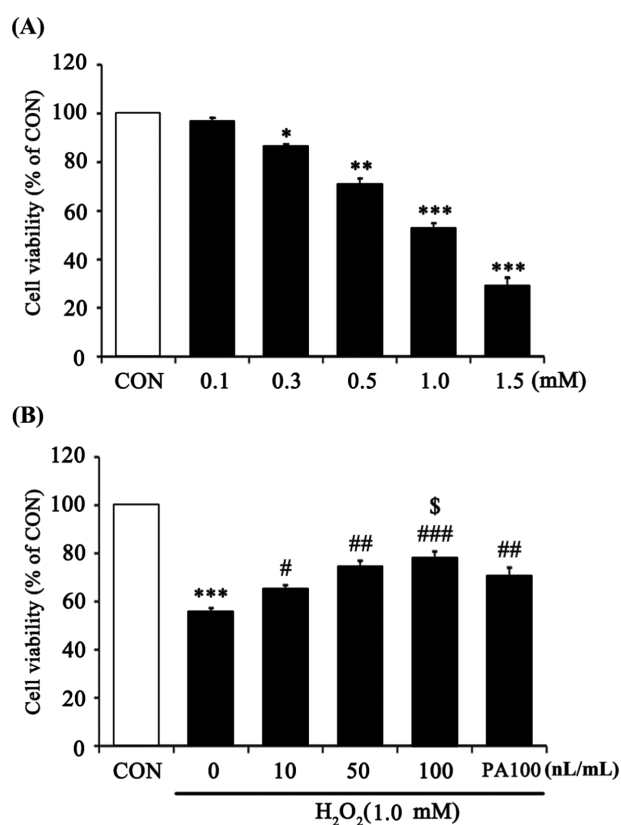


Fig. 2. Protective effect of 7-MEGA on cell viability against oxidative stress induced by H₂O₂ in HaCaT cells. Viability of HaCaT cells after treatment with increasing concentrations of H₂O₂ (0.1~1.5 mM) for 24 hr. (B) Viability of HaCaT cells after treatment with 1.0 mM H₂O₂ for 24 hr after pretreatment with 7-MEGA (10~100 nL/mL) for 1 hr. Data are expressed as the mean \pm SEM of three independent experiments, * p < 0.05, ** p < 0.01, *** p < 0.001 vs CON, # p < 0.05, ## p < 0.01, ### p < 0.001 vs. H₂O₂, § p < 0.05 vs. PA100.

Anti-oxidative effect of 7-MEGA in HaCaT cells under oxidative stress. To confirm the anti-oxidant capacity of 7-MEGA to eliminate oxidative stress, ROS, SOD, and GSH were measured in HaCaT cells treated with various concentrations of 7-MEGA and 1.0 mM H₂O₂. ROS are a

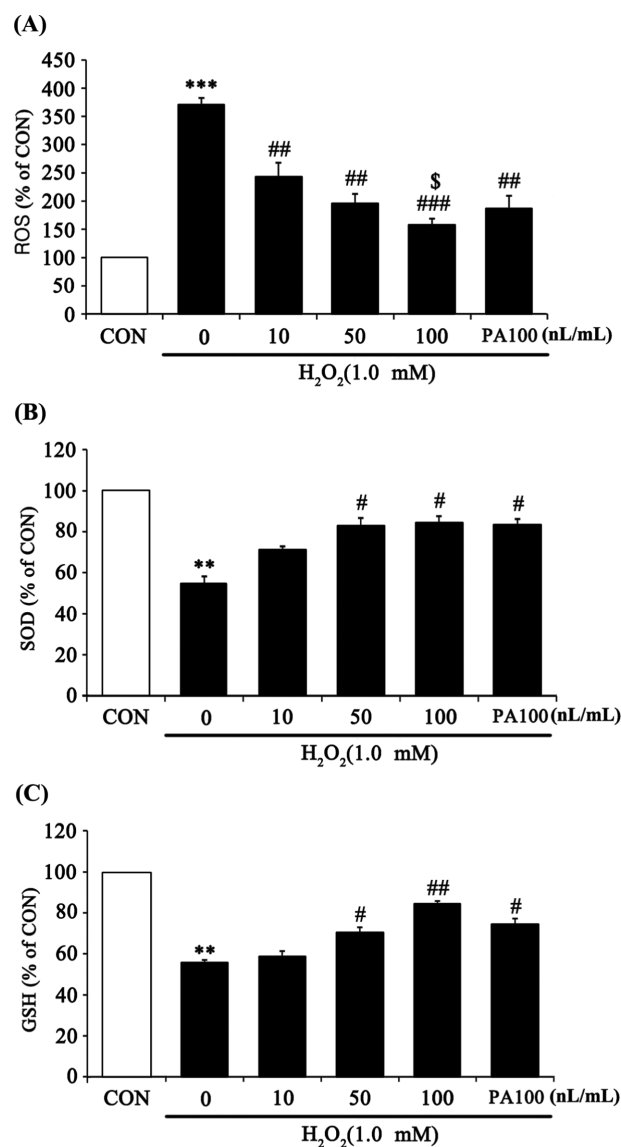


Fig. 3. Effect of 7-MEGA on reactive oxygen species (ROS) generation and anti-oxidative activity (SOD, GSH) in H₂O₂-treated HaCaT cells. (A) HaCaT cells were pretreated with 7-MEGA (10~100 nL/mL) for 1 hr, then oxidative stress was induced using H₂O₂ (1.0 mM) for 5 min. ROS generation was evaluated by DCF-DA. (B-C) HaCaT cells were pretreated with 7-MEGA (10~100 nL/mL) for 1 hr, then oxidative stress was induced using H₂O₂ (1.0 mM) for 24 hr. SOD and GSH expression were measured in cell lysates by ELISA. Data are expressed as the mean \pm SEM of three independent experiments, ** p < 0.01, *** p < 0.001 vs. CON, # p < 0.05, ## p < 0.01, ### p < 0.001 vs. H₂O₂, § p < 0.05 vs. PA100.

highly active intermediate product of oxygen molecules that are incompletely reduced during respiration (3). When reacting with surrounding biomolecules, such as lipids, nucleic acids and proteins, ROS interrupt normal cellular function, thus inhibiting skin regeneration and promoting skin aging (14). From the experimental results, ROS production significantly increased after H_2O_2 treatment compared with untreated controls. However, ROS production in 7-MEGA and H_2O_2 co-treated cells significantly decreased in a dose-dependent manner compared with cells treated with H_2O_2 alone. Especially, compared to cells treated with the same concentration (100 nL/mL) of PA, those treated with 7-MEGA showed a significant decrease in ROS production (Fig. 3A). SOD is an enzyme that catalyzes the process of defending cells from oxidative toxicity by catalyzing a disproportionation reaction that converts excess oxidizing ions into oxygen and hydrogen peroxide in the first step of anti-oxidation (15). GSH is an enzyme that prevents peroxidative damage in organisms by catalyzing the reaction which produces oxidized glutathione and water from H_2O_2 and reduced glutathione (16). In this study, SOD and GSH production in cells treated with H_2O_2 alone were significantly decreased compared to untreated control

cells. On the other hand, cells co-treated with 7-MEGA and H_2O_2 showed a significant increase in the production of these two enzymes in a dose-dependent manner compared with cells treated with H_2O_2 alone. When compared to cells treated with the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA didn't show significantly increased SOD or GSH production (Fig. 3B, 3C).

Anti-inflammatory effect of 7-MEGA in HaCaT cells under oxidative stress.

To assess the anti-inflammatory effect of 7-MEGA, pro-inflammatory factors were measured in HaCaT cells treated with various concentrations of 7-MEGA and 1.0 mM H_2O_2 . An inflammatory reaction occurs when there is too much active oxygen in the body (17,18). TNF- α and IL-1 β are representative inflammatory cytokines, and act in the early stage of the inflammatory reaction. These cytokines are produced by activation of COX-2. COX-2 is an enzyme that catalyzes the production of PGE_2 and plays a major role in controlling the inflammatory reaction, cell proliferation and necrosis, and cytokine generation (19,20). In this experiment, COX-2 and PGE_2 expression in cells treated with H_2O_2 alone were significantly increased, whereas cells co-

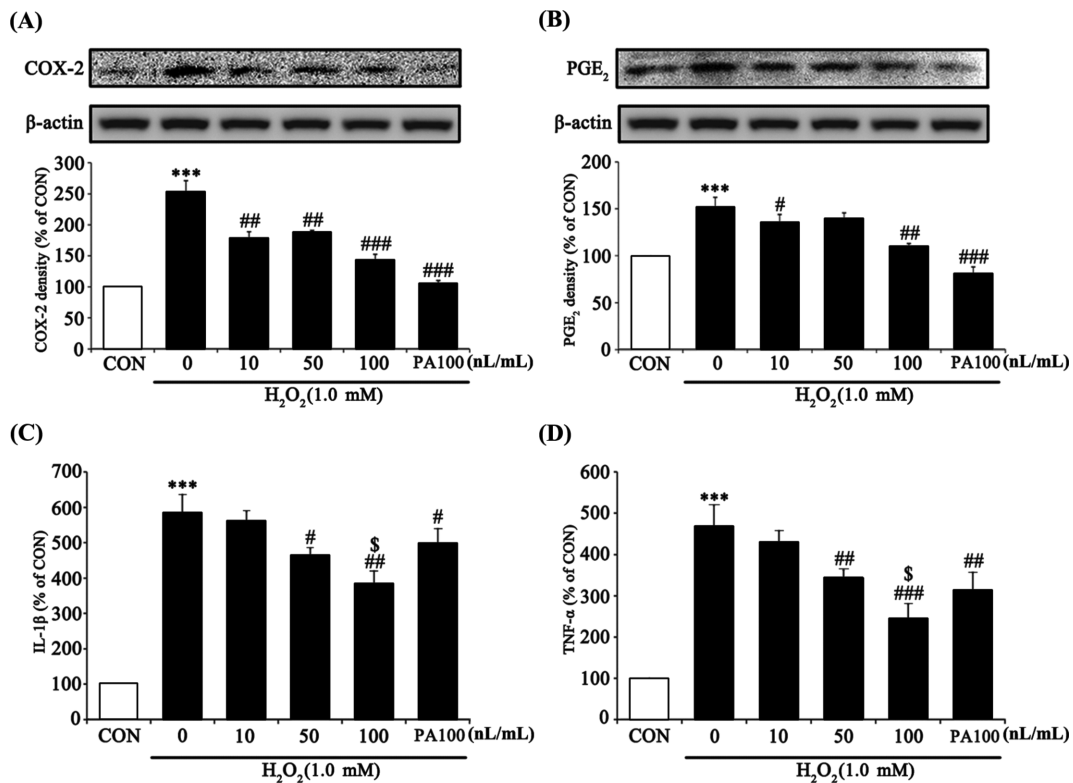


Fig. 4. Effect of 7-MEGA on the protein expression of pro-inflammatory markers (TNF- α , IL-1 β), COX-2, and PGE_2 in H_2O_2 -treated HaCaT cells. HaCaT cells were pretreated with 7-MEGA (10~100 nL/mL) for 1 hr, then oxidative stress was induced using H_2O_2 (1.0 mM) for 24 hr. (A-B) Whole cell lysates were subjected to Western blot analysis to evaluate COX-2 and PGE_2 expression. (C-D) IL-1 β and TNF- α were measured in the culture supernatant by ELISA. Data are expressed as the mean \pm SEM of three independent experiments, *** p < 0.001 vs. CON, * p < 0.05, ## p < 0.01, ### p < 0.001, vs. H_2O_2 , § p < 0.05 vs. PA100.

treated with 7-MEGA and H_2O_2 exhibited significantly decreased expression of these factors in a dose-dependent manner compared with the cells treated with H_2O_2 alone. When compared to cells treated with the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA didn't show significantly different COX-2 and PGE_2 expression (Fig. 4A, 4B). IL-1 β and TNF- α generation in cells treated with H_2O_2 alone were significantly increased, whereas cells co-treated with 7-MEGA and H_2O_2 showed significantly decreased expression of these two pro-inflammatory cytokines in a dose-dependent manner compared to cells treated with H_2O_2 alone. Especially, when compared to cells treated with the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA showed significantly decreased IL-1 β and TNF- α generation (Fig. 4C, 4D). Together, these results demonstrate that 7-MEGA induced strong anti-inflammatory activity in HaCaT cells.

Skin regenerative effect of 7-MEGA in HaCaT cells under oxidative stress. To evaluate the skin regeneration effect of 7-MEGA, MMP-1, PCOL1 and Elastin were measured in HaCaT cells treated with various concentrations of 7-MEGA and 1.0 mM H_2O_2 . Collagen is one of the main constituents of connective tissue, and a balance between the activity of PCOL1, a synthesizing enzyme, and MMP-1, a catabolic enzyme, is maintained in skin cells (21,22). An imbalance in the activity of both of these enzymes leads to a decrease in collagen and elastin production, followed by the formation of wrinkles and a reduction in skin regeneration (23-27). In this experiment, the protein expression of skin regeneration factors was measured to examine how 7-MEGA impacts the H_2O_2 -induced oxidative stress response. According to the results, MMP-1 expression in cells treated with H_2O_2 alone was significantly increased, whereas co-treatment with 7-MEGA and H_2O_2 significantly decreased the expression of MMP-1 in a dose-dependent manner when compared to treatment with H_2O_2 alone. When compared to cells treated with the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA didn't exhibit significantly decreased MMP-1 expression (Fig. 5A). PCOL1 and Elastin expression in cells treated with H_2O_2 alone were significantly decreased, whereas in cells co-treated with 7-MEGA and H_2O_2 there was significantly increased expression of these markers in a dose-dependent manner compared with the cells treated with H_2O_2 alone. Especially, when compared to cells treated with the same concentration (100 nL/mL) of PA, cells treated with 7-MEGA showed significantly increased PCOL1 expression. However, there was no significant difference in Elastin expression between these two cell populations (Fig. 5B, 5C). The ability of MMP-1 to regulate collagen synthesis was reduced by oxidative stress induced by H_2O_2 . Pretreatment with 7-MEGA inhibited collagen degradation caused by MMP-1 downregulation, increased

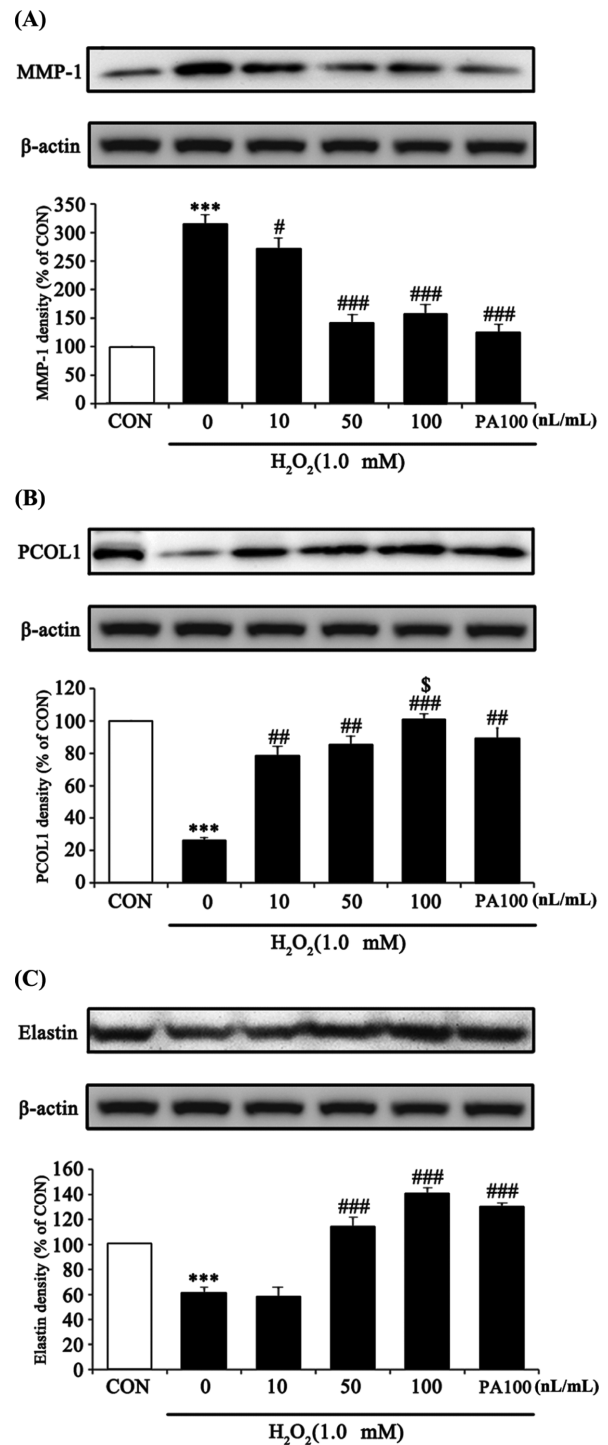


Fig. 5. Effect of 7-MEGA on MMP-1, procollagen type 1, Elastin protein expression in H_2O_2 -treated HaCaT cells. HaCaT cells were pretreated with 7-MEGA (10~100 nL/mL) for 1 hr, then oxidative stress was induced using H_2O_2 (1.0 mM) for 24 hr. (A-C) Whole cell lysates were subjected to Western blot analysis to evaluate MMP-1, PCOL1 and Elastin expression. Data are expressed as the mean \pm SEM of three independent experiments, *** p < 0.001 vs. CON, # p < 0.05, ## p < 0.01, ### p < 0.001, vs. H_2O_2 , § p < 0.05 vs. PA100.

collagen synthesis by increasing PCOL1 expression, and increased skin elasticity through an increase in Elastin. Thus, 7-MEGA can be used to improve the functionality of skin as a protective barrier, as well as to improve wrinkles and prevent skin aging.

In conclusion, our results suggest that 7-MEGA has anti-inflammatory effects in HaCaT cells, where it promotes collagen regeneration in the presence of H₂O₂-induced cytotoxicity. In addition, present study was showed that 7-MEGA has higher DPPH free radical scavenging activity and protective effect against cell damage from oxidative stress than PA. We could have expected this result that 7-MEGA, which contains various substances including omega-7, is more effective than omega-7 as a single substance. Our results provide strong evidence for 7-MEGA as a functional food for promoting skin health to prevent aging.

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