A valuable model of UVB-induced oxidative damage in HaCaT cells

Yaling Song[1]; Yang Ouyang[2]; Zhe Feng[1]; Qiang Wu[1]; Xin Yang[1*]

[1] Proya Cosmetics Co., Ltd, Hang Zhou, China;
[2] Hangzhou Jenover Biotechnology Co., Ltd., Hang Zhou, China

*Xin Yang, Proya Cosmetics Co., Ltd, Hang Zhou, China, +86-18500465159, yangxin@proya.com

Abstract

Background: Many cosmetic companies are working on skin care products that can effectively resist UV light damage. In order to provide an efficacy evaluation method for the development of cosmetic ingredients that can effectively resist UV damage and photoaging, we have developed a UV simulator for cells and established a UV-induced oxidative stress model of skin-related cells.

Methods: The UVA/UVB simulator uses LED lamp beads, which have the advantage of good wavelength controllability. Human keratinocytes HaCaT were irradiated with a certain dose of UVB. The cell proliferation after UVB irradiation was detected by RTCA technology. The apoptosis degree, cell cycle, and reactive oxygen species level of HaCaT cells after UVB irradiation were detected by flow cytometry. The effects of UVB irradiation on the production of inflammatory factors TNF-α, IL-6, and IL-8 in HaCaT cells were confirmed by qPCR.

Results: A single short-duration UVB irradiation had no significant effect on cell morphology and cell cycle. However, with the increase in irradiation dose, the apoptosis rate and doubling time of HaCaT cells increased significantly, while promoting the release of inflammatory factors and the production of ROS from HaCaT cells.

Conclusion: We developed a UV simulator for cells and conducted an experimental study of UVB-induced oxidative stress in HaCaT cells. The UVB-induced HaCaT cell ROS-damage model was successfully constructed, providing a powerful evaluation method for the development of cosmetic raw materials that effectively resist UV damage and photoaging.

Keywords: UVB; ROS; inflammatory factors; apoptosis; cell cycle.

Introduction

The skin is located on the surface of the human body. As the largest organ of the human body, the skin has become the first line of defence against foreign pathogens, particularly the stratum corneum, which has an essential function[1,2]. The stratum corneum is the outermost layer of the epidermis, which has a certain toughness and can withstand mild scratching and friction. The stratum corneum is elastic and works in conjunction with the more elastic dermal fibrous tissue and subcutaneous adipose tissue, which can buffer external shocks and bruises[3]. Keratinocytes in the stratum corneum can absorb a large number of ultraviolet rays and protect organs and tissues in the body from light damage. However, lifestyle changes involving frequent outdoor activities are contributing to increased exposure to harmful ultraviolet light, which leads to earlier skin aging such as wrinkles and scars[4-6]. Therefore, more and more people are beginning to pay attention to skin care.

At the moment of "effective skincare", the biological effect of UV has been paid more and more attention. Numerous studies have demonstrated that UV is the main environmental factor leading to skin photoaging. Ultraviolet (UV) light is a physical carcinogen and UV irradiation from sunlight has profound effects on the human skin[7,8], causing various biological events such as sunburn, inflammation, cellular/tissue injury, cell death, and skin cancer[9]. Solar UV light reaching earth is a combination of both UVB (290–320 nm) and UVA (320–400 nm) wavelengths. Among UV rays, UVB is considered the most damaging and genotoxic component of sunlight, making up 4%–5% of UV light. It is 1,000 times more potent at causing sunburn than UVA and is responsible for photo-induced skin damage[10,11]. UVB induces various cell modifications including mutations in DNA, cell cycle arrest, apoptotic responses through various signaling pathways, and formation of reactive oxygen species (ROS).

Since excessive UV exposure can cause a variety of skin damage, many cosmetic companies are working on skin care products that can effectively resist UV light damage. In order to establish a biological evaluation system for the cell-level evaluation method of cosmetic raw materials stably responding to photoaging, we have developed a UV simulator for cells that contain both UVB and UVA light. This simulator simulates UVA and UVB ultraviolet radiation in sunlight and is designed for in vitro evaluation of chemical and cosmetic phototoxicity safety and anti-ROS effect. We measured the oxidative damage marker ROS

level, the release level of cellular inflammatory factors, cell apoptosis, and cell cycle changes in human keratinocyte HaCaT after UVB irradiation, and comprehensively evaluated the physiological changes of HaCaT cells after UVB irradiation. A model of UV-induced oxidative stress in skin-related cells was successfully established.

Materials and Methods

Cells and Cell Culture Human keratinocyte cell line (HaCaT), obtained from iCell Bioscience Inc. HaCaT were grown in Dulbecco's modified Eagle's medium (Gibco, Paisley, Scotland, UK) supplemented with 1× penicillin-streptomycin (Gibco) and 10% fetal bovine serum (Corning, Australia). All cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

UV Simulator The UVA/UVB simulator can simulate UVA and UVB UV radiation in sunlight. The simulator uses LED lamp beads and has the advantage of accurate wavelength controllability. By setting several sensors to collect the radiation intensity, the device can provide real-time feedback and accurately calibrate the irradiation power of the LED lamp beads to realize the uniformity and controllability of the illumination in the irradiation areas. The device has a compact structure with three independent irradiation areas, each of which can accommodate a standard culture plate. The irradiation intensity and energy of UVA and UVB of three independent irradiation areas can be precisely adjusted respectively. For the traceability of test data, the device is equipped with an experimental process recording module, to record the operating parameters after each startup for subsequent experimental analysis.

UV Irradiation HaCaT cells were seeded in 6-well plates(4×10^5 cells/well) and grown in Dulbecco's modified Eagle's medium supplemented with 1× penicillin-streptomycin and 10% fetal bovine serum in a CO_2 incubator for 24 hr. Prior to UVB irradiation, cells were washed with PBS and then added with 800μL HBSS buffer. Cells were exposed to various intensities of UVB (300, 600, and 900 mJ/cm^2) . After irradiation, cells were collected to detect cell viability, apoptosis degree, cell cycle, reactive oxygen species level and the release of inflammatory factors.

Cell Viability of HaCaT cells Cell viability of HaCaT after UVB exposure at various intensities was determined using Real Time Cellular Analysis(RTCA). UVB-irradiated HaCaT cells with medium were seeded in E-Plate (ACEA Biosciences) at 1×10^4 cells per well and incubated for 24 hr. The cell viability was quantified by measuring the electrical impedance using a cell function analyzer(iCELLigence, ACEA Biosciences, USA). Real-time label-free dynamic cell analysis technology (RTCA, Real Time Cellular Analysis) integrates the microelectronic cell sensor chip into the bottom of the cell detection plate through a special process to construct a real-time, dynamic and quantitative cell impedance detection sensing system for tracking cells. When adherent cells growing on the surface of the microelectrode caused changes in the interface impedance of the adherent electrodes, the changes were correlated with the changes in the real-time functional state of the cells. Through real-time dynamic electrode impedance detection, biological information related to cell physiological functions can be obtained, including cell growth, extension, morphological changes, death and adherence.

ROS detection in HaCaT cells DCFH-DA(S0033S, Beyotime Biotechnology, China)was used as probes for ROS detected in HaCaT cells. After irradiation, cells were washed with PBS and followed by a 30-min incubation with 10-μM DCFH-DA in the dark at 37°C. Intracellular ROS was quantified by the intensity of DCFH-DA. The intensity of the fluorophore was measured by flow cytometer (NovoCyte, ACEA Biosciences, USA).

Detection of HaCaT cells Apoptosis Annexin V-FITC Apoptosis Detection Kit(C1062S, Beyotime Biotechnology, China)was used to detect apoptotic cells after UVB exposure. Cells dissociated with trypsin were collected and washed twice with PBS after irradiation. After centrifuging cells at 1000 r.p.m, cells were resuspended in 1× binding buffer. Then 5μl of annexin V-FITC and 10μl of propidium iodide (PI) were added. Cells were mixed gently and incubated for 15 min at room temperature in the dark. Samples were detected by flow cytometer.

Cell Cycle Assay Cell cycle phases were determined using cell cycle staining kit(CCS012, Beyotime Biotechnology, China). In brief, The UVB-irradiated cells were harvested, washed

twice with PBS, fixed with 70% ethanol, and stained with DNA staining solution supplied by the kit for 30 min at temperature. The cell cycle profile was measured using a flow cytometer. For each sample, 10,000 ungated events were acquired.

Cytokine release profiles in HaCaT cells The Cytokine/chemokine release profiles in HaCaT cells were detected by using quantitative real-time RT-PCR analysis. After treatment, cells were harvested and subjected to the total RNA extraction using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa). First strand cDNAs were synthesized using the PrimeScriptII1st Strand cDNA Synthesis Kit (TaKaRa), according to the manufacturer's instructions. PCR was performed in 96-well plates using the TB Green® Fast qPCR Mix (TaKaRa) and determined using the Applied Biosystems® QuantStudio® 3 Real-Time PCR Detection System (QuantStudio 3, Thermo, USA). The mRNA expression levels of TNF-α, IL-6, and IL-8 were analyzed by normalizing with the housekeeping gene, β-actin.

Statistical Analysis Data are expressed as mean ± SD. Each experiment was repeated in triplicate (n=3). The student's unpaired t-test was used to compare groups, and $p < 0.05$ was considered significant.

Results

Viability of HaCaT cells to UVB irradiation HaCaT cells were irradiated with UVB at 300, 600 and 900 mJ/cm^2 and cell viability after different doses of UVB irradiation was estimated by RTCA assay for 46h, as described above. The results of RTCA can reflect the proliferation of cells (Fig. 1). The CI value is proportional to the number of cells in this experiment, and the more cells, the higher the CI value. The results showed (Fig. 1a) that HaCaT cells without UVB irradiation grew well on RTCA. The proliferation curve of HaCaT cells after UVB irradiation was lower than that of the control group. The doubling time of cells in each group was counted (Figure 1b), and the results showed that the higher the UVB irradiation dose, the greater the cell doubling time, reflecting a good gradient.

a b

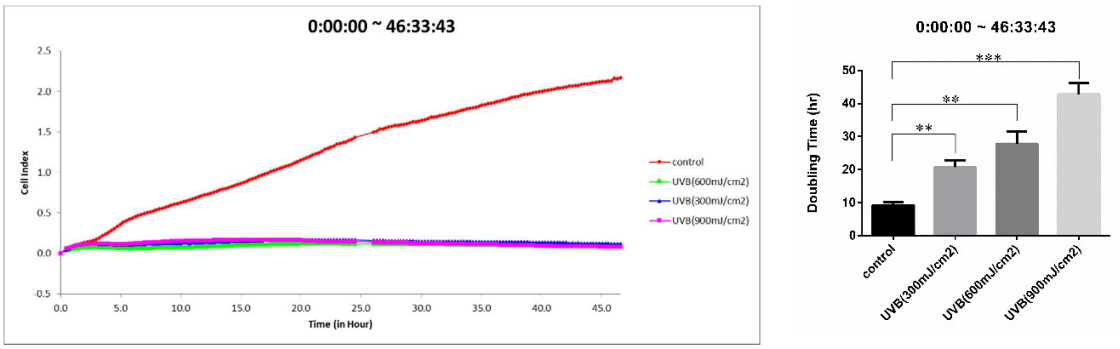


Figure 1. Cellular activity and doubling time. a) RTCA real-time detection of the proliferation curve of HaCaT cells irradiated with different doses of UVB. The horizontal axis is time, and the vertical axis is Cell Index (CI value). b) ** means p-value less than 0.05 and *** means p-value less than 0.01. The doubling time of HaCaT cells irradiated with different doses of UVB in the three groups was significantly greater than that in the control group, indicating that the proliferation rate of HaCaT was significantly reduced after UVB irradiation. Among them, the doubling time of HaCaT cells after high-dose(900mJ/cm^2) UVB irradiation was significantly greater than that of the control group(P=0.0053).

ROS productions of UVB-exposed HaCaT cells The intracellular ROS level is an important index to evaluate the damages led by UVB exposure. Levels of intracellular ROS were assessed in HaCaT cells treated with or without UVB using ROS sensitive probe DCFH-DA, as described above. The results showed that intracellular ROS increased to varying degrees after different doses of UVB irradiation (Fig. 2a), and high dose UVB irradiation led to a significant increase in ROS in HaCat cells (p=0.0077, Fig. 2b).

a b

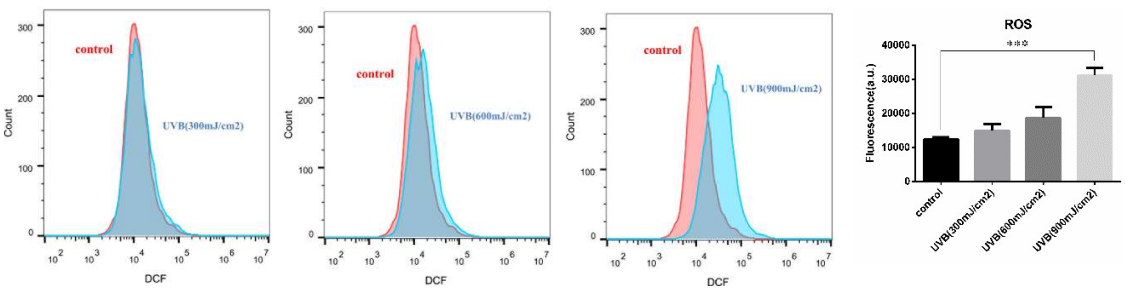
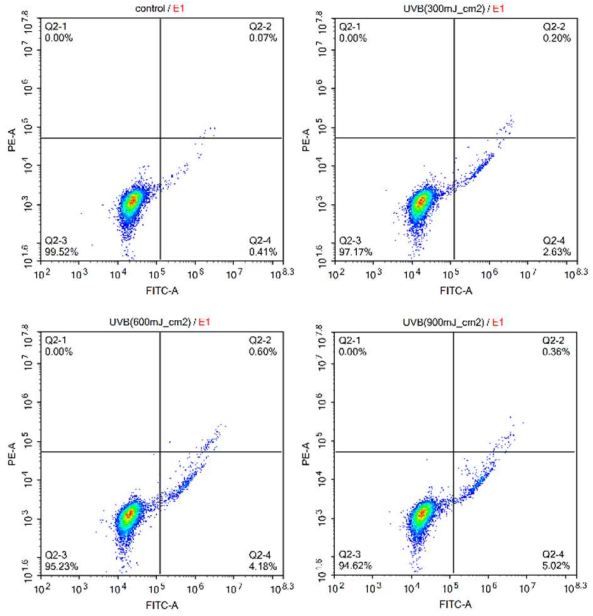


Figure 2. Elevated intracellular ROS level in HaCaT cells after UVB irradiation. a) The results of flow cytometry contain 10,000 ungated events. b)Representative histograms of triplicate experiments plot the relative green DCF fluorescence intensity.

Flow cytometric analysis of Apoptosis Annexin V-FITC Apoptosis Detection Kit(C1062S, Beyotime Biotechnology, China)was used to detect apoptotic cells after UVB exposure as above. Flow cytometry results showed that the apoptosis rate of HaCaT cells increased with the increase of UVB irradiation dose(Fig. 3a). We performed a statistical analysis on the apoptosis of HaCaT cells and found that the apoptosis rate was significantly increased after high-dose(900mJ/cm^2) UVB irradiation(P=0.008, Fig. 3b).

a



b

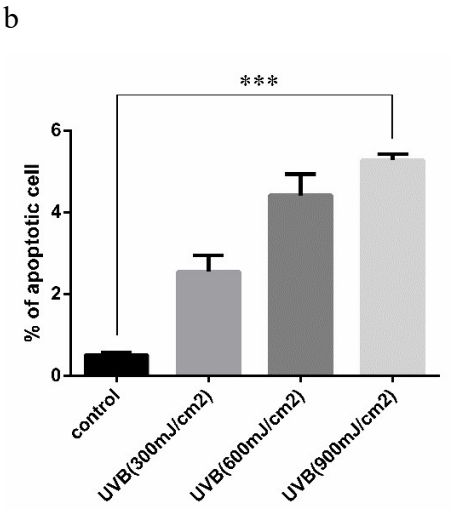


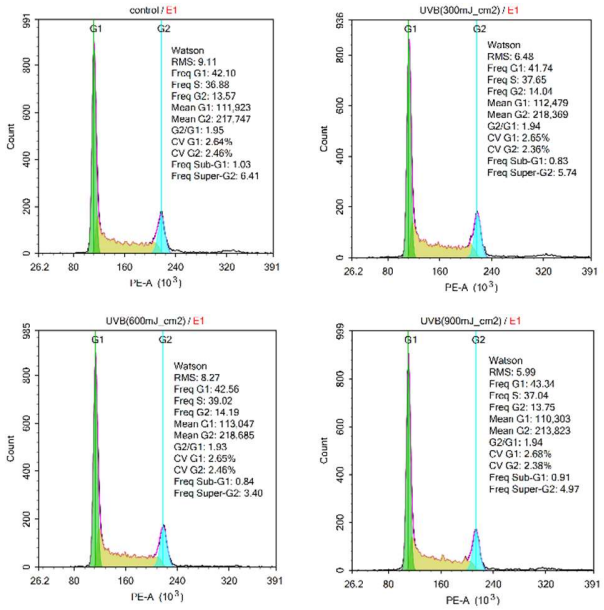
Figure 3. Flow cytometry analysis of UVB irradiated HaCaT. a) Percent of apoptotic cells are calculated as the summation of PE positive quadrant and annexin-V positive quadrant. The dot plot analysis of annexin-V apoptosis assay with our triplicate experiments. b) The quantitative results of annexin V apoptosis assay and the error bar represents mean ± SD of triplicate samples.

Cell cycle progression of HaCaT cells by UVB irradiation To monitor the cell cycle progression after UVB irradiation, we implemented the cell cycle analysis assay with PI (Fig. 4a). There was 43.34% of cells in subG1 phase with high-dose(900mJ/cm^2) exposure,

comparing to 42.10 (control) , 41.74% (low-dose, 300mJ/cm^2) and 42.56%(medium-dose, 600mJ/cm^2). It can be seen that a single short-time UVB irradiation did not significantly change the mitotic state of HaCaT cells.

Cytokine release profiles in UVB-irradiated HaCaT cells There is accumulating evidence that the expression of inflammatory factors were upregulated in UVB induced HaCaT keratinocytes. To determine the effects of UVB on inflammatory factor expression, a qRT-PCR assay was performed. According to the previous findings, we carried out the detection of the secretion levels of TNF-α, IL-6 and IL-8 after high-dose UVB irradiation of cells. As shown in Fig. 4b, high-dose UVB irradiation promoted the release of TNF-α, IL-6 and IL-8 from HaCaT cells, but not significantly.

a



b

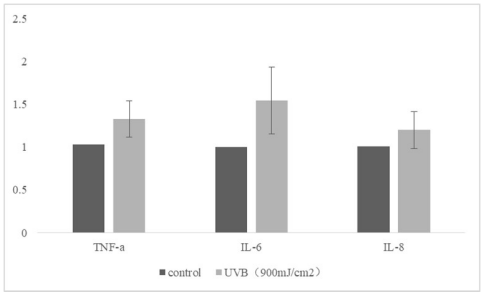


Figure 4a. Cell cycle analysis of UVB irradiated HaCaT cells.

Figure 4b. Release of various cytokines from UVB-treated HaCaT cells.

Discussion

Overexposure to UVB can cause damage to epidermal cells, including structural changes in proteins, DNA, lipid membranes, and many other important biological molecules. Ultimately, these injuries can lead to a variety of pathological changes, such as sunburn inflammation, hyperplasia, skin aging, and skin cancer[12,13]. In this study, we have developed a UV simulator for cells that contain both UVB and UVA light to establish a biological evaluation system for the cell-level evaluation method of cosmetic raw materials stably responding to

photoaging. This simulator simulates UVA and UVB ultraviolet radiation in sunlight and is designed for in vitro evaluation of chemical and cosmetic phototoxicity safety and anti-ROS effect. The UVA/UVB simulator uses LED lamp beads, which have the advantages of good wavelength controllability. Then human keratinocytes HaCaT were irradiated with a certain dose of UVB. We utilized several assays to confirm the dose response and viability of HaCaT cells to UVB irradiation. Both RTCA and Annexin-V apoptosis assays showed lower cell viability at high irradiation doses, whereas subG1 proportion showed no significant change at different irradiation doses. This indicated that a single short-duration UVB irradiation only moderated the cell proliferation rate and partially apoptosis, but did not significantly alter the cell mitotic cycle, which was different from previous studies[14,15].

UV-induced apoptosis is a complex process involving multiple pathways such as massive production of ROS and NO, activation of cell surface death receptors, and induction of genomic DNA damage [16-18]. It has been reported that at least 50% of UVB-induced cellular damage is caused by elevated ROS levels [19]. At the same time, excessive release of ROS caused by UV irradiation can also trigger cellular inflammation. We, therefore, tested the intracellular ROS levels after different doses of UVB irradiation. The results showed that intracellular ROS increased to varying degrees after different doses of UVB irradiation, and high dose UVB irradiation led to a significant increase in ROS in HaCat cells. Then we carried out the detection of the secretion levels of TNF-α, IL-6 and IL-8 after high-dose UVB irradiation of cells. High-dose UVB irradiation promoted the release of TNF-α, IL-6 and IL-8 from HaCaT cells, but not significantly, indicating that the cells have a certain ability to automatically clear cytokines.

In conclusion, UVB irradiation induces apoptosis, overproduction of ROS, and release of various cytokines/chemokines in keratinocytes. These cytokines/chemokines may act as promoters of excessive inflammation. Therefore, sunburn may be partly responsible for the overproduction of inflammatory cytokines/chemokines in the skin [20]. However, due to the ability of cells to self-renew and scavenge free radicals, a single short-term UVB irradiation will not block the mitotic cycle of cells, although some cells will undergo apoptosis and generate ROS, thereby reducing the rate of cell proliferation.

Conclusion

We developed a UV simulator for cells and conducted an experimental study of UVB-induced oxidative stress in HaCat cells. The UVB-induced HaCat cell ROS-damage model was successfully constructed, providing a powerful evaluation method for the development of cosmetic raw materials that effectively resist UV damage and photoaging. At the same time, this study further proves that short-term UVB exposure does not change the mitotic cycle of cells and causes irreversible cell damage. We can protect the skin by using sunscreens and avoiding long-term UVB exposure.

Acknowledgments

This research was supported by the International Academy of Sciences, Proya Cosmetics Co., Ltd, Hang Zhou, China. We thank the company for its great support. In addition, thanks for the helpful assistance and discussion from Hangzhou Jenover Biotechnology Co., Ltd., Hang Zhou, China.

Conflict of Interest Statement NONE.

References.

[1] Zhang S , Duan E . Fighting against Skin Aging: The Way from Bench to Bedside[J]. Cell Transplantation, 2018, 27(2):096368971772575.

[2] Rev A , Dev C , Downloaded B . Epidermal Stem Cells of the Skin. 2006.

[3] Pensalfini M , Rotach M , Hopf R , et al. How cosmetic tightening products modulate the biomechanics and morphology of human skin[J]. Acta Biomaterialia, 2020.

[4] Chen B Y , Lin D P , Wu C Y , et al. Dietary zerumbone prevents mouse cornea from UVB-induced photokeratitis through inhibition of NF-κB, iNOS, and TNF-α expression and reduction of MDA accumulation.[J]. Molecular Vision, 2011, 17:854-863.

[5] Im A R , Kim Y M , Chin Y W , et al. Protective effects of compounds from Garcinia mangostana L. (mangosteen) against UVB damage in HaCaT cells and hairless mice[J]. International Journal of Molecular Medicine, 2017.

[6] Mcdaniel D , Farris P , Valacchi G . Atmospheric skin aging-Contributors and inhibitors.[J]. J Cosmet Dermatol, 2018, 17(2):124-137.

[7] Yang S J , Han T , Choi E M . the protective effect of laver extract against the uva-and uvb-induced damage in hacat cells effect of laver extract on uv damage in hacat cells 187[J]. Toxicology and Environmental Health Sciences, 2012, 4(3):186-193.

[8] Spencer D , Rachel O , Zhou L , et al. Natural Antioxidants: Multiple Mechanisms to Protect Skin From Solar Radiation[J]. Frontiers in Pharmacology, 2018, 9:392.

[9] Assefa Z , An V L , Garmyn M , et al. Ultraviolet radiation-induced apoptosis in keratinocytes: on the role of cytosolic factors.[J]. Biochim Biophys Acta, 2005, 1755(2):90-106.2.

[10] Fisher G J , Kang S , Varani J , et al. Mechanisms of Photoaging and Chronological Skin Aging[J]. Archives of Dermatology, 2002, 138(11):1462-1470.

[11] Perluigi M , Domenico F D , Blarzino C , et al. Effects of UVB-induced oxidative stress on protein expression and specific protein oxidation in normal human epithelial keratinocytes: a proteomic approach[J]. Proteome Science, 2010, 8(1):13.

[12] Lee C W , Ko H H , Chai C Y , et al. Effect of Artocarpus communis Extract on UVB Irradiation-Induced Oxidative Stress and Inflammation in Hairless Mice[J]. International Journal of Molecular Sciences, 2013, 14(2):3860-3873.

[13] Tiraravesit N , Yakaew S , Rukchay R , et al. Artocarpus altilis heartwood extract protects skin against UVB in vitro and in vivo[J]. Journal of ethnopharmacology, 2015, 175:153-162.

[14] Luangpraditkun K , Charoensit P , Grandmottet F , et al. Photoprotective Potential of the Natural Artocarpin against In Vitro UVB-Induced Apoptosis[J]. Oxidative Medicine and, Cellular Longevity, 2020, 2020(16):1-17.

[15] Chang RS, Chen CS, Huang CL, Chang CT, Cui Y, Chung WJ, Shu WY, Chiang CS, Chuang CY, Hsu IC. Unexpected dose response of HaCaT to UVB irradiation. In Vitro Cell Dev Biol Anim. 2018 Sep;54(8):589-599.

[16] Assefa Z , An V L , Garmyn M , et al. Ultraviolet radiation-induced apoptosis in keratinocytes: on the role of cytosolic factors.[J]. Biochim Biophys Acta, 2005, 1755(2):90-106.

[17] Ryu H C , Kim C , Kim J Y , et al. UVB Radiation Induces Apoptosis in Keratinocytes by Activating a Pathway Linked to "BLT2-Reactive Oxygen Species"[J]. Journal of Investigative Dermatology, 2010, 130(4):1095-1106.

[18] Kulms, Dagmar, Zeise, et al. DNA damage, death receptor activation and reactive oxygen species contribute o ultraviolet radiation-induced apoptosis in an essential and independent way.[J]. Oncogene, 2002.

[19] Wölfle U, Esser P R , Simon-Haarhaus B , et al. UVB-induced DNA damage, generation of reactive oxygen species, and inflammation are effectively attenuated by the flavonoid luteolin in vitro and in vivo.[J]. Free Radical Biology & Medicine, 2011, 50(9):1081-1093.

[20] Kabashima K , Nagamachi M , Honda T , et al. Prostaglandin E2 is required for ultraviolet B-induced skin inflammation via EP2 and EP4 receptors.[J]. Laboratory investigation; a journal of technical methods and pathology, 2007, 87(1):49.