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Baicalin Scavenges Reactive Oxygen Species and Protects Human Keratinocytes Against UVC-induced Cytotoxicity

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Abstract. *Long-term exposure to solar ultraviolet (UV) radiation can cause multiple skin disorders, including skin cancer. Protection against UV-induced damage is, therefore, a worldwide concern. Baicalin, a major component of traditional Chinese medicine Scutellaria baicalensis, has been reported to have antioxidant and cytostatic effects on normal epithelial and normal peripheral blood and myeloid cells. In the current study, we examined whether baicalin could also effectively protect human keratinocytes from damaging short-wave UVC irradiation. Baicalin-scavenged reactive oxygen species increased within 2 h after UVC radiation. Baicalin also abrogated UVC-induced apoptosis. In addition, we identified the major products after UVC radiation with T4 UV endonuclease, finding that baicalin prevented cyclobutane pyrimidine dimer formation induced by UVC. Furthermore, baicalin also prevented formation of oxidative adducts induced by UVC. Our results demonstrated the utility of baicalin in assessing the potential contribution of traditional Chinese medicinal agents in therapy of UVC-induced genomic damage to skin and suggest potential application of these agents as pharmaceuticals in prevention of solar-induced skin damage.*

Ultraviolet (UV) light, which can be further divided into UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280 nm), is a component of solar radiation that significantly affects the human skin and various organisms (1, 2). Among the three kinds of UV, UVC is most mutagenic and well-analyzed in the laboratory. When cells are irradiated under the UVC wavelength range, adjacent pyrimidines in DNA become covalently linked by the formation of a four-membered ring structure which results from saturation of their respective-5,-6 double bonds (3). The structure formed by this photochemical cyclo-addition is referred to as a cyclobutane di-pyrimidine or cyclobutane pyrimidine dimer (CPD). In humans, exposure to sunlight may cause skin cancer and UV-induced DNA adducts. Genome instability can then occur which affects the development of skin carcinogenesis. Clearly the least damaging UVA may alter cell membrane components (4), induce DNA–protein crosslinking (5), and increase reactive oxygen species (ROS). UVC, the strongest kind of UV radiation, may also induce ROS and CPDs, which should be repaired *via* the cells nucleotide excision repair pathway (6).

The traditional Chinese medicine *Scutellaria* (common Chinese name: Huang-Qin) is especially highly rich in flavonoid components, which have been reported to have antitumor effect (7, 8). Flavones isolated from the roots of *Scutellaria* have multiple potential in exerting anti-oxidant (9), anti-viral (10-14), anti-thrombotic (15, 16), anti-inflammatory (17), anti-cardiovascular illness (16, 18), and neuroprotective (19-24) effects. Baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone) is reported to be one of the major flavones in *Scutellaria baicalensis* Georgi, comprising of 5.41% relative dry matter (25).

The UV-protective effects of baicalin on skin cells have not yet been well-described. For instance, previous studies have mainly focused on the protective effects of baicalin on UVB in cellular (26) and animal (27-29) models but never on UVC

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alone. In this study, we aimed to investigate UVC-induced damage to HaCaT cells and the effects and mechanisms of action of baicalin on UVC-induced damage to HaCaT cells.

Materials and Methods

Chemicals. All chemicals and solvents used throughout this study, including balicalin, dimethyl sulfoxide (DMSO), propidium iodide (PI), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich Chemical Co. (Milwaukee, WI, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Cambrex, Walkersville, MD, USA). T4 UV endonuclease V was purchased from Epicentre Technologies (Madison, WI, USA). Formamidopyrimidine-DNA glycosylases (FPG) and endonuclease III were purchased from Trevigen (Gaithersburg, MD, USA).

Cell culture. HaCaT human keratinocyte cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 mM non-essential amino acid, 2 mM glutamate, 100 U/ml penicillin, and 100 μg/ml stryptomycin. The cultures were incubated at 37˚C in a humidified atmosphere with 5% CO_2. Cells were sub-cultured every 2–3 days to obtain exponential growth.

Cell viability measurement. For determining cell viability, the MTT assay was performed as previously published (30-32). Briefly, cells were plated onto 24-well plates at a density of 3×10^4 HaCaT cells/well, grown for another 24 h, then treated with 0.1% DMSO or different concentrations of baicalin. After treatment, MTT was added to each well at a final concentration of 0.5 mg/ml and the mixture of MTT and cells was further incubated in 37˚C for 4 h. The viable cell number was directly proportional to the production of formazan following solubilization with isopropanol. The color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed in triplicate.

Cell-cycle distribution determination. About 2×10^6/ml HaCaT cells were seeded in 10-cm dishes and 0-200 μM baicalin added then, all cells were incubated for 24 h. After incubation, the cells were harvested and fixed gently with 70% ethanol, washed twice with phosphate buffered saline (PBS) and then incubated with PI buffer (4 μg/ml PI, 0.5 μg/ml RNase, and 1% Triton X-100 in PBS) for 30 min in the dark at room temperature. The cells were filtered through a 40-μm nylon filter and the PI-stained cells were analyzed for cell cycle distribution and apoptosis (sub-G1 phase) using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software as described previously (32). The sub-G_1 group was representative of the mean apoptosis.

UV density measurement and UV exposure. A UV light crosslinker (Spectrolinker XL-1000. Spectronics Co., Westburg, NY, USA) was used, and the UV 254 dose measured by a sensor in a UVC light box. All cells were washed with PBS, drained in a dish, and exposed to UVC radiation at a dose-rate of 0.5 W/m^2 of UVC on ice for the indicated UV doses (HaCaT cells were irradiated with UVC of 0, 5, 10, 20, 40, 80 and 160 J/m^2), and the Comet assay was performed immediately.

ROS analysis. About 2×10^5 HaCaT cells/well in 12-well plates were pre-treated for 2 h with 0-200 μM baicalin, UVC irradiated, and re-incubated with baicalin immediately after UVC irradiation for 0-4 h to determine the productions of ROS. Cells from each treatment were harvested, washed twice with PBS, then re-suspended in 500 μl of 2', 7'-dichlorfluorescein-diacetate (DCFHDA; 10 μM) for ROS in the dark for 30 min. Then all samples were analyzed immediately by flow cytometry as previously described (32).

Enzyme-incorporated comet assay. The standard comet assay without enzyme digestion was as follows: Agarose gels were made using PBS. A glass microscope slide was placed on a hot plate at 60˚C, and 100 μl of 1% normal-melting point agarose at 65˚C was applied to cover an area of 6.0×2.5 cm. A coverslip was applied immediately and the slide placed on ice to set the gel. Coverslips were then removed by dipping slides into water containing ice. To each gel surface, a second layer of agarose containing cells (50 cells/μl) in a total volume of 80 μl was applied. This second layer was prepared by mixing 400 μl of 1.5% low-melting point agarose at 40˚C with 200 μl of PBS containing earthworm cells. A coverslip was then applied to each slide. Coverslips were removed as described above. In a similar manner as the first layer, a 100 μl of 1.5% low-melting-point agarose was then applied as a third layer. After removing coverslips, slides were immersed in cell lysis solution at 4˚C for 18 h or more. Cell lysis solution contained 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH adjusted to 10.0 with NaOH, and 1% N-laurylsarcosine, 1% Triton X-100, and 10% DMSO added immediately before use. DNA was then denatured by incubating in 0.3 M NaOH, 1 mM EDTA pH 13.4 for 20 min. Electrophoresis was performed at 25 V, 300 mA for 25 min.

Slides were washed briefly in distilled water, blotted, and transferred to 0.4 M Tris-HCl, pH 7.5. DNA was stained by adding 12.5 μl of 200x Sybr green I to slides. A coverslip was applied and comets were observed under a fluorescence microscope (wavelength 450-490, farb teiler (FT) 510, long pass filter (LP) 520). An image of 50 cells per treatment was recorded with a digital camera (Kodak DCS-420, Kodak Digital Science, Rochester, NY). The migration of DNA from the nucleus of each cell was measured with a computer program using the parameter of comet moment. The comet moment was calculated using the formula $\sum 0 \rightarrow n$ [(amount of DNA at distance X)×(distance X)]/total DNA. This protocol was referred to as the standard comet assay.

For enzyme digestion, as performed elsewhere (11, 12), slides were washed after lysis treatment with distilled water then incubated at 37˚C for 30 min in enzyme reaction buffer. Then 0.5 unit of UV endonuclease V (for CPD), or one unit of FPG followed by one unit of endonuclease III (for oxidative adducts) in 10 μl of enzyme reaction buffer was added to the center of each half of the gel. A coverslip was applied and the slides were incubated at 37˚C for 2 h in a sealed box containing wet tissue paper to maintain humidity. Slides were prepared for electrophoresis as described above. In the experiments, we used the enzyme buffers supplied by Trevigen (Gaithersburg, MD, USA), namely 10 mM HEPES-KOH (pH 7.4), 100 mM KCl; 10 mM EDTA, 0.1 mg/ml bovine serum albumin as FPG digestion buffer; 10 mM HEPES-KOH (pH 7.4), 100 mM KCl, and 10 mM EDTA as endonuclease III digestion buffer.

Statistical analysis. Data were expressed as the mean±SEM and analysis of variance (ANOVA) was employed. Student's unpaired *t*-test was used in two-group comparisons. A value of $p<0.05$ was considered to be statistically significant.

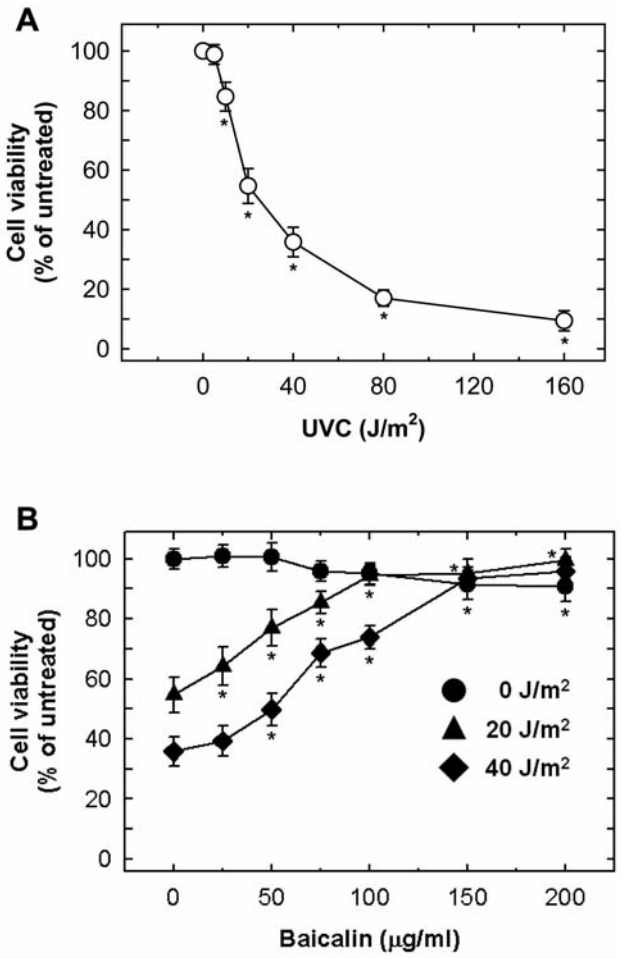

Figure 1. *Baicalin reversed UVC-induced cytotoxicity of HaCaT cells. A: HaCaT cells were irradiated with 0-160 J/m^2 UVC, harvested and analyzed for cell viability by MTT assay. *p<0.05, with versus without UVC irradiation. B: HaCaT cells were pre-treated for 2 h with 0-200 μg/ml baicalin, irradiated with UVC, and post-treated with the same dose of baicalin for 24 h, then harvested and analyzed for cell viability by MTT assay. *p<0.05, with versus without baicalin treatment. Data are mean±SD.*

Results

Baicalin induces non-toxic effects and abrogates the cytotoxicity of UVC on viable HaCaT cells. As shown in Figure 1, UVC reduced cell viability of HaCaT cells dose-dependently (Figure 1A).

The effects of baicalin on the UVC cytotoxicity were observed by adding baicalin 2 h, and irradiated with UVC 20 or 40 J/m^2 UVC, then re-incubated with baicalin immediately after irradiation for 24 h. At 20 J/m^2 UVC, which induced 45.3% reduction in cell viability, 25 μg/ml of baicalin began to abrogate UVC-induced cytotoxicity and at 100 μg/ml, baicalin restored cell viability almost to the level of the non-UVC treated control cells (Figure 1B). At 40 J/m^2 UVC, which reduced cell viability by 64.2%, baicalin started to be effective at 50 μg/ml and 150 μg/ml of baicalin restored cell viability almost to that of the non-UVC treated control cells (Figure 1B). At the same time, 25 to 200 μg/ml baicalin treatment alone was non-toxic to HaCaT cells (Figure 1B).

Baicalin effectively protected against UVC-induced apoptosis of HaCaT cells. The number of cells in each the cell-cycle phase and the typical sub-G_1 phase shown in Figure 2(A-C) were calculated as a percentage of the total number of cells and, the final results are presented in Figure 2D. The results indicated that 20 (Figure 2B) and 40 J/m^2 (Figure 2C) of UVC induced apoptosis in 20.7% and 40.3% of cells respectively after 24 h. Baicalin-alone did not induce HaCaT cell apoptosis (Figure 2A). The quantitative data showed that apoptosis of HaCaT cells induced by UVC was abrogated dose-dependently by baicalin and even to the basal level by 2-h pre- and 24-h post-treatment of 150 μg/ml baicalin (Figure 2D).

Baicalin effectively reduced the UVC-induced formation of ROS in HaCaT cells. Exposure to 20 or 40 J/m^2 of UVC induced a rapid increase of ROS in HaCaT cells within 1 h and to a plateau at 4 to 8 h (Figure 3A). Therefore, we pre-treated HaCaT cells with baicalin for 2 h before UVC irradiation and post-treated them for 4 h to investigate the effects of baicalin on UVC-induced ROS in HaCaT cells. The results showed that baicalin effectively reduced UVC-induced ROS in HaCaT cells in a dose-dependent manner (Figure 3B).

Baicalin prevented the formation of UVC-induced CPDs and oxidative DNA adducts. We were interested in the effects of baicalin on the formation and removal of UVC-induced DNA adducts. For this purpose, the enzyme-incorporated comet assay system was established, using UV endonuclease V to identify UV-induced CPD (31, 33), and FPG and endonuclease III to identify H_2O_2-induced oxidative DNA adducts, respectively (31, 34, 35).

After pre-treatment of HaCaT cells with different doses of baicalin, and exposure UVC, the enzyme-digestible adducts were detected to investigate the preventive effects of balcalin on UVC irradiation. The results showed that pre-treatment of baicalin dose-dependently reduced the formation of UVC-induced CPDs, and FPG-digestible and endonuclease III-digestible adducts (Figure 4A, C, E).

We also treated HaCaT cells post UVC-irradiation with baicalin for 24 h to investigate the effects of baicalin on removal of UVC-induced DNA adducts. The results showed that baicalin at up to 150 μg/ml could not enhance the efficiency of removal of either CPD, FPG-digestible or endonuclease III-digestible adducts in HaCaT cells (Figure 4B, D, F).

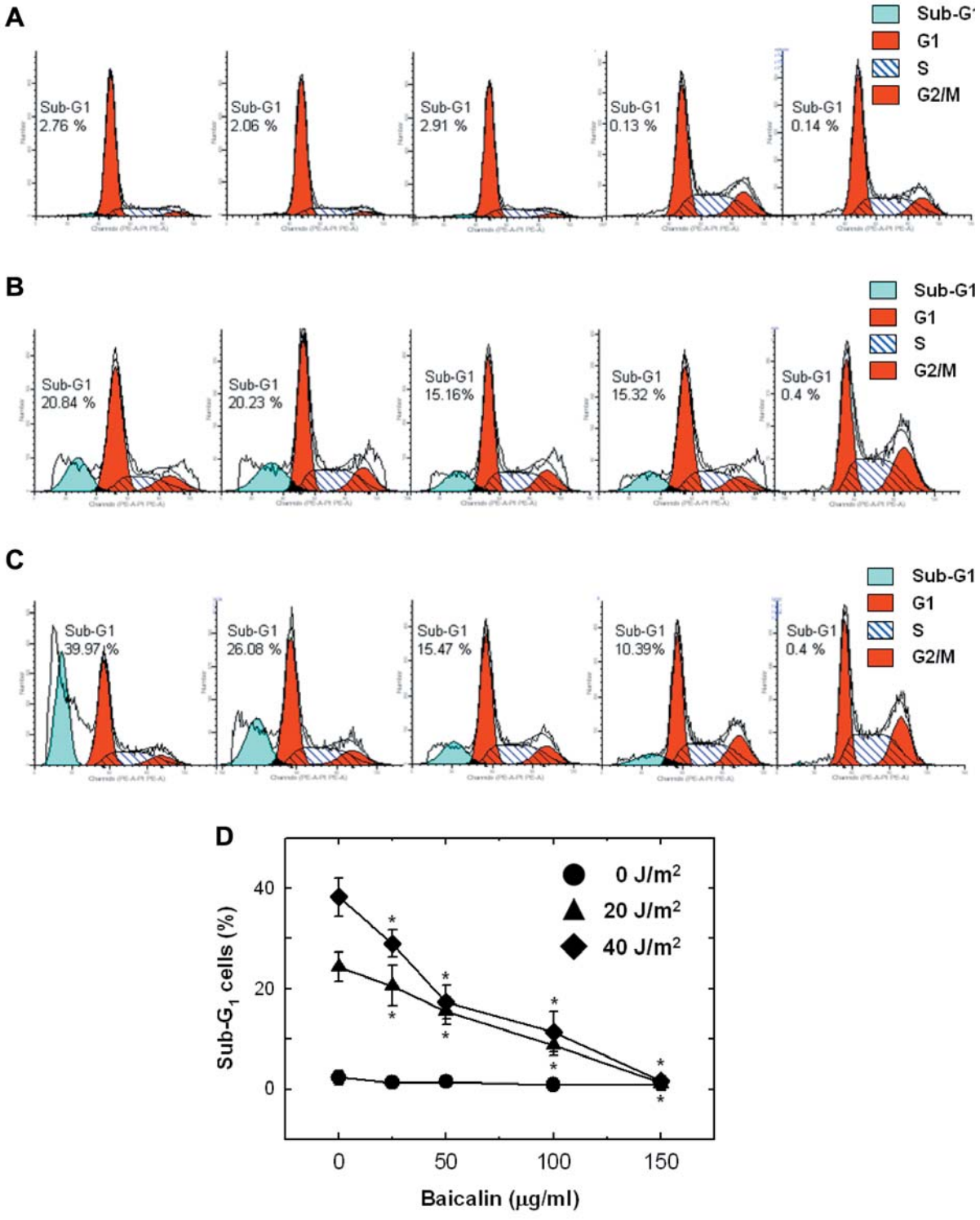

Figure 2. *Baicalin effectively reversed UVC-induced apoptosis of HaCaT cells. A: HaCaT cells were treated with 0, 25, 50, 100, 150 μg/ml baicalin for 24 h and the harvested cells were examined and analyzed for cell-cycle phase and sub-G_1 (apoptosis) proportion by flow cytometry as described in the Materials and Methods. Each point is the mean±SD of three experiments. B: HaCaT cells were pre-treated with 0, 25, 50, 100, 150 μg/ml baicalin for 2 h, irradiated with UVC at 20 J/m^2, and post-treated with the same concentration of baicalin for 24 h. The harvested cells were then examined and analyzed for cell-cycle phase and sub-G1 (apoptosis) proportion. Each point is the mean±SD of three experiments. C: Protocols were followed as in (B) except for the dose of UVC which was 40 J/m^2. D: The average percentages of sub-G1 cells in triple repeats of each protocol are presented and compared. *p<0.05, with versus without baicalin treatment.*

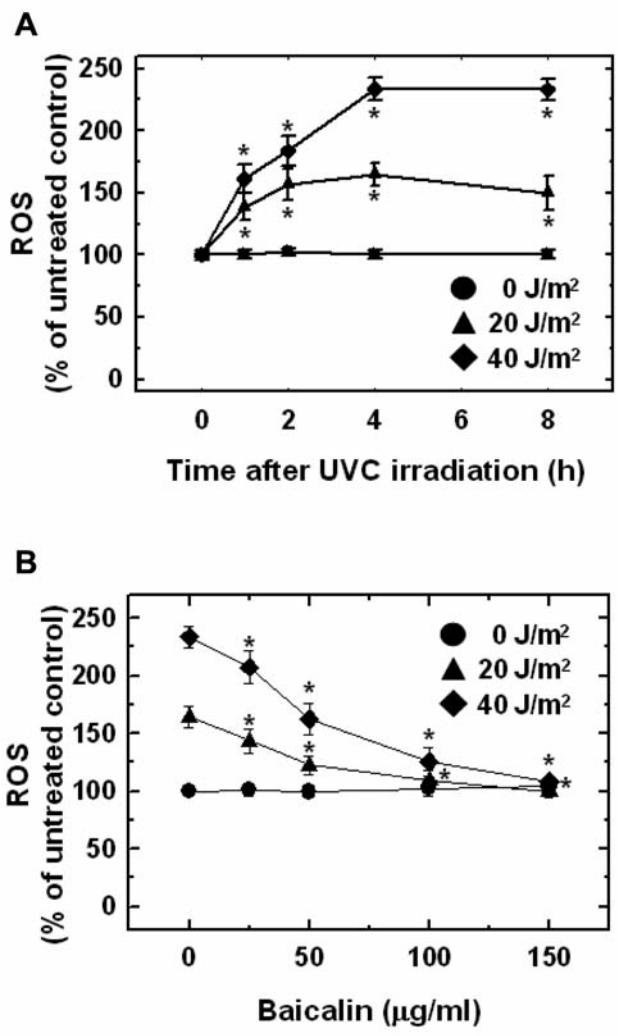

Figure 3. *Baicalin effectively reduced UVC-induced production of reactive oxygen species (ROS) in HaCaT cells. A: HaCaT cells were irradiated with UVC, harvested and analyzed for ROS production. *p<0.05, with versus without re-incubation after UVC 0, 20 or 40 J/m^2 irradiation. B: HaCaT cells were pre-treated for 2 h with 0-150 μg/ml baicalin, irradiated with UVC and post-treated with the same dose of baicalin for 4 h, then harvested and analyzed for ROS production. *p<0.05, with versus without baicalin treatment. Data are mean±SD.*

Discussion

Human skin cells are continuously exposed to outdoor solar UV radiation, which can cause cellular alterations, such as free radical formation and ROS generation, cytokine secretion, immunosuppression, genomic damage and mutations, leading to photo-aging and photo-carcinogenesis (36-38). The formation of photoproducts in the genome, mainly CPDs, plays a critical role in the initiation of UV-induced skin carcinogenesis (39). Traditional Chinese medicine, such as *Scutellaria baicalensis*, has been utilized clinically for centuries and is of great interest in chemoprevention of skin cancer. Baicalin is the most effective component isolated from the root of *Scutellaria baicalensis*, and mounting evidence from *in vivo* and *in vitro* studies have confirmed that baicalin has many biological effects, including improvement of microcirculation, elimination of oxygen-free radicals, anti-inflammation and anti-carcinogenesis (16, 40-42). Baicalin has been shown to inhibit UVB-induced photo-damage *via* blocking the relevant cytokine secretion and reducing the expression of *p53, p21, FBJ murine osteosarcoma viral oncogene homolog (c-FOS), proliferating cell nuclear antigen (PCNA)* and *replication protein A (RPA)* genes (26, 27, 29). In this study, we firstly investigated whether baicalin also protected human skin cells against UVC irradiation and its underlying molecular mechanisms.

There are three main end-points of UVC-induced cellular events and the effects of baicalin on these events were investigated in the current study: cell death, especially apoptosis; the production of ROS; and genomic damage and repair. As to the first point, UVC-induced cytotoxicity and apoptosis was abrogated by baicalin dose-dependently (Figures 1 and 2). For the second point, rapid UVC-induced production of ROS was also reduced by baicalin dose-dependently (Figure 3). For the last point, baicalin successfully prevented the formation of UVC-induced CPD and oxidative DNA adducts, but did not enhance them removal of these adducts after 24 h (Figure 4).

The loss of cell viability of HaCaT cells by 20 and 40 J/m^2 UVC were very consistent with previous findings (43). Although baicalin seemed to rescue all of UVC-damaged HaCaT cells, preventing them from dying, the baicalin rescued UVC-treated HaCaT cells did not exhibit exactly the same morphology under microscopy (data not shown).

Balicalin was capable of reducing UVC-induced generation of intracellular ROS strengthens which may cause oxidative damage to cell components, including genomic DNA, RNA, proteins, lipids and small molecules. The reduction of cytotoxicity and apoptosis might partly be due to the strong antioxidant activity of baicalin toward ROS. Consistent with the alterations in cellular viability (Figure 1 and 2), CPDs and oxidative DNA adducts (Figure 4), the results showed UVC-induced ROS production in cells, and that ROS were reduced in baicalin-treated groups in a dose-dependent manner (Figure 3). Subsequently we further investigated whether baicalin had a direct effect on the DNA damage/repair process and the relevant mechanisms. We believe that ROS production is also as important as UVC-induction of DNA adducts, such as CPDs, in carcinogenesis and apoptosis initiation. Therefore, we not only focused on genomic damage induced by UVC through the well-known photoproducts, CPDs, but also on oxidative DNA adducts.

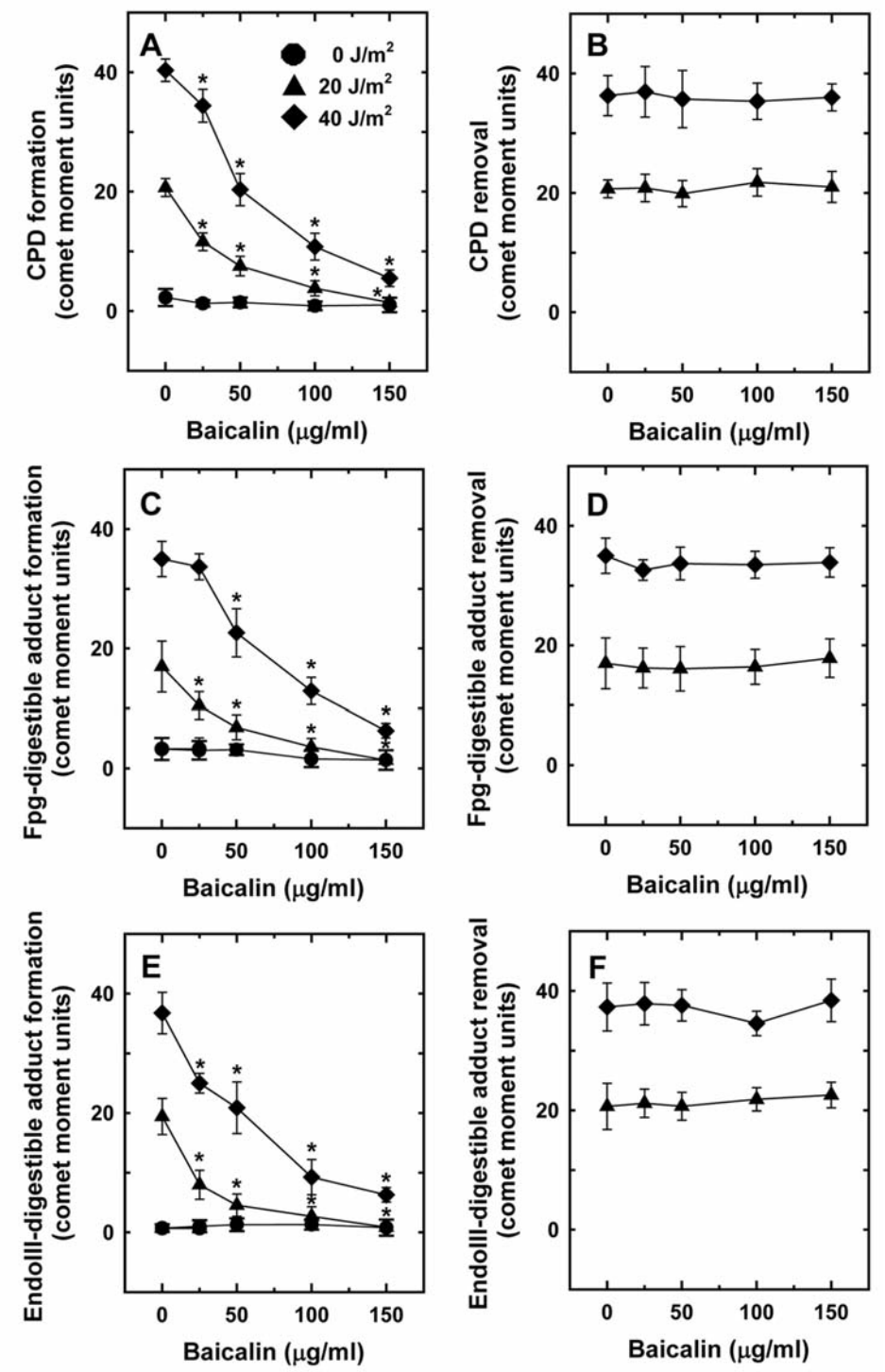

Figure 4. *Baicalin prevented the formation of UVC-induced cyclobutane pyrimidine dimers (CPDs) and oxidative DNA adducts. A: Cells were irradiated with indicated doses of UVC on ice. Then the cells were subjected to comet slide preparation and the slides were sham-digested or digested with UV endonuclease V for twice to detect DNA strand breaks by comet assay. The CPD formation was the difference in comet moments between sham-digested and digested slides. *p<0.05, with versus without baicalin pre-treatment. B: Cells were irradiated with UVC and re-incubated for 24 h in baicalin before slide preparation. The slides were then sham-digested or digested with UV endonuclease V twice to detect DNA strand breaks by comet assay as in (A). The CPD removal was the difference in comet moments between the resulting maximum CPD formation and that in (A). *p<0.05, with versus without baicalin. C: The same protocol was used as in (A) except for the digestion enzyme that was changed to formamidopyrimidine-DNA glycosylases (FPG). D: The same protocol was used as in (B) except for the digestion enzyme that was changed to FPG. E: The same protocol was used as in (A) for except the digestion enzyme that was changed to endonuclease III. F: The same protocol was used as in (B) except the digestion enzyme was changed to endonuclease III.*

Baicalin prevented the formation of UVC-induced CPDs and oxidative DNA adducts, but the pilot results here showed that baicalin had no effects on their removal. The dynamic and complicated DNA repair processes are worthy of attention. Interestingly, we found that after 24 h, all the CPD and oxidative adducts induced by UVC were repaired. In fact, baicalin increased the speed of UVC-induced DNA adduct removal within the first 4 h after UVC irradiation (data not shown). The CPDs are repaired by the nucleotide excision repair pathway, while the oxidative adducts are repaired by the base-excision pathway. In the near future, our team hopes to reveal the detailed DNA repair mechanisms affected by baicalin.

Treatment with baicalin alone, up to 150 μg/ml, was non-toxic to HaCaT cells (Figures 1 and 2), and induced production of neither ROS (Figure 3), CPD (Figure 4A), nor oxidative adducts (Figure 4C, E). The finding that baicalin is non-toxic to HaCaT cells is consistent with that of Min and colleagues (26).

In conclusion, our findings indicated that baicalin has a promising UVC-protective capacity *via* reducing UVC-induced HaCaT cell cytotoxicity, apoptosis, ROS production, and CPD and oxidative DNA adduct formation.

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