Letter to the Editor

Combination of PDTC and GBE Could Better Alleviate the Damage to HaCaT Cells Caused by UVB*

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Ultraviolet radiation (UVR) is a public health concern due to the depletion in ozone layer and the excessive exposure to UVR has been recognized as one of the major environmental risk factors for various skin diseases including the damage on keratinocyte which leading to formation of cutaneous malignancies[1]. UVR consists of UVA (320–400 nm), UVB (290–320 nm) and UVC (200–290 nm)[2].

Even though UVB radiation is considered as a minor source of solar radiation, it has been found to be much more harmful than UVA radiation[2]. It has been found keratinocytes in the epidermis are more mainly affected by UVB[3]. The NF-κB signalling pathway has been implicated to be responsible for the UVR damage on keratinocytes[4]. In brief, the reactive oxygen species (ROS) generation following the UV radiation exposure has been found to affect NF-κB expression level which eventually leading to a higher intracellular level of ROS production[4]. The high level of ROS will induce ER stress causing accumulation of proteins within the ER lumen and imbalance between unfolded proteins[5], which may be responsible for disorders such as cancers and aging in human beings[6].

Antioxidants are able to attenuate the damaging effects of ROS and can reverse many of the events that contribute to epidermal toxicity and disease[7]. *Ginkgo biloba L.* extract (GBE) is a traditional herbal medicine and has been commonly used to protect skin cells from the damages resulted from oxidative stress[8]. Pyrrolidine dithiocarbamate (PDTC) is a thiol-containing compound which have antioxidant properties and is also capable of inhibiting IκB ubiquitin ligase activity, which is often used in inhibition of NF-κB[9]. Therefore, we hypothesize that combination of GBE and PDTC will produce better effects to alleviate the damages to keratinocytes caused by UVB radiation.

The study will explore whether or not a combination of PDTC and GBE will produce better effects to attenuate the damages to the HaCaT cells exposed to the UVB.

The power supply of UVB light is in the range of 290–320 nm with an emission peak at 312 nm (UVB 313EL, the photoelectric instrument factory of Beijing Normal University, China). *Ginkgo biloba L.* extract produced by Chi Sheng Pharma & Biotech Co. Ltd, Taiwan, China (Package of 5 mL containing 17.5 mg extract with 4.2 mg Ginkgo flavonoids glycosides). The HaCaT cell line was purchased from Shanghai Cell Bank (China). The cell line was grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin 100 IU/mL, streptomycin 100 ug/mL at 37 °C with 5% CO_2. HaCaT cells were allowed to grow to the confluence of 80%, different groups of HaCaT cells were pre-treated with PBS, PDTC (75 μmol/L), GBE (100 μg/L), or PDTC+GBE for 2 h, then the cells were washed with PBS, covered with a thin layer of PBS and exposed to UVB for 3 min (90 J/m^2) at a fixed distance of 50 cm. Three min later, after the addition of medium to the cells, the cells were further incubated for 24 h prior to the determination of experimental parameters.

The morphological change of HaCaT cells was observed under microscope (1×100), and photomicrographs were taken with Nikon digital camera (Nikon, Eclipse TS 100-F, Japan).

HaCaT cells were seeded into 6-well culture dishes and allowed to expand to confluence. Confluent cells were pre-treated with PBS, PDTC,

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GBE, GBE+PDTC respectively for 2 h and a standardized scratch was established in the monolayer using a 20 μL loading tip. Following scratch formation HaCaT cells were irradiated with UVB (90 J/m^2) for 3 min and then incubated in culture media. The extent of scratch closure was quantified by measuring the area of the scratch prior to (0 h) and 24 h following wounding, using a phase-contrast microscope (Nikon, Eclipse TS 100-F, Japan). The ROS level in HaCaT cells was determined with a DCFH-DA Fluorescent Kit. The intracellular fluorescence intensity of the cells were analyzed by flow cytometry (Ex/Em = 488/525). The protein expression level was determined by Western blot and the images were obtained by Chemiluminescence imaging analysis system (ECL, Tanon 5200, Shanghai Tianneng Technology Corp. Limited, China) and band intensity was analyzed with Tanon Gis analytical software (Shanghai Tianneng Technology Corp., Limited, Shanghai, China). Statistical evaluations were performed with SPSS 21.0 software using Student's *t*-test, or one-way or two-way ANOVA.

The HaCaT cells pre-treated with PBS prior to UVB irradiation were ruptured, the cytoplasm was stained red and the cell membrane was broken, the outline of the cells was unclear, and the density of viable cells was obviously decreased. In contrast, the outline of the HaCaT cells pre-treated with PDTC, GBE, and PDTC+GBE prior to exposure to UVB irradiation was clear with red staining cytoplasm. The density of viable cells was similar to that of the unexposed control cells (Supplementary Figure S1, available in www.besjournal.com).

As demonstrated in Figure 1, the HaCaT cells pre-treated with PDTC, GBE, or PDTC+GBE produced less ROS than those pre-treated with PBS, moreover, those pre-treated with PDTC+GBE had significantly lower level of ROS compared with those pre-treated with either PDTC or GBE ($P < 0.05$), which suggests that HaCaT cells with pre-treatment with GBE or PDTC will lower the production of ROS, and combination of GBE and PDTC will be more potent in reducing the production of ROS.

Significant closure of the scratch was seen in the cells pre-incubated with PDTC, GBE, and PDTC+GBE while no marked closure was observed in cells pre-treated with PBS 12 h after UVB irradiation. Moreover, on 24 h, an almost complete closure of the scratch was demonstrated in the cells pre-incubated with PDTC, GBE, and PDTC+GBE, however, the closure for the PBS-pre-treated cells was not complete. PBS-pre-treated cells demonstrated only a weak change in the extent of cell migration between 0 h and 24 h (Supplementary Figure S2, available in www.besjournal.com).

The expression of p-NF-κB and NF-κB was significantly higher in HaCaT cells pre-treated with PBS than that in those pre-treated with PDTC, GBE, PDTC+GBE. Moreover, the expression of p-NF-κB and NF-κB in HaCaT cells pre-treated with PDTC+GBE was significantly lower than that in those pre-treated with GBE or PDTC (Figure 2).

Western blot results showed that a significant ER

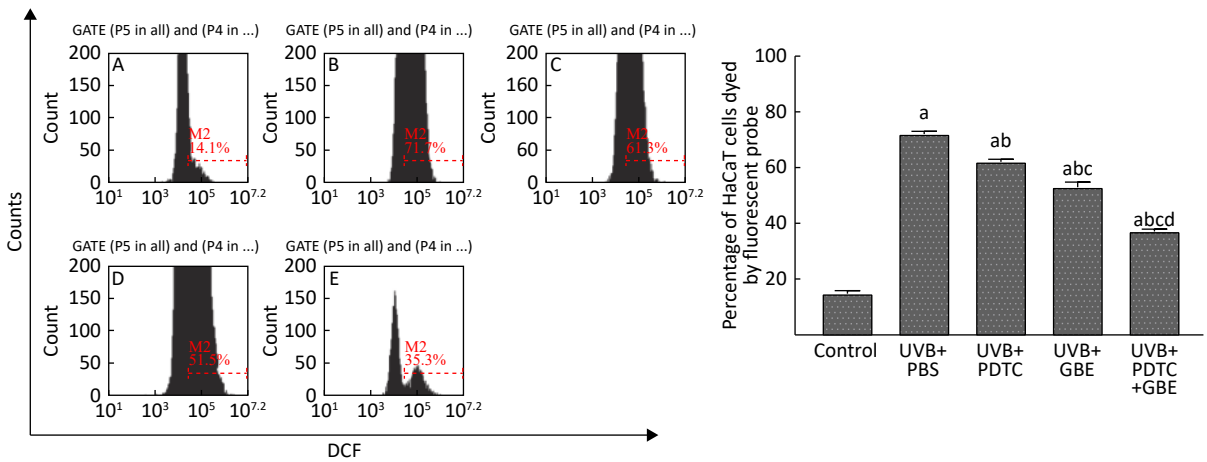

Figure 1. ROS production by HaCaT cells pre-treated with PDTC, GBE, or PDTC+GBE. (A) Unexposed control; (B) HaCaT cells pre-treated with PBS before being irradiated with UVB; (C) HaCaT cells pre-treated with PDTC before being irradiated with UVB; (D) HaCaT cells pre-treated with GBE before being irradiated with UVB; (E) HaCaT cells pre-treated with PDTC+GBE before being irradiated with UVB. [a]$P < 0.05$ *vs*. control; [b]$P < 0.05$ *vs*. UVB; [c]$P < 0.05$ *vs*. PDTC; [d]$P < 0.05$ *vs*. GBE.

stress was induced by UVB irradiation, which was demonstrated in Figure 3. We used BIP, IRE-1α, eIF-2α, CHOP, and PERK as indicators of ER stress and the expression of BIP, IRE-1α, eIF-2α, and PERK was significantly increased in HaCaT cells pre-treated with PBS prior to UVB irradiation while the expression of these indicators was significantly decreased in HaCaT cells pre-treated with PDTC, GBE, or PDTC+GBE prior to UVB irradiation (Figure 3). All these results indicate that UVB induces ER stress, and PDTC or GBE could alleviate the activation of ER stress and PDTC+GBE could produce a better protective effects against UVB irradiation.

We observed the higher expression of Caspase-8, Cytc, FADD, and Bax in HaCaT cells pre-treated with PBS than that in those pre-treated with PDTC, GBE, PDTC+GBE, which suggests that UVB irradiation was able to trigger both extrinsic and intrinsic apoptosis (Supplementary Figure S3, available in www.besjournal.com).

Different levels of intensity and duration of UVR irradiation will trigger varying levels of intricate stress responses that may become pathologic[1]. One of the significant stress responses involved in the damage of keratinocytes is oxidative stress which is associated with multiple skin conditions including aging, inflammation and skin cancer[7]. Moreover, oxidative stress will activate NF-κB pathway[4] and trigger endoplasmic reticulum stress, which are involved in many human pathologies such as cancers

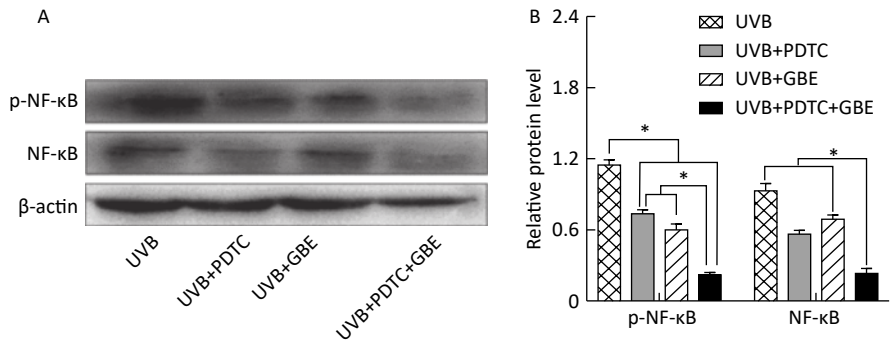

Figure 2. Comparison of expression of p-NF-κB and NF-κB in HaCaT cells pre-treated with PBS, PDTC, GBE, or PDTC+GBE. (A) Western blot analysis of p-NF-κB and NF-κB expression in HaCaT cells treated with PBS, PDTC, GBE or both PDTC and GBE. (B) The comparison of p-NF-κB and NF-κB relative expression level. $^{*}P < 0.05$.

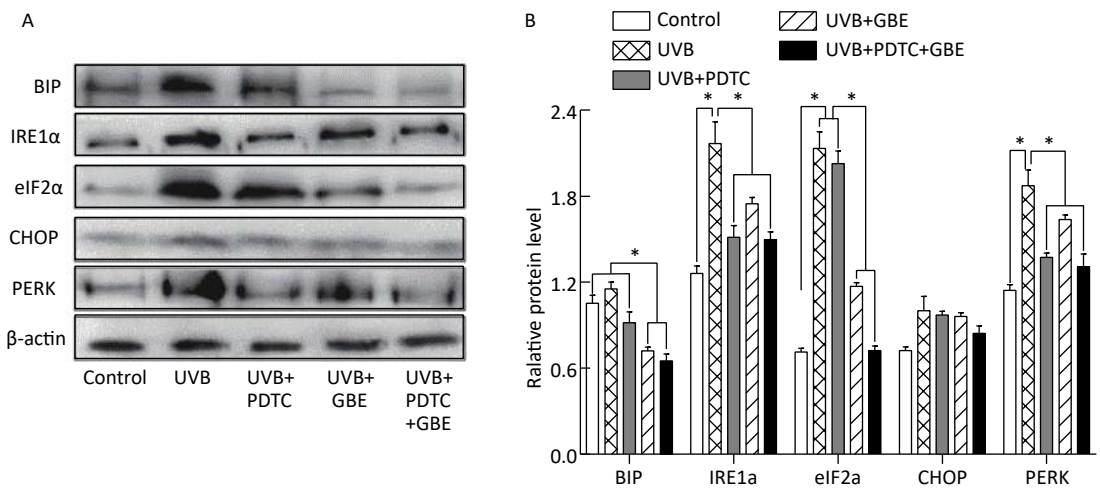

Figure 3. Comparison of expression of BIP, IRE 1α, eIF2α, CHOP, and PERK in HaCaT cells pre-treated with PBS, PDTC, GBE, or PDTC+GBE. (A) Western blot analysis of BIP, IRE 1α, eIF2α, CHOP, and PERK in HaCaT cells pre-treated with PBS, PDTC, GBE, or PDTC+GBE. (B) The comparison of BIP, IRE 1α, eIF2α, CHOP, and PERK relative expression level. $^{*}P < 0.05$.

and aging[6].

The novelty and significance of the present study is the application of combination of PDTC and GBE in protecting HaCaT cells from UVB irradiation. PDTC is often employed in inhibition of NF-κB[9] while GBE is applied for skin cell protection from oxidative stress[8]. Due to the different mechanisms of PDTC and GBE in alleviating oxidative stress, we are to test whether or not the combination of PDTC and GBE will produce more potent protective effects on HaCaT cells against UVB irradiation.

The major finding of our study reveal that combination of PDTC and GBE is able to produce a better protection for HaCaT cells against UVB, and our results are in accordance with other researchers'[8].

Systematic application of PDTC will produce side effects to other cells or tissues[10], but with limited dosage, local application of PDTC is unlikely to cause adverse effects systematically. GBE has been applied in clinics systematic for many years without observable unwanted effects. Therefore, it is possible to apply the combination of PDTC and GBE locally to fight against UVB irradiation.

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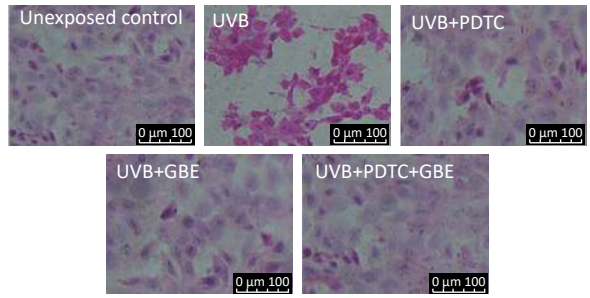
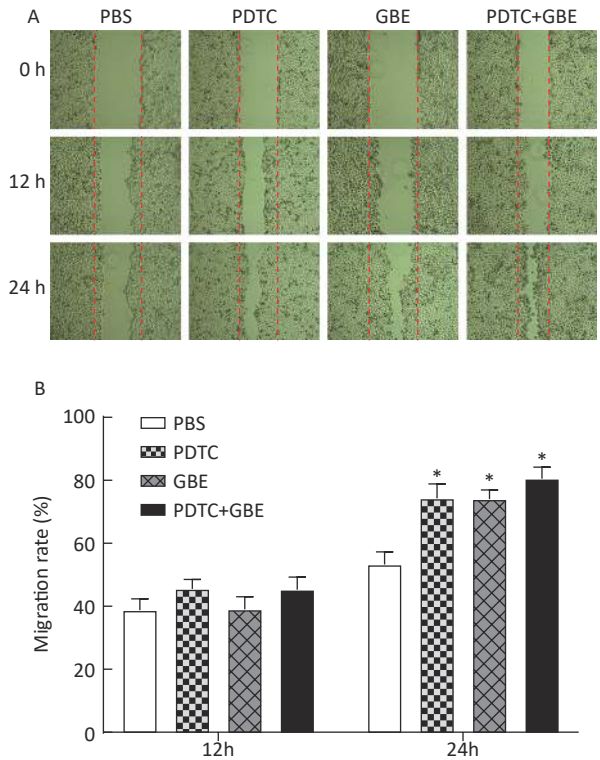
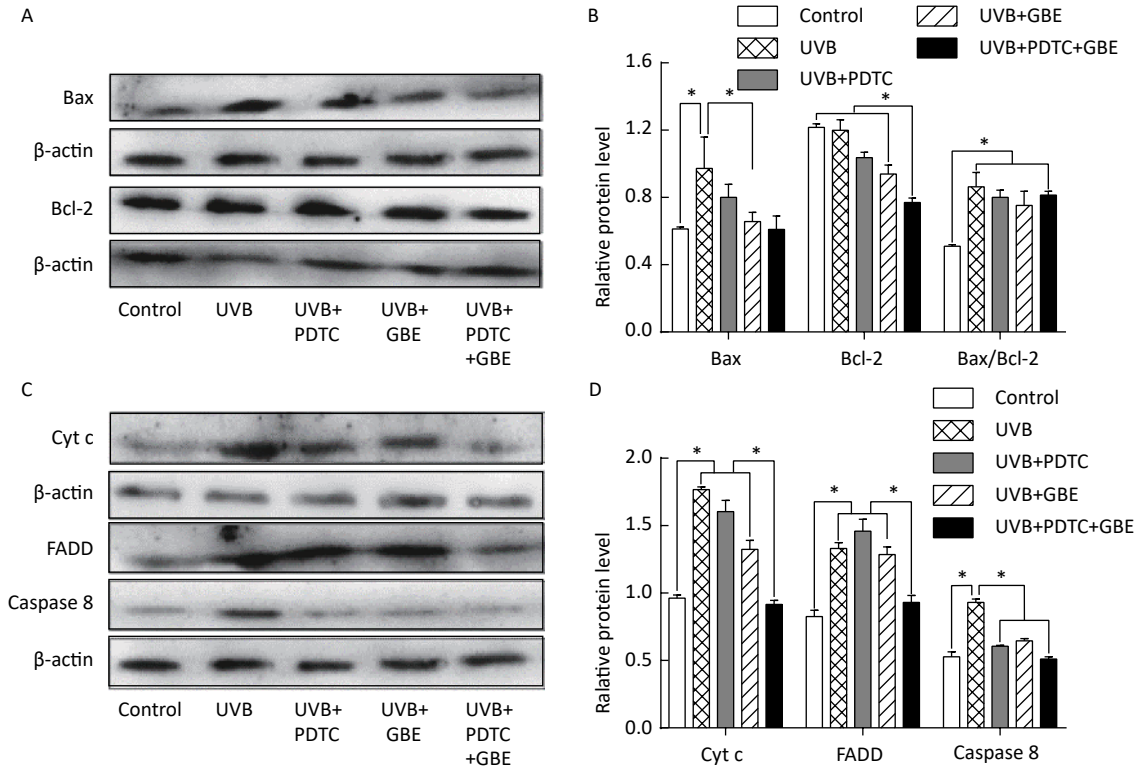


Supplementary Figure S1. Morphology of HaCaT cells pre-treated with PBS, PDTC, GBE, or PDTC+GBE prior to UVB irradiation (×100).



Supplementary Figure S2. Migration assay of HaCaT cells pre-treated with PBS, PDTC, GBE or PDTC+GBE prior to UVB irradiation. (A) Representative images at 0 h, 12 h, and 24 h of a scratch test assay. (B) Comparison of scratch close. $^{*}P < 0.05$ *vs*. PBS.



Supplementary Figure S3. Comparison of apoptosis-related proteins in HaCaT cells pre-treated with PBS, PDTC, GBE or PDTC+GBE. (A) Western blot analysis of Bax and Bcl-2 in HaCaT cells pre-treated with PBS, PDTC, GBE or PDTC+GBE. (B) The comparison of Bax and Bcl-2 relative expression level. (C) Western blot analysis of Cyt C, FADD and Caspase-8 in HaCaT cells pre-treated with PBS, PDTC, GBE, or PDTC+GBE. (D) The comparison of Cyt C, FADD and Caspase-8 relative expression level.