Pearl extract protects HaCaT cells from UV radiation-induced apoptosis through mitochondrial pathway regulation

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Abstract

Background: Previous studies demonstrated that pearl extract (PE) promotes wound healing and skin whitening. However, whether PE can inhibit ultraviolet (UV) photodamage in HaCaT cells remains unclear. In this study, an in vitro photoaging cell model was established to observe the effect of PE on UV-induced damage and apoptosis of HaCaT cells. The aim was to provide a reference for future development of natural sunscreen agents.

Results: PE concentrations of 0.1 and 1 μg/mL were considered as the most effective and safe concentrations. Compared to the control group, superoxide dismutase and glutathione peroxidase activities in the photoaging group were significantly reduced, while malondialdehyde and reactive oxygen species content, along with tumor necrosis factor-alpha (TNF-a) and interleukin (IL)-10 mRNA and protein levels were markedly increased. In contrast, Bcl-2 protein expression was significantly decreased, while caspase-3, caspase-9, and Bax protein expression levels were significantly increased. Compared to the photoaging group, HaCaT cell proliferation was significantly increased in the PE group. Both PE concentrations significantly increased superoxide dismutase and glutathione peroxidase activities in cells, reduced malondialdehyde and reactive oxygen species content, decreased TNF-a and IL-10 mRNA expression in cells, and reduced TNF-a and IL-10 protein levels in the supernatant. Additionally, Bcl-2 protein expression levels were significantly increased, while caspase-3, caspase-9, and Bax protein expression levels were significantly reduced by PE treatment.

Conclusions: PE can inhibit UV-induced apoptosis by inhibiting mitochondria-mediated apoptosis and regulating TNF-a and IL-10 expression.

Background
Human keratinocytes (HaCaT cells) are the main cellular constituent of the epidermis (outermost layer of skin), accounting for more than 90% of epidermal cells [1]. Keratinocyte cells prevent external physical, chemical, and microbial damage and maintain the stability of the body’s internal environment [2]. They can also protect the skin by absorbing 95% of the ultraviolet (UV) radiation that reaches the skin [3]. Human keratinocytes participate in various cellular and biological processes, such as apoptosis and inflammation [4]. Damage caused by UV radiation to human epidermal keratinocytes occurs mainly because of the production of reactive oxygen species (ROS) [5], which induce DNA damage, enzyme activity, and mitochondrial dysfunction, resulting in damage to various cell functions [6]. To maintain the normal function of human epidermal keratinocytes, the skin can be covered by clothing, which reduces UV radiation exposure and oxidative damage [7]. Additionally, antioxidants are effective in reducing oxidative damage [8]. Jian-min et al. found that the 50% ethanol macroporous resin elution site of *Eucommia ulmoides* effectively protected against UVA and UVB-induced photoaging in HaCaT cells [9]. Zhiwu et al. reported that rose water inhibited UV-induced apoptosis of HaCaT cells by regulating the nuclear factor-kappa B (NF-κB) nuclear transcription factor pathway [10]. Min et al. demonstrated that hesperidin antagonized the decreased antioxidant enzyme activity in HaCaT cells caused by UVB and showed photoprotective effects [11].

Pearl powder is used as a traditional Chinese medicine to moisturize the heart, liver, and muscle [12] and retard skin aging [13]. Anti-inflammation and anti-apoptosis properties have also been described [14,15]. We previously reported that pearl extract (PE) effectively reduced the melanin content in cells by inhibiting the activity of intracellular tyrosinase, suggesting that PE has a whitening effect [16].

The inhibitory effect of PE on UV photodamage-induced HaCaT has not been reported. In
this study, an *in vitro* photoaging cell model was established to evaluate the effect of PE on UV-induced damage and apoptosis of UV-irradiated HaCaT cells and explore the molecular mechanisms involved.

Results

Effect of PE on HaCaT Cell Proliferation Rate

Compared to the blank group, 0.01 μg/mL PE significantly promoted cell proliferation (*P* < 0.01; Figure 1). PE concentrations of 0.1 and 1 μg/mL PE showed no obvious enhancement and there was no significant effect on the cell proliferation rate. In contrast, 10 μg/mL PE significantly inhibited cell proliferation (*P* < 0.01). Therefore, 0.1 and 1 μg/mL PE were used in subsequent experiments.

Effect of PE on Proliferation Rate of Photoaged HaCaT Cells

Compared to the blank group, the proliferation rate of the model group was significantly reduced (*P* < 0.01), suggesting that UV radiation inhibited cell proliferation (Figure 2). Compared to the model group, 0.1 and 1 μg/mL PE significantly promoted cell proliferation (*P* < 0.01), suggesting that PE protects against photoaging in cells in a concentration-dependent manner.

Effect of PE on Cytokine mRNA Levels

Total cellular RNA was extracted after UV irradiation, and cDNA for β-actin was used as an internal control. The qRT-PCR results were converted to fold-changes. Significantly higher levels of tumor necrosis factor-alpha (TNF-α; Fig. 3A) and interleukin (IL)-10 (Fig. 3B) mRNA were evident in the photoaging group compared to the levels in the blank group (*P* < 0.01). Compared to the photoaging group, after the addition of 0.1 and 1 μg/mL PE, the TNF-α mRNA expression level was decreased significantly in a concentration-dependent manner (*P* < 0.05 and *P* < 0.01, respectively). Similar results were observed for IL-10.
mRNA expression (P < 0.05 and P < 0.01, respectively), suggesting that PE downregulated TNF-α and IL-10 expression.

**Effect of PE on Cytokine Protein Expression**

The levels of IL-10 and TNF-α were measured by enzyme-linked immunosorbent assay. As shown in Figure 4, the protein expression levels of IL-10 and TNF-α in the photoaging group were significantly increased compared to those in the control group (both P < 0.01). Compared to the photoaging group, the expression of TNF-α protein was significantly reduced in the presence of 0.1 and 1 μg/mL PE (P < 0.05 and P < 0.01, respectively), and the IL-10 protein level results were similar (P < 0.05 and P < 0.01, respectively), suggesting that PE decreased the inflammatory response in cells.

**Effect of PE on Antioxidant Indices of UV-Irradiated HaCaT Cells**

The effects of different concentrations of PE on the contents of ROS, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) of UV-irradiated HaCaT cells are shown in Figure 5. Statistical variance analysis revealed that the measurement indices between different groups were significant. Activities of GSH-Px and SOD in the photoaging group were significantly lower than those in the control group (both P < 0.01). In the 0.1 and 1 μg/mL PE treatment groups, SOD levels (both P < 0.05) and GSH-Px levels were higher in the photoaging group (P < 0.05 and P < 0.01, respectively). The ROS and MDA levels in the photoaging group were significantly increased compared to the levels in the control group (P < 0.01). Treatment with 0.1 and 1 μg/mL PE produced significant decreases compared to that in the photoaging group (P < 0.05 and P < 0.01, respectively) in a concentration-dependent manner.

**Effect of PE on Caspase-3, Caspase-9, Bax, and Bcl-2 Protein Expression Levels in HaCaT Cells**
The expression levels of caspase–3, caspase–9, Bax, and Bcl–2 were measured by western blot analysis (Figure 6A). Compared to the control group, the Bcl–2 protein expression level in the photoaging group was significantly decreased (P < 0.05), while caspase–3, caspase–9, and Bax protein expression levels were significantly increased (P < 0.01, P < 0.05 and P < 0.05, respectively; Figure 6B). Compared to the photoaging group, Bcl–2 protein expression in the 1 μg/mL PE group was increased significantly (P < 0.05). In contrast, in the 0.1 and 1 μg/mL PE treated groups, there were significant decreases in caspase–3 (P < 0.05 and P < 0.01, respectively), caspase–9 (P < 0.05 and P < 0.05, respectively), and Bax protein expression levels (both P < 0.01).

Discussion

HaCaT cells reside in the most superficial layer of human skin and are the main target cells of UV radiation [17]. Long-term UV radiation can lead to skin photoaging and even cancer [18]. Apoptosis refers to the gene-regulated process of autonomous and orderly cell death to maintain a stable state in the internal environment [19]. Because of changes in the cellular internal and external environment, as well as stimulation of death signals, this process eliminates aging cells and other cells with potential abnormal growth to maintain a stable state in the cell population [20]. Apoptosis is mainly controlled by three pathways: mitochondrial signaling, death receptor-mediated signaling, and endoplasmic reticulum-mediated signaling [21]. Apoptosis is closely related to changes in mitochondrial structure and function. Long-term UV radiation causes excessive accumulation of ROS in the cell, which damages DNA and the mitochondrial inner membrane [22], ultimately resulting in loss of mitochondrial function, lipid peroxidation, proteins, and nucleic acids, and directly promotes apoptosis [23]. The level of MDA often reflects the degree of lipid peroxidation in the cell and indirectly reflects the degree of cell damage [24]. As such, MDA and SOD assays are often used interchangeably. GSH-PX is an enzyme that is widely
found in cells. It catalyzes the breakdown of hydrogen peroxide and protects the integrity and function of the cell membrane [25].

As a key pro-inflammatory factor widely distributed in the cell, TNF-α has a variety of biological effects, which include mediating inflammation, the immune response, and apoptosis [26]. UV radiation causes an increase in secretory TNF-α protein and total TNF-α expression [27]. Increased TNF-α can accelerate the induction of keratinocyte apoptosis after binding to the corresponding receptor. Additionally, UV radiation causes increased secretion of TNF-α and IL-10 [28], which in turn promotes apoptosis of UV-induced keratinocytes, thus playing an important role in UV-induced skin radiation damage [29].

In the mitochondrial apoptosis pathway, the Bcl–2 protein family, including pro-apoptotic and anti-apoptotic factors [30], plays a very important role in apoptosis regulation by controlling the permeability of the mitochondrial membrane [31]. The caspase family is a series of cysteine amino acid proteases with similar amino acid sequences and secondary protein structures. Caspase proteins can block the cell cycle, label apoptotic cells, break down structural proteins in the cytoskeleton, and inactivate DNA repair enzymes, leading to apoptosis [32]. Under physiological conditions, apoptosis-promoting Bcl–2 family members are located in the cytoplasm; however, they activate apoptotic signals through external stimuli, which are transferred to mitochondria, leading to changes in mitochondrial membrane permeability [33]. These changes prompt transfer of cytochrome C (CytoC) from the mitochondrial inner membrane to the cytosol [34]. Caspase–9 acts as an apoptotic neutron that plays a key role in the mitochondrial apoptotic pathway [35]. Caspase–9 is located upstream of the cascade reaction, forming an active complex with the apoptosis protease activator with CytoC [36]. Subsequently, the complex activates downstream apoptosis-inducing caspase–3, which hydrolyzes apoptosis-inhibiting proteins and repair-related molecules, ultimately activating mitochondria-mediated apoptosis [37].
The anti-apoptotic factor Bcl–2 suppresses caspase activation in the aforementioned process, reducing CytoC release. In these processes, Bcl–2 can inhibit apoptosis [38].

In summary, after UV irradiation at 10 J/cm², SOD and GSH-Px activities in the photoaging group were decreased. In contrast, the contents of ROS and MDA and protein expression levels of IL–10 and TNF-α in the photoaging group were increased. However, after PE treatment, the activity of SOD and GSH-Px was increased, and the expression levels of ROS, MDA, IL–10, and TNF-α were decreased. These results suggest that PE can effectively improve the activity of SOD and GSH-Px, remove oxygen free radicals from the cell, and inhibit lipid peroxidation. Further, GSH-Px can lessen the effects of UV radiation in cells by reducing the production of inflammatory IL–10 and TNF-α. Additionally, the western blot results demonstrated that PE upregulated the expression level of anti-apoptotic factor Bcl–2 protein and downregulated that of caspase–3, caspase–9, and Bax. These results suggest that PE effectively protects HaCaT cells against photoaging damage through a mechanism that involves apoptotic mitochondrial pathways. Thus, PE may be utilized as a new natural sunscreen agent to prevent skin damage triggered by UV (Fig. 7).

Conclusion

Our study shows, for the first time, that PE can reduce HaCaT cell damage caused by UV radiation, which is mainly regulated by reducing the ROS and MDA content, increasing the activity of SOD and GSH-Px, and inhibiting the inflammatory response and mitochondria-mediated apoptosis. The findings indicate that PE can effectively prevent cell damage caused by UV.

Methods

PE

PE containing 2.1% total protein was kindly provided by Zhejiang Osmum Biological Co.,
Ltd. (Huzhou, China). The main preparation method involves grinding of freshwater pearls to the nanometre scale (10–100 nm), followed by hydrolysis with neutral protease to obtain various amino acids, trace elements, and polypeptides.

Cell Culture and Subgroups

HaCaT cells (purchased from Shanghai GeFan Biotechnology Co., Ltd., Shanghai, China) were cultured in RPMI–1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO₂ at 37°C. Under a biological-inverted microscope, round-shaped newly subcultured HaCaT cells were observed. After 4 h of culture, the cells began to adhere to the culture plate, and after 24 h of growth, the cells were completely adherent. The cells were collected during exponential growth for subsequent experiments and were divided into the following subgroups: control group, photoaging cell group (irradiated with 10 J/cm² UV), and PE + UV group (pre-treated with PE before irradiation with 10 J/cm² UV). According to our previous study, PE was added to the culture medium for 48 h before UV irradiation at different concentrations (0, 0.01, 0.1, 1, and 10 μg/mL).

UV Irradiation of Cells

Prior to UV irradiation, the cells were washed with phosphate-buffered saline (PBS) and covered with a thin layer of PBS. The cells were irradiated on ice-cold plates to eliminate UV thermal stimulation. Monolayers of HaCaT cells in a thin layer of PBS were irradiated with 10 J/cm² UV and incubated with culture medium contained PE for 24 h.

CCK8 Assay of Cell Viability

HaCaT cells (1 × 10⁵/mL) were cultured in 96-well plates. Different concentrations of PE (0, 0.01, 0.1, 1, and 10 μg/mL) were added to the cell suspension and incubated for 48 h at 37°C in a 5% CO₂ incubator. The cells were irradiated with 10 J/cm² UV, while control
cells were sham-irradiated by covering them with tin foil. Further, the cells were incubated for 24 h in a cell incubator at 37°C in 5% CO₂. Cell viability was assessed by the CCK8 assay. After the indicated treatment, 10 μL of CCK8 was added to each well for 4 h at 37°C, and the absorbance of each well was measured with a plate reader at a test wavelength of 490 nm. Finally, the concentration of PE showing a significant protective effect against UV radiation-induced cell damage was selected for further experiments. The experiment was repeated five times.

**Assays of Cellular ROS, GSH-Px, SOD, and MDA**

Cells were seeded into 6-well plates (2 × 10⁴ cells/well) and treated with different concentrations of PE (0.1 and 1 μg/mL) for 48 h prior to UV irradiation. Subsequently, the cell suspensions were collected and assayed for ROS and MDA levels and GSH-Px and SOD activities using assay kits in accordance with the manufacturer’s instructions (Jian Cheng Bioengineering Co., Nanjing, China).

**Cytokine mRNA Levels**

Total RNA was isolated from HaCaT cells after treatment using the RNAiso Plus kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s guidelines. For each RT-PCR sample, 1 μg of total RNA was added, and the purity of the RNA was determined as the ratio of the optical density reading at 260 nm to that at 280 nm. The ratio of the RNA used for RT-PCR was 1.8 to 2.0. TNF-α, IL-10, and β-actin mRNA levels were determined by real-time quantitative PCR using a SYBR® Premix Ex Taq™ Kit (TaKaRa Bio) according to the manufacturer’s instructions. The cDNA amplification of a specific sequence of human TNF-α, IL-10, and β-actin was performed by PCR using the primer sequences shown in Table 1. PCR was conducted at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s in the StepOne plus real-time PCR system (Applied Biosystems, Foster City, CA, USA).
The qRT-PCR results were analyzed and expressed as relative mRNA expression of CT (threshold cycle) value, which were then converted to fold-changes. Quantitative real-time RT-PCR assay was performed to detect β-actin expression to normalize the amount of cDNA in each sample.

Cytokine Protein Expression

After 48 h of the indicated treatment, the supernatant of each group of cells in the 6-well plate was collected. TNF-α and IL-10 were detected in accordance with the enzyme-linked immunosorbent assay kit instructions (Jian Cheng Bioengineering Co.). The cytokine concentration was measured three times.

Western Blot Analysis

After appropriate treatment for 48 h, the HaCaT cells were collected using RIPA mixed with phenylmethylsulphonyl fluoride to extract protein (Solarbio Science & Technology Co., Ltd., Beijing, China). Protein levels were measured by bicinchoninic acid assay (Jian Cheng Bioengineering Co.). Briefly, 50 μg of protein was resolved by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis for 60 min at 140 V, and the resolved proteins were transferred to a polyvinylidene fluoride membrane for 45 min at 60 V. The membrane was blocked with 5% fat-free dried milk powder in TBST (1× Tris buffered saline, 0.1% Tween-20) at room temperature for 2 h and incubated with primary antibody diluted 1:1000 in fresh blocking buffer overnight at 4°C with gentle shaking (rabbit anti-caspase–3, rabbit anti-caspase–9, rabbit anti-Bcl–2, rabbit anti-Bax, and rabbit anti-actin antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA). Goat anti-rabbit secondary antibodies (Abmart, Shanghai, China) were diluted at 1:8,000 in fresh blocking buffer and incubated for 1 h at room temperature. The membranes were washed five times for 10 min each in TBST, and the bands were detected using the ECL Plus kit.
The membranes were exposed to Tanon 5200 Multi (Tanon Science & Technology Co., Ltd., Shanghai, China), and TanonImage analysis software was used for quantitative analysis.

**Statistical Analysis**
All data are expressed as the mean ± SD. Experiments were independently repeated at least three times. P < 0.05 indicated significant differences between the experimental and control groups, which were analyzed by one-way analysis of variance. Representative western blots from three independent experiments are shown.

**Abbreviations**
PE: pearl extract
UV: ultraviolet
SOD: superoxide dismutase
GSH-Px: glutathione peroxidase
MAD: malondialdehyde
ROS: reactive oxygen species
TNF-α: tumor necrosis factor-α
IL-10: interleukin-10

**Declarations**

*Ethics approval and consent to participate:* Not applicable

*Consent for publication:* Not applicable

*Availability of data and material:* All data analyzed during this study and material are included in this article

*Competing interests:* The authors declare that they have no competing interests

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Authors’ contributions: ZXC performed the design of experiment and performed the Cytokine expression by mRNA Levels and western blot analysis. JW analyzed and interpreted the patient data regarding the photoaging by the mitochondrial pathway regulation and was a major contributor in writing the manuscript. AQY performed the sample preparation of the PE, LHZ and YJL established photoaging cell model and conducted the antioxidant indices. MX and XZS performed cell culture. All authors read and approved the final manuscript.

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Table 1
| Primer pairs | Forward Primer  | Reverse Primer              |
|--------------|----------------|-----------------------------|
| TNF-α        | 5′-CTGCTGCACTTTGGAGTGAT-3′ | 5′-AGATGATCTGACTGCTGGG-3′ |
| IL-10        | 5′-GAACCAAGACCCACAGACATC-3′ | 5′-GCATTCTTCACCTGCTCCAC-3′ |
| Actin        | 5′-CATGTCACATGCCTACGGTCA-3′ | 5′-CTCCTTAATGTCACCGGTAT3′ |

Figures
Effect of pearl extract (PE) on proliferation rate of normal HaCaT cells. Cell viability was determined by the CCK8 assay. HaCaT cells were treated with PE (0-10 μg/mL) for 48 h. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. **P < 0.01 indicates significant differences between experimental and control groups.
Effects of different treatment conditions on viability in UV-irradiated HaCaT cells. Cell viability was determined by the CCK8 assay. HaCaT cells were treated with PE (0, 0.1, and 1 μg/mL) for 48 h. The cells were exposed to 10 J/cm² UV, and the control group was sham-irradiated by covering with tin foil. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. **P < 0.01 and ***P < 0.01 indicate significant differences between experimental and control groups.
Figure 3

Effect of pearl extract (PE) on mRNA expression. (A) TNF-α mRNA expression level. (B) IL-10 mRNA expression level. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. *P < 0.05 and **P < 0.01 indicate significant differences between photoaging and other groups.
Effect of pearl extract (PE) on TNF-α and IL-10 content in HaCaT cell supernatant. (A) TNF-α protein expression and (B) IL-10 protein expression. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. *P < 0.05, **P < 0.01, and ***P < 0.01 indicate significant differences between photoaging and other groups.
Effects of pearl extract (PE) on antioxidant index in HaCaT cells. (A) ROS levels, (B) MDA levels, (C) SOD activity, and (D) GSH-Px activity. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. *P < 0.05, **P < 0.01, and ***P < 0.01 indicate significant differences between photoaging and other groups.
Effect of pearl extract (PE) on UV-induced protein expression. Caspase-3, caspase-9, Bax, and Bcl-2 in HaCaT cells, as determined by (A) western blotting and (B) band densitometry analysis. Data are expressed as the mean ± SD. The experiments were performed at least three times independently. *P < 0.05 and **P < 0.01: indicate significant differences between photoaging and other groups.
Figure 7

Schematic illustration of photoaging-protective properties of pearl extract.