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Juglans mandshurica leaf extract protects skin fibroblasts from damage by regulating the oxidative defense system

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ABSTRACT

Skin is mainly damaged by genetic and environmental factors such as ultraviolet light, xenobiotics, hormonal changes, heat, and smoking. ROS production is commonly involved in the pathogenesis of skin damage induced by these factors, causing skin aging, including wrinkling, by activating the metalloproteinases (MMP-1) that break down type I collagen (COL1A1). The walnut tree *Juglans mandshurica* MAX. (JM) is found in China, Siberia and Korea. JM has been reported to have various pharmacological activities, such as anti-tumor, anti-oxidative, and anti-bacterial effects. In the present study, we investigated the protective effect of JM leaf extract (JME) against oxidative stress in HS68 human skin fibroblasts. JME significantly and dose-dependently protected HS68 cells against H_2O_2-induced damage, as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase assay. Other assays demonstrated that JME protected HS68 cells by regulating ROS production and increasing levels of glutathione, heme oxygenase-1, and activated NF-E2-related factor 2. JME additionally prevented the elevation of MMP-1 and reduction of COL1A1 induced by H_2O_2. It also inhibited H_2O_2-induced phosphorylation of ERK, p38, and JNK. These results indicate that JME protects human skin fibroblasts from H_2O_2-induced damage by regulating the oxidative defense system.

1. Introduction

Skin aging is commonly characterized by wrinkling, sagging, and laxity and is influenced by several factors, including genetic and environmental factors such as ultraviolet (UV) light, xenobiotics, and hormonal changes [1]. Histological studies have revealed that the major changes in aged skin occur in the dermis, which is predominantly composed of type I and III collagens, elastin, proteoglycans, fibronectin, and other extracellular matrix (ECM) proteins [1,2]. Because collagen and elastin are responsible for the strength and resilience of skin, their disarrangement with age causes skin to appear aged [3].

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen [4]. They are formed as natural byproducts of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis [4,5]. However, during times of environmental stress, ROS levels can increase dramatically [5]. This may result in significant damage to cell structures [6]. Additionally, previous studies reported that excessive ECM degradation by UV-induced collagenase secreted by various cells, including keratinocytes, fibroblasts, and inflammatory cells, contributes substantially to the connective tissue damage that is responsible for wrinkle formation in aged skin [7,8]. Generally, the skin defends itself against ROS-induced damage with enzymes such as superoxide dismutases, catalases, lactoperoxidases, glutathione peroxidases and peroxiredoxins [9,10]. Small-molecule antioxidants such as ascorbic acid (vitamin C), tocopherol, uric acid, and glutathione also play important roles as cellular antioxidants [11,12]. Recently, the transcription factor nuclear factor erythroid-derived 2-related factor 2 (Nrf2) was reported to play a key role in the regulation of oxidative stress response systems in the skin [13]. Nrf2 is normally only located in the cytoplasm, where it binds to Kelch-like ECH-associated protein 1 (Keap1) [14]. Under oxidative stress, oxidative modification of Keap1 allows Nrf2 to be released from Keap1 and to translocate to the nucleus, where it binds to antioxidant response elements and potentiates the expression of multiple genes that protect cells against oxidative stress, including those encoding heme oxgenase-1 (HO-1), glutathione (GSH), and thioredoxin [15,16].

The Manchurian walnut, *Juglans mandshurica* MAX. (JM), is a fast-growing deciduous tree that is widely distributed in China, Siberia and Korea [17]. It has been used as a folk medicine plant for the treatment of esophageal, gastric, cardiac, and lung cancer

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[17–19]. JM is known to contain juglone, β-sitosterol, naphthoquinones, naphthalenyl glycosides, α-tertalonyl glucopyranosides, flavonoids, and diarylheptanoyl glucopyranosides [20]. Additionally, JM, has been shown to have anti-tumor, anti-oxidative, antibacterial, and anti-inflammatory effects [19,21,22]. However, it is not known whether matrix metalloproteinase-1 (MMP-1) expression in fibroblasts is related to the skin antioxidant defense system.

In this study, we evaluated the effects of JM leaf extract (JME) on H_2O_2-induced toxicity in HS68 human skin fibroblasts, and the possible mechanisms underlying its effects, by performing 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, and by measuring levels of intracellular ROS, microtubule-affinity-regulating-kinases (MARKs), oxidative defense proteins such as Nrf2, GSH, and HO-1, and skin aging-related proteins such as MMP-1 and type I collagen (COL1A1).

2. Materials

2.1. Materials and methods

2.1.1. Chemical

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Hyclone laboratories. Inc. (Logan, UT). MTT, dimethylsulfoxide (DMSO), and 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, USA). GSH quantitation kit was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Tetramethylethylenediamine, protein standards dual color, western view marker, protein assay, Tween-20, acrylamide, ammonium persulfate, skim milk, and ECL reagent were purchase from Bio-Rad Laboratories (Hercules, USA). Nuclear/cytosol fraction kit was purchased from BioVision (Mountain View, USA). Rabbit anti-Nrf2 was obtained from Abcam (Cambridge, UK). Rabbit anti-HO-1 was obtained from assay designs (Ann Arbor, USA). Rabbit anti-extracellular signal-regulated kinases (ERK), phosphorylation (p)-ERK, p38 mitogen-activated protein kinases (p38), pp38, c-Jun N-terminal kinases (JNK), and pJNK were obtained from cell signaling (Beverly, MA). Mouse anti-β-actin was obtained from Santa Cruz (Santa Cruz, CA). Anti-rabbit and mouse-horseradish peroxidase (HRP) secondary antibody was purchased from Assay Designs (Ann Arbor, USA). The other reagents used were of guaranteed or analytical grade.

2.2. Preparation of the JME

Fresh JM was obtained from a producing district (Gangwon, Korea) and a voucher specimen (KHUOPS-GCH003) was deposited in the herbarium at the College of Pharmacy, Kyung Hee University (Seoul, Korea). 100 g of JM leaf were ground with 1 L of 70% ethanol for 24 h at room temperature. Then, the extract was filtered, evaporated on a rotary vacuum evaporator, and lyophilized (yield; 5.15%). The powder (JME) was kept at 4 °C before use.

2.3. Cell culture

The HS68 cell line, human skin fibroblast cell, was obtained from the American Type Culture Collection (ATCC; Rockville, USA). Cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in condition of 95% air and 5% CO_2 at 37 °C. All experiments were carried out 12 h after cells had been seeded in 96- and 24-well plates, at densities of 1×10^4 and 2×10^4 cells/well, respectively.

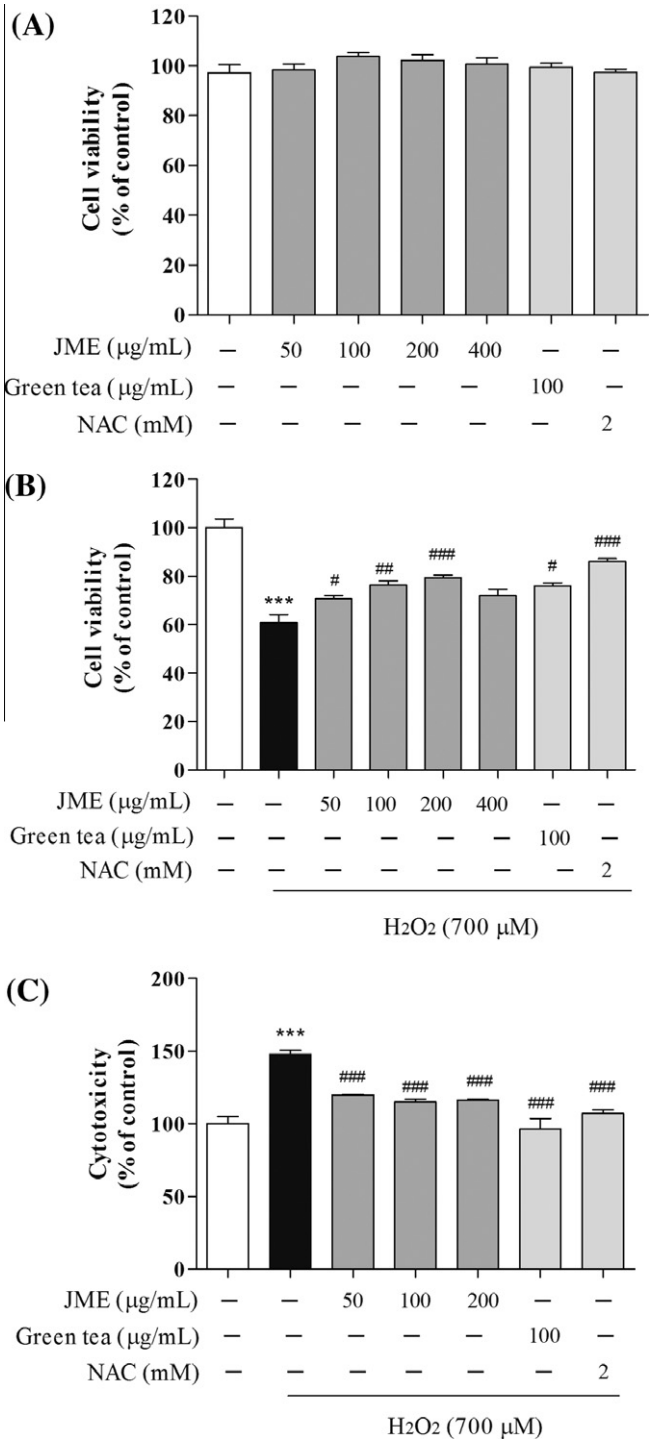

Fig. 1. Effects of JME on H_2O_2-induced toxicity in HS68 cells. After cells reached confluence, they were treated with JME, NAC, or green tea extract for 1 h and incubated without (A) or with 700 μM H_2O_2 (B) for a further 6 h. Cytotoxicity was determined based on the level of LDH (C). Cell viability and cytotoxicity are expressed as percentages of controls. Values are the mean ± S.E.M. $^{***}p < 0.001$ vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 alone group.

2.4. Measuring cell viability

Cell viability was measured using the MTT assay. HS68 cells were seeded on 96-well plates and treated with JME at doses of 50, 100, 200, and 400 μg/mL or for 7 h or pretreated with JME for 1 h, and then stimulated with 700 μM H_2O_2 for an additional 6 h. The treated cells were incubated with 1 mg/mL MTT for 2 h. The

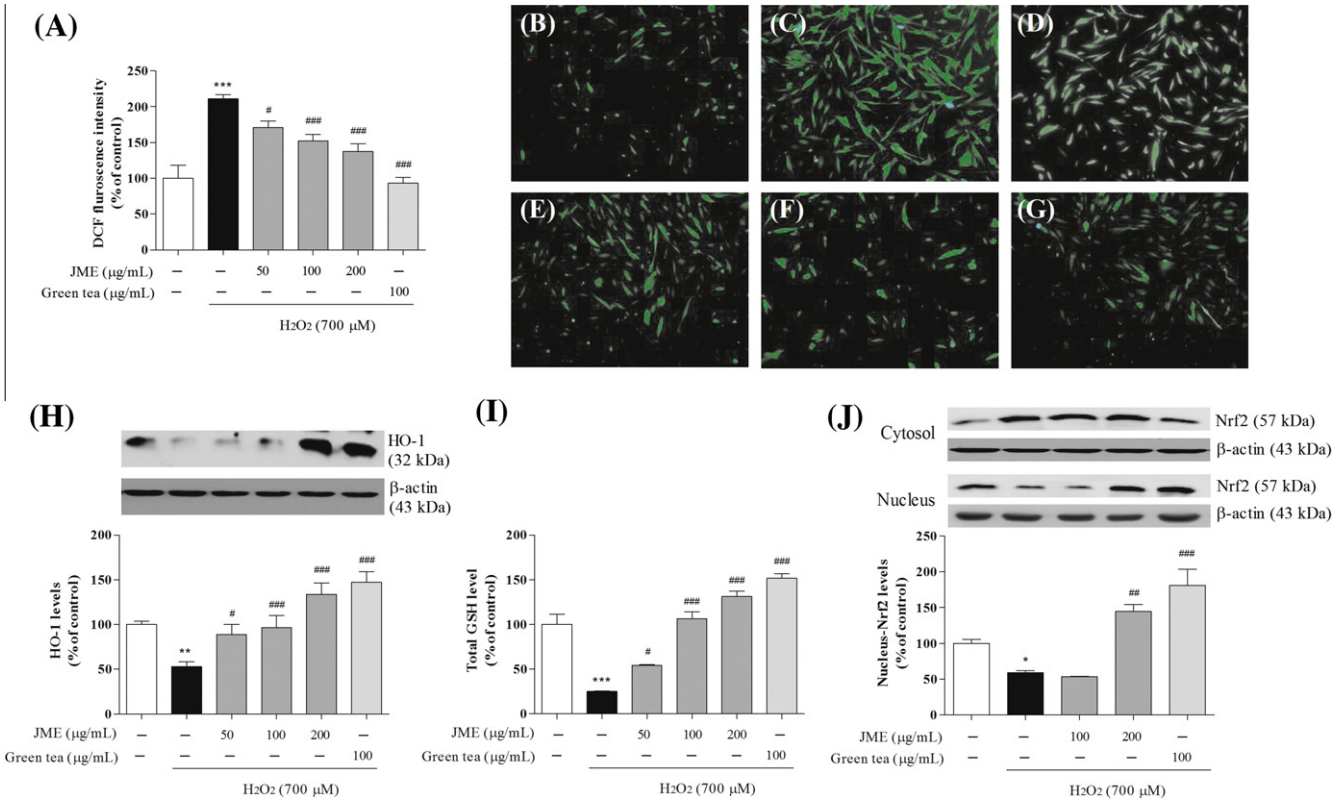

Fig. 2. Effect of JME on H_2O_2-induced ROS generation. Cells were treated with JME for 1 h and then stimulated with 700 μM H_2O_2. ROS generation was measured by measuring the fluorescence intensity of DCF-DA after stimulation with H_2O_2 for 30 min (A). Representative images for the control group (B), H_2O_2 alone group (C), H_2O_2 + 100 μg/mL green tea extract group (D), H_2O_2 + 50 μg/mL JME group (E), H_2O_2 + 100 μg/mL JME group (F), and H_2O_2 + 200 μg/mL JME group (G). Then, effect of JME on ROS-regulatory proteins in HS68 cells. Cells were treated with JME (50, 100, or 200 μg/mL) or green tea extract (100 μg/mL) for 1 h, and were then stimulated with 700 μM H_2O_2 for an additional 6 h. HO-1 (H), GSH (I), and Nrf2 translocation (J) were measured. Scale bar = 50 μm. The values are the mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.01$ vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 alone group.

MTT medium was aspirated carefully from the wells, and the formazan dye was eluted using DMSO. The absorbance was measured using a spectrophotometer (Versamax microplate reader; Molecular Device, Sunnyvale, CA, USA) at a wavelength of 570 nm and was expressed as a percent of the value for the control.

2.5. Measurement of cytotoxicity

Release of LDH was determined using a CytoScanTM LDH-cytotoxicity assay kit, according to the instruction manual (Bioworld, USA). Briefly, the supernatants (100 μL) were centrifuged, transferred, and reacted with 100 μL of the mixture of dye solution in the dark for 30 min. Absorbance at 490 nm was measured.

2.6. Measuring intracellular ROS

Intracellular ROS generation was measured using DCFH-DA fluorescence dye. DCFH-DA enters cells passively and is converted into non-fluorescent DCFH, which reacts with ROS to form the fluorescent product DCF. HS68 cells were seeded onto coverslips in 24-well plates and treated with JME at doses of 50, 100, and 200 μg/mL for 1 h. Then, they were stimulated with 700 μM H_2O_2 for an additional 30 min. The cells were incubated with 25 μM DCFH-DA for 30 min. The fluorescence intensity was determined at 485 nm excitation and 535 nm emission using a fluorescence microplate reader (SpectraMax Gemini EM, Molecular Device; Sunnyvale, CA, USA). Representative images were taken using a fluorescence microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan).

2.7. Measuring the MAPKs, Nrf2, HO-1, and COL1A1 levels

The cells were seeded on a 100 mm dish and treated with JME at doses of 100 and 200 μg/mL for 1 h. Then, they were stimulated with 700 μM H_2O_2 for an additional 6 h. The cells were lysed with protein extraction buffer for whole protein. Nuclear and cytosol were lysed with nuclear/cytosolic fraction kit according to the manufacturer's protocol. Cell lysates were separated on 15% or 10% SDS–polyacrylamide gel electrophoresis, and then separated proteins were electrophoretically transferred to a membrane. The membranes were incubated with 5% skim milk in TBST (25 mM tris–Cl, 150 mM NaCl, 0.005% Tween-20) for 45 min. Then they were incubated with rabbit anti- ERK, pERK, p38, pp38, JNK, pJNK, Nrf2, HO-1, and COL1A1 (1:500–1000 dilution) and mouse anti-β-actin (1:2000 dilution) primary antibody overnight at 4 °C, followed by incubated with HRP-conjugated anti-rabbit IgG for 1 h, respectively. Immunoreactive bands were detected using an ECL detection kit and visualized using the LAS-4000 mini system (Fujifilm corp., Japan).

2.8. Measuring the total GSH level

Total GSH levels were determined using a total glutathione quantitation kit, according to the instruction manual (Dojindo molecular technologies, Japan). Briefly, cells were seeded on a 100 mm dish and treated with JME at doses of 100 and 200 μg/mL for 1 h. Then, they were stimulated with 700 μM H_2O_2 for an additional 6 h. The treated cells were lysed in 10 mM hydrochloric acid solution by freezing and thawing. Then, they were treated

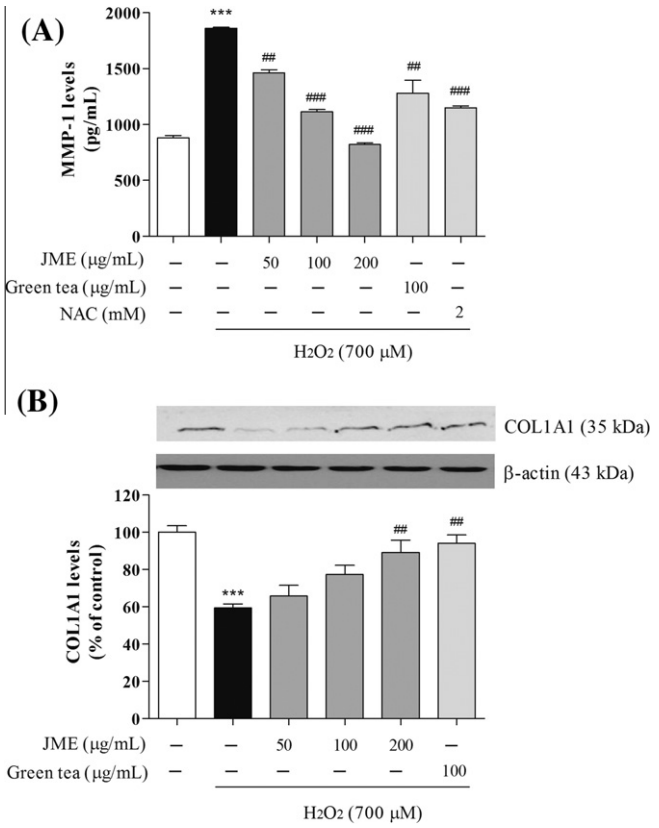

Fig. 3. Effect of JME on MMP-1 and collagen type I levels in HS68 cells. Cells were treated with JME (50, 100, or 200 μg/mL) or green tea extract (100 μg/mL) for 1 h, and were then stimulated with 700 μM H_2O_2 for an additional 6 h. MMP-1 levels (A) were measured using a kit and the collagen type I levels (B) were assessed by Western blotting. The values are the mean ± S.E.M. $^{**}p < 0.01$, $^{***}p < 0.01$ vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 alone group.

with 5% 5-sulfosalicyclic acid. After centrifugation at 8000 × *g* for 10 min at 4 °C, the supernatant was used to assess the GSH level. A co-enzyme working solution, buffer solution, and enzyme working solution were added to the wells containing the supernatant incubated at 37 °C for 5 min. Then, GSH standard, sample, and substrate working solutions were added for 10 min each. The absorbance was measured using a spectrophotometer at a wavelength of 405 nm, and the GSH concentrations were determined using a GSH standard calibration curve.

2.9. Measuring the MMP-1 level

MMP-1 levels were measured using a Human MMP-1 ELISA kit (RayBiotech, USA) according to the instruction manual. Briefly, cells were seeded on a 100 mm dish and treated with JME at doses of 50, 100, and 200 μg/mL for 1 h. They were then stimulated with 700 μM H_2O_2 for an additional 6 h. Then, 100 μL of supernatant or standard was added to each well and incubated overnight at 4 °C. Each well was then treated with biotin antibody for 1 h, and with streptavidin solution and TMB One-Step Substrate Reagent for 30 min at room temperature. Then, 50 μL of stop solution was added to each well. Absorbances at 450 nm were then measured using a spectrophotometer, and MMP-1 concentrations were determined using a standard curve.

2.10. Statistical analysis

All statistical parameters were calculated using Graphpad Prism 4.0 software. Values were expressed as the mean ± standard error of the mean (S.E.M.). The results were analyzed by one-way analysis of variance. Differences with a *p*-value less than 0.05 were considered statistically significant.

3. Results and discussion

In this study, we evaluated the protective effects of JME against H_2O_2-induced stress in HS68 human skin fibroblasts by measuring oxidative defense-related proteins and skin aging-related proteins. Then, to identify the possible mechanisms involved, we measured MAPK protein expression.

First, to investigate the effect of JME on H_2O_2-induced cell toxicity, we measured cell viability and cytotoxicity using MTT and LDH assays, respectively. MTT, commonly used to measure cell viability, produces purple formazan in response to mitochondrial enzymes in live cells; LDH is produced as a result of cell breakdown [23]. The results showed that treatment with 50–200 μg/mL JME or 2 mM NAC alone had no effect on the cells (Fig. 1A). Treatment with 1 mM H_2O_2 for an additional 6 h reduced cell viability to 60.77 ± 3.60%, as compared to control cells. Pre-treatment with JME limited this reduction in cell viability. Cell viability in cells pretreated with 50–200 μg/mL JME was 70.61 ± 1.47–79.33 ± 1.93% of control values. In this regard, 200 μg/mL JME had an effect similar to those of 100 μg/mL green tea and 2 mM (positive controls). (Fig. 1B). Pre-treatment with 50–200 μg/mL JME also reduced H_2O_2-triggered LDH release (Fig. 1C). These results

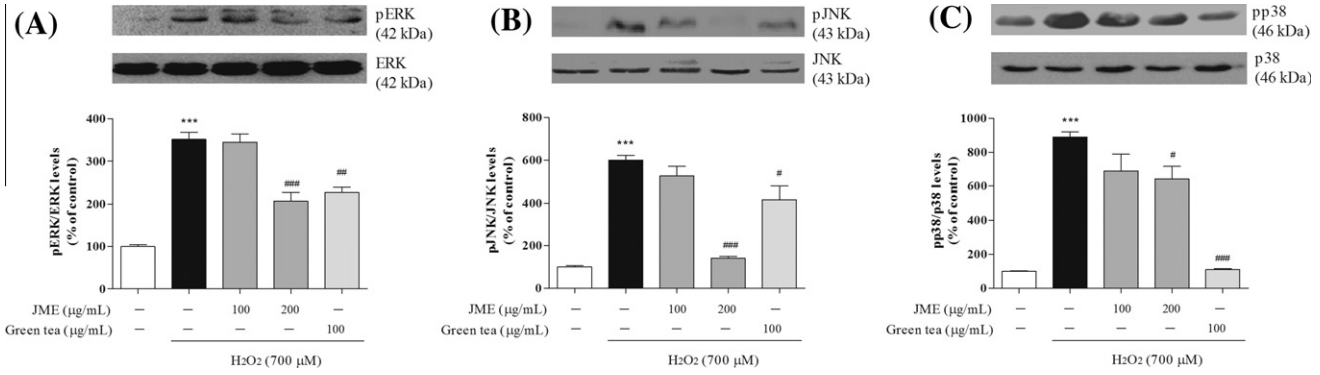

Fig. 4. Effect of JME on H_2O_2-induced MAPK levels in HS68 cells. Cells were treated with JME (100 or 200 μg/mL) or green tea extract (100 μg/mL) for 1 h, and were then stimulated with 700 μM H_2O_2 for an additional 1 h. Effect of JME on H_2O_2-induced MAPKs levels in HS68 cells. Cells were treated with JME (100 or 200 μg/mL) or green tea extract (100 μg/mL) for 1 h, and were then stimulated with 700 μM H_2O_2 for an additional 1 h. For MAPKs detection, phospho form of ERK, JNK, and p38 (pERK, pJNK, and pp38) and total form of ERK, p38, and JNK antibodies were used in whole protein lysate. Levels of pERK, pJNK, and pp38 were normalized to ERK, JNK, and p38, respectively. The representative band images of experiments are shown. The values are the mean ± S.E.M. $^{***}p < 0.001$ vs. the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the H_2O_2 alone group.

indicate that JME exhibits protective effects against H_2O_2 in HS68 cells.

Next, to examine whether the protective effect of JME against H_2O_2-induced cell toxicity is related to antioxidant effects, we assessed intracellular ROS generation using DCFH-DA. ROS are potential inducers of skin aging-related protein and cause intracellular oxidative damage in human skin fibroblasts [24]. In this study, treatment with 1 mM H_2O_2 for 30 min significantly increased ROS generation 2.11-fold compared with control cells, while treatment with 50, 100, and 200 μg/mL JME reduced H_2O_2-induced ROS generation 1.70-, 1.52-, and 1.37-fold, respectively. The effect of 200 μg/mL JME was similar to that of 100 μg/mL green tea (Fig. 2A-G). These results indicate that JME protected HS68 cells from the H_2O_2-induced ROS.

Next, to investigate whether JME affected the ROS-regulatory enzyme system, we measured levels of HO-1 and GSH, which repair or protect cells by reducing the skin damage caused by superoxide, the most common free radical [25,26]. In this study, H_2O_2 caused significant HO-1 and GSH depletion (by 53.16 ± 5.31% and 25.32 ± 0.34%, respectively), while JME increased HO-1 and GSH levels (by 88.96 ± 11.27–133.98 ± 12.65% and 54.34 ± 0.68–131.20 ± 6.34%, respectively) (Fig. 2H and I). These results suggest that JME protects HS68 cells from ROS by regulating the oxidative defense enzyme. Nrf2, a central transcription factor regulating anti-oxidative genes, is activated upon exposure to oxidative stress and translocates to the nucleus, where it interacts with antioxidant response elements to activate antioxidant-related gene transcription, resulting in HO-1 and GSH expression [15,27]. We investigated the effect of JME on Nrf2 translocation. The activation of Nrf2 protected skin cells and neuronal cells from H_2O_2-induced toxicity, and the loss of Nrf2 increased vulnerability to H_2O_2. As shown in Fig. 2J, treatment with 1 mM H_2O_2 significantly reduced nuclear Nrf2 protein levels compared with control cells (by 59.29 ± 2.97%), whereas JME at 200 μg/mL significantly attenuated nuclear Nrf2 protein levels (by 53.58 ± 0.58–181.14 ± 22.24%, respectively) (Fig. 2J).

Taken together, these results indicate that the reduction of ROS levels by JME is related to up-regulation of HO-1 and GSH levels via Nrf2 translocation.

To examine the anti-aging effects of JME on H_2O_2-damaged human skin fibroblasts, we measured cellular COL1A1 levels and MMP-1 secretion. MMPs are a family of structurally-related matrix-degrading enzymes that play important roles in various destructive processes, including skin aging [28]. Notably, MMP-1, also known as interstitial collagenase, initiates the degradation of collagen types I, II, and III in the skin [29]. Type I collagen is synthesized as procollagen type I, a soluble precursor secreted by fibroblasts when organizing the main ECM components [30]. H_2O_2 caused significant MMP-1 elevation and COL1A1 depletion (by 1860.83 ± 8.40% and 59.39 ± 2.21%, respectively), while JME attenuated MMP-1 and COL1A1 levels (by 1462.80 ± 25.58–821.58 ± 16.51% and 65.73 ± 5.83–89.03 ± 6.03%, respectively) (Fig. 3).

These results provide evidence that JME prevents the H_2O_2-induced elevation of MMP-1 levels and reduction in COL1A1 levels, which are strongly related to anti-wrinkling effects.

Finally, because MAPK-dependent pathways are involved in both Nrf2 activation and MMP-1 secretion, we measured MAPKs protein levels [31,32]. Phosphorylated MAPKs regulate ROS generation, Nrf2, phase 2 enzymes such as HO-1 and GSH, and MMP-1 secretion in skin fibroblast cells [33,34]. H_2O_2 caused significant activation of pERK, pJNK, and pp38 protein (by 351.96 ± 16.90%, 600.13 ± 23.41%, and 888.67 ± 32.61%, respectively), while JME at 200 μg/mL decreased levels of pERK, pJNK, and pp38 protein (by 345.21 ± 19.15–206.76 ± 21.23%, 525.51 ± 45.71–416.02 ± 63.97%, and 589.05 ± 18.18–111.94 ± 3.10%, respectively) (Fig. 4).

These observations suggest that the inhibitory effects of JME on MAPK activation may be due, partially, to its antioxidant properties.

In conclusion, the present study shows that JME protects against H_2O_2-induced skin cell damage by regulating antioxidant-related proteins. JME is well known to have free radical scavenging effects, and has been reported to protect against X-ray-induced toxicity by attenuating oxidative stress [35,36]. Furthermore, JME was previously reported to contain well-known antioxidants such as flavonoids, gallotannins, and β-sitosterol [36,37]. Therefore, antioxidant compounds in JME may contribute to the observed protection of skin from H_2O_2-induced stress. Further studies on the active compounds in JME are needed.

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