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Hesperidin ameliorates UV radiation-induced skin damage by abrogation of oxidative stress and inflammatory in HaCaT cells

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ABSTRACT

Ultraviolet A (UVA) radiation contributes to skin photoaging. Hesperidin which is a flavanone glycoside found in citrus fruit peels, have been intensively studied for their UVA-protective activity, but its effects and mechanisms on UVA irradiation-induced inflammation and oxidative stress have never been described. Thus, the purpose of this study was to evaluate the effects of hesperidin in skin oxidative stress and inflammation induced by UVA irradiation. In this study, we firstly examined whether hesperidin may exert direct protective effects on the UVA-induced in human keratinocytes (HaCaT) cell injury *in vitro*. Cell viability was determined by MTT assay. The levels of superoxide dismutase (SOD), malondialdehyde (MDA) and total antioxidative capacity (T-AOC) were measured by using a commercially available kits. Quantitative reverse transcriptase PCR (qRT-PCR) and ELISA were used to determine messenger RNA (mRNA) and protein levels of the tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. UVA significantly decreased the cell viability ($P < 0.05$). In our study, hesperidin (220 μg/ml) significantly reduced UVA-induced oxidative stress and inflammatory response. In conclusion, hesperidin treatment effectively protected HaCaT keratinocytes from these UVA radiation-induced skin injuries, suggesting that the underlying mechanism involves the anti-oxidative and anti-inflammatory capacities, it is possible to be used as a sunscreen agent.

1. Introduction

Sunlight is one of the most common environmental agents to which humans are exposed. Ultraviolet (UV) radiation from the sunlight was associated with the pathogenesis of sunburn cells, photoaging and acute inflammation in skin tissue [1]. Nevertheless the rest of coming UV radiation, part of UVB and all UVA, is responsible for manifold skin diseases [2]. UVA radiation is an oxidizing agent that causes significant damage to cellular components and that leads to photoaging [3]. Reactive oxygen species (ROS) and cytokine [4] are an important factor in the development of skin photodamage after UVA radiation.

Human skin is the largest body organ that serves as an important environmental interface by providing a protective envelope that is crucial for homeostasis. On the other hand, the skin is a major target for toxic insults by a broad spectrum of physical UV radiation that are capable of altering its structure and function. Thus, human skin is routinely and pathologically exposed to many oxidative stresses and inflammation [4]. UV radiation [5] can cause sunburn, skin cancer, skin aging and immune suppression in skin. Among the types of solar radiation, UVA is a potent inducer of various ROS and seriously damages keratinocytes [6]. As an essential cell, keratinocytes play an important role in the inflammatory response to UV irradiation of skin. UVA irradiation was increased the production of some pro-inflammatory cytokines [3,6–7], such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6, leading to the cell death in HaCaT keratinocytes.

In our previous studies in keratinocytes demonstrated that the UVA stimulates the production of ROS and cytokine [8–9]. Hesperidin is a flavone glycoside present in citrus fruits. Many of the beneficial effects obtained through the consumption of orange juice have been attributed to the high contents of flavonone hesperidin [10–11]. *In vitro* studies have revealed that hesperidin is an antioxidant [12]. Hesperidin exhibits antioxidant and anti-inflammatory properties [10–12], however, whether hesperidin could protect HaCaT keratinocytes from UVA radiation-induced oxidative damage and inflammatory has not been investigated. In the present study, we used human skin keratinocyte line HaCaT. We tested the hypothesis the potential UVA (photo)protective effects of hesperidin reduces oxidative stress and inflammatory in the UVA radiation-induced HaCaT cell injury *in vitro*.

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2. Materials and Methods

2.1. Materials

The following materials were used in the current study: SUV-100 solar simulator and radiant emittance monitor (Sigma ShangHai); ABI 7300 RealTime PCR System (Applied biotechnology company, USA); Dulbecco's modified eagle medium (DMEM, Gibco/BRL, USA); SOD, MDA, and T-AOC assay kits (Nanjing Jiancheng Bioengineering Institute, China); reverse transcription kit and real-time quantitative PCR kit (TaKaRa, DaLian); Hesperidin (Procter & Gamble Company); The human keratinocyte cell line HaCaT was a gift from Department of Dermatology, Changhai Hospital of Shanghai.

2.2. Cell Culture and Subgroups

Human skin keratinocyte line HaCaT cells were routinely cultured as described previously [8–9]. Briefly, the HaCaT cells were cultured in a cell incubator at 37 °C in 5% CO_2, in DMEM medium containing 10% fetal bovine serum, 1% penicillin and streptomycin. After the cells became polygonal, arranged as a single layer, they were vaccinated at a density of 1×10^9 cells/l with 0.25% trypsin solution. Culture medium was replaced every 2–3 days and cells passaged once a week. The cultured cells were used for the following experiments when they adhered to the culture plate and the confluence reached 70–80%.

They were then divided into the following subgroups: control group, hesperidin group(HPD), UVA irradiation group (UVA) and UVA + hesperidin group(UVA + HPD). According to our previous study [8–9], hesperidin was added into culture medium for 24 h before UVA irradiation at a 220 μg/ml concentration. All experimental protocols were performed in accordance with the guidelines for the human use of laboratory cells of our Institute and approved by the Nanjing Medical University Experimental Ethic Committee.

2.3. Irradiation of Cells With UVA

The UVA source consisted of a bank of 9 fluorescent bulbs (TL12, Philips) that emitted most of their energy within the UVA range (320–400 nm). The intensity of irradiation was measured with an IL443 phototherapy radiometer and an SED240/UV/W photodetector. Prior to UV irradiation, cells were washed with phosphate-buffered saline (PBS) and covered with a thin layer of PBS. Keratinocytes were irradiated on ice-cold plates to eliminate UVA thermal stimulation. In parallel, non-irradiated cells were treated similarly and were kept in the dark in an incubator for the time of UVA treatment. Monolayers of fibroblasts in a thin layer of PBS were irradiated with 10 J/cm^2 UVA and then they were incubated with culture medium contained hesperidin for 6 or 24 h.

2.4. MTT Assay for Cell Viability

The HaCaT cells (100 μl 10^5/ml) were cultured in 96-well plates. Different concentrations of hesperidin (0, 10, 20, 40, 80, 160, 220, 320 μg/ml)were added to the suspension of cells and incubated for 24 h at 37 °C in a 5% CO_2 incubator. Then cells were irradiated with 10 J/cm^2 UVA. The control cells were sham-irradiated for an identical period of time. Subsequently, the cells were incubated for 24 h in the growth medium with hesperidin. Cell viability was assessed using MTT (3-[4,5-dimethyl-thia-zol-2-yl]-2,5-diphenyl tetrazolium bromide). After the indicated treatment, 20 μl of MTT tetrazolium salt (Sigma-Aldrich) was added to each well for 4 h at 37 °C. Afterwards, the growth medium was replaced with dimethyl sulfoxide, and the absorbance of each well was measured with a plate reader using a test wavelength of 490 nm which concentration of hesperidin have a significant protective effect against UVA radiation-induced cells damage, was then selected for further experiments. The experiment was repeated three times.

2.5. Assays of Cellular Superoxide Dismutase (SOD), Malondialdehyde (MDA) and Total Antioxidative Capacity (T-AOC) Concentrations

Cells were seeded in 24-well plates (2×10^4 cells/well) and subjected to the various treatments for 24 h after UVA irradiation. Then, the cell suspensions were collected and assayed for the enzymatic activity of SOD, MDA, and T-AOC, by using assay kits in accordance with the manufacturer's instructions (Jiancheng Bioengineering Co., Nanjing, China).

2.6. Cytokine mRNA Levels and Protein Expression

2.6.1. RNA Extraction and Real-time Quantitative RT-PCR

Total RNA was isolated from HaCaT cells after appropriate treatment using the RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's guidelines. For each RT-PCR, 1 μg of total RNA was used, and the purity of the RNA was determined by the ratio of the optical density reading at 260 nm to the optical density reading at 280 nm. The ratio of the RNA used for RT-PCR was 1.8–2.0. Levels of TNF-α, IL-1β, IL-6 and GAPDH mRNA were determined by real-time quantitative PCR using a SYBR® Premix Ex Taq™ Kit (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA amplification of a specific sequence of mouse TNF-α, IL-1β, IL-6 or GAPDH was performed by PCR using the primer sequences in the Table 1. The PCR reaction was conducted at 95 °C for 30 s and followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s in the ABI 7500 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 was then converted to fold changes. Quantitative real-time RT-PCR assay was performed to detect GAPDH expression that was used to normalize the amount of cDNA for each sample.

2.6.2. Measure of Cytokines Protein Expression

We examined the levels of Pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) at 6 h after UVA irradiation by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). Protein samples of HaCaT cells were prepared for assay using CytoBuster™ Protein Extraction Reagent (Novagen). The total protein concentration of each sample was analyzed using a modified Bradford assay. The supernatant was collected and stored at −80 °C until use. The levels of TNF-α, IL-1β and IL-6 were measured according to the manufacturer's instructions. All assays were done in duplicate. The average absorbance values for each set of reference standards, control and samples were calculated and a standard curve was constructed. Using the mean absorbance value for each sample, the corresponding concentration of it from the standard curve was determined. The cytokine concentration was performed in duplicate and repeated three times.

2.7. Statistical Analysis

Data were represented as mean ± S.D. (standard deviation) and were examined for the homogeneity of variance. Comparisons between

Table 1
Real-time PCR primers.

Primer pairs	
TNF-α	5′-CTGCTGCACTTTGGAGTGAT-3′
	5′-AGATGATCTGACTGCCTGGG-3′
IL-1β	5′-AAGCTGATGGCCCTAAACAG-3′
	5′-AGGTGCATCGTGCACATAAG-3′
IL-6	5′-AAGCCAGAGCTGTGCAGATGAGTA-3′
	5′-TGTCCTGCAGCCACTGGTTC-3′
GAPDH	5′-ACCCACTCCTCCACCTTTGA-3′
	5′-CTGTTGCTGTAGCCAAATTCGT-3′