

Protective Effects of Pyridoxamine Against UVC-induced Programmed Cell Death in HaCaT Cells

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Abstract. *Background: Exposure to ultraviolet (UV) light is closely related to human diseases, such as skin cancer, due to irreversible injuries to the skin cells. The UV-induced DNA damage and programmed cell death are important determinants for skin carcinogenesis. The aim of the present study was to investigate the anti-ultraviolet-C (UVC) effects of pyridoxamine in human keratinocyte HaCaT cells and its mechanisms of action. Results: UVC-induced programmed cell death in HaCaT cells was abrogated by treated the cells immediately after UVC irradiation with 40, 80 and 160 μ M of pyridoxamine. Monitoring the UVC-induced-specific reactive oxygen species, we found that 20, 40, 80 and 160 μ M of pyridoxamine was also effective in suppressing the induction of reactive oxygen species by UVC. Conclusion: Overall, our results provided evidence showing that pyridoxamine was effective in protecting HaCaT cells from UVC-induced programmed cell death and may be a potential anti-UVC agent in life and clinical practice.*

Ultraviolet (UV) light is a component of solar radiation that significantly affects the human skin, as well as other organs (1). According to spectrum wavelength, UV can be divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). Among them, UVC is most mutagenic and it was

reported that adjacent pyrimidines in DNA become covalently linked by the formation of a four-membered ring structure when the cells were irradiated with UVC. The structure formed by this photochemical cyclo-addition is referred to as a cyclobutane di-pyrimidine or cyclobutane pyrimidine dimer (CPD) (2). In humans, unprotected long-term exposure to sunlight may cause irreversible DNA damage and accumulated genome instability can then occur and initiate skin tumorigenesis. Even the least damaging UVA was reported to alter cell membrane components (3), induce DNA-protein crosslinking (4) and elevate the level of intracellular reactive oxygen species (ROS) (5). UVC, the strongest kind of UV radiation, may also induce ROS and CPDs (6), which should be repaired *via* the cells' nucleotide excision repair pathway (7).

Pyridoxamine, together with pyridoxal and pyridoxine, are the three natural forms of vitamin B6 (8). In a rat model, pyridoxamine is effective in preventing the occurrence of diabetes (9, 10) by mechanisms that may be closely related to the decrease in plasma levels of the reactive carbonyl compounds glyoxal and methylglyoxal, the precursors of advanced glycation end-products (11).

In the present study, we aimed at examining the effects of pyridoxamine on UVC-induced DNA adducts and programmed cell death of human keratinocyte HaCaT cells.

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Materials and Methods

Chemicals. All chemicals and solvents used throughout this study, including pyridoxamine, 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). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, 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).

Keratinocyte HaCaT cell culture. Human keratinocyte HaCaT 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 μ M non-essential amino acids, 2 μ M glutamate, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Cells were sub-cultured every 2-3 days to obtain exponential growth.

UV density measurement and UVC exposure. A UV light crosslinker (Spectrolinker XL-1000; Spectronics Co., Westburg, NY, USA) was used and the UV 254 dose was measured by a sensor in a UVC light box. All HaCaT cells were washed with PBS, drained in a dish and exposed to UVC radiation at a dose-rate of 0.5 W/m² of UVC on ice for the indicated UV doses (0, 5, 10, 20, 40, 80 and 160 J/m²). All assays were performed with or without re-incubation with pyridoxamine-containing medium for the indicated time periods.

Cell viability measurement. For determining cellular viability, the MTT assay was performed as previously described (12). Briefly, HaCaT cells were plated into 24-well plates at a density of 3×10^4 cells per well, grown for another 24 h and, then, treated with 0.1% DMSO or indicated concentrations of pyridoxamine. After the 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 the solubilization with isopropanol. The color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). Each experiment was repeated at least three times.

Cell cycle distribution determination. About 2×10^6 /ml HaCaT cells were seeded in 10-cm dishes and treated with 0-160 μ M pyridoxamine; all cells were incubated for 0, 6, 12, 24, 48 and 72 h. Then the HaCaT cells were harvested and fixed gently with 70% ethanol, washed twice with PBS and incubated with PI buffer (4 μ g/ml PI, 0.5 μ g/ml RNase and 1% Triton X-100 in PBS) for 30 min in the dark at room temperature. After the fixation and staining processes, the cells were filtered through a 40- μ m nylon filter and 10,000 single PI-stained cells were analyzed for cell cycle distribution and programmed cell death (appearance of sub-G₁ phase) by using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with the Cell Quest software, as described previously (6, 13). The sub-G₁ group was representative of the percentage of apoptosis.

Reactive oxygen species (ROS) measurement. About 2×10^5 HaCaT cells/well in 12-well plates were incubated with 0-160 μ M pyridoxamine for 24 h to determine the level of ROS. After cells were incubated for various time periods, all the HaCaT cells in each treatment were harvested, washed twice by PBS and re-suspended in dichloro-dihydro-fluorescein diacetate (10 μ M) for ROS at 37°C in the dark for 30 min. Then, all samples were analyzed immediately by flow cytometry as previously described (14).

Statistical analysis. The quantitative data are shown as mean \pm standard deviation (SD). The statistical differences between the pyridoxamine-treated and control samples were calculated by one-way analysis of variance (ANOVA) and Student's *t*-test. Any *p*-values less than 0.05 were considered statistically significant. Results are representative of at least three independent experiments.

Results

The structure of pyridoxamine and its effects on HaCaT cell viability. The structure of pyridoxamine is presented in Figure 1A. Twenty-four h treatment of 20 and 40 μ M of pyridoxamine did not induce loss of cell viability; however, 80 and 160 μ M of pyridoxamine significantly decreased cell viability by 10.67 and 15.67%, respectively (Figure 1B).

UVC induced dose-dependent loss of HaCaT cell viability, which could be abrogated by pyridoxamine. The protective effects of pyridoxamine on the UVC-induced cytotoxicity were evaluated by adding pyridoxamine immediately after UVC irradiation into the medium, re-incubating the cells for 24 h and analyzing the cell viability by MTT assay. Without the treatment of pyridoxamine, 20 and 40 J/m² UVC induced 45.33 and 64.17% of cell death after 24 h (Figure 2A). The treatment of 10- μ M pyridoxamine was effective in reversing the UVC-induced cytotoxicity for both 20 and 40 J/m² UVC (Figure 2B). The protective effects of pyridoxamine were dose-dependent; however, at high doses of 80 and 160 μ M, cell viabilities were at levels similar to those of pyridoxamine-alone seen in Figure 1B (Figure 2B).

Pyridoxamine was effective in protecting HaCaT cells from UVC-induced programmed cell death. The UVC-induced loss of cell viability was certified by the appearance of sub-G₁ to be programmed cell death. The results indicated that 20 and 40 J/m² of UVC induced apoptosis in 42.0% and 60.17% of cells, respectively, after 24 h (Figure 3A). Pyridoxamine was effective in abrogating the apoptosis induced by 20 and 40 J/m² UVC in HaCaT cells dose-dependently (Figure 3B). Pyridoxamine-alone did not induce significant HaCaT cell apoptosis except at high doses of 80 and 160 μ M (Figure 3B).

UVC-induced formation of ROS in HaCaT cells, which was reduced by pyridoxamine. Figure 4A shows that both 20 and 40 J/m² UVC induced a significant increase of ROS in HaCaT cells 8 h after irradiation. Subsequently, the cells were treated with 0-160 μ M of pyridoxamine for 8 h after UVC irradiation and then intracellular ROS levels were evaluated. The data (Figure 4B) show that pyridoxamine itself could not induce any ROS but significantly decreases the level of UVC-induced ROS in a dose-dependent manner.

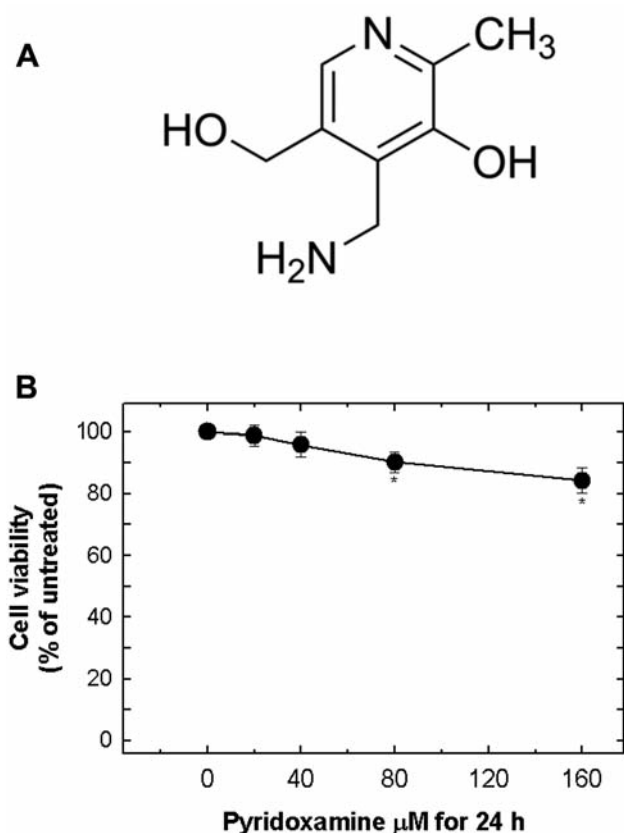


Figure 1. Structure of pyridoxamine and its action on pyridoxamine-induced cytotoxicity of HaCaT cells. A: Structure of pyridoxamine. B: HaCaT cells were treated in the culturing medium with 0-160 μM of pyridoxamine for 24 h, then, harvested and analyzed for cell viability by the MTT assay. * $p < 0.05$, with versus without pyridoxamine treatment (data are mean \pm SD).

Discussion

As UV irradiation is almost inevitable, skin cancer is one of the leading types of cancer in USA and worldwide (15). Human skin cells, such as keratinocytes and fibroblasts, are subjects to UV irradiation, which may cause direct and indirect alterations and damage to the cells. Direct damage to genomic DNA will lead to the formation of DNA adducts, including cyclobutane-pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts, whereas indirect signaling alterations will include the increases in ROS levels and other free radicals (6, 16). Any effective agent promoting protection of the cells from UVC-induced cell injury is potentially winning the war of photo-aging and photo-carcinogenesis.

The aim of the present study was to determine whether pyridoxamine was effective in protecting human keratinocytes, HaCaT cells, against UVC-induced cell apoptosis and ROS. HaCaT cells possess the well-recognized

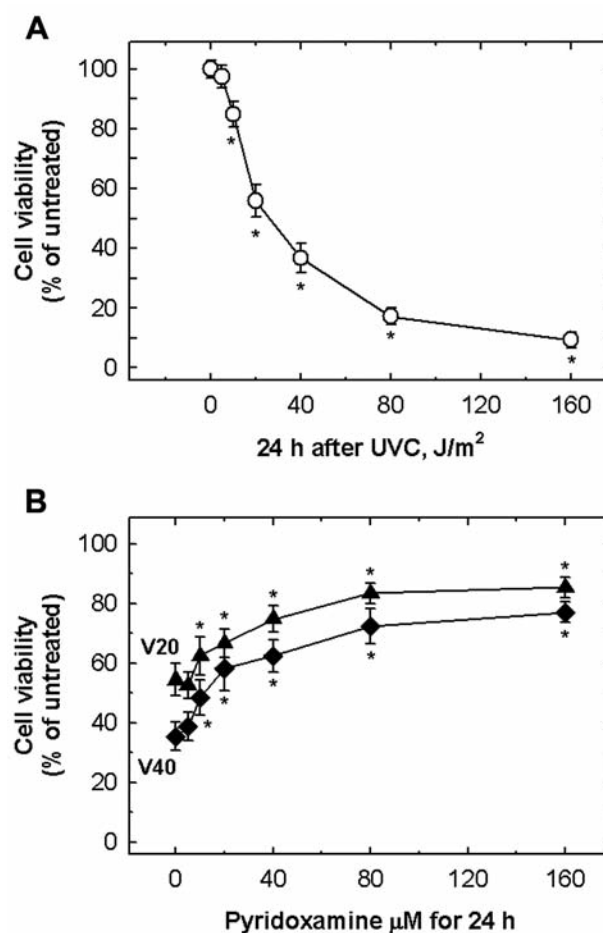


Figure 2. UVC induced dose-dependent loss of HaCaT cell viability, which could be abrogated by pyridoxamine. A: HaCaT cells were irradiated with 0-160 J/m^2 UVC, harvested and analyzed for cell viability by MTT assay. * $p < 0.05$, with versus without UVC irradiation. B: HaCaT cells were irradiated with 0, 20 or 40 J/m^2 UVC and post-treated with the indicated doses of pyridoxamine for 24 h, then, harvested and analyzed for cell viability by MTT assay. * $p < 0.05$, with versus without pyridoxamine treatment (data are mean \pm SD).

characteristics of being stable, non-tumorigenic keratinocytes and with a largely preserved differentiation capacity (17).

Clinically, pyridoxamine was marketed as a dietary supplement with a good safety record, as confirmed from Phase I and II clinical trials evaluating its effects on serum creatinine levels in patients with type-2 diabetic nephropathy (18, 19). However, the detailed intracellular mechanisms for pyridoxamine against aging-associated diseases are not well-clarified. Previously, pyridoxamine was reported to be a potential radio-protector for guarding normal epithelial cells from ionizing radiation (20). The detailed mechanisms for pyridoxamine as a radio-protector were closely related to the scavenging capacity of pyridoxamine against ROS and

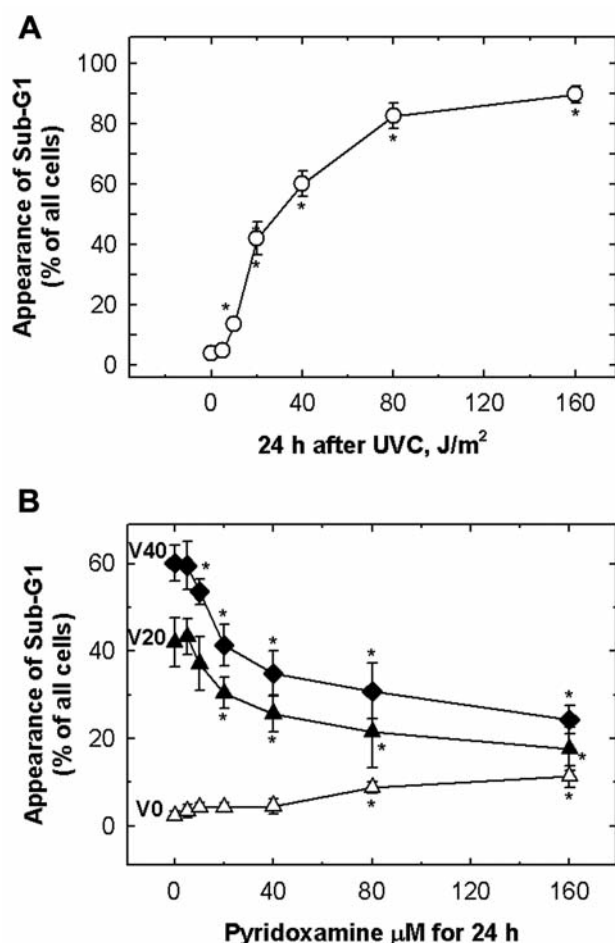


Figure 3. Pyridoxamine effectively reversed UVC-induced apoptosis of HaCaT cells. A: HaCaT cells were irradiated with 0-160 J/m² UVC, examined and analyzed for cell cycle phase and sub-G₁ (apoptosis) proportion by flow cytometry as described in the Materials and Methods. B: HaCaT cells were irradiated with 0, 20 or 40 J/m² UVC, re-incubated with 0, 5, 10, 20, 40, 80 and 160 µM of pyridoxamine for 24 h and the harvested cells were subjected to flow cytometry as described in A. The average percentages of sub-G₁ cells in experiments performed in triplicates are presented and compared. **p*<0.05, with versus without pyridoxamine treatment.

reactive carbonyl species (20). In this article, we have provided evidence showing that pyridoxamine was capable of protecting another normal human cell line, keratinocyte HaCaT cells, from another environmental radiation stress, UVC, with respect to UVC-induced apoptosis (Figure 3) and ROS (Figure 4). Our results provided another piece of supporting evidence for pyridoxamine as a natural antioxidant beneficial agent protecting normal tissues and cells of the whole body. These data are also consistent with those reporting that pyridoxamine was capable of preventing H₂O₂-induced cytotoxicity in HepG2 cells (21) and light-induced cytotoxicity in murine retinal cells (22). Further

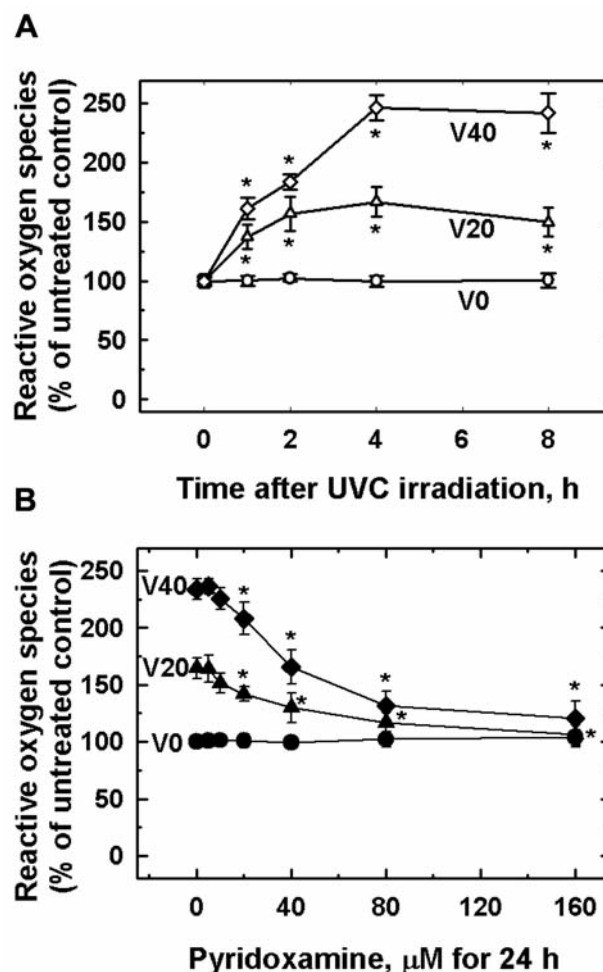


Figure 4. Pyridoxamine effectively reduced UVC-induced production of reactive oxygen species (ROS) in HaCaT cells. A: HaCaT cells were irradiated with UVC, re-incubated and analyzed for ROS production after 0, 1, 2, 4 and 8 h. **p*<0.05, with versus without re-incubation after irradiation. B: HaCaT cells were irradiated with 0, 20 or 40 J/m² UVC and post-treated with the indicated doses of pyridoxamine for 24 h, then, harvested and analyzed for ROS production. **p*<0.05, with versus without pyridoxamine treatment (data are mean ± SD).

investigations of the signaling network and targeting cascade for pyridoxamine against UVC are needed. In particular, it is of interest to determine whether pyridoxamine may influence the production of photoproducts (cyclobutane pyrimidine dimers) and the interaction between pyridoxamine and repair proteins for UVC irradiation. Also, as caspases are involved in UVC-induced apoptosis, their potential alterations to pyridoxamine should be investigated and revealed in the near future.

In conclusion, the present pilot study indicated that pyridoxamine, as a nutrition supplement, may act as a potential anti-photo-aging and anti-photo-carcinogenesis agent.

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