DOI: 10.26599/FSHW.2024.9250122

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

Moringia oleifera Lam. seeds isothiocyanate against skin photoaging by regulating p38MAPK-dependent autophagy signalling pathway

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ABSTRACT: People living long-term in areas with UV will cause premature photoaging. An abnormal reduction in autophagy is a key feature of photoaging, and p38MAPK has been regarded as a key regulator of autophagy. Isothiocyanate is one of the main active components of *Moringa oleifera* Lam. seeds. Studies have reported that MITC has anticancer, anti-inflammatory, cardiometabolic repair, nervous system protection, blood lipid regulation and diabetes prevention properties. However, the molecular mechanisms of MITC with protective effects against skin photoaging have not been studied thus far. In this study, we aimed to evaluate the antiphotoaging activity of MITC and to investigate the effect of p38MAPK-dependent autophagy *in vivo* and *in vitro* models of photoaging. In this research we found that MITC can reverse the intracellular ROS content and inhibit the activation of p38MAPK to improve the autophagy level, reduce the expression of MMPs, and finally protect against photoaging by UV. Our results will uncover the molecular mechanisms of MITC that play a role in the protective effects against skin photoaging, provide helpful information for developing MITC as an ant-photoaging plant material and improve the utilization of *Moringa oleifera* Lam. seeds.

Key words: *Moringa oleifera* Lam. seeds, Ant-photoaging, ROS, MMPs, p38MAPK-dependent autophagy pathways.

1. Introduction

The skin is the largest and most complex organ of the human body. It is in direct contact with the external environment and protects the internal structures of the body from the external environment [1,2]. Skin ageing is a complex biological phenomenon caused by both internal (genetically determined physiological ageing) and external factors (temperature, pollution, and ultraviolet radiation) [2]. UV radiation is harmful to human skin, and long-term exposure to UV radiation can cause sunburn immunosuppression,

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Received 29 August 2022
Received in revised from 19 December 2022
Accepted 21 May 2023

cancer and skin photoaging. UV radiation can be divided into UVA (320 nm-400 nm), UVB (280 nm-320 nm) and UVC (200 nm-280 nm). UV radiation causes significant biological damage to the skin, significantly affecting the epidermis and causing premature skin ageing [3,4]. Skin photoaging is a common skin disease, and its clinical manifestations include loss of skin elasticity, epidermal atrophy, deep wrinkles and pigmentation, which can lead to cell apoptosis or cancer [5,6]. Studies have demonstrated that UV radiation can induce the skin to produce MMPs, which can degrade skin-related collagen and other extracellular matrix proteins [7]. Moreover, UV radiation repeatedly causes many ROS in skin tissue, and excessive ROS can cause cell damage and even lead to cell apoptosis.

Studies have shown that reduced autophagy activity is an important cause of skin ageing [8,9], and autophagy is considered to be an important antiaging mechanism. The reduction in autophagy leads to accelerated ageing, while stimulating autophagy enhances antiaging effects and prolongs one's lifespan [10]. In contrast, overexpression of autophagy-related genes can delay ageing. Moreover, autophagy induced by rapamycin can also delay UVB-induced photoaging by reducing ROS production and activating autophagy [11]. MAPK is a highly conserved serine/threonine protein kinase consisting of JNK, p38MAPK kinase and ERK [12,13]. Studies have shown that ROS produced by UV radiation can promote the phosphorylation of p38MAPK kinase by activating the MAPK upstream kinase MKK6. Therefore, p38MAPK is the most susceptible to UV stimulation of MAPK and is extremely sensitive to ROS [14]. Activated p38MAPK kinase activates downstream AP-1 protein, which promotes the secretion and expression of collagenase MMP-1 and MMP-9, thereby decomposing the ECM in skin cells, degrading skin collagen, and aggravating skin photoaging damage [15]. Moreover, p38MAPK is also a key enzyme that regulates autophagy. After UV radiation, p38MAPK kinase expression increases and autophagy levels decrease, thus exacerbating skin inflammation after UV radiation. In addition, p38MAPK can also induce apoptosis and cell cycle arrest by regulating P53 protein expression, leading to an abnormal decrease in intracellular autophagy activity [16,17]. These results suggest that UV radiation may decreases autophagy levels by activating p38MAPK activity, inducing cell apoptosis and leading to skin photoaging. Therefore, decreasing p38MAPK activity and increasing autophagy activity may play important roles in delaying the UV-induced photoaging process.

At present, VC and retinoic acid are often used in the market to treat skin aging [18, 19], but excessive retinoic acid may lead to osteoporosis [19]. Vc is considered to be an important antioxidant that protects the skin by releasing solvated electrons during singlet excitation and neutralizing ROS generated when exposed to sunlight. In biological systems, it reduces oxygen and nitrogen free radicals through redox reactions, thereby delaying the ageing process [18]. Vc has multiple functions in the skin, such as collagen synthesis, decolorization and antioxidant activity. Studies have found that the combination of collagen peptide and Vc can promote the proliferation of HaCaT cells induced by ultraviolet radiation [20]. Gavage administration of Vc and collagen peptide can also increase the content of type I collagen and the expression of antioxidant enzymes, inhibit the expression of metalloproteinases, and regulate the Nrf2/ARE and TGF-β/Smad pathways, thereby improving the ultraviolet-induced mouse skin photoaging model [19]. Some clinical

studies have also shown that the application of VC can significantly alleviate wrinkles around the orbit. Therefore, due to its antioxidant properties and protective effect on the skin, Vc can be used as an additive for commercial cosmetics [21].

Many studies have shown that various parts of *Moringa oleifera* Lam. (especially seeds and leaves) contain a large number of nutrients and active ingredients, and these factors show different antioxidant, antibacterial, antitumour, anti-inflammatory, heart protective, liver protective and neuroprotective effects [22,23,24]. Isothiocyanate is one of the main active components (content 2%~4%) of *Moringa oleifera* Lam. seeds [25]. Studies have reported that MITC has anticancer, anti-inflammatory, cardiometabolic repair, nervous system protection, blood lipid regulation and diabetes prevention activities [26,27]. MITC are organic compounds with an (R N=C=S) group in their structures, but their biological activity and chemical stability are higher than those of isothiocyanate compounds in other plants due to the presence of aromatic rings and rhamnoses, among which 4-[(α -L-rhamnoxyl) benzyl] isothiocyanate has the highest content and the best activity [28].

However, the molecular mechanisms of MITC with protective effects against skin photoaging have not been studied thus far. In this study, we aimed to evaluate the antiphotoaging activity of MITC and to investigate the effect of p38MAPK-dependent autophagy *in vitro* and *in vivo* models of photoaging. Based on the *in vivo* animal models, we first clarified the role of MITC in anti-photoaging. Then, we reconstructed the regulatory network that controls p38MAPK-dependent autophagy *in vivo* models of photoaging by using RNA-seq. Moreover, we defined the role of MITC in anti-photoaging through an *in vitro* model. The results of this study provide insight for the preparation of MITC with potential applications in anti-photoaging functional foods and drugs.

2. Materials and methods

2.1. Plant materials and reagents

MITC was prepared by the development and utilization of food and drug homologous resources from the engineering research centre of the ministry of education (Yunnan agriculture university, Kunming, China) (Fig. 1A); MTT, Penicillin–streptomycin, DMSO, and PBS were purchased from Solarbio Technology Co., Ltd. (Beijing, China) and stored at -20 ℃. Mouse hyaluronic acid ELISA kit (CSB-E08121m) and mouse hydroxyproline ELISA kit (CSB-E08839m) were purchased from Cusabio Technology Co., Ltd. (Wuhan, China). Human MMP-1(MM-0072H2) and human MMP-3 (MM-0108H2) ELISA kits were purchased from MeiMian Co., Ltd. (Jiangsu, China). Mouse MMP-1(SEKM-0165) and mouse MMP-3 (SEKM-0167) ELISA kits were purchased from Solarbio Technology Co., Ltd. (Beijing, China). FBS and Dulbecco's Modified Eagle's Medium were obtained from Gibco Co., Ltd. (Carlsbad, CA, USA). The annexin-V/PI apoptosis kit and BCA protein quantitative kit were acquired from Biyuntian Biotechnology Co., Ltd. Primary antibodies against p38MAPK, p-p38MAPK, JNK, p-JNK, Erk, p-Erk, P21, p-mTOR, p62, AP-1, LC3 I, and LC3 II were acquired from Proteintech Inc. (Rosemont, United States). Mitochondrial membrane

potential assay kits with JC-1 (M8650), ROS assay kits (CA1410), SOD assay kits (BC0170) and MDA assay kits (A003-1-2) were purchased from Solarbio Technology Co., Ltd. (Beijing, China). Nunc™ Lab-Tek™ II Chamber Slide™ were obtained from Thermo Fisher Scientific Inc. (NY, USA).

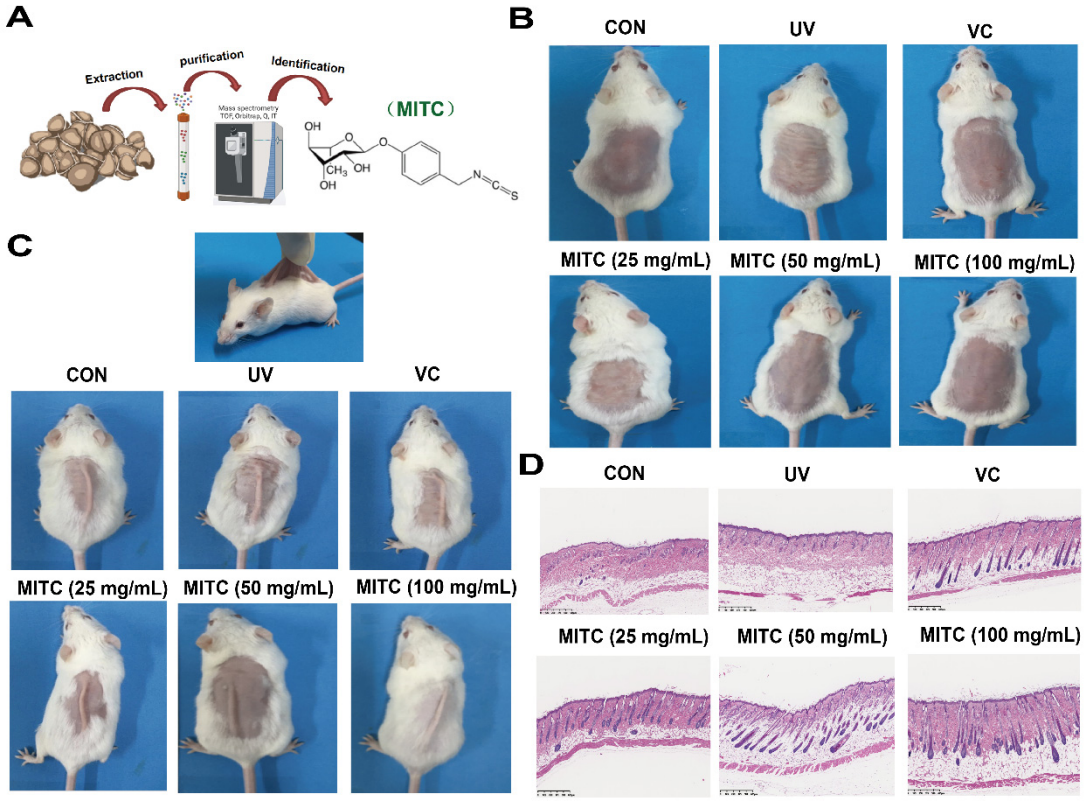

Fig. 1. MITC is involved in UV-irradiated photoaging of skin in mice. (A) Extract identification and molecular structure formula of MITC. (B) The dorsal skin of all groups of mice was taken for phenotype analysis. (C) Dorsal skin elasticity recovery test of mice. (D) H&E-stained histological images of skin sections from all groups of mice were captured (N=8 per group).

2.2. In vivo animal photoaging model

To determine the effects of MITC on photoaging *in vivo*, forty-eight healthy male ICR mice (four weeks old, (20±2) g in weight) were provided by Charles River Experimental Animal Technology Co., Ltd. (Beijing China) and maintained in a specific pathogen-free (SPF) environment. The mice were housed under a 12 h/12 h light/dark cycle and given access to standard laboratory food and water.

After 7 days, the mice were randomly divided into 6 groups with 8 animals in each group as follows: the normal control group (without any treatment); model group (UV irradiation); positive control group (UV+ VC); low-dose group (UV+25 mg/mL MITC); medium-dose group (UV+50 mg/mL MITC); and high-dose group (UV+100 mg/mL MITC). All drugs were dissolved in trace 1% DMSO and then diluted with sterile water. After that, the samples were wrapped in tin paper and stored in the dark.

The experiment was carried out by smearing drugs on the backs of the mice every day. The control group and the model group were smeared with sterile water, the positive control group was smeared with Vc, and the MITC group was smeared with different concentrations of MITC, except for the control group, the mice in the other groups were application after UV irradiated.

Before irradiation, an area of 2 cm~3 cm of hair was shaved on the backs of the mice in each group so that the skin in the shaved area was completely exposed to UV irradiation. UV irradiation was started at week 2 by using an SS-04AB UV phototherapy instrument (UVA emission spectrum 340 nm~400 nm, peak 365 nm; UVB emission spectrum 260 nm~350 nm, peak 311 nm). The irradiator was used to irradiate the dorsal skin of mice from Monday to Friday every week for 40 minutes, and after 10 weeks, the irradiation time was adjusted to 50 minutes and continued until the end of the 12th week, for a total of 12 weeks of UV irradiation (the accumulated UVA radiation dose was 100.35 J/cm^2, and the accumulated UVB radiation dose was 9.50 J/cm^2). Some reports have pointed out that ICR mice are suitable for constructing irradiated photoaging models. After UV irradiation, the skin of mice shows typical characteristics of chronic photoaging skin, such as obvious thickening of the skin, dry and rough epidermis, and obvious relaxation. In addition, long-term chronic irradiation can also lead to red spots, pigmentation and desquamation on the skin surface, and obvious lesions in the skin, indicating that the photoaging mouse model is successful [29, 30].

After 12 weeks of the experiment, the mice were euthanized with carbon dioxide, and dorsal skin tissue was obtained and quickly frozen in liquid nitrogen and formalin for additional analysis. The animal experiment was reviewed and approved by the Yunnan Agricultural University Animal Ethics Committee (Approval Number YNAU-2020-015).

2.3. Skin tissue histological analysis

Skin tissue samples from the treatment group were fixed in 10% formalin. Skin samples were embedded in paraffin, and 5 μm sections were stained with haematoxylin and eosin. Epidermal pathological conditions were measured under optical microscopy (Olympus, Tokyo, Japan).

2.4. Skin tissue Masson analysis

Skin tissue samples were fixed with 10% formalin. The sample slices were embedded in paraffin and stained with Bouin's solution, haematoxylin, phosphomolybdic acid, and aniline blue. Then, the slices were treated with glacial acetic acid solution. Finally, the slices were dehydrated and sealed, and an optical microscope (Olympus, Tokyo, Japan) was used to observe the distribution of collagen, which was analysed by *ImageJ*.

2.5. Skin tissue immunohistochemistry

Skin tissue samples were removed from formalin and embedded in paraffin. Sections were treated with 3% H_2O_2, and after 10 min of treatment, skin sections were incubated with TBS, Triton-X-100 and normal goat serum and then incubated with MMP-1 and MMP-3 primary antibodies (1:200 dilution, Abcam, MA, United States). Sections were then further incubated with secondary goat anti-mouse IgG-horseradish peroxidase-conjugated antibody (1:500 dilution, Abcam, MA, United States). Then, the sections were washed, stained with DAB, counterstained with haematoxylin, and sealed after dehydration. All images were captured using an inverted fluorescence microscope (Nikon, Japan) and analysed by *ImageJ*.

2.6. RNA sequencing

Total RNA was extracted from skin tissue samples according to the manufacturer's instructions (Invitrogen). Then, RNA quality was determined by 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). The RNA-seq transcriptome library was prepared following the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA) using RNA samples with high quality. The paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq X Ten/NovaSeq 6000 sequencer.

2.7. Cell line culture and UV irradiation

Normal human keratinocyte cell lines (HaCaT cells) were obtained from the Kunming Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences.

Cells were cultured in DMEM High medium with 10% FBS and 1% antibiotics (penicillin and streptomycin). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ℃. HaCaT cells were starved for 24 h in serum-free DMEM, the medium was discarded, and each well was replaced with 100 μL of PBS. The UV irradiation cell model was established using an SS-04AB UV phototherapy instrument (UVA emission spectrum 340 nm-400 nm, peak 365 nm; UVB emission spectrum 260 nm-350 nm, peak 311 nm) with an irradiation dose of 30 mJ/cm^2. Cells were treated with three different concentrations of MITC (2 μmol/L, 4 μmol/L or 8 μmol/L) immediately after UV irradiation. The cells were cultured for 24 h and then washed with PBS, and the cells or cell supernatants were collected for subsequent experiments. The MTT method was used to detect the cell survival rate. When the survival rate of HaCaT cells was 50% and the expression of related indicators could be measured, the photoaging cell model was considered successfully modelled [31, 32].

2.8. MTT cell viability assay

HaCaT cells were treated with different concentrations of MITC. Next, the cells were incubated for 24 h, and then 5 μg/mL MTT was added to each well. After incubation for an additional 4 h, the medium was replaced with DMSO, and OD values were measured at a wavelength of 490 nm by a microplate reader. The cell inhibition rate was calculated by the following equation:

Cell inhibition rate of colon cancer proliferation = 1-(OD value of the drug treatment group/OD value of the cell control group) × 100%.

2.9. Cell apoptosis analysis

HaCaT cells (9×10^3 cells per well) were inoculated in a six-well plate for 24 h. After treatment with three different concentrations of MITC (2 μmol/L, 4 μmol/L or 8 μmol/L) for 24 h, the cells were washed three times with cold PBS. For the cell apoptosis assay, the incubated cells were collected and stained with an annexin-V/propidium iodide (PI) apoptosis kit. Cells were resuspended in 100 μL of 1X binding buffer containing 5 μL of FITC annexin V and 5 μL of PI and incubated for 30 min in the dark. Apoptosis of HCT116 cells was detected and analysed by flow cytometry and *FlowJo 9.0*.

2.10. ROS analysis

The level of intracellular ROS induced by UV irradiation was determined with the fluorescent probe DCFH-DA. Briefly, HaCaT cells were cultured in DMEM supplemented with UV continued irradiation. After the last radiation and MITC treatment, the cells were incubated with 10 μmol/L DCFH-DA in DMEM (1:1000) without FBS for 30 min. To remove DCFH-DA, the cells were washed with DMEM without FBS three times. Fluorescence intensity was measured with an Olympus fluorescence microscope (Olympus, Tokyo, Japan).

2.11. Mitochondrial membrane potential

The level of intracellular mitochondrial membrane potential induced by UV irradiation was determined with the fluorescent probe JC-1. Briefly, HaCaT cells were cultured in DMEM supplemented with UV irradiation. After the last radiation and MITC treatment, the cells were incubated with 1 mL JC-1 staining solution in 1 mL DMEM for 20 min. To remove JC-1 staining solution, the cells were washed with DMEM without FBS three times. Fluorescence intensity was measured with an Olympus fluorescence microscope (Olympus, Tokyo, Japan).

2.12. Enzyme-linked immunosorbent assay (ELISA)

For cell samples, HaCaT cells were inoculated in a six-well plate for 24 h and treated with MITC. Then, the cell supernatant was extracted and stored at -80 ℃. Mouse blood samples were collected through a heparinization tube collection, then centrifuged at 3500 *r/min* for 30 min and stored at -80 ℃. For mouse skin samples, dorsal skin tissue was obtained and quickly frozen in liquid nitrogen.

The levels of MMP-1, MMP-3, HA, and HYP were investigated by ELISA kits according to the kit instructions. The colour reaction was developed by a chromogenic agent by a microplate reader.

2.13. Immunofluorescence staining of LC3 in cell analysis

HaCaT cells were inoculated in a six-well plate for 24 h. Then, the cells were treated with MITC and UV irradiation. Treated cells were washed with cold PBS three times, fixed in 4% paraformaldehyde for 30 min at RT, permeabilized with 0.2% Triton X-100 (Sigma, Louis, MO, United States) for 10 min, washed with PBS and blocked with 5% PBS for 1 h. The LC3 antibody was added to 1% BSA (1:100), and the cells were incubated overnight at 4 ℃. After washing with PBS three times, the cells were incubated with a goat-antirabbit IgG secondary antibody (1:200, Cell Signaling Technology, Beverly, United States) for 30 min. Then, DAPI was added to stain the cell nucleus. Fluorescent cells were observed and photographed under a confocal fluorescence microscope (Olympus, FV 1000).

2.14. Western blotting

After treatment with MITC for 24 h, the phosphorylation of the p38MAPK pathway was observed. Cell total protein was extracted by RIPA buffer (Beyotime, Shanghai, China), and protein concentrations were assessed by the BCA assay kit. Target protein samples were separated by 10% SDS–PAGE and transferred by electroblotting to polyvinylidene difluoride membranes (Millipore, MA, USA). After blocking with 5% skim milk for 2 h, the proteins were incubated with primary antibodies against p38 MAPK, p-p38 MAPK,

JNK, p-JNK, Erk, p-Erk, P21, p-mTOR, p62, AP-1, LC3 I, LC3 II, GAPDH and β-actin overnight at 4 ℃. The PVDF membranes were then washed three times with TBST (Tris Hcl Tween) each time and incubated for 1 h with goat-anti-rabbit-HRP or goat-anti-mouse-HRP (1:10000, Abcam, MA, USA). The signal was detected using ECL western blotting substrate and analysed by *ImageJ*.

Skin tissues were collected, and the protein was determined by western blotting.

2.15. Statistical analysis

The experimental data were analysed by using GraphPad Prism 7.0. Values are expressed as the mean ± standard error of the mean (SEM). The results comparisons were performed using Student's t test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ were considered to indicate statistically significant differences.

3. Results

3.1. MITC alleviates skin photoaging damage caused by UV irradiation in vivo

UV radiation from sunlight can penetrate the dermis, and some of it can reach the subcutaneous layer, which is considered to be the main factor leading to skin photoaging [2,13]. To investigate the effect of MITC on UV-induced photoaging *in vivo*, we used ICR mice to measure wrinkle formation and skin conditions after hair removal. The results indicated that after UV irradiation, the surface skin of mice showed obvious wrinkling, peeling and red spots, and the tissue structure was damaged, including loss of skin elasticity, severe skin keratinization with increased erythema and wrinkles, and increased thickness of the epidermis and dermis (Fig. 1B). Repeated UV irradiation can lead to structural changes in the skin. However, the skin of model mice showed structural changes under UV irradiation. UV irradiation caused a loss of skin elasticity in mice, while MITC treatment restored the level of skin elasticity in mice (Fig. 1C). According to the results of HE staining, the skin of normal mice showed a relatively intact structure compared with that of the UV group, and MITC treatment improved the average length and depth of wrinkles (Fig. 1D). Previous studies have shown that UV exposure can cause the skin to dehydrate and lose water, leading to noticeable dryness and wrinkles [1,6]. Compared with the control group, different doses of MITC after UV irradiation could significantly reduce the thickness of the epidermis and dermis and alleviate skin keratinization and wrinkles.

3.2. MITC alleviates UV-induced skin photoaging and is associated with MMP responses and oxidative stress in vivo

To investigate the inhibitory effect of MITC on UV-induced photoaging, oxidative stress and MMPs reactions were further demonstrated to cause collagen degradation by using masson staining and immunohistochemistry in an *in vivo* skin photoaging model.

Compared with the control group, the UV group had reduced levels of collagen fibres in the upper dermis, distorted and disorganized dermal fibres, reduced hair follicles, and increased pore closure. In contrast, MITC treatment alleviated dermal collagen fibres in mice with extensive collagen repair,

suggesting that MITC inhibited UV-induced collagen degradation in mouse skin (Fig. 2A-B). The expression of MMPs under UV irradiation is the main reason for the destruction of collagen. MMP-1 and MMP-3 can decompose type I, II, III or X collagen. The increased expression of the collagenous hydrolytic proteases MMP-1 and MMP-3 will destroy and hydrolyse skin collagen [6,7]. Therefore, immunohistochemical staining was used to determine whether the photoprotective effect of MITC was related to UV-induced changes in MMPs expression. The contents of MMP-1 and MMP-3 in the control group were significantly lower than those in the model group (p<0.001), indicating that the photoaging model of mouse skin was successfully constructed (Fig. 2C-F). The contents of MMP-1 and MMP-3 in the skin of mice in the VC group and MITC groups were significantly lower than those in the model group (Fig. 2C, 2E). Compared with the UV group, the UV-induced expression of MMP-1 and MMP-3 was decreased by 3.2- and 4.9 times, respectively (Fig. 2D, 2F). After repair by MITC, the surface skin folds of mice were relieved, and the skin was smooth. The expression of collagen hydrolytic protease was downregulated, and the degree of skin collagen hydrolysis decreased and recovered. The expression of MMP-1 and MMP-3 in mouse skin detected by ELISA also reflected the same results (Fig. 2K, 2L). In conclusion, MITC can alleviate the contents of MMP-1 and MMP-3 in the skin of photoaged mice, thus inhibiting the degradation degree of skin collagen caused by MMPs.

Moreover, after MITC treatment, the content of HA (Fig. 2G) and HYP (Fig. 2H) in the skin of mice also increased significantly, indicating that the moisture content and moisture degree of the skin of mice were improved. SDO and CAT are important antioxidant indices in skin oxidative damage. SOD activity in the skin homogenate of mice in the control group was significantly higher than that in the UV group, SOD activity in the skin of mice in the VC group and MITC group was significantly higher than that in the UV group, and SOD activity in the skin of mice treated with MITC (100 mg/kg) was significantly higher than that in the low-dose and medium-dose MITC groups (Fig. 2I). The results showed that MITC treatment could increase the activity of SOD enzyme in the skin of photoaged mice to improve UV-induced photoaged damage. On the other hand, CAT enzyme activity in the skin homogenate of mice in the control group was significantly higher than that in the model group, and CAT activity in the VC and MITC groups was significantly higher than that in the UV group (Fig. 2J). The results suggest that MITC can resist skin photoaging caused by UV irradiation. Immunohistochemical staining also showed that skin collagen was restored and hair follicles were repaired in mice treated with MITC.

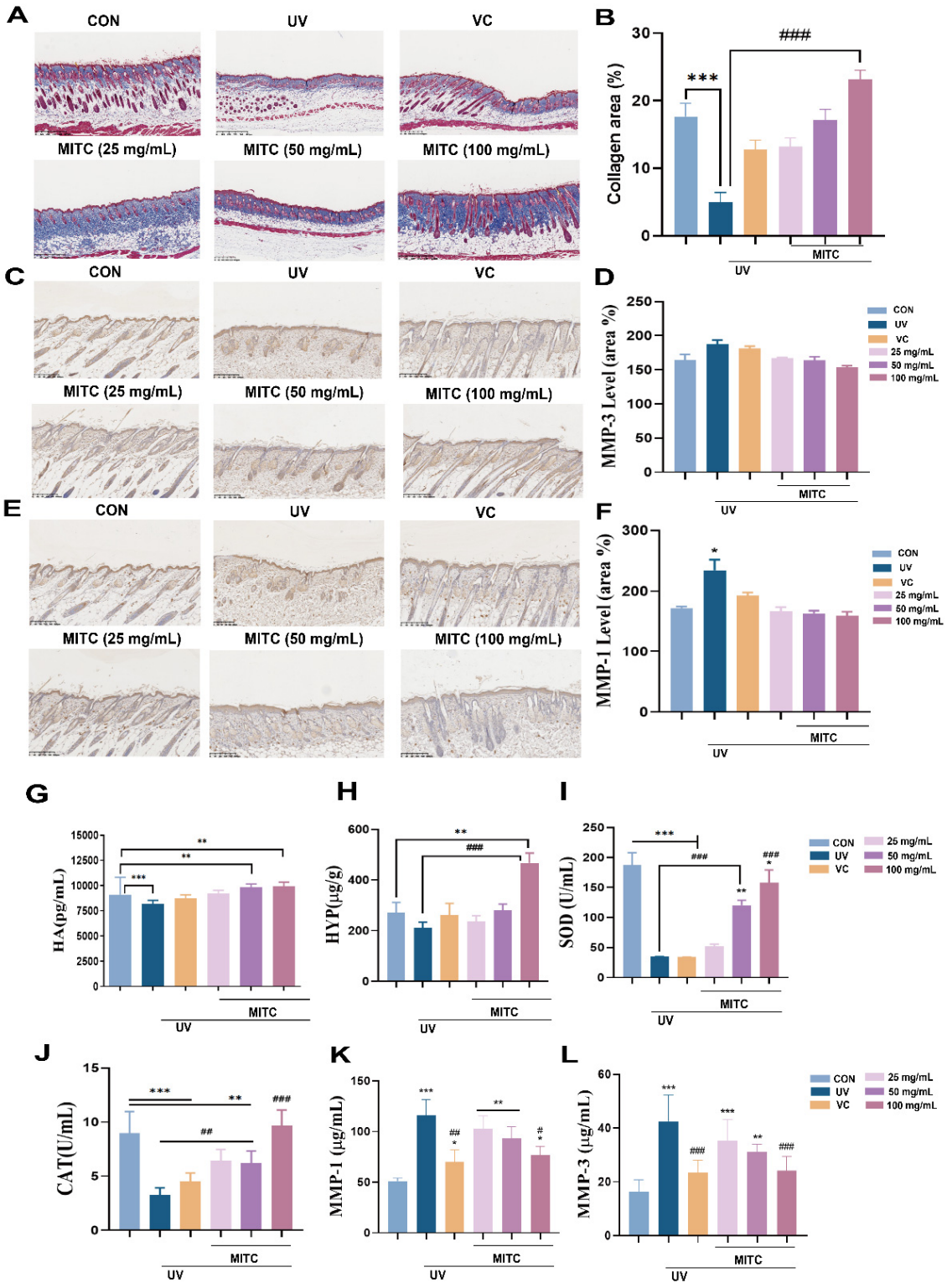

Fig. 2. MITC alleviates UV-induced skin photoaging and is associated with MMP responses and oxidative stress *in vivo*. (A) Masson staining images of skin sections from all groups of mice were captured. (B) Quantitative analysis of collagen content in Masson staining analyses in mouse skin. (C) Immunohistochemical staining histological images of all mouse skin sections of MMP-3 were captured for all the groups. (D) Quantitative analysis of MMP-3 immunofluorescence in mouse skin. (E) Immunohistochemical staining histological images of mouse skin sections of MMP-1 were captured for all the groups. (F) Quantitative analysis of MMP-1 immunofluorescence in mouse skin. (G) Expression levels of HA were determined by ELISA. (H) Expression levels of HYP were determined by ELISA. (I) Expression levels of SOD were determined by ELISA. (J) Expression levels of CAT were determined by ELISA. (K) Expression levels of MMP-1 were determined by ELISA. (L) Expression levels of MMP-3 were determined by ELISA. All data are expressed as the mean ± SEM from three independent experiments, and significance was determined using an unpaired t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. UV group.

3.3. MITC ameliorates photoaging by inhibiting activation of the p38MAPK pathway and promoting autophagy

To determine the underlying biological processes and pathways by which MITC ameliorates anti-photoaging in mice, RNA-Seq analyses were conducted in this study. Significant differences in transcriptional profiles were detected among the control UV and UV +MITC mouse groups (Fig. 3A-C).

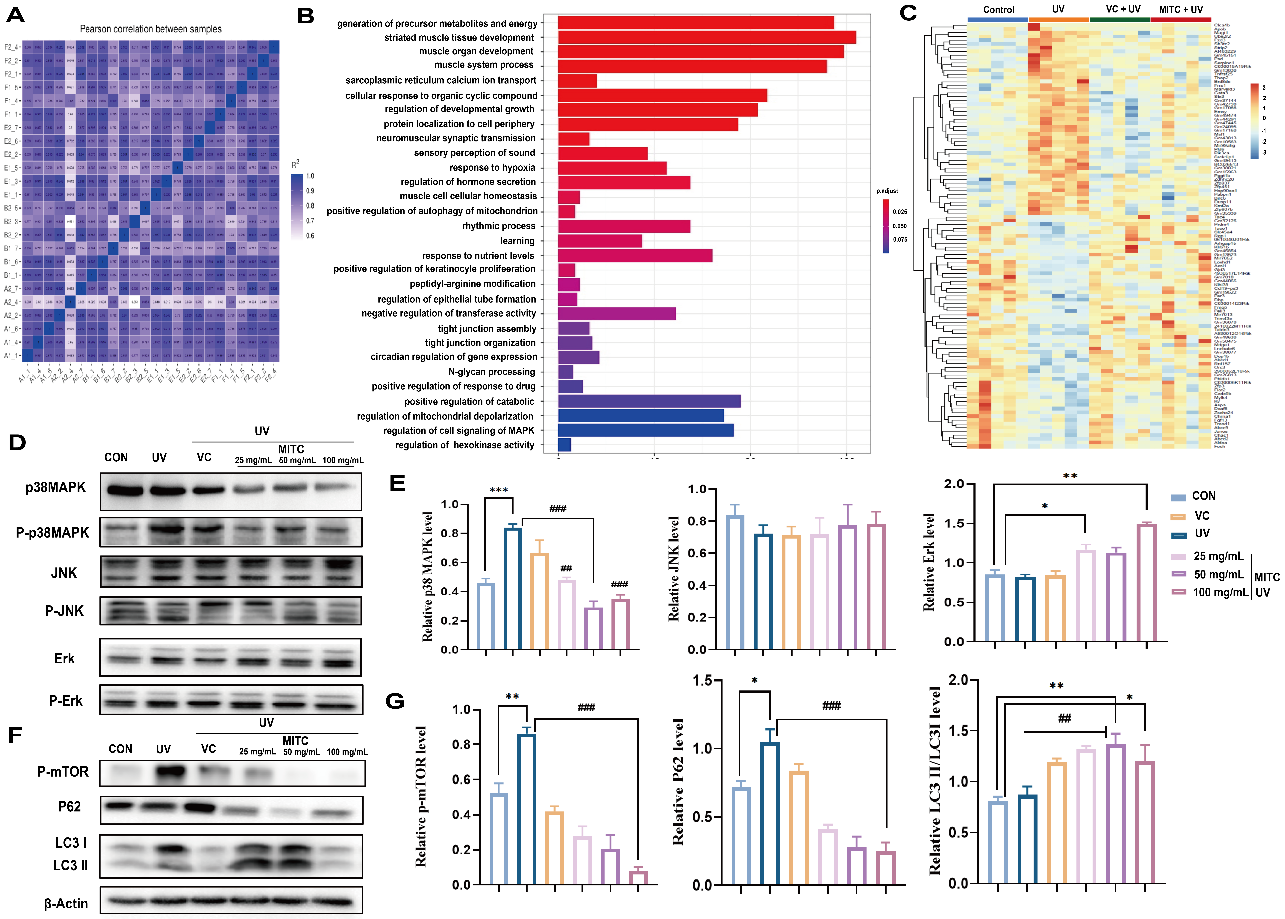

Fig. 3. MITC ameliorates photoaging by inhibiting activation of the p38MAPK pathway and autophagy pathway *in vivo*. (A) Pearson correlation of dorsal skin in the four groups of mice. (B) KEGG enrichment analysis of MITC between the UV and UV+MITC groups. (C) Heatmap of the dorsal skin (differentially expressed genes) of the four groups of mice. The colour in the graph indicates the expression value of the gene after normalization in each sample, with red indicating higher expression of a given gene in that sample and blue indicating lower expression. (D) The protein expression levels of p38MAPK, P-p38MAPK, JNK, P-JNK, Erk, P-Erk, and β-actin were detected by using western blotting. (E) Quantitative analysis of p38MAPK, P-p38MAPK, JNK, P-JNK, Erk, and P-Erk by using *ImageJ*. (F) The protein expression levels of P-mTOR, P62, LC3I, and LC3II were detected by using western blotting. (G) Quantitative analysis of P-mTOR, P62, LC3I, and LC3II by using *ImageJ*. All data are expressed as the mean ± SEM from three independent experiments, and significance was determined using an unpaired t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. UV group.

Specifically. The follow-up GO enrichment analysis indicated that the apoptotic response to UV-induced photoaging. Interestingly, KEGG pathway analysis of the differentially expressed genes indicated that the MAPK pathway and autophagy pathway were highly enriched functional pathways (Fig. 3B). The heatmap results showed that UV induced the upregulation of MAPK pathway-related gene expression in mice, but MITC administration significantly downregulated MAPK pathway-related gene

expression (Fig. 3C). This suggested that MITC intervention could exert its antiphotoaging effects by inhibiting the MAPK signalling pathway.

To determine the role of the MAPK pathway in alleviating skin photoaging by MITC, this study used western blotting technology to detect the expression levels of key proteins in the MAPK signalling pathway and autophagy pathway. The results showed that MITC alleviated UV-induced skin photoaging in mice by regulating the MAPK pathway. Interestingly, compared with the JNK and ERK proteins, MITC had a strong regulatory effect on the p38MAPK protein (Fig. 3D-E). Compared with the UV group, MITC significantly downregulated p38MAPK protein expression in a dose-dependent manner. The results were further verified in phosphorylated proteins (Fig. 3D-E). The results showed that compared with the control group, the expression of p38MAPK protein in the skin was upregulated by UV radiation and significantly downregulated after MITC treatment in a dose-dependent manner. These results indicated that MITC inhibited UV-induced p38MAPK phosphorylation but did not inhibit JNK and Erk phosphorylation. To further verify that MITC had stronger anti-photoaging activity, as shown in Fig. 3F-G, the expression of P62 and mTOR in the UV-damaged *in vivo* model was lower than that in the UV group, while the ratio of LC3I/LC3II was higher. The results showed that MITC can increase the level of autophagy *in vivo* after UV irradiation. This study revealed that the P38MAPK and autophagy pathways may act as UV-induced regulatory pathways for skin photoaging and play an important role in the mechanism of MITC in alleviating skin photoaging.

3.4. MITC restored antioxidant enzyme activity and reduced the high expression of MMPs induced by UV irradiation

Cells are the most basic structures that make up living organisms, and the ageing process of the organism is often accompanied by the onset of cellular senescence [9]. In our study, the antiphotoaging activity of MITC was determined by using a UV-induced HaCaT cell model. MITC had no toxic effect on HaCaT cells, and subsequent experiments were performed at low, medium and high doses at concentrations of 2, 4 and 8 μmol/L, respectively (Fig. 4A). As shown in Fig. 4B, normal HaCaT cells showed shuttle-shaped growth, but after UV irradiation, the cells were crinkled, the cell membrane was broken, and the intracellular cytosol was exposed, which was repaired by MITC treatment. The activities of SOD and CAT were measured in the present study because previous studies indicated that antioxidative enzymes protect the skin from UV-induced photodamage by scavenging ROS. It was observed that the activities of CAT and SOD were significantly decreased after continuous UV exposure compared to the control group. However, when treated with MITC, the decrease in CAT and SOD induced by UV irradiation was gradually mitigated (Fig. 4C-D). This result indicated that MITC had the best antioxidative activity. Thus, MITC had a certain ability to maintain the balance of oxidation and antioxidation in HaCaT cells.

Proteolytic enzymes such as MMPs are produced by epidermal keratinocytes and fibroblasts in the mediation of ECM remodelling. MMPs initiate the photoaging of the skin by acting as collagenases. Collagen and elastin are the major structural proteins in the ECM. However, UV radiation induces high

expression of MMP-1 and MMP-3. MMP-1 is the most important enzyme for degrading the components of the ECM and breaking the normal structure of collagen fibres and elastic fibres [5, 6]. MMP-3 is a stromelysin enzyme that degrades denatured collagens [12]. In our study, the expression levels of MMP-1 and MMP-3 were significantly higher in the UV group. Furthermore, when treated with MITC, the expression levels were significantly decreased compared with those in the UV group (Fig. 4E-F). This indicates that MITC can reduce collagen loss caused by UV irradiation. Moreover, the contents of oxidation factors in the body indicate that MITC can reduce the oxidative stress response.

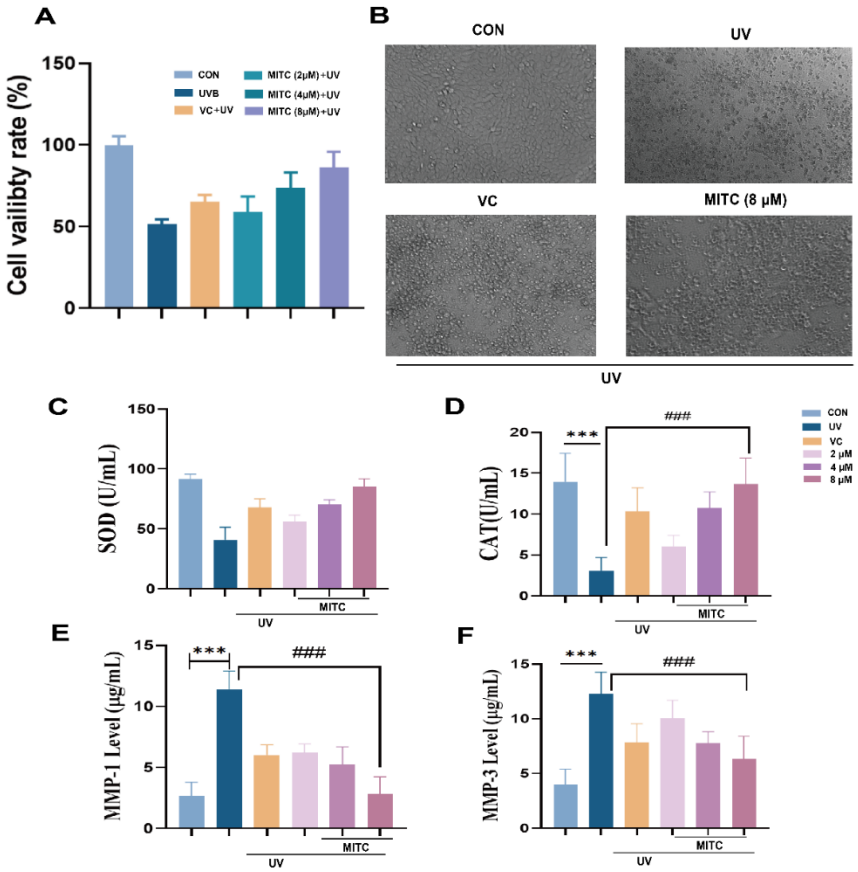

Fig. 4. MITC restored cell antioxidant activity and reduced the high expression of MMPs induced by UV irradiation. (A) Inhibition of the proliferation of UV-induced HaCaT cells treated with MITC. (B) Cell morphology of UV-induced HaCaT cells treated with MITC. (C) Expression levels of SOD were determined by ELISA. (D) Expression levels of CAT were determined by ELISA. (E) Expression levels of MMP-1 were determined by ELISA. (F) Expression levels of MMP-3 were determined by ELISA. All data are expressed as the mean ± SEM from three independent experiments, and significance was determined using an unpaired t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. UV group.

3.5. MITC can decrease ROS levels and inhibit apoptosis in cells after UV irradiation

According to the theory of free radical ageing, oxidative stress caused by ROS accumulation can interact directly with lipids, proteins, and nucleic acids or promote cellular ageing through a series of signalling pathways, which is widely considered one of the core mechanisms mediating skin ageing. Overexpression of MMPs stimulates ROS secretion and accelerates oxidative damage in the skin [4, 25].

As shown in Fig. 5A-B, the ROS level in UV-irradiated cells was increased significantly compared with that in the control group, but the production of intracellular ROS decreased after MITC treatment, proving indicating that UV-irradiated cells caused cell damage similar to the increase in ROS caused by photoaging. The results showed that MITC could significantly remove UV-induced ROS and protect cells from damage caused by photoaging. In addition, UV-irradiated cells produce a large amount of ROS, and excess ROS also lead to apoptosis, while MITC treatment repairs cell growth and reduces apoptosis (Fig. 5C-D). Mitochondrial membrane potential plays an important role in apoptosis and ROS generation [5]. The effect of MITC on UV-induced HaCaT cells was further examined. As shown in Fig. 5E, after UV irradiation of damaged cells, the green fluorescence intensity was much higher than that of the control group. However, after MITC treatment, the red fluorescence increased, the green fluorescence decreased, and the green–red fluorescence ratio decreased. These results indicated that the antioxidant effect in cells is related to the recovery of mitochondrial function. Subsequently, we found that UV irradiation could significantly increase ROS release in HaCaT cells and promote cell apoptosis. After treatment with MITC, ROS levels and the cell apoptosis rate were reduced.

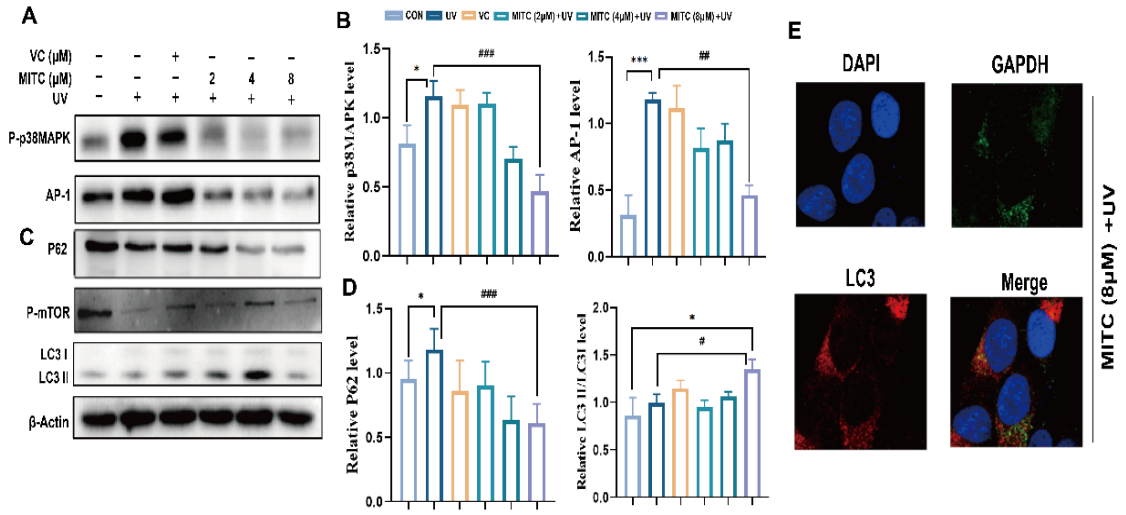

Fig. 5. MITC decreased the ROS level and inhibited apoptosis of cells after UV irradiation. (A) ROS production was determined by measuring the DCF fluorescence level in HaCaT cells. (B) Quantitative analysis of ROS immunofluorescence levels in cells. (C) The apoptosis test group of HaCaT cells treated with MITC. (D) Quantitative analysis of the ratio of apoptosis levels in cells. (E) The mitochondrial membrane potential test of HaCaT cells treated with UV radiation and MITC. All data are expressed as the mean ± SEM from three independent experiments, and significance was determined using an unpaired t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. UV group.

3.6. MITC ameliorates skin photoaging by regulating p38MAPK-dependent autophagy pathways

Autophagy is a highly conserved degradation mechanism widely present in cells from yeast to higher vertebrates that removes damaged organelles and biomolecules, such as proteins and lipids, to maintain the homeostasis of the internal environment [2, 3]. In addition, the autophagic lysosomal pathway is currently the only pathway capable of clearing entire organelles such as mitochondria, and studies have shown that autophagy is closely related to senescence and shares many common features, with activation of autophagy delaying senescence and overexpression of autophagy-related genes inhibiting stress-induced cellular

senescence [7]. Furthermore, autophagy is involved in skin ageing regulation, and the MAPK signalling pathway may be a potential connection between skin ageing and autophagy. To further verify that MITC had stronger antiphotoaging activity, we treated UV-damaged cells with different doses of MITC.

In our study, compared with that in the control group, the expression of P-p38MAPK in HaCaT cells was increased after UV irradiation but significantly decreased after MITC treatment. The AP-1 protein also showed some results that were similar to those of p38MAPK (Fig. 6A-B). Compared with the UV group, MITC significantly downregulated p38MAPK protein expression in a dose-dependent manner. As shown in Fig. 6 C-D, the expression of LC3, P62 and mTOR in UV-damaged HaCaT cells was higher than that in the control group. Confocal fluorescence microscopy showed that MITC treatment promoted the expression of LC3 in UV-induced cells (Fig. 6E). The results showed that MITC can increase the level of autophagy in photoaging cell models after UV irradiation, mainly manifested in the significant downregulation of P62 protein expression and upregulation of LC3B II protein expression. MITC treatment promoted the conversion of LC3 I to LC3 II.

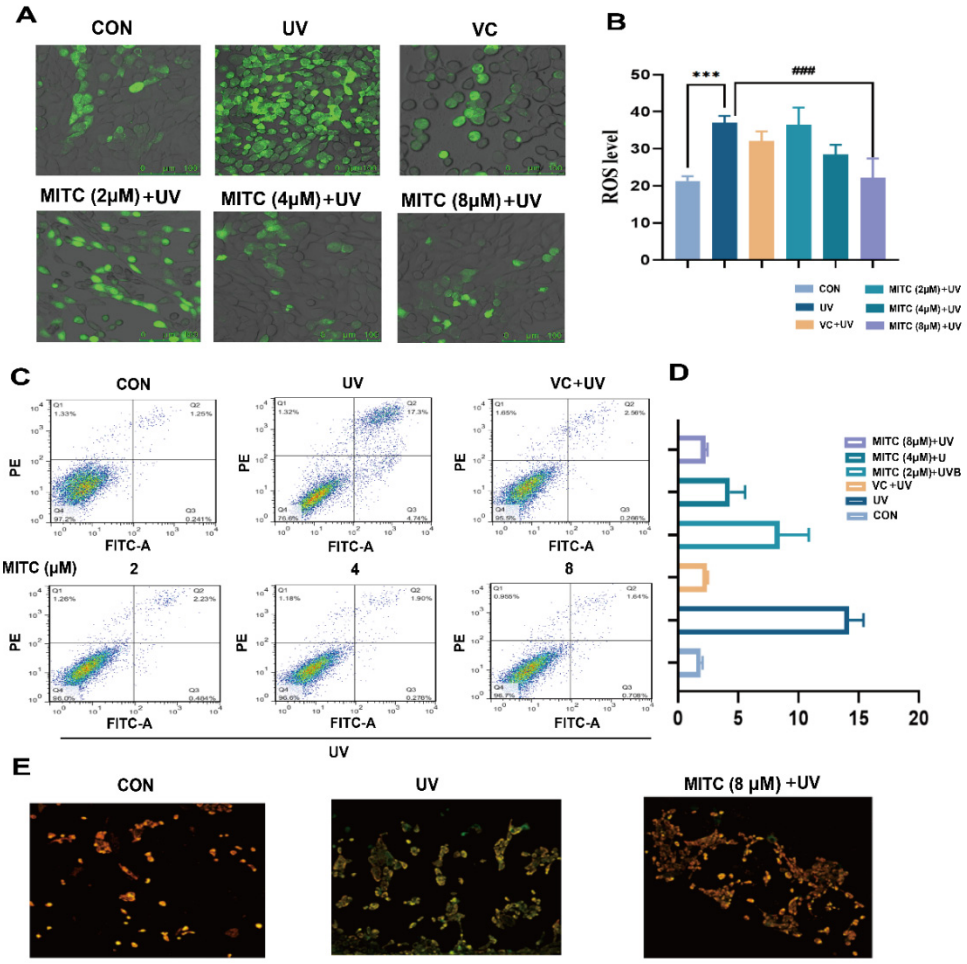

Fig. 6. MITC ameliorates skin photoaging by regulating the p38MAPK-dependent autophagy pathway. (A) The protein expression levels of P-p38MAPK, AP-1, and β-actin were detected by using western blotting. (B) Quantitative analysis of P-p38MAPK and AP-1 by using *ImageJ*. (C) The protein expression levels of P-mTOR, P62, LC3I, and LC3II were detected by using western blotting. (D) Quantitative analysis of P-mTOR, P62, LC3I, and LC3II by using *ImageJ*. (E) Immunofluorescence confocal staining for determining LC3 (red) in HaCaT cells. All data are expressed as the mean ± SEM from three independent experiments, and significance was determined using an unpaired t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. UV group.

4. Discussion

Skin photoaging is a phenomenon of skin ageing caused by long-term and repeated exposure to ultraviolet rays [33]. The common clinical manifestations of skin photoaging include loss of skin elasticity, epidermal atrophy, abnormal pigmentation, and even cell necrosis, apoptosis or cancer [34, 35]. Skin photoaging not only impairs aesthetic appearance and mental quality of life but is also associated with the development of skin cancer, posing a serious health risk. However, in contrast to irreversible skin ageing, skin photoaging is a preventable and treatable skin disease [36]. Laser treatment and surgery are effective methods to improve the appearance of photoaged skin at present. However, due to the strong penetration of lasers, the heat generated on deep tissue or skin surfaces may cause soft tissue burns if the energy is not controlled properly, and there are certain risks in surgical scrubbing. Chemical retinoic acid is currently the only product approved by the US Food and Drug Administration for photoaging treatment, but it may cause side effects such as erythema [37,38]. Studies have found that plant natural active substances have a good biological effect on skin photodamage protection [39]. Thus, the exploration of natural active substances with fewer side effects in the treatment of skin photoaging diseases has become the focus of antiphotoaging research.

Moringa oleifera Lam. is a plant with a short growth cycle and high economic value that is widely planted in tropical and subtropical regions. Studies have found that *Moringa oleifera* Lam. can be used as an edible and medicinal resource [40]. *Moringa oleifera* Lam. is rich in active ingredients, which exhibit good pharmacological activities, such as hypoglycaemia, antitumour, lipid regulator, antioxidant and hepatoprotective activities [22, 23, 24]. Isothiocyanates are a class of sulfur-containing plant secondary metabolites that are widely found in Brassicaceae of the cruciferous family [26, 27]. Isothiocyanates are one of the main active ingredients of *Moringa oleifera* Lam. and the highest content (2%-4%) was found in *Moringa oleifera* Lam. seeds. The presence of aromatic rings and rhamnose fractions in MITC has higher biological activity and chemical stability than isothiocyanate compounds from other plants, with the highest content of 4- [(α-L-rhamnose oxy) benzyl] isothiocyanate (MITC) (Fig. 1A). Our previous studies have found that MITC has anti-skin photoaging activity, but the exact mechanism is not clear [41]. Thus, the present study reveals the key role of MITC in anti-photoaging, which regulates the activation of the p38MAPK pathway-mediated autophagy signalling pathway to enhance cellular autophagy and inhibit apoptosis, thereby inhibiting UV-induced photoaging.

Oxidative stress is a key factor in skin photoaging and is also considered a major cause of intrinsic ageing in addition to genetic factors [42]. Skin is exposed to damage from environmental UV radiation, causing oxidative damage to macromolecules by ROS, ultimately leading to cellular ageing and subsequent metabolite accumulation in cells [43, 44]. Long-term exposure to UV radiation will increase the level of ROS in skin, and this oxidative stress reaction will also decrease oxidative stress factors, such as SOD and CAT [45]. Our research confirms that UV radiation drives oxidative stress in the skin, promoting increased levels of oxidative factors in the skin and that MITC significantly ameliorates this occurrence to defend against

potentially oxidatively damaging irritants in the skin (Fig. 2I-J, Fig. 4C-D, Fig. 5A-B). In addition, excessive oxidative stress leads to apoptosis in many normal cells, which eventually disrupts homeostasis [46]. MITC can maintain cell survival and avoid the occurrence of apoptosis produced by UV radiation (Fig. 5C-D).

In addition, chronic UV exposure can lead to undesirable changes in the extracellular matrix, leading to skin photoaging due to an excess of ROS-mediated MMPs, such as MMP-1 and MMP-3 [47, 48]. When the skin is exposed to long-term UV radiation for a long time, the skin epidermis will produce MMPs, which can degrade the extracellular matrix, cause inflammatory reactions and skin photoaging, and degrade skin collagen, especially type I collagen in skin cells [40, 45]. When MMPs in the skin increase greatly, MMP-1 and MMP-3 can jointly degrade collagen and turn it into a small molecule peptide, resulting in a large loss of collagen. After the loss of collagen, the skin will suffer serious ageing phenomena, such as wrinkles, freckles and loss of skin elasticity [49]. In addition, the self-repair ability of damaged collagen is weakened, so the content of collagen fibre and elastic fibre decreases, so that the synthesis ability of collagen in the skin is reduced, and the elastic fibre is abnormal [48, 50]. Among them, hydroxyproline is an important component of collagen and is mainly found in skin collagen, while hyaluronic acid is an acidic mucopolysaccharide secreted by fibroblasts in the skin and has an important water retention function [51]. According to the experimental results, the expression levels of MMP-1 and MMP-3 in the MITC group were significantly reduced compared with those in the UV group, indicating that MITC can reduce matrix lysin and interstitial collagenase to achieve antiphotoaging effects (Fig. 2 C-F, Fig. 2K-L). Moreover, skin collagen was repaired and elevated in MITC-treated mice (Fig. 2A-B). At the same time, MITC can also increase the contents of hydroxyproline and hyaluronic acid, and these factors can improve the self-repair ability of skin collagen (Fig. 2G-H). These results highlight the role of MITC in the regulation of oxidative stress and matrix metalloproteinase responses during skin photoaging.

Although there are many studies on skin photoaging, the free radical ageing theory is currently the most recognized theory [2,52]. Repeated UV irradiation generates a large amount of ROS in skin tissue, and excessive ROS can cause damage to biological macromolecules such as nucleic acids and proteins in cells, leading to cellular ageing and even apoptosis [53, 54]. MAPK is a highly conserved serine/threonine protein kinase that mainly includes JNK, p38MAPK kinase, and ERK. It was shown that ROS generated by UV radiation can promote p38MAPK kinase phosphorylation but not JNK kinase or ERK kinase phosphorylation by activating MKK6, the upstream kinase of MAPK [12, 13, 14]. Therefore, p38MAPK is the most UV-stimulated and extremely ROS-sensitive class of MAPK kinases, and activated p38MAPK kinase will activate the downstream AP-1 protein, which can promote the secretory expression of collagenases, thereby breaking down the skin ECM, degrading skin collagen and elastin, and aggravating skin photoaging damage [55, 56]. Using photoaged mice, we demonstrated that p38MAPK phosphorylation and oxidative stress were reversed after MITC treatment (Figure 3), inducing downregulated oxidative factor and matrix metalloproteinase expression and upregulated hyaluronic acid and hydroxyproline contents in the skin (Fig. 2).

Numerous studies have shown that an abnormal reduction in autophagic activity is an important cause of photoaging. In the absence of autophagy in keratinocytes, oxidative stress, DNA damage and cellular ageing are increased [57, 51]. Knockdown of autophagy-related genes accelerated senescence in human primary fibroblasts, mouse fibroblasts and melanocytes. Conversely, overexpression of autophagy-related genes delays ageing [58, 52]. In the in *vivo* photoaging model, we examined the expression of autophagy-related proteins in the skin and showed that excessive UV irradiation led to downregulation of autophagy proteins, which was reversed by MITC treatment. MITC activated autophagy by downregulating the expression of upstream regulators of autophagy, such as mTOR and p62 proteins, and increased the LC3I/LC3II protein ratio (Fig. 3, Fig. 6). Some research has indicated that the autophagy inducer rapamycin is also able to delay UV-induced photoaging by decreasing ROS production and activating autophagy [59]. Studies have reported that autophagy is closely related to MAPK signalling, and the MAPK signalling pathway may be a potential link between oxidative stress-induced skin ageing and the autophagic response. Propolis exerts anti-inflammatory effects by inhibiting the MAPK/NF-κB pathway and autophagy [60, 61]. These findings underscore the role of MAPK-mediated activation of autophagy in the skin photoaging process. The roles of the MAPK and autophagy signalling pathways have been reported. Our results showed that UV may contribute to skin photoaging by activating p38MAPK activity, inhibiting autophagy levels, and inducing apoptosis. In keratinocyte cells, treatment of normal cells with UV confirmed the *in vivo* results (Fig. 4). MITC treatment decreased p38MAPK protein expression levels, increased autophagy-related protein expression (Fig. 6A-E), reduced cell apoptosis and downregulated intracellular oxidative factor expression (Fig. 5). Therefore, reducing p38MAPK activity and increasing autophagic activity against apoptosis are important for delaying the UV-induced photoaging process. For the first time, our study investigated and revealed that MITC activates the p38MAPK/autophagy pathway and inhibits apoptosis by inhibiting UV-induced ROS production, confirming the upstream regulatory role of MITC in the oxidative response to skin photoaging, and further studies are necessary to examine the deep modification of the p38MAPK/autophagy-mediated oxidative apoptotic response in the skin photoaging process.

5. Conclusion

In conclusion, repeated UV irradiation will lead to the production of many ROS in skin tissues, which will eventually induce cell senescence and apoptosis. Our recent study found that MITC can significantly inhibit photoaging *in vivo* and *in vitro* and reduce ROS levels. Moreover, MITC inhibited the activation of p38MAPK and improved autophagy levels in the photoaging model, ultimately reducing the expression of MMP-1 and MMP-3, reducing the degradation of collagen, and inhibiting cell apoptosis in the photoaging model. Based on previous studies and our recent experimental results, we speculated that MITC could inhibit the production of UV-induced ROS, thereby inhibiting the activity of p38MAPK, activating the autophagy pathway and inhibiting cell apoptosis to play an anti-skin photoaging role (Fig. 7). This study not

only provides a theoretical and experimental basis for the development of MITC as a new antiaging material and confirms that the p38MAPK-mediated autophagy signalling axis may be a potential therapeutic target for the management of skin photoaging but also provides new ideas for the high-value utilization and development of *Moringa oleifera* Lam. seeds resources.

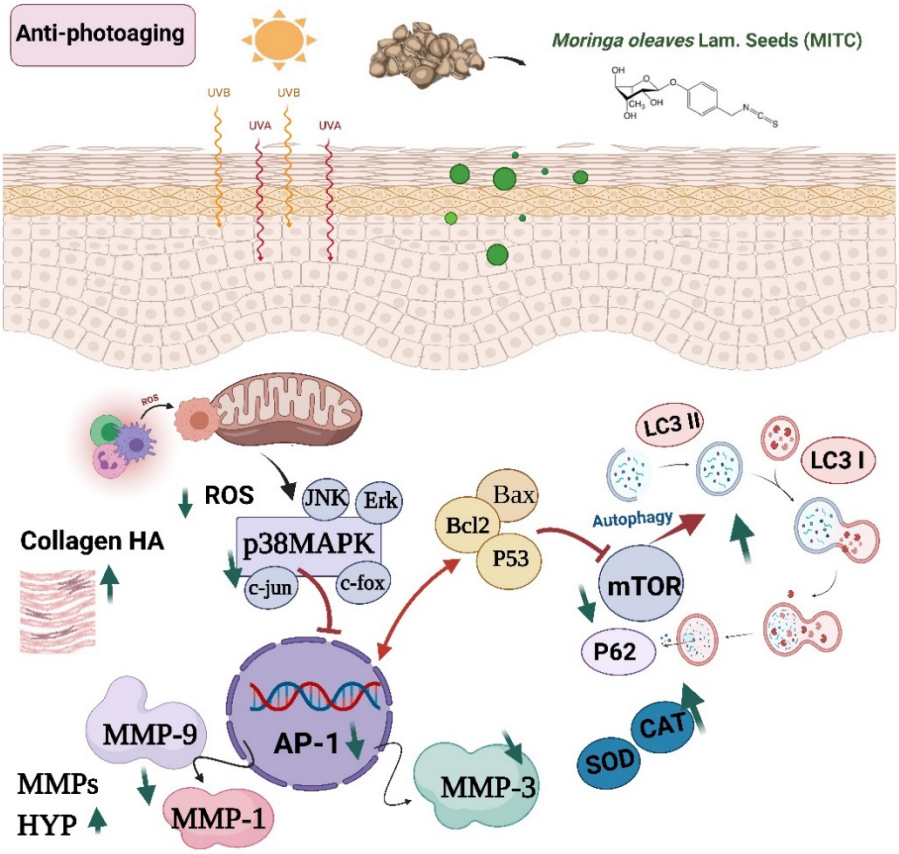

Fig. 7. Graphical description of MITC inducing ROS-mediated apoptosis by regulating the p38MAPK-dependent autophagy pathway during skin photoaging.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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