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ORIGINAL RESEARCH

Ergothioneine Protects Against UV-Induced Oxidative Stress Through the PI3K/AKT/Nrf2 Signaling Pathway

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Background: Ergothioneine (EGT) is an antioxidant, which could be detected in human tissues, and human skin cells could utilize EGT and play an anti-oxidative role in keratinocytes. And in this study we are going to elucidate whether EGT could protect the skin from photoaging by Ultraviolet (UV) exposure in mice and its molecule pathway.

Methods: Histological analysis was performed for evaluating the skin structure change. Malondialdehyde (MDA) and superoxide dismutase (SOD) levels were measured with biological assay for evaluating oxidative and antioxidative ability of skin exposed to UV light. And the level of marker molecules in mouse skin were detected by hydroxyproline (Hyp) assay, immunohistochemical analysis, Western blot, and quantitative real-time PCR (qRT-PCR). The markers of skin aging and cell death were tested by cell culture and treatment, Western blot and qRT-PCR.

Results: EGT decreased the levels of inflammatory factors induced by UV exposure in mouse skin. MDA and SOD activity detection showed that EGT decreased MDA levels, increased SOD activity, and upregulated PI3K/Akt/Nrf2 signals in mouse skin exposed to UV, which further activated Nrf2 in the nucleus and enhanced the expression of Nrf2 target genes. In the cell model, we revealed that EGT could inhibit the increase in senescence-associated β-galactosidase-positive cells and p16 and γ-H2A.X positive cells induced by etoposide and activate PI3K/Akt/Nrf2 signaling. Moreover, a PI3K inhibitor blocked EGT protection against etoposide-induced cell death.

Conclusion: The study showed EGT may play an important protective role against cell damage or death through the PI3K/Akt/Nrf2 signaling pathway in skin.

Keywords: ergothioneine, skin aging, PI3K/Akt, Nrf2

Introduction

EGT is a small molecule antioxidant isolated from microorganisms and has been showed to have varied levels in human tissues and blood after dietary intake.[1] The levels of EGT ranges from 100 μM to 2 mM in human tissues.[1–3] Previous studies showed that human skin cells could utilize EGT, playing an anti-oxidative role in keratinocytes.[4] However, it was unknown whether EGT could protect the skin against photoaging caused by UV light.

The UV light from the sun consists of UVA, UVB, and UVC.[5] The UVA could alter DNA through the formation of reactive oxygen species (ROS).[5] Although the skin possesses higher antioxidant activities, it has been demonstrated that ROS levels increase and antioxidant defenses decline during aging.[6,7] ROS is a main cause of skin aging. It also inhibited collagen generation in fibroblast cells.[5] In addition, UVB can promote age-related signal transduction, resulting in photoaging.[8] Keratinocytes are the main cell type affected by solar UVB radiation.[8] UVB irradiation-induced skin aging is mediated by reactive oxygen species (ROS) from keratinocytes.[9]

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UV radiation is primarily absorbed by the skin and induces skin aging.[8] During the skin aging process, there is a dramatic decrease in the percentage of collagen I and III in the skin.[10–12] The elastase from neutrophils that migrates to the dermis after UV exposure, as well as the activation of matrix metalloproteases (MMPs), further disrupts the extracellular matrix, especially with significant up-regulation of MMP1, 3 and 9.[13,14] At the same time, excessive ROS activates the NF-κB signaling pathway, leading to an increase in MMP expression.[15] Collagen is cleaved by MMP1 and completely degraded by MMP3 and MMP9.[16]

The skin has an antioxidant system that maintains oxidative hemostasis and resists excess ROS,[4] which includes antioxidants such as vitamin C, glutathione, and superoxide dismutase (SOD).[4] UVA radiation could upregulate the central molecule NF-E2-related factor-2 (Nrf2).[1] Under unstimulated conditions, Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1). Keap1 interacts with Nrf2, and then they are translated and degraded via ubiquitinated proteasomal degradation.[17] Under stimulus conditions, Nrf2 translocates into the nucleus and activates the downstream antioxidative genes.[1] The target genes of Nrf2 include glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase (NQO1), heme oxygenase-1 (HO-1), and thioredoxin reductase (Txnrd1), which are activated by nuclear Nrf2 and play antioxidative roles in skin.[18,19]

In our study, we tested that the role of EGT in protecting against skin aging in mouse and cell model, and researched its molecular pathway. And we found that EGT could protect the skin or cells from damage caused by UV or DNA-damaging drugs. Additionally, EGT activates PI3K/Akt/Nrf2 signaling to protect the skin against UV exposure and maintain cell viability from etoposide-induced cell death. Overall, EGT would play an important protective role against skin photoaging through the PI3K/Akt/Nrf2 signaling pathway.

Materials and Methods

Animal Ethics

The study was approved by the Institutional Animal Care and Use Committee of Guangdong Laboratory Animal Monitoring Institute (IACUC no. 2021203, AAALAC accredited), and adhered the "Care and Use of Laboratory Animals" guidelines. Female ICR mice were purchased from the Guangdong Medical Laboratory Animal Center. The mice were fed and drank water randomly under specific pathogen-free conditions, and the light–dark shift was 12 h.

Cream Preparation and UV Irradiation

The EGT was prepared as described previously.[20] The matrix consisted of stearic acid, glycerin monostearate, liquid paraffin, white petrolatum, menthol, ethylparaben, triethanolamine, lauryl sodium sulfate, glycerin, and deionized water. The matrix was mixed with quercetin or EGT to form Que and EGT creams, respectively.

According to the experimental design, mice were divided into control group, UV group, UV and Matrix group (UV + Matrix), UV and 0.4 mM Que group (UV + Que), UV and 10 mM EGT group (UV + EGT). The UV irradiation dosage was referred to in a previous report.[21] Briefly, hair was removed from the back of mouse using depilatory cream. The control group was only treated with depilation without other treatments, and the other groups were treated with the matrix or cream and exposed to UV irradiation using the Skin Phototoxic Aging Integrated Experimental Detector (HOPE-MED8134, Tianjin Hope Industry & Trade Co. LTD). The matrix or cream was applied to the back of the mice 30 min before UV irradiation and was erased softly 30 min after UV irradiation. Mice were exposed as follows: to UVA 8 mJ/cm^2 and UVB 30 mJ/cm^2 on the first day; to UVB 30 mJ/cm^2 on the second day; to UVA 10 mJ/cm^2 and UVB 40 mJ/cm^2 on the third day; to UVB 40 mJ/cm^2 on the fourth day; and to UVA 12 mJ/cm^2 and UVB 50mJ/cm^2 on the fifth day. On the sixth day, the skin on the back was sampled and analyzed.

Histological Analysis

The mice were euthanized after being anesthetized. Then, skin tissues were sampled from the back of the mouse and fixed in 4% paraformaldehyde. The skin tissues were dehydrated and hyalinized (Leica ASP300S, Germany), then embedded in wax blocks, cut into 4 μm sections, and stained with hematoxylin and eosin (H&E). After staining, the skin sections were scanned with a Digital Pathology Scanner Versa 8 (Leica, Germany), and skin characteristics were assessed using a Leica Aperio Versa 8 microscope (Leica, Germany). The thickness of the skin epidermis was assessed in four randomly selected areas and analyzed using GraphPad Prism 8.3.0 (USA).

Immunohistochemical Analysis

Immunohistochemical staining was performed according to the manual of the SP Rabbit & Mouse horseradish peroxidase (HRP) Kit (DBA) (Cowin Biotech, China). Tissue sections were dewaxed, then recovered in the citrate buffer solution, and placed in a pressure cooker for 5 min. Solution A (endogenous peroxidase blocking solution) and Solution B (goat serum) were added and blocked. Primary antibodies (Nrf2 1:300, CST) were incubated at 4°C overnight, then Solution C (Biotin labeled goat-anti-rabbit and mouse secondary antibody working solution) was added and incubated for 1 h, followed by adding Solution D (HRP-labeled streptavidin) and incubating for 30 min. Development was performed using a DBA working solution. The desired stain was controlled with a microscope, and the sections were sealed with neutral gum. Finally, the sections were scanned and analyzed using Aperio ImageScope v12.4.3 software (Leica, Germany).

Cell Culture and Treatment

The HaCaT cells were obtained from BeNa Culture Collection (Beijing, China) and were cultured in DMEM medium (Gibco) containing 10% fetal blood serum (FBS, Gibco) and 1% penicillin-streptomycin solution (Gibco) at 37°C in a 5% CO_2 incubator (Thermal Fisher, USA). Briefly, the cells were incubated by the trypsin, counted, and seeded at a density of 1×10^5 cells in 24-well plates. The cells were then cultured overnight, the medium was replaced with fresh medium, and they were treated with etoposide for 48 h. DMSO was used as a control. After that, the cells were treated with quercetin or EGT for 48 h. Finally, the cells were harvested for SA-β-gal activity analysis, immunofluorescence staining, RT-PCR detection, and Western blotting.

Senescence-Associated β-Galactosidase (SA-β-Gal) Staining

SA-β-gal staining is based on the increased activity of senescence-associated β-galactosidase in senescent cells or tissues and was stained using the Senescence β-Galactosidase Staining Kit (Beyotime, China). Briefly, cells were washed with 1× PBS and fixed with β-Gal fixation solution for 15 min at room temperature. The cells were then stained with the working solution and sealed with parafilm at 37°C overnight. The next day, the cells were photographed under a microscope (Leica, Germany), and the SA-β-gal-positive cells were calculated using ImageJ (National Institutes of Health, USA) and analyzed using GraphPad Prism 8.3.0 (USA).

Western Blot

Skin protein lysates were extracted using RIPA (Thermo Fisher, USA), and nuclear proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech (Shanghai) Co., Ltd., China). The protein concentration was measured using a BCA assay kit (Thermo Fisher Scientific). After SDS-PAGE, the proteins were transferred to PVDF membranes (Immobilon, USA). The membranes were blocked with 5% nonfat dry milk in TBST (TBS buffer containing 0.1% Tween-20) and incubated with primary antibodies and secondary antibody conjugated with horseradish peroxidase (HRP). The membranes were then incubated for 2 min with Immobilon Western Chemiluminescent HRP substrate (Millipore, USA) and developed using Azure Biosystems c600 (USA). The gray value ratio of the target protein was analyzed using ImageJ software. Gapdh and Lamin b were used as internal controls. Details of the antibodies used are listed in Supplementary Table 1.

RNA Extraction and qRT-PCR

Total RNA was extracted from skin tissue or HaCaT cells using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Then cDNA was reverse transcripted using A PCR reverse transcription kit (TaKaRa, China), then the TB Green® Premix Ex Taq™ II kit (TaKaRa, China) was used to detect the relative mRNA levels. *Actb* was used as the internal control. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows: *Tnfα* (forward primer: 5'-ATGA GAAGTTCCCAAATGGC-3'; reverse primer: 5'-CTCCACTTGGTGGTTTGCTA-3'). *HO-1* (forward primer: 5'- AAG CCGAGAATGCTGAGTTCA-3'; reverse primer: 5'- GCCGTGTAGATATGGTACAAGGA-3'). *Txnrd1* (forward primer: 5'- GGGTCCTATGACTTCGACCTG-3'; reverse primer: 5'- AGTCGGTGTGACAAAATCCAAG-3'). *Nqo1* (forward primer: 5'- AGGATGGGAGGTACTCGAATC-3'; reverse primer: 5'- TGCTAGAGATGACTCGGAAGG-3'). *Gpx2* (forward primer: 5'- GCCTCAAGTATGTCCGACCTG-3'; reverse primer: 5'- GGAGAACGGGTCATCATAAGGG-3'). *Actb* (forward primer:

5’- GGCTGTATTCCCCTCCATCG-3’; reverse primer: 5’- CCAGTTGGTAACAATGCCATGT-3’). *IL-6* (forward primer: 5’-ACTCACCTCTTCAGAACGAATTG-3’; reverse primer: 5’-CCATCTTTGGAAGGTTCAGGTTG-3’). *TNFα* (forward primer: 5’-CCTCTCTCTAATCAGCCCTCTG-3’; reverse primer: 5’-GAGGACCTGGGAGTAGATGAG-3’). *ACTB* (forward primer: 5’-CATGTACGTTGCTATCCAGGC-3’; reverse primer: 5’-CTCCTTAATGTCACGCACGAT-3’).

Statistical Analysis

Data were analyzed using GraphPad Prism 8.3.0 (USA). Results are shown as mean ± standard error of the mean (SEM). Statistical significance of differences was analyzed using Student’s *t*-test (two-way, unpaired) or two-way ANOVA (*Bonferroni*). Probability values (*P*) of <0.05 were considered significant. For comparisons between the control group and UV group or UV + Matrix group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. For comparisons between the Que group or EGT group and UV + Matrix group: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Results

EGT Reduces the Change of Skin Structure Induced by UV Irradiation

In this study, we found that UV exposure could decrease mouse weight and increase epidermal thickness (Figure 1, and Supplementary Figure 1A and B). Compared with the control group, the epidermis was thickened, with incomplete keratosis, and inflammatory cells gathered in the dermis in the UV and UV + Matrix groups. In the Que and EGT groups, the epidermis thickness and inflammatory cells decreased compared with that in the UV + Matrix group (Figure 1 and Supplementary Figure 1B). The results showed that EGT could reduce skin damage induced by UV exposure, providing protection comparable to that of Que.

EGT Could Reduce Oxidative Stress and Inflammation in Skin Induced by UV Exposure

UV exposure induces the production of ROS and regulates proinflammatory cytokines.[22,23] UVB irradiation increases inflammatory factor TNFα.[24] Therefore, we investigated whether EGT could regulate ROS and TNF-α in mouse skin exposed to UV light.

Malondialdehyde (MDA) is a marker of oxidative stress and an enzyme biomarker, as well as an end product of oxidative stress.[25] The antioxidant defense system of the skin includes lipophilic radical scavengers, like superoxide

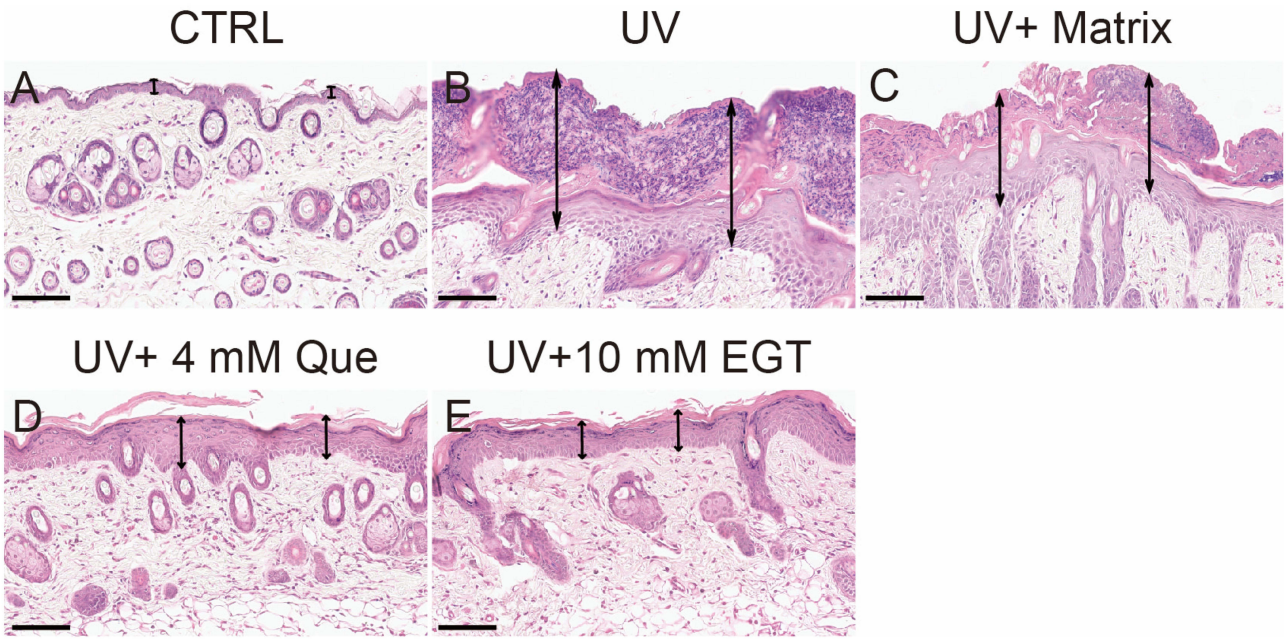

Figure 1 EGT reduce the increase of epidermis thickness under UV irradiation. H&E staining of back skin of control (CTRL) group (**A**), UV group (**B**), UV+Matrix group (**C**), UV+Que group (**D**), UV+EGT group (**E**). The scale bar was 100 μm, and the black double arrows indicate the thickness of epidermis.

dismutase (SOD).[25–27] ROS can induce the activity of the antioxidant enzyme SOD.[9] The UV and UV+ Matrix groups showed higher MDA levels and lower SOD activity than those of the control group. In the Que and EGT groups, the results show MDA levels decreased significantly, and SOD activity increased significantly in the EGT group (Supplementary Figure 2A and B).

Compared to the control group, UV irradiation significantly increased the level of *Tnfα* mRNA in the UV and UV+ Matrix groups. In comparison with the UV+ Matrix group, the levels of *Tnfα* in the skin of the Que and EGT groups decreased significantly, and the EGT group showed lower *Tnfα* levels than the Que group (Supplementary Figure 3). Therefore, EGT decreased ROS and proinflammatory factors and enhanced SOD activity in skin exposed to UV.

EGT Could Downregulate MMPs and Upregulate Collagens

High ROS production results in the overexpression of MMPs (especially MMP1), leading to collagen and elastin degradation.[25] Compared with the control group, levels of collagen I and collagen III decreased in the UV and UV+Matrix groups, while they were significantly upregulated in the Que and EGT groups (Figure 2A–C). Additionally, MMP1, MMP3, and MMP9 levels increased in the UV and UV+Matrix groups and decreased in the Que and EGT groups. And MMP3 expression was significantly downregulated in the EGT group compared to that in the Que group (Figure 2A–F). The Hyp is a collagen-specific amino acid that reflects changes in collagen content.[26] The Hyp content decreased by almost 40% in the UV group compared with the control group (Figure 2G). Compared with the UV+Matrix group, Hyp content was significantly increased in the Que and EGT groups (Figure 2G). These results suggest that EGT decreases MMP levels and increases collagen levels in mice exposed to UV radiation (see Supplementary Methods and Materials).

EGT Could Upregulate PI3K Signals and Increase Nrf2 Level

The PI3K/Akt signaling pathway plays an important role in UVB-induced skin malignancies and is involved in various cellular processes.[28] Additionally, ROS can induce Keap1 and Nrf2 signals.[29] In the UV and UV+Matrix groups, the levels of PI3K, Akt, and their phosphorylated proteins were downregulated upon UV exposure (Figure 3A, B, C, E and F). In the Que group, there was no significant change in the level of PI3K and p-PI3K, Akt, p-Akt or the ratio p-Akt/Akt (Figure 3B–G). However, there was a significant increase in p-PI3K level and the ratio of p-PI3K/PI3K in EGT group (Figure 3C and D).

Previous studies have shown that UVA can induce Nrf2 upregulation and enhance its presence in the nucleus.[1] Keap1 levels decreased in the UV and UV+Matrix groups, while in the Que and EGT groups, Keap1 showed a slight decrease compared to the UV+Matrix group (Figure 4A and B). Nrf2 protein was slightly upregulated by UV exposure, activating skin defense. However, Nrf2 levels were significantly increased in the Que and EGT groups, as were Nrf2 levels in the nucleus (Figure 4A, C and D). Immunohistochemical results also indicated that EGT could enhance the transfer of Nrf2 into the nucleus (Supplementary Figure 4).

In addition, Nrf2 can translocate into the nucleus to activate the expression of anti-oxidative genes (*HO-1, Txnrd1, Nqo1* and *Gpx2*, et al), playing a key role in antioxidation.[30] qRT-PCR results showed that, in the UV and UV+Matrix groups, the levels of *HO-1, Txnrd1, Nqo1* and *Gpx2* decreased significantly. The transcription levels of Nrf2 and its target genes were not significantly activated in the UV+Matrix group (Supplementary Figure 5). In the Que and EGT groups, *Nqo1, Gpx2* and *Txnrd1* levels increased. EGT significantly upregulated the levels of *Gpx2 and Txnrd1*, while Que upregulated *Gpx2* expression (Supplementary Figure 5).

These results suggest that EGT would play an important role in protecting the skin from UV exposure through PI3K signaling and upregulating Nrf2 and its target genes.

EGT Could Reduce p16 and H2AX Levels in HaCaT Cells

To confirm the protective effects of EGT against cell damage, we examined whether EGT could attenuate etoposide-induced cell death in HaCaT cells. Etoposide is a DNA-damaging drug that induces cell death.[31,32] The human keratinocytes cell line HaCaT treated with 10 μM etoposide increased the number of SA-β-Gal-positive cells (Figure 5A), and the levels of p16 and γ-H2A.X, and Que and EGT treatments decreased SA-β-Gal-positive cells and downregulated p16 and γ-H2A.X (Figure 5B and C). Meanwhile, the qRT-PCR results showed that etoposide treatment induced *IL-6* and *TNFα* mRNA transcription in HaCaT cells. EGT and Que downregulated the mRNA levels of *IL-6* and *TNFα* (Supplementary Figure 6).

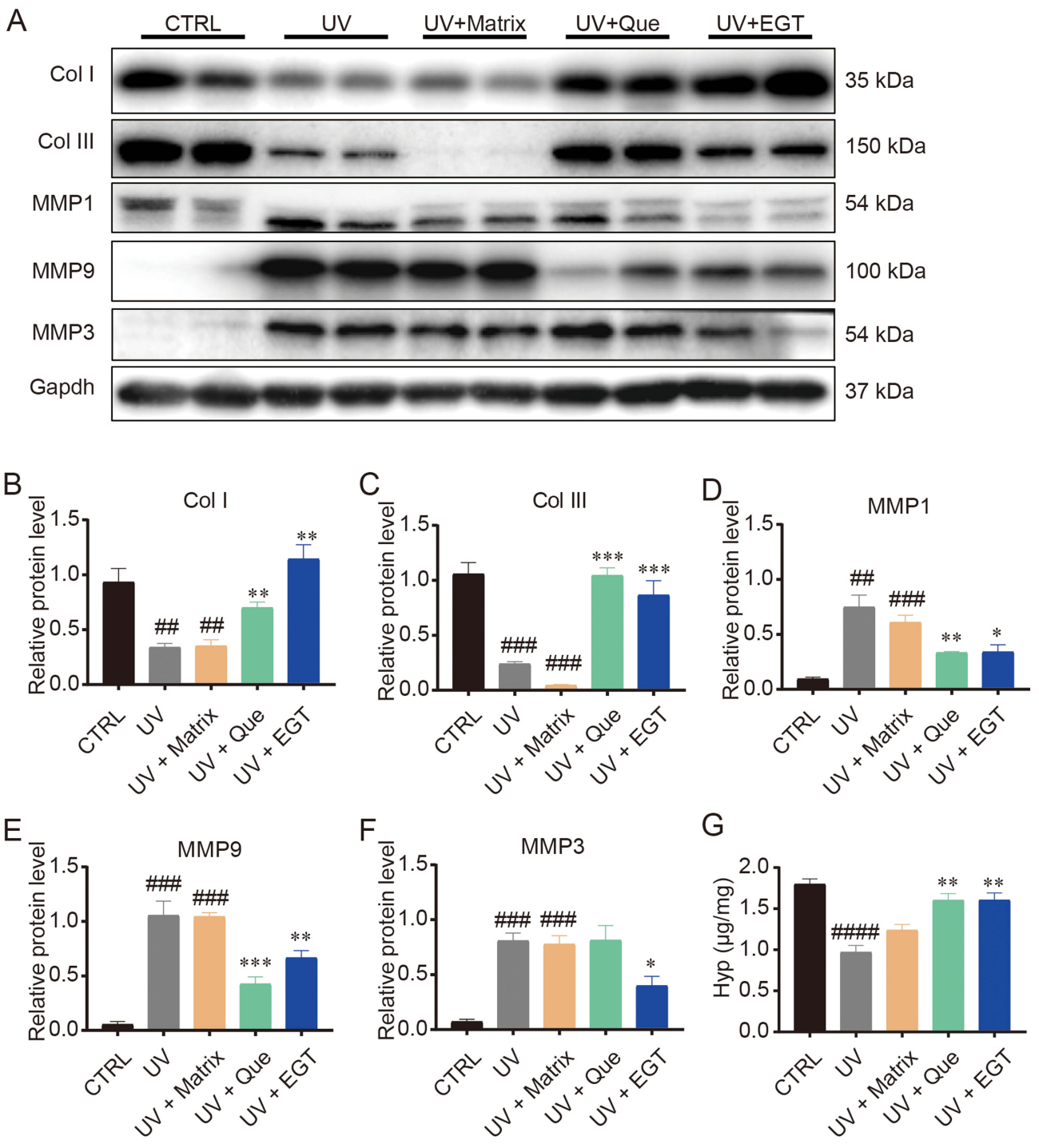

Figure 2 EGT significantly could reduce MMPs level and maintain collagen protein in mouse skin exposed to UV. (**A**) The protein levels of collagens and MMPs, the protein levels relative to Gapdh of collagen I (**B**), collagen III (**C**), MMP1 (**D**), MMP9 (**E**) and MMP3 (**F**). (**G**) Hyp level was detected using Hyp kit. Control group vs UV group or UV + Matrix group: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$. Que group or EGT group vs UV + Matrix group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

EGT Could Upregulate p-PI3K and p-Akt in HaCaT Cells

Etoposide treatment decreased p-PI3K and p-Akt levels in HaCaT cells, while EGT upregulated these levels. Additionally, etoposide reduced Nrf2 levels, whereas EGT increased Nrf2 levels in a dose-dependent manner (Figure 6).

To determine whether EGT induces Nrf2 signaling through the PI3K pathway, we used an inhibitor LY294002 that blocks PI3K to confirm the target molecule of EGT. EGT inhibited p16 and γ-H2AX expression, and LY294002 (a PI3K

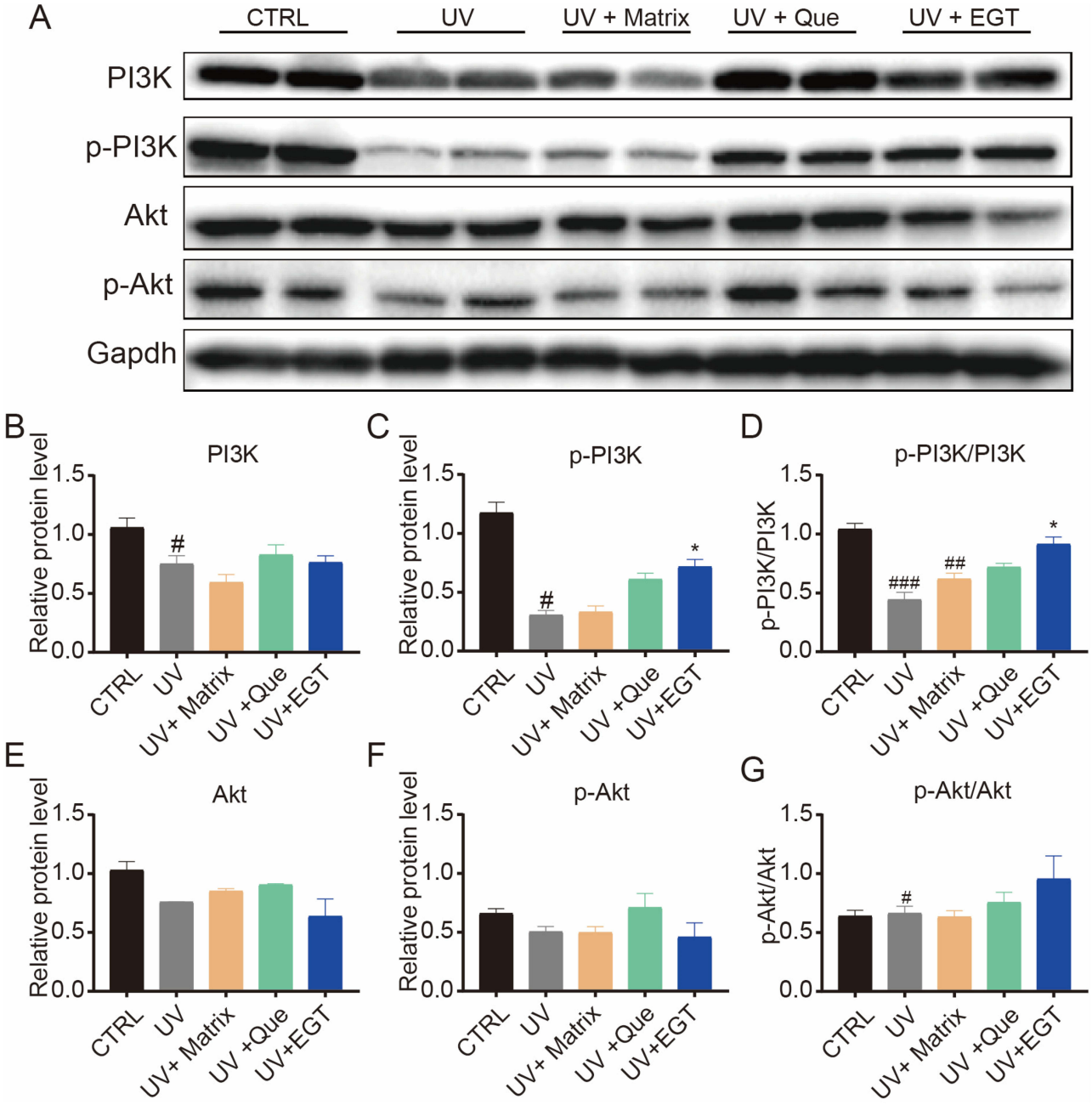

Figure 3 EGT could upregulate the levels of p-PI3K and p-Akt, and activate PI3K/Akt signals. (**A**) Western blot bands of PI3K, p-PI3K, Akt and p-Akt. (**B, C, E** and **F**) The histograms of PI3K, p-PI3K, Akt and p-Akt relative to Gapdh, and (**D** and **G**) the ratio value of p-PI3K/PI3K and p-Akt/Akt. Control group vs UV group or UV + Matrix group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$. Que group or EGT group vs UV + Matrix group: $^{*}P < 0.05$.

inhibitor) antagonized the expression of Que and EGT proteins (Figure 7A–C). These results suggest that EGT protects cells from etoposide-induced cell death via PI3K-Akt-Nrf2 signaling.

Discussion

UV exposure could induce suntan and sunburn, and increase skin wrinkling and photoaging.[13,21,33] In this study, we also found that UV exposure caused erythema in mouse skin, and the epidermis of the back skin in ICR mice was significantly thickened, accompanied by dry skin and exfoliation (data not shown). Oxidative stress is one of the most important factors driving disease or the aging process.[7] As previously mentioned, the skin was damaged under UV exposure leading

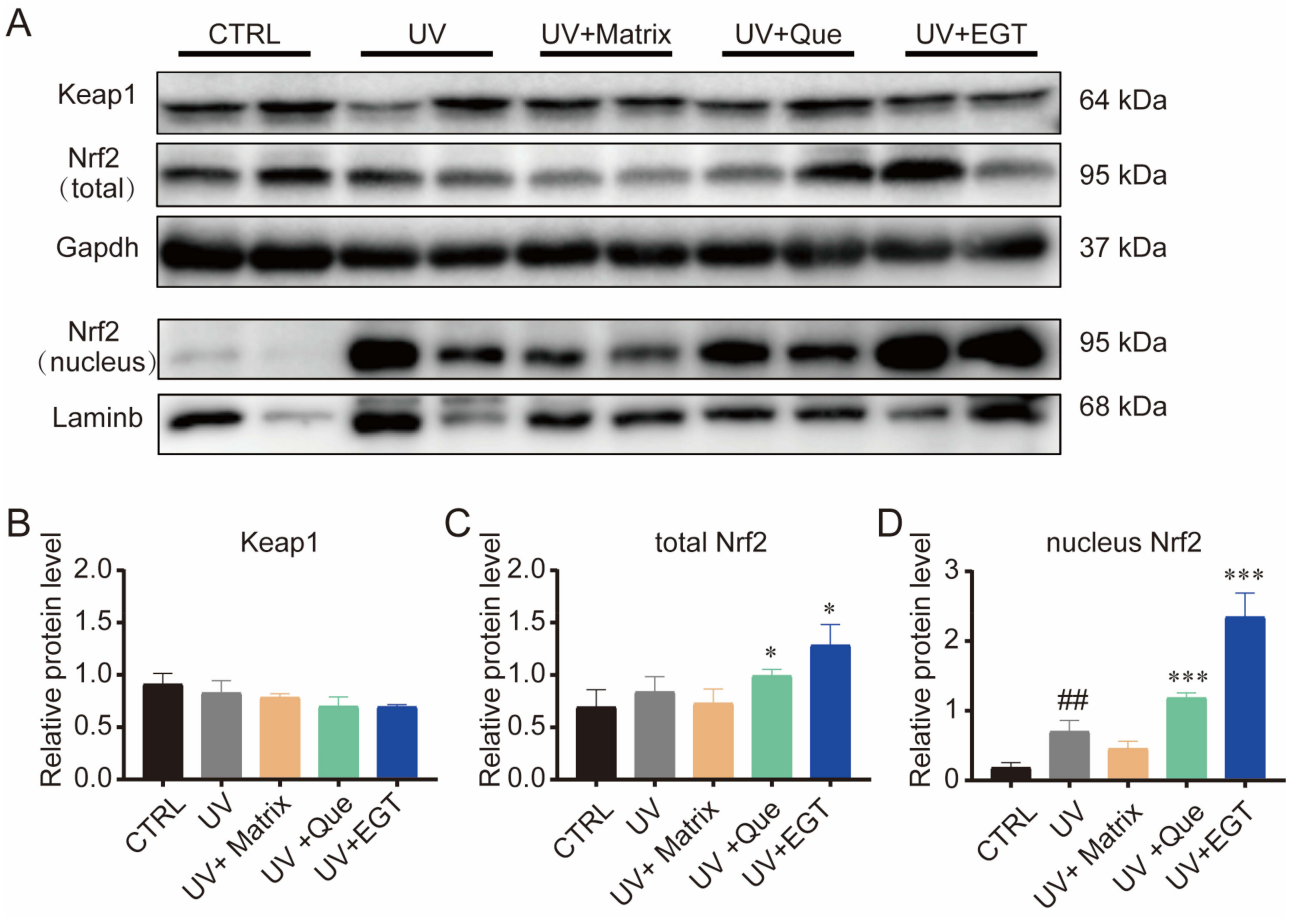

Figure 4 Ergothioneine promoted Nrf2 expression into the nucleus. (**A**) The levels of protein Keap1, total Nrf2, nucleus Nrf2. (**A**) The bands of Keap1, Nrf2 (total), Nrf2 (nucleus) and Lamin b. The gray values of Keap1 (**B**), total Nrf2 (**C**) and nucleus Nrf2 (**D**) relative to Gapdh or Lamin b. Control group vs UV group or UV + Matrix group: $^{\#\#}P < 0.01$. Que group or EGT group vs UV + Matrix group: $^{*}P < 0.05$, $^{***}P < 0.001$.

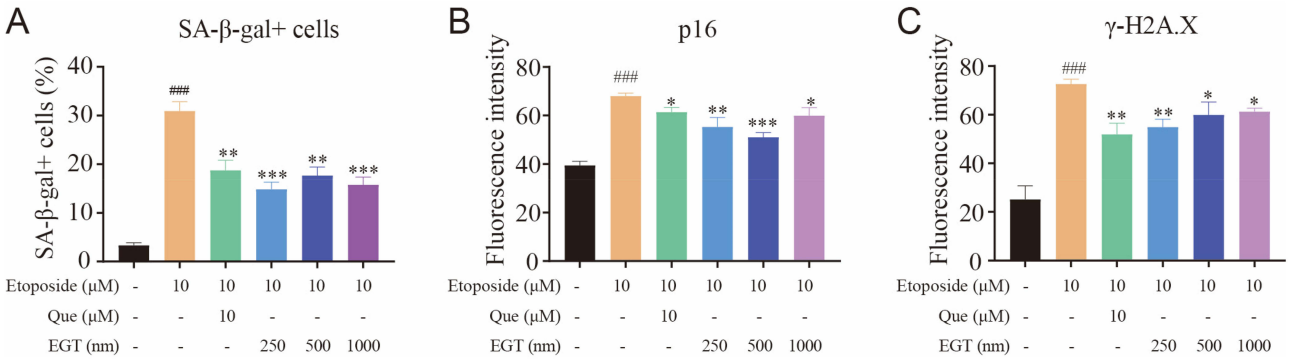

Figure 5 EGT could reduce the number of SA-β-Gal positive cells, and downregulate p16 and γ-H2A.X in HaCaT cells treated with etoposide. (**A**) the number of SA-β-gal positive cells, (**B**) the fluorescence intensity of p16 in HaCaT cells, (**C**) the fluorescence intensity of γ-H2A.X in HaCaT cells. Control group vs etoposide group, $^{\#\#\#}P < 0.001$. Etoposide group vs Que or EGT groups, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to excessive production of ROS, as indicated by increased MDA level and decreased SOD activity. Exposure of the skin to UV can lead to the increased degradation of collagen type I caused by MMP-1, which preferentially cleaves collagen type I.[34] The decrease in Hyp content is a striking feature of skin collagen loss, as the content of collagen I and III decreases, skin wrinkles and loss of elasticity occur. Moreover, the UV-induced increase in MMP protein expression is

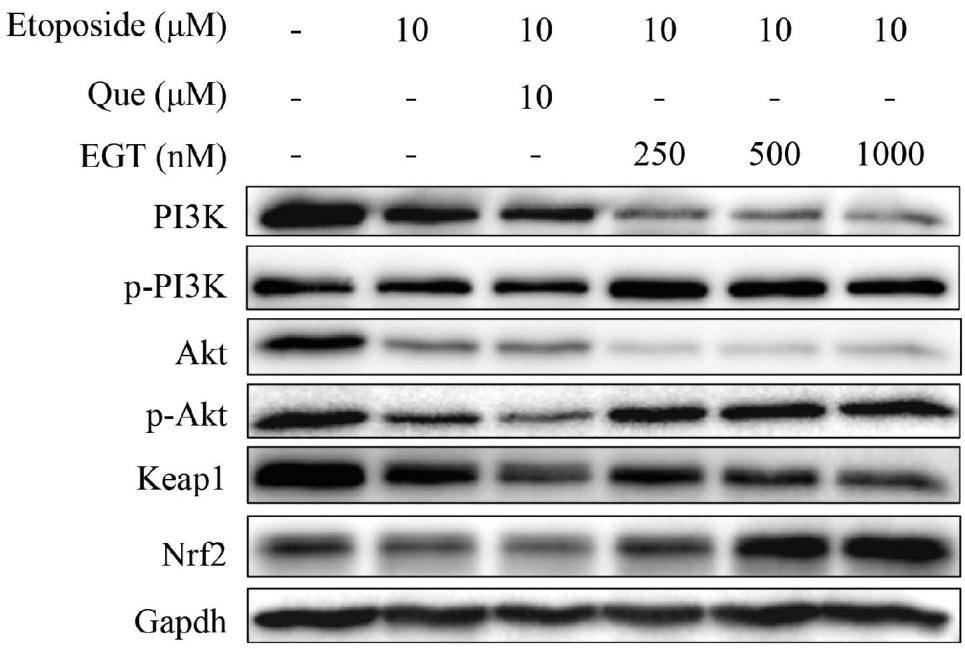

Figure 6 EGT could increase p-PI3K and p-Akt, Nrf2 levels in HaCaT cells treated by etoposide. The HaCaT cells were treated by etoposide for 48 h, then adding Que and EGT for 48 h. Finally, the cells were harvested for Western blot. DMSO was used as a control treatment.

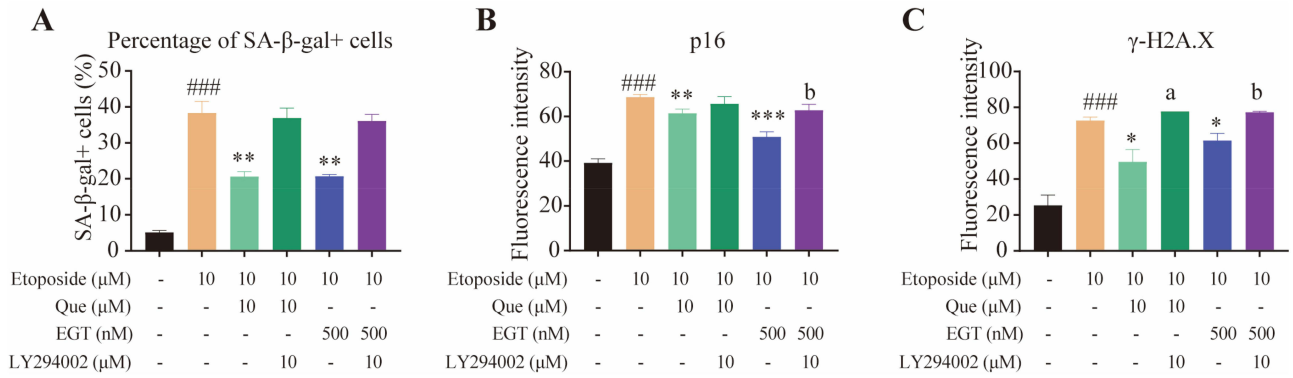

Figure 7 PI3K inhibitor (LY294002) could repress EGT protection in etoposide-induced cell death. The percentage of SA-β-gal cells (**A**) were based on SA-β-gal staining, and the fluorescence intensity of p16 (**B**) and γ-H2A.X (**C**) were from the immunofluorescence of p16 and γ-H2A.X. Control group vs etoposide group: $^{\#\#\#}P < 0.001$. Etoposide group vs Que or EGT groups: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Que group vs Que+LY294002 group, $^{a}P < 0.05$. EGT group vs EGT+LY294002 group, $^{b}P < 0.05$.

also an important reason for accelerated collagen degradation. Therefore, anti-UV and anti-oxidation methods are important means of protecting the skin from photoaging or damage caused by UV exposure.

EGT is a powerful antioxidant and non-toxic to rats,[35] which can protect fibroblasts and keratinocytes against UV damage.[5,8] OCTN1 is an EGT-specific transporter expressed in HaCaT cells that can be absorbed, accumulated, and utilized in HaCaT cells as an important part of the antioxidant defense system.[4,36] EGT and DNA share a similar absorption wavelength in the UV absorption range, which could protect against damage from UV radiation.[4] In the study, EGT attenuated UV-induced skin damage in UV exposure, similar to quercetin.

After EGT treatment, the increase in collagen proteins and Hyp levels indicated that EGT could play a protective role in maintaining skin elasticity. Furthermore, the increase in SOD activity in mouse skin suggests that EGT could enhance the regulation of antioxidant signals and protect the skin against oxidative stress, along with a decrease in MDA levels, MMPs, and the transcription level of *Tnfα*. EGT also inhibited *IL-6* and *TNFα* expression and downregulated SA-β-gal activity, p16, and γ-H2A.X in HaCaT cells treated with etoposide. Previous studies have reported that EGT pretreatment

could significantly inhibit the increase in ROS and the expression of inflammatory factors in human keratinocytes induced by UVA or UVB.[1,8] Therefore, we hypothesized that EGT could inhibit inflammation and protect cells against DNA damage induced by UV radiation or drugs.

PI3K/Akt-Nrf2 is one of the important antioxidant signal pathways involved in cell proliferation, apoptosis, and senescence as well as being an important mediator of UV-induced cellular responses.[1,5,28,37] In this study, UV radiation inhibited PI3K/Akt signaling, resulting in an imbalance in the skin antioxidant system and ultimately leading to skin aging. Nrf2 expression was slightly activated in the UV and UV+Matrix groups, while levels of p-PI3K and p-Akt decreased, which could be attributed to ROS in response to UV radiation. In the EGT group, EGT treatment resulted in higher levels of p-PI3K/PI3K, p-Akt/Akt, and nuclear Nrf2 protein than in the UV+Matrix group, which indicates that EGT makes a significant contribution to enhancing Nrf2 protein translocation into the nucleus. EGT upregulates Nrf2 target genes, especially *Txnrd1* and *Gpx2*. We also observed that EGT and Que have different effects on the activation of Nrf2 target genes in the skin. PI3K/Akt/Nrf2 signaling was also verified in etoposide-induced cell death, and EGT upregulated the levels of p-PI3K, p-Akt, and Nrf2, findings consistent with previous reports.[1,8] Meanwhile, a PI3K inhibitor (LY294002) inhibited EGT and Que protection against etoposide-induced cell death. Thus, we confirmed that EGT was the main activator of PI3K/Akt/Nrf2 signaling against cell damage or aging.

Conclusion

In summary, our results confirmed that EGT decreased MMPs levels and attenuated collagen loss, thereby enhancing the antioxidant capacity of the skin and cells. EGT activates the PI3K/Akt/Nrf2 signaling pathway and plays an important role in protecting the skin from UV irradiation. Thus, EGT is a potential agent for preventing skin aging caused by UV exposure and cell death.

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Disclosure

Ergothioneine production patent (WO-2014100752-A1) has been assigned from Boston University to Ergo-health LLC and Pinghua Liu is one of the co-founders of Ergo-health. The authors declare that they have no other competing interests in this work.

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