Handelin Reduces Ultraviolet A-Induced Photoaging by Inhibiting Reactive Oxygen Species Generation and Enhancing Autophagy

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Photoaging is mainly caused by the exposure of the skin to ultraviolet (UV) radiation. Among them, damage to human dermal fibroblast (HDF) cells caused by ultraviolet A (UVA) is the main cause of skin aging. Researchers have dedicated to identifying natural compounds from plants to fight against UV radiation-induced photoaging. We previously found that extracts from wild chrysanthemum could prevent acute damage and photoaging induced by UV irradiation. As one of the most abundant ingredients in wild chrysanthemum extract, handelin was hypothesized to have the potential to prevent UVA-induced photoaging of skin fibroblast. In the present study, we report the great potential of handelin in combating UVA-induced photoaging of fibroblasts. We firstly demonstrated that handelin was safe for skin fibroblast as high as a concentration of 0.0125 μM, showing no toxicity on the cells and improved cell viability. Furthermore, handelin can reduce UVA-induced cellular senescence, indicated by a reduced proportion of senescence-associated beta-galactose positive cells and the expression of P21. We then verified that handelin pretreatment markedly attenuated the production of reactive oxygen species (ROS) generation after UVA irradiation. Meanwhile, we found that handelin enhances autophagy after UVA irradiation, and autophagy is involved in the quality control of intracellular proteins after UV-induced damage (partially indirectly via ROS). Therefore, these results suggest that handelin has a very high potential as an effective ingredient against UVA-induced skin aging. Moreover, this provides an important basis for further research on the photoprotective mechanism of handelin.

Keywords: handelin; human dermal fibroblast cell; photoaging; ultraviolet A
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Introduction

Ultraviolet (UV) radiation is part of the electromagnetic radiation spectrum emitted by the sun, consisting of UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm) of different wavelengths. Oxygen and ozone in the atmosphere completely absorb UVC and approximately 90% of UVB; therefore, the components that are closely associated with human health are mainly UVA and UVB. The longer wavelengths of UVA penetrate the skin epidermis to reach the dermis and affect the dermal and epidermal skin structures (Battie et al. 2014), leading to the aging of skin tissues (Yaar and Gilchrest 2007). In addition to direct cellular damage, UVA induces the production of reactive oxygen species (ROS) in a large amount (Wondrak et al. 2006), which can further damage cellular DNA, proteins, lipids, and other macromolecules, aggravating cellular damage and aging (Gęgotek et al. 2020).

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To counteract the effects of UVA on cells, the body needs to activate autophagy to remove damaged cellular components and harmful substances. Autophagy is a crucial lysosomal degradation pathway with highly conserved properties. It is a process wherein phagocytes engulf harmful components or senescent cells to form autophagosomes, which are hydrolyzed in lysosomes to release macronutrients to nourish surrounding normal cells. Moreover, autophagy can occur in normal cells under physiological conditions and can be induced when cells are exposed to external stimuli (Ravikumar et al. 2010), promoting cell survival and adaptive responses under stressful conditions (Rubinsztein et al. 2011; Zhang et al. 2016).

To minimize UVA-induced skin damage, it is necessary to develop bioactive agents with anti-aging, anti-oxidant, and anti-inflammatory properties (Rubinsztein et al. 2012; Grether-Beck et al. 2014). Numerous studies have found some natural chemical components to prevent and treat skin diseases caused by UVB (El-Mahdy et al. 2008). Wild chrysanthemum, a traditional Chinese herb, is used by local people in some high-altitude areas exposed to high UV rays for the prevention and treatment of sunburn. Previous studies have found that its extracts have excellent anti-inflammatory effects; however, there are insufficient studies on the specific components of wild chrysanthemum and its mechanism of action. In this study, we aimed to investigate the effect of wild chrysanthemum lactone (handelin) in wild chrysanthemum extract on the cellular activity of human dermal fibroblast (HDF) cells and UVA-induced cellular photoaging and further explore its mechanism of action.

Materials and Methods

Cell culture

Primary HDF cells were obtained from the laboratory of the Department of Dermatology, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan Province, China. The cells were cultured in the complete Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin/amphotericin B (Biological Industries, Kibbutz Beit Haemek, Israel), in a cell culture incubator at 37°C and 5% CO_2. The medium was changed once every two to three days, and the cells were passaged when they reached 70%-80% confluence of the T25 cell culture flask. HDF cells in the logarithmic growth phase and well-grown condition were selected for the experiment.

Preparation of handelin

Handelin was purchased from Yuanye, Shanghai, China. Moreover, 10 mg of drug powder was dissolved in 1.4 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) at a stock drug concentration of 12.8 mmol/L, and dispensed and stored at −20°C. The maximum final concentration of the drug in this study was 1.6 μM, and the volume percentage of DMSO in the culture medium was 0.1%. The effect of DMSO concentration on the cells under this experimental condition was either extremely low or negligible; therefore, a blank control was used as the experimental control group (Qi et al. 2008).

Experimental grouping and UVA irradiation

This study included the control and experimental groups. The control group was untreated. The experimental group was divided into the handelin-treated group, the UVA-exposed group, and the group that was pretreated with handelin before the UVA exposure.

UVA irradiation was performed using a UV light therapy instrument SS-04A (Sigma, Shanghai, China) at an irradiation distance of 25 cm, and the irradiation dose was monitored with the UVA irradiance monitor (Sigma). The irradiation intensity of 4 J/cm^2 for 16 min 40 s is based on the results of the previous experiment (Tu et al. 2020), using the following formula: UV intensity = irradiation time × UV energy. The medium was removed and covered with a thin layer of phosphate-buffered saline (PBS; Gibco) before irradiation to avoid cell drying. After irradiation, the next experiment was performed according to the experimental needs or the DMEM medium was changed, and the experiment was continued for 24 h.

Cellular viability assays

Cell counting kit 8 (CCK8; Proteintech, Wuhan, Hubei, China) contains the monosodium salt of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazole, which is reduced by dehydrogenase in the cell mitochondria to a highly water-soluble orange-colored methanogenic substance in the presence of an electron carrier. The amount of methanogenic substances produced is proportional to the number of living cells.

HDF cells were inoculated in 96-well plates at a density of 4 × 10^3 cells/well and incubated overnight. After the cells were completely attached, different concentrations of handelin (0.0015625, 0.003125, 0.00625, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 μM) were added. Three replicate wells were set up for each concentration. After 24 h, the medium was removed, and 100 μL of serum-free medium containing CCK8 (1:10 volume ratio) was added to each well and incubated in a cell incubator for 1 h. The absorbance of each well was measured at 450 nm with an enzyme marker to calculate the cell viability and screen the concentration required for subsequent experiments. The calculation formula was as follows.

Cell viability = [(absorbance of experimental wells − absorbance of blank wells) / (absorbance of control wells − absorbance of blank wells)] × 100%.

Counting senescent cells

SA-X-GAL is the specific senescence-associated beta-galactosidase produced by senescent cells to produce a dark

blue product. This product reflects cellular senescence.

HDFs were inoculated in 6-well plates at a density of 1×10^5 cells/well and incubated overnight. After the cells had fully adhered to the wall, 1,000 μL of handelin at a concentration of 0.0125 μM was added for 24 h. The cells were then irradiated by UVA; after the irradiation, the medium was changed and incubated for 24 h. The cell culture medium was aspirated and washed once with PBS, and 1 mL of fixative was added to each well and fixed at room temperature for 15 min. After aspirating the fixative, the cells were washed three times with PBS for 3 min each time. Moreover, 1 mL of SA-β-GAL staining solution (Solarbio, Beijing, China) was added to each well after aspirating PBS, and cultured overnight in an incubator at 37°C. The cells were observed and photographed the next day using a light microscope at 10 × 10.

Measurement of ROS level

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a fluorescent probe that can freely cross cell membranes and is hydrolyzed by an esterase inside cells to produce DCFH that cannot pass through cell membranes, thus loading the probe into cells. The intracellular ROS can oxidize DCFH to generate fluorescent dichlorofluorescein (DCF), and the level of intracellular ROS can be indicated by detecting the fluorescence intensity of DCF.

HDF cells were inoculated in 6-well plates at a density of 1×10^5 cells/well and incubated overnight, and then treated with 1,000 μL of handelin at a concentration of 0.0125 μM for 24 h after the cells were completely attached to the cell walls, followed by UVA irradiation. After the irradiation, PBS was aspirated, and 100 μL of serum-free medium containing DCFH-DA (Solarbio) was added to each well; DCFH-DA was diluted at 1:1,000 with serum-free medium to a concentration of 10 μM and incubated in a cell incubator for 20 min. The cells were washed thrice with serum-free medium, observed, and photographed at 10 × 10 magnification using a fluorescence microscope.

Staining of intracellular autophagosomes

HDF cells were seeded into 24-well plates at a density of 3×10^5 cells/well overnight. The tandem fluorescent GFP-LC3 adenoviral vectors (Ad-tf-LC3) were purchased from HanbioCo (HanbioCo. Ltd, Shanghai, China). Cells cultured on coverslips were transduced with Ad-tf-LC3 at 100 MOI for 6 h. The transfection should be continued 48 h after adenovirus transduction. Then, the cells were treated with different concentrations of handelin and UVA irradiation. Next, the cells were washed with PBS, fixed with 4% paraformaldehyde for 20 min, mounted with a reagent, and viewed with confocal microscopy (Nikon, Tokyo, Japan). Autophagy was determined by analyzing the formation of fluorescence dots of autophagosomes in cells.

Determination of protein expression

HDF cells were inoculated in 3-cm flat dishes and cultured until cells were 50%-60% fused. To evaluate the relationship between the drug concentration and the protein of interest, the cells were treated with handelin at a concentration of 0.0125 and 0.025 μM for 24 h, followed by UVA irradiation. After irradiation, the medium was changed, and the cells were further cultured for 24 h. The cells were collected and lysed on ice for 20 min by adding radioimmunoprecipitation assay lysis buffer (Solarbio), containing 1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was extracted, and the protein concentration of the supernatant was determined using the bicinchoninic acid assay kit (Beyotime, Shanghai, China).

Approximately 20 μg of protein was separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were closed with 5% skim milk at room temperature for 60 min, after which the membranes were incubated with anti-LC3B (1:1,000; #3868, Cell Signaling Technology, Danvers, MA, USA), anti-Hsp70 (1:1,000; #4873, Cell Signaling Technology), anti-P21 (1:1,000; #2947, Cell Signaling Technology), and beta-actin (1:5,000; ABclonal, Wuhan, Hubei, China) antibodies and incubated overnight at 4°C. The membranes were washed three times with Tris-buffered saline with Tween 20 (TBST) for 10 min each time on the following day. After incubation with the respective species-specific secondary antibodies for 60 min at room temperature, the membranes were washed three times with TBST for 10 min each time. The color was developed using Immobilon ECL (Millipore, Boston, MA, USA) chemiluminescence and photographed using a luminescence imager 5200 multi (Tennant, Shanghai, China) to observe protein expression. Protein bands were analyzed using Image J software 1.8.0 (NIH Image J system, Bethesda, MD, USA), and beta-actin was used as an internal reference.

Statistical analysis

The data were analyzed using SPSS software 20.0 (International Business Machines Corporation, Armonk, NY, USA). The measurement data were expressed as mean ± standard (SD) deviation, and all experimental data were independently replicated three times. Different treatment groups were compared using Student's t-test, and within-group comparisons were made using one-way analysis of variance. A statistical significance was considered at $P < 0.05$.

Results

Handelin has a bidirectional effect on the proliferative activity of HDF cells

After treating HDF cells with different concentrations of handelin for 24 h, the proliferation activity of HDF cells was enhanced by handelin at concentrations of 0.0125,

0.025, and 0.05 μM (relative cell proliferation activity of 137.4% ± 2.9%, 147.1% ± 8.1%, and 134.4% ± 10.7%, respectively), whereas it was inhibited by 0.8 and 1.6 μM concentration of handelin (relative cell proliferation activity of 67.4% ± 0.7% and 66.1% ± 7.8%, respectively), with statistically significant differences ($F = 28.7$, $P < 0.01$) (Fig. 1). Therefore, handelin has certain bidirectional regulatory effects on the proliferative activity of HDF cells. On the basis of the above experimental results, we selected 0.0125 and 0.025 μM concentrations for the follow-up experiments.

Handelin ameliorates the effects of UVA irradiation on cell morphology

The number and morphology of HDF cells were altered with different treatments compared with the control group as observed by inverted phase-contrast microscopy. In the control group, the cells were tightly connected, and the cells were structured in elongated strips. Under the same observation conditions and after irradiation with 4 J/cm², the number of cells in the UVA group decreased, the gap widened, and the cells became thicker and larger, consistent with the aging characteristics of HDF cells (Khan et al. 2018). In contrast, treatment with handelin at 0.0125 and 0.025 μM concentrations significantly increased the number of cells and swirling arrangement, with no significant changes in cell morphology. On comparison between the handelin + UVA and UVA groups, handelin at concentrations of 0.0125 and 0.025 μM still promoted cell proliferation activity and maintained cell morphology; however, the effect of handelin at a concentration of 0.0125 μM was more evident than that at 0.025 μM. Moreover, the number of cells increased significantly, and only a few cells changed their shape (Fig. 2).

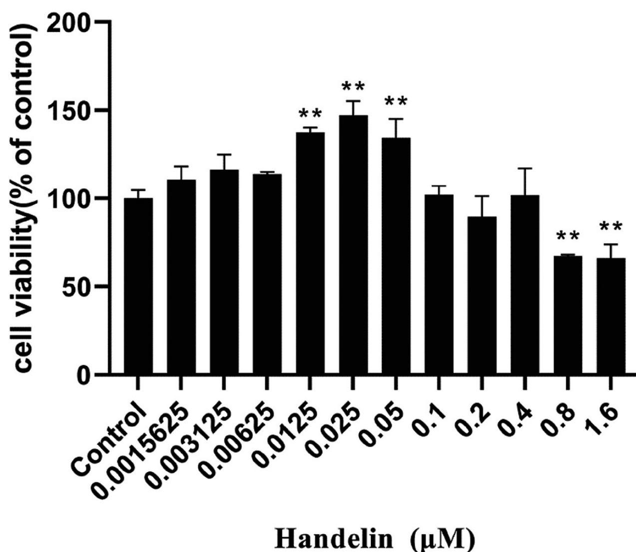


Fig. 1. The effect of handelin at different concentrations on the proliferation activity of human dermal fibroblast (HDF) cells.
Cell viability was measured using the CCK-8 assay, and the results were calculated as mean ± SD. **$P < 0.01$ using One-way ANOVA.

Handelin reduces the number of senescent cells and inhibits the protein expression of P21 after UVA exposure

As handelin ameliorates the cellular morphology using UVA irradiation, extremely similarly to cellular senescence alterations, the effect of cellular senescence was further verified at the molecular level. We stained the treated HDF cells with SA-β-GAL, and after data collation and analysis, the proportion of senescent cells to normal cells in each group was as follows: control group: 13.2% ± 2.2%; UVA group: 21.4% ± 1.5%; handelin group: 12.0% ± 1.8%; and handelin + UVA group: 13.4% ± 0.9% (Fig. 3A, right). Compared with the control group, the number of cells in the UVA group decreased, wherein the number of senescent cells was significantly higher ($P < 0.05$), and the number of cells in the handelin group increased, wherein the change in the number of their senescent cells was not significant ($P > 0.05$). Compared with the UVA group, the number of HDF cells in the handelin + UVA group increased, and the number of senescent cells significantly reduced ($P < 0.05$). The results of this study were consistent with the changes in cell morphology presented in Fig. 3A (left), confirming the occurrence of cellular senescence and the ability of handelin to improve the phenomenon of cellular senescence induced by UVA at the molecular level.

To further verify that handelin could reduce UVA-induced cellular senescence, P21, a protein related to cellular senescence, was examined. The results showed that the expression of P21 protein was significantly increased in the UVA group compared with the control group ($P < 0.01$); the expression of P21 protein was also increased in the handelin group at different concentrations (Fig. 3B). Compared with the UVA group, the expression of P21 in the handelin + UVA group pretreated with different concentrations decreased significantly at 0.0125 μM ($P < 0.01$) (Fig. 3B). In addition, it was observed that the expression of P21 protein was correlated with the change in handelin concentration.

Handelin effectively reduces ROS production in HDF cells after UVA irradiation

Numerous studies have shown that damage to the skin due to UVA mainly damages the genetic material by increasing intracellular ROS levels to induce cellular senescence or death (Bosch et al. 2015; Redza-Dutordoir and Averill-Bates 2016). To investigate the effect of handelin on cellular ROS levels, the ROS levels of cells in each group were further examined using the fluorescent probe method (Fig. 4A). In addition, the experimental results showed that the ROS production by HDF cells in the handelin group was significantly lower ($P < 0.05$) than the control group; whereas, the ROS production by cells in the UVA group was significantly higher ($P < 0.05$) (Fig. 4B). Compared with the UVA group, the ROS production in the

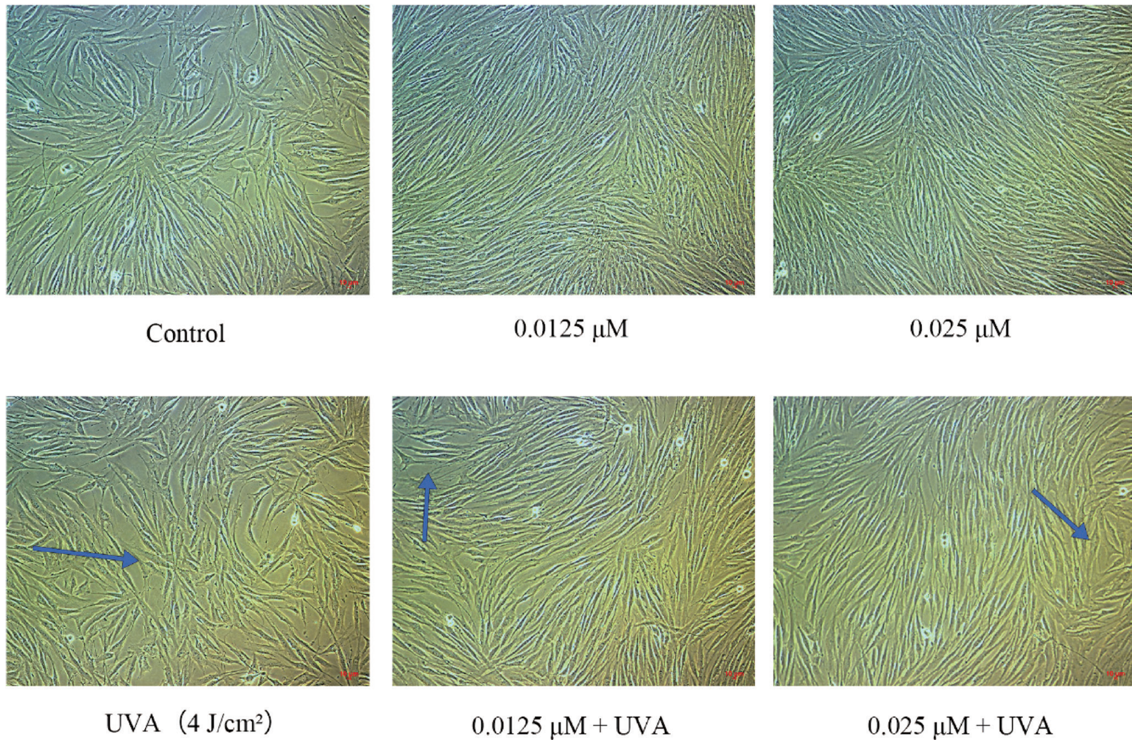
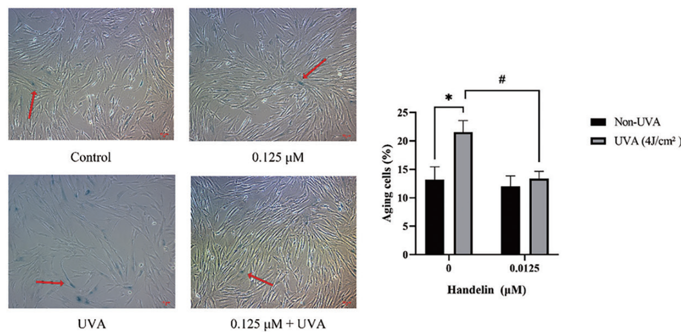


Fig. 2. Protective role of handelin in HDF cells following ultraviolet A (UVA) irradiation.
Handelin prevents morphological and ultrastructural alterations in UVA irradiated HDF cells. The blue arrows show HDF cells with senescent features. Original magnification 10 × 10; scale bar = 10 μm.

(A)



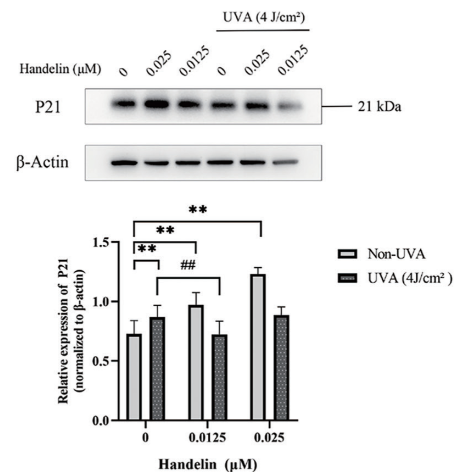


Fig. 3. Handelin reduces the number of senescent cells and inhibits the protein expression of P21 after UVA irradiation.
(A) The number of senescent cells was evaluated after handelin-treated (0.0125 μM) and UVA irradiation (4 J/cm²) using the SA-β-GAL assay. Red arrows indicate stained aging HDF cells. Original magnification 10×10; scale bar = 10 μm. (B) The protein expression levels of P21 in HDF cells were determined by western blot, and were normalized to β-actin. The results were calculated as mean ± SD. *P < 0.05, **P < 0.01 compared to the control group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 compared to the UVA group using Student's t-test.

handelin + UVA group was significantly reduced (P < 0.01); however, it was slightly higher than that in the control group (P < 0.01) (Fig. 4B). The pathway of handelin to attenuate UVA-induced cellular senescence may inhibit ROS production by HDF cells; however, this may not be the only pathway of action.

Handelin promotes autophagy and enhances the expression of autophagy-related proteins LC3B II and HSP70

Several studies have found that autophagy plays a very crucial role in reducing cellular senescence (Sample and He 2017; Wang et al. 2019). To investigate whether the molecular mechanism of handelin anti-UVA-induced cellular senescence was associated with cellular autophagy, we first

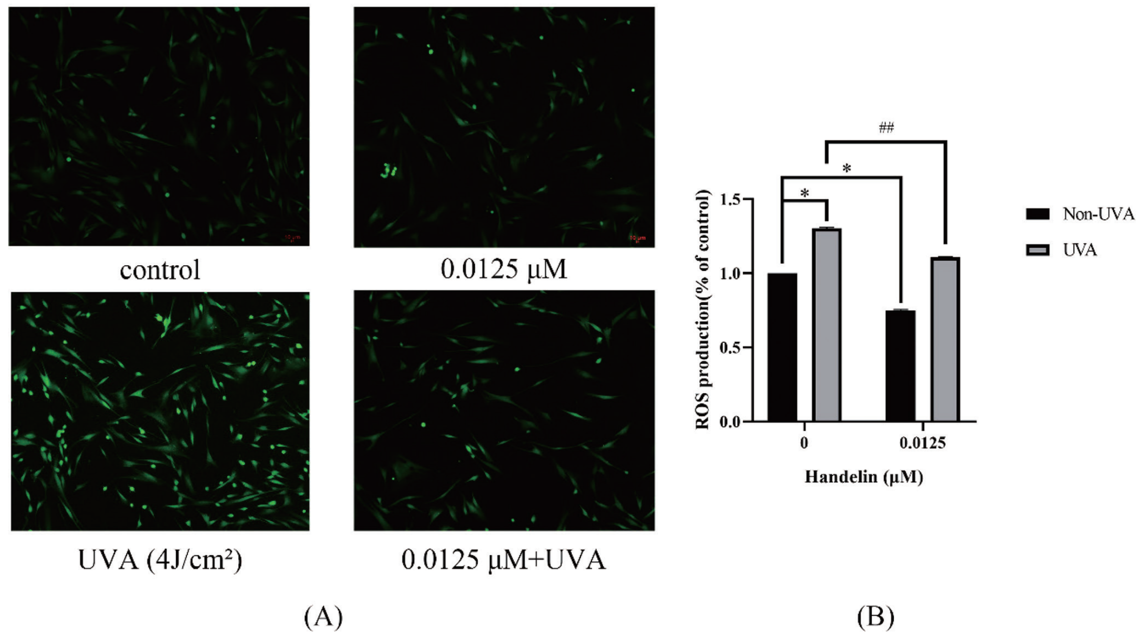


Fig. 4. Handelin can reduce intracellular reactive oxygen species (ROS) activity in HDF cells.
(A) Intracellular ROS generation was evaluated after handelin-treated (0.0125 μM) and UVA irradiation (4 J/cm²) by relative fluorescence intensity using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay. Original magnification 10 × 10; scale bar = 10 μm. (B) The 488 nm and 525 nm were the excitation and emission wavelengths, respectively. The results were calculated as mean ± SD. *P < 0.05 compared to the control group; $^{##}$P < 0.01 compared to the UVA group using Student's t-test.

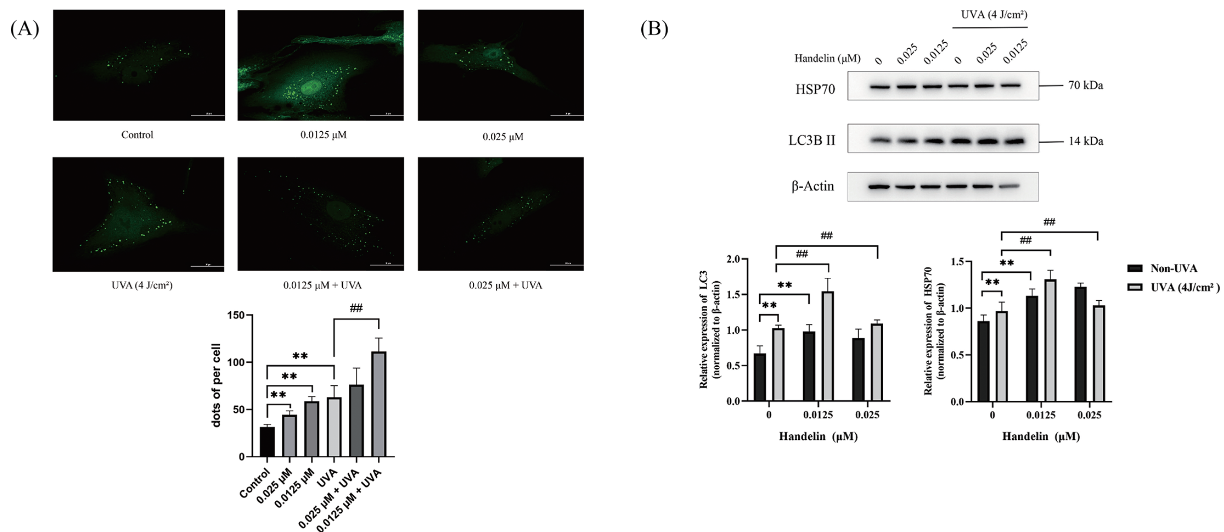


Fig. 5. Handelin enhances the expression of LC3B II and HSP70 expression in HDF cells.
(A) Intracellular autophagosomes were visible as light green dots after fluorescent staining. Original magnification 60 × 10; scale bar = 50 μm. (B) The protein expression levels of LC3B II and HSP70 in HDF cells were determined by western blot, and were normalized to β-actin. The results were calculated as mean ± SD. **P < 0.01 compared to the control group; $^{##}$P < 0.01 compared to the UVA group using Student's t-test.

assayed intracellular autophagosomes. The autophagosomes in the cells could be observed to be stained fluorescence dots under confocal microscope after the GFP-LC3 staining HDF cells. The confocal results showed that two consecutive concentrations of 0.0125 μM and 0.025 μM in the handelin group promoted the autophagy level of normal cells compared with the control group (P < 0.01); while the autophagy level of cells in the UVA group was significantly higher compared with the control group (P < 0.01) (Fig. 5A). The autophagy level of cells in the 0.0125 μM + UVA group was also significantly higher compared with the UVA irradiation group (P < 0.01).

Next, we performed an assay for LC3B II and HSP70, two proteins associated with cellular autophagy (Fig. 5B). The results showed that the protein expression of HSP70 was significantly higher ($P < 0.01$) in the UVA group compared to the control group. The handelin group showed a dose-related expression of HSP70 after treatment with two consecutive concentrations of 0.025 and 0.0125 μM of handelin. Among them, the expression of HSP70 was positively correlated with dose. Compared with the UVA group, the expression of HSP70 increased significantly at 0.025 and 0.0125 μM ($P < 0.01$) and was negatively associated with the concentration. Therefore, we proposed that handelin can also promote autophagy by altering the expression of HSP70, which are autophagy-related proteins, to achieve anti-UVA-induced cellular senescence.

Discussion

Skin aging is a complex process that includes natural aging and photoaging associated with UV exposure, demonstrated by increased wrinkles, reduced elasticity, and hyperpigmentation of the skin. HDF is a crucial cellular component of the skin dermis that produces collagen, fibronectin, glycosaminoglycan, and other extracellular matrices to support the entire dermal structure and maintain skin elasticity. UVA can damage HDF cells, leading to a decrease in cell number, abnormal secretion/synthesis function, and eventually aging of the skin.

Many studies have shown that some plants growing at high altitudes and in areas with strong UV radiation contain more substances that protect against UV damage than those growing at lower altitudes. In fact, we chose handelin based on the fact that it is an extract of wild chrysanthemum that grows at high altitudes. Moreover, local people use wild chrysanthemum to cure skin damage caused by sun exposure.

In this study, handelin reduced UVA-induced photoaging of HDF cells. SA-β-GAL senescent cell staining showed that the number of HDF cells in the UVA group decreased, the cell shape was round and large, and the proportion of senescent cells increased. In contrast, pretreatment with handelin effectively reduced the number of senescent cells after irradiation and maintained the original elongated cell shape of HDF. In addition, handelin inhibited the expression of P21, a protein associated with cellular senescence, which shows its biological activity by binding to cell cycle protein-dependent kinases (Pontén et al. 1995; Abbas and Dutta 2009; Chen et al. 2015). Sustained DNA damage can lead to cellular senescence and induce P21 upregulation to promote permanent cell cycle arrest and inhibit the activity of senescent cells (Burton and Krizhanovsky 2014). In this experiment, P21 expression was significantly increased after UVA irradiation and significantly decreased on pretreatment with handelin at a concentration of 0.0125 μM before UVA irradiation. Therefore, handelin can reduce cellular DNA damage caused by UVA irradiation, decrease senescent cell production, and inhibit P21 expression. To investigate the mechanism by which handelin weakens cellular photoaging, the relationship between the cellular ROS levels and autophagy was further characterized, which is associated with cellular photoaging.

Cells can produce a certain amount of ROS in their physiological state, and the cells have an antioxidant system that consists of redox-active substances and enzymes which can antagonize ROS production and maintain redox homeostasis (Brem et al. 2017). When UVA irradiation time is extremely long or the irradiation dose is extremely high, UVA interacts with various chromophores (such as porphyrins, bilirubin, and melanin) in HDF cells and generates a large amount of ROS. When the level of ROS generation exceeds the load of the antioxidant system, a large amount of ROS cannot be removed in time, leading to oxidative stress (Poljšak and Dahmane 2012). This, in turn, damages cellular DNA and macromolecules, oxidizes cellular membrane lipids, and forms oxidized phospholipid-protein complexes and other oxidized complexes, which can aggravate cellular damage when deposited in the cells (Zhao et al. 2013). This experimental study found that HDF cells can produce ROS in their physiological state and are in redox homeostasis because of their antioxidant system. ROS production decreased only in cells treated with handelin, suggesting that handelin maintains cellular redox homeostasis at a lower state and reduces the stress on the body's antioxidant system. After UVA irradiation, cells produced a large amount of ROS in a short duration when the redox homeostasis was disrupted and the stress on the antioxidant system increased. Compared with the unspiked irradiation group, the cellular ROS production in the UVA group irradiated with handelin was significantly reduced, close to the ROS level in the physiological state of the cells. This indicated that handelin has strong antioxidant properties and can effectively inhibit ROS generation in HDF cells before and after UVA irradiation; particularly, it can significantly reduce ROS levels after UVA irradiation, attenuate oxidative stress and oxidative complex generation, and maintain redox homeostasis (Tyrrell 2012). We hypothesize that the antioxidant properties of handelin act mainly through the absorption of UVA. It enhances the antioxidant capacity of the cells and directly reduces the cellular damage caused by UVA. Also, ROS levels were reduced in the cells treated with handelin compared to normal cells, suggesting that handelin may include other mechanisms of action in addition to UVA absorptance to enhance the antioxidant capacity of cells.

The cells under various stress conditions such as heat stress, oxidative stress, nutrient deficiency, and organelle damage, activate autophagy to remove damaged cellular components and oxidative complexes and reduce the effects of UVA irradiation on cells (Hansen et al. 2018). However, the effect of autophagy decreases over time, and the damaged fraction and oxidative complexes that are not completely removed accumulate and damage the cells again (Widmer et al. 2006; Olivier et al. 2017). The amount of

autophagosome fluorescence was significantly elevated after UVA irradiation, suggesting that cell damage caused by UVA irradiation can lead to enhanced autophagy. Pretreatment with handelin before UVA irradiation showed a significant increase in the number of intracellular autophagosomes, suggesting that handelin promotes the occurrence of autophagy after UVA irradiation to enhance and maintain this self-cleaning process. Autophagy occurs in three parts: initiation, elongation, and autophagosome degradation. The initiation of autophagy depends on phagocytosis, formed by the action of class III phosphatidylinositol 3-kinase on a macromolecular complex of autophagy-linked proteins. In contrast, the elongation of the phagocytic cell membrane is mainly because of the cleavage of LC3 by Atg4 to LC3-I, then LC3-I ubiquitin-like binding through Atg7 (E1 ubiquitin activator) and binding to phosphatidylethanolamine through Atg3 (E2 ubiquitin ligase) to form LC3-II. LC3-II promotes the targeted degradation of proteins, their aggregates, and damaged or dead organelles by interacting with junctional proteins (Tanida et al. 2004). Thus, LC3 plays a crucial role in the autophagic process. Meanwhile, we found that normal cells can undergo autophagy under physiological conditions, indicating that the cells themselves have strong autophagic activity (Mizushima et al. 2004). The expression of LC3B II was significantly increased after UVA irradiation, suggesting that cell damage caused by UVA irradiation can lead to enhanced autophagy. Pretreatment with handelin before UVA irradiation showed a significant increase in the expression of this protein, suggesting that handelin promotes the occurrence of autophagy after UVA irradiation. Based on these experimental results, we speculate that the effect of handelin on reducing cellular photoaging is very important related to its ability to enhance cellular autophagy. Autophagy is also activated in normal cells after UVA irradiation, but the effect is limited and maintained for a short period of time. However, handelin can greatly increase the intensity of cellular autophagy, which means that the effect of autophagy can be maintained for a long time under the action of handelin. The harmful substances produced after UVA irradiation can be completely removed to avoid their accumulation. In other words, handelin can protect cells indirectly by enhancing cellular autophagy and reducing the effects of UVA on cells.

In addition, HSP70 is a heat shock protein associated with autophagy and plays a very crucial role in controlling protein folding and clearing damaged cellular components (Evans et al. 2010; Dokladny et al. 2015). Moreover, HSP70 plays a key role in several aging-related diseases, and using exogenous recombinant human HSP70 in adult mice prolongs their lifespan (Bobkova et al. 2015; Shirato et al. 2018). HSP70 can bind to denatured proteins, preventing the formation of associated protein aggregates and facilitating protein remodeling or degradation (Walther et al. 2015). Meanwhile, HSP70 can bind to normal proteins and stabilize the protein structure from being destroyed (Reeg et al. 2016). Moreover, handelin could upregulate the level of HSP70, particularly at a concentration of 0.0125 μM; it could increase the expression of HSP70 after UVA irradiation to approximately twice the level of UVA irradiation group. This showed that handelin could specifically act on HSP70 to enhance the effect of autophagy and promote the repair of proteins and clearance of damaged cellular components. Therefore, handelin is photoprotective against UVA-induced cellular senescence. Notably, Wang et al. (2017) reported that handelin can bind directly to HSP70 by targeting to cysteine 306. This is expected to be used for the treatment of inflammation-related diseases. In our experiments, we also found that handelin was able to stabilize HSP70. So, handelin is indeed a small molecule with a large potential for development. However, the deeper molecular mechanism of its action needs further investigation.

We also compared the effect of different concentrations of handelin. Although the effect of handelin at 0.025 μM in maintaining cell morphology and promoting cell proliferation activity was comparable to that at 0.0125 μM, the effects produced by successive concentrations of handelin showed dose-dependence in terms of the expression of those proteins. However, the effect of 0.025 μM of handelin on two proteins, P21 and HSP70, in normal cells was not as expected, whereas 0.0125 μM of handelin had a more favorable effect on all indices. We speculate that this may be due to the fact that handelin itself is more effective at lower concentrations and that lower effective concentrations mean lower costs and lower potential for side effects. This suggests that handelin is safer to use at effective drug concentrations.

In conclusion, in this study, the effects of handelin were observed on the proliferative activity of normal HDF cells and UVA-induced cellular senescence, ROS generation, and autophagy by pretreating HDF cells with handelin before UVA irradiation. Moreover, handelin at a concentration of 0.0125 μM enhanced the proliferative activity of normal HDF cells, maintained the morphology of HDF cells independent of UVA, and reduced the generation of senescent cells. This is because of the antioxidant property of handelin itself, which can act as an exogenous oxidant on cells and inhibit the production of cellular ROS and deposition of oxidized substances after UVA irradiation. In addition, it effectively increases the level of cellular autophagy after UVA irradiation so that harmful substances produced after light exposure, including damaged cells and oxidation products, can be completely removed. Moreover, it provides a photoprotective effect on cells against cellular photoaging.

In summary, handelin is a natural plant extract with UV-absorbing, anti-oxidant, cell autophagy-promoting, and anti-inflammatory properties, which can be used as a photoprotective agent to prevent and reduce UV damage to the skin.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yi Duan and Sujiao Sun. The first draft of the manuscript was written by Yi Duan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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