



Astaxanthin Protects Ultraviolet B-Induced Oxidative Stress and Apoptosis in Human Keratinocytes via Intrinsic Apoptotic Pathway

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Background: Ultraviolet radiation causes skin damage due to increased production of reactive oxygen species (ROS) and inflammatory intermediates and direct attack of DNA of skin cells. Astaxanthin is a reddish pigment that belongs to a group of chemicals called carotenoids and has protective effects as an antioxidant.

Objective: To determine the beneficial effects of astaxanthin on damaged human skin after exposure to ultraviolet radiation.

Methods: Normal human epidermal keratinocytes (NHEKs) were pre-treated with astaxanthin for 24 hours and exposed to ultraviolet B (UVB) irradiation. After 24 hours, the Cell Counting Kit-8 (CCK-8) assay measured cell viability, ROS assay and flow cytometry analysis assessed apoptosis, and western blotting was performed to determine expression of apoptosis-related proteins.

Results: Astaxanthin significantly inhibited UVB-induced NHEKs cytotoxicity. Pretreatment of NHEKs with astaxanthin reduced UVB-induced ROS production. Astaxanthin caused significant inhibition of UVB-induced apoptosis, as evidenced by flow cytometry analysis and western blotting.

Conclusion: These results suggest that astaxanthin has a beneficial effect of reducing damage caused by UVB by effectively inhibiting cell death and reducing ROS production in keratinocytes.

Keywords: Apoptosis, Astaxanthin, Keratinocytes, Reactive oxygen species, Ultraviolet rays

INTRODUCTION

The skin is a front-line barrier to protecting our bodies from foreign organisms and serves as a shield against heat and ultraviolet (UV) radiation. UV radiation causes structural and functional changes in cells. UV-induced skin damage ranges from aging of skin and pigmentation including melasma to skin cancers such as basal cell carcinoma, squamous cell carcinoma, and melanoma¹. UV rays are divided into UVA (320~400 nm), UVB (280~320 nm), and UVC (200~280 nm)². UVC rays are blocked by the ozone layer, while UVB and UVA cross the epidermis to reach the dermis. UVB rays are

considered the most dangerous due to increased production of reactive oxygen species (ROS) and inflammatory intermediates and direct attack of DNA of skin cells³. ROS include singlet oxygen, superoxide radicals, hydroxyl radicals, and hydrogen peroxide that cause damage to DNA, proteins, and lipids of human cells. ROS are a major factor of skin aging and cause inflammation and apoptotic cell death of keratinocytes by activating transcription factors⁴. The mitochondrial membranes disrupted by ROS release cytochrome C and activate the caspase that induces apoptosis. ROS-induced apoptosis can be inhibited by antioxidative substances or enzymes⁵.

Astaxanthin is a keto-carotenoid that belongs to a larger



class of chemical compounds known as terpenes, comprised of five carbon precursors, isopentenyl diphosphate, and dimethylallyl diphosphate. Astaxanthin is classified as a xanthophyll and is found in salmon, krill, and lobsters⁶. Like many carotenoids, astaxanthin is a lipid-soluble pigment. Its red-orange color is due to the extended chain of conjugated double bonds at the center of the compound that are responsible for the antioxidant function of astaxanthin⁷.

Previous studies have reported that UVB causes ROS production and apoptosis of keratinocytes⁸⁻¹¹. Herein, this study aimed to investigate the effects of astaxanthin on normal human epidermal keratinocytes (NHEKs) irradiated with UVB.

MATERIALS AND METHODS

Reagents and antibodies

Astaxanthin and dimethyl sulfoxide were purchased from Sigma-Aldrich Inc. (Taufkirchen, Germany). Cell Counting Kit-8 (CCK-8) was purchased from DOJINDO Inc. (Kumamoto, Japan). ROS Detection Assay Kit was purchased from Biovision Inc. (Milpitas, CA, USA). PE Annexin V apoptosis detection kit was purchased from BD Biosciences Inc. (San Jose, CA, USA). Anti-BAX pAb, anti-BCL2 mAb, anti-cleaved CASP3 mAb, and anti-cleaved PARP mAb were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-ACTB pAb were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

NHEKs, obtained from Promocell Inc. (Heidelberg, Germany), were cultured in serum-free keratinocyte growth medium 2 (Promocell) supplemented with bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, epinephrine, transferrin, and CaCl₂ at standard cell culture conditions (37°C, 5% CO₂ in a humidified incubator). All experiments were performed using cells between the 2nd and 7th passages.

UVB source

The UVB light source was a G15T8E (Sanyo Denki, Tokyo, Japan) lamp that emitted 15 W of UV radiation between 280 and 360 nm (peak 306 nm). UVB irradiance was measured using the UV light meter UV-340A (Lutron Electronics, Coopersburg, PA, USA).

Treatment of normal human epidermal keratinocytes

NHEKs were seeded in culture plates, allowed to attach for 24 hours, and pre-treated with astaxanthin (0, 10, 20, 30 μM for 24 hours). Post incubation, cells were exposed to UVB irradiation (0, 20, 40, 60 mJ/cm²) and moved to a cell incubator. After 24 hours, analysis was performed.

Measurement of cell viability

The cells were seeded in 96-well plates (2×10⁴ cells/well) to 70%~80% confluency. The CCK-8 solution (DOJINDO) was added to each well. The cells were incubated for 1 hour at 37°C. The optical density was measured at 450 nm using a Molecular Devices SpectraMax i3 multimode microplate reader.

Detection of ROS production

Intracellular ROS was measured using ROS Detection Assay Kit (Biovision). The cells were seeded in 96-well plates (2×10⁴ cells/well) to 70%~80% confluency. The cells were washed with ROS assay buffer, incubated with 1X ROS label for 45 minutes at 37°C in the dark, and then washed with ROS assay buffer. Finally, fluorescence was measured at 495 nm excitation and 529 nm emission to quantify the ROS production.

Flow cytometric analysis

Flow cytometry analysis for detection of apoptosis was performed with 7-amino-actinomycin (7-AAD) and phycoerythrin (PE)-labeled annexin V in a PE Annexin V Apoptosis Detection kit (BD Biosciences). Samples were washed in cooled phosphate buffered saline (PBS) and centrifuged at 220×g for 3 minutes. The pellets were resuspended and adjusted to 1×10⁶ cells/ml with the binding buffer of the kit. PE-labeled annexin V (5 μl) and 7-AAD (5 μl) were added to the suspension. After incubation for 15 minutes at room temperature, the suspensions were analyzed with a flow cytometer (BD biosciences Inc.).

Western blot analysis

Cold PBS-washed cells were harvested and lysed in PRO-PREP™ Protein Extraction Solution (Intronbio, Seongnam, Korea). Protein concentration was measured using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). The protein lysates were denatured at 97°C for 5 minutes after mixing with 2 μl of sodium dodecylsulphate loading buffer, applied on a sodium dodecylsulphate polyacrylamide gel for electrophoresis, and transferred to nitrocellulose membranes. Western blot analysis was carried out

to detect the expression levels of *BAX*, *BCL2*, cleaved *CASP3*, and cleaved *PARP* using specific antibodies. Band signals were visualized on X-ray film using ECL Western Blotting Substrate (Promega, Madison, WI, USA). The relative amounts of proteins associated with specific antibodies were normalized according to the intensity of *ACTB*. Band density was quantified using ImageJ.

Statistical analysis

Differences between treatments were statistically analyzed using the Kruskal–Wallis test. For comparisons of multiple groups, Wilcoxon signed rank test was applied to the data. Furthermore, p -values <0.05 were considered statistically significant. Data in the figures are shown as mean \pm standard deviation.

Ethical approval

The study protocol was approved by the Institutional Review Board (IRB) of the Catholic University Uijeongbu St. Mary's Hospital (approval no. UC21ZESE0116). The informed consent was waived.

RESULTS

Protective effects of astaxanthin on UVB-induced cytotoxicity in NHEKs

To evaluate the protective effect of astaxanthin against UVB-induced cytotoxicity in NHEKs, we assessed the viability of cells treated with UVB and/or astaxanthin using the CCK-

8 assay. NHEKs incubated with astaxanthin (0, 10, 20 and 30 μM) for 24 hours showed no cytotoxic effect on viability (Fig. 1A). To examine the cytotoxicity to UVB irradiation, NHEKs were pretreated for 24 hours with astaxanthin (0, 20 μM) and subsequently exposed to UVB irradiation (0, 20, 40, 60 mJ/cm^2), and the cell viability was measured 24 hours post-irradiation. Data showed that UVB irradiation induced dosage-dependent viability loss, and astaxanthin significantly rescued UVB-induced cell cytotoxicity in NHEKs (Fig. 1B).

Effects of astaxanthin supplementation on UVB-induced ROS production in NHEKs

To investigate whether astaxanthin could inhibit UVB-induced ROS production, NHEKs were exposed to UVB (0, 20, 40, 60 mJ/cm^2) with or without pretreatment with astaxanthin (20 μM) for 24 hours. Then, ROS production was investigated 24 hours post-irradiation. The NHEKs exposed to UVB displayed an increase in ROS, and astaxanthin significantly decreased ROS production (Fig. 2).

Effects of astaxanthin on apoptosis of UVB-exposed NHEKs

Cells pretreated with astaxanthin at 0 or 20 μM for 24 hours were exposed to UVB 0, 40, or 60 mJ/cm^2 . Then, the flow cytometry analysis was carried out after 24 hours of incubation at 37 $^{\circ}\text{C}$. The results showed that the number of early apoptotic cells increased after UVB (40 and 60 mJ/cm^2) irradiation. Treatment with astaxanthin before UVB irradiation reduced the

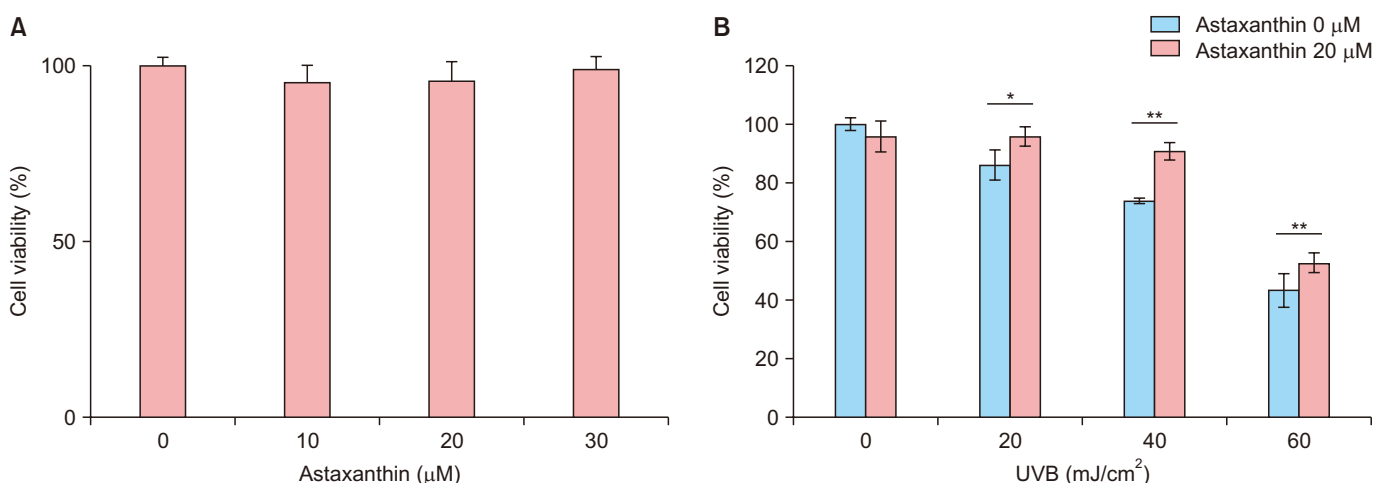


Fig. 1. The cytotoxicity of normal human epidermal keratinocytes (NHEKs) after ultraviolet B (UVB) exposure and astaxanthin treatment. (A) NHEKs were treated with various concentrations of astaxanthin (0, 10, 20, and 30 μM) for 24 hours. (B) NHEKs were pretreated for 24 hours with or without astaxanthin (20 μM) and subsequently exposed to UVB (0, 20, 40, and 60 mJ/cm^2). Cell viability was determined by Cell Counting Kit-8. Significant difference compared to the untreated control group. Each bar represents the mean \pm standard deviation ($n=5$); * $p<0.05$, ** $p<0.01$ (the experiments were repeated three times and produced similar results).

number of early apoptotic cells compared to UVB alone (Fig. 3).

Effects of astaxanthin on apoptosis-related protein signal

As apoptosis was induced by UVB irradiation, the expression of BAX, CASP3, and PARP increased and the expression of BCL2 decreased. Treatment with astaxanthin significantly inhibited these UVB irradiation-induced changes in the expression of apoptosis-related proteins (Fig. 4).

DISCUSSION

Antioxidants are actively used to prevent skin aging. There are many natural antioxidants such as coenzyme Q10, tocopherol (vitamin E), ascorbic acid (vitamin C), ergothioneine, and Zn(II)-glycine. Epigallocatechin gallate and resveratrol are representative polyphenol antioxidants derived from plants. Also, carotenoids, β -carotene, and lycopene protect against UV-induced skin damage¹². Astaxanthin has been researched as an antioxidant and has more potent capacity than carotene carotenoids. Astaxanthin has anti-inflammatory and immunosuppressive functions that result from ROS scavenging ability in addition to antioxidants¹³.

This study confirmed the antioxidant effect of astaxanthin

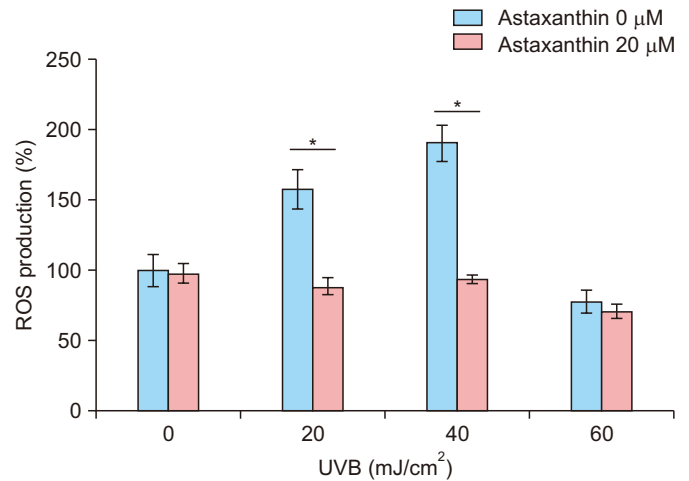


Fig. 2. The effects of astaxanthin on the ultraviolet B (UVB)-induced reactive oxygen species in normal human epidermal keratinocytes (NHEKs). Cells were treated with or without astaxanthin (20 μM) for 24 hours and then irradiated with UVB (0, 20, 40, and 60 mJ/cm²). The data shown are representative of three independent experiments. Astaxanthin decreased reactive oxygen species (ROS) production in UVB-irradiated NHEKs. Each bar represents the mean ± standard deviation (n=5), *p<0.01 (the experiments were repeated three times and produced similar results).

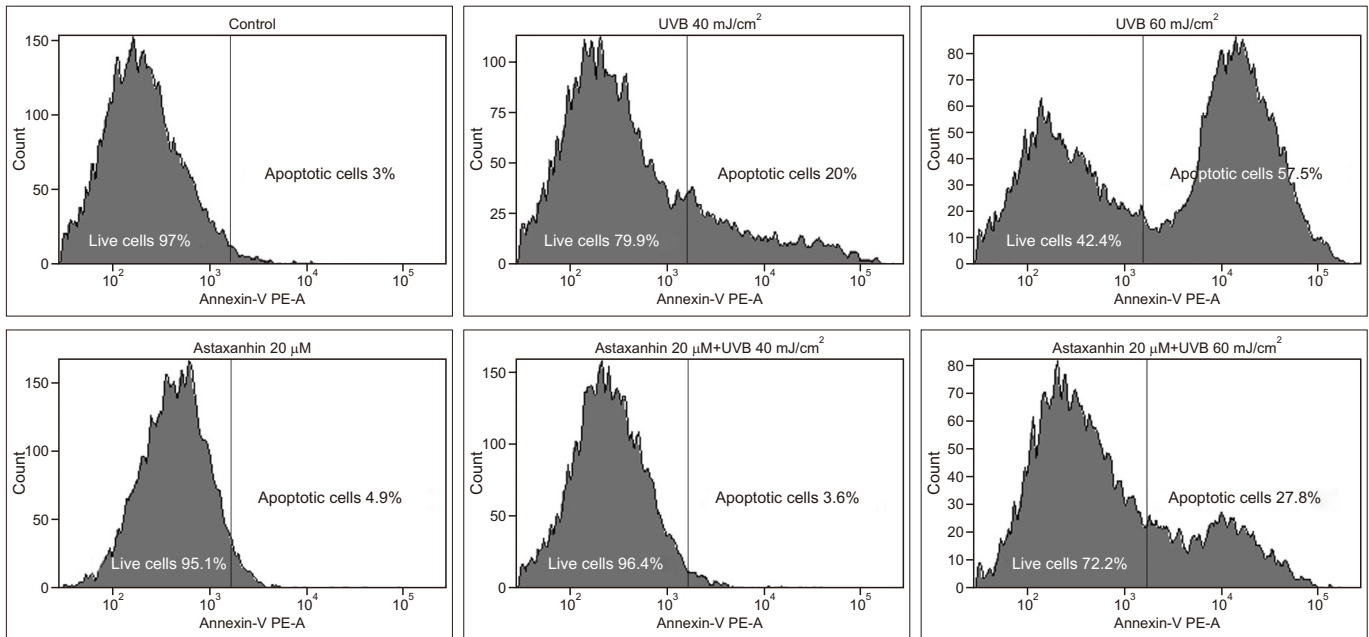


Fig. 3. The effects of astaxanthin on ultraviolet B (UVB)-induced apoptosis. Cells were treated with or without astaxanthin (20 μM) for 24 hours and then irradiated with UVB (0, 40, and 60 mJ/cm²). Flow cytometry analysis was performed after 24 hours incubation at 37°C. The experiments were repeated three times and produced similar results.

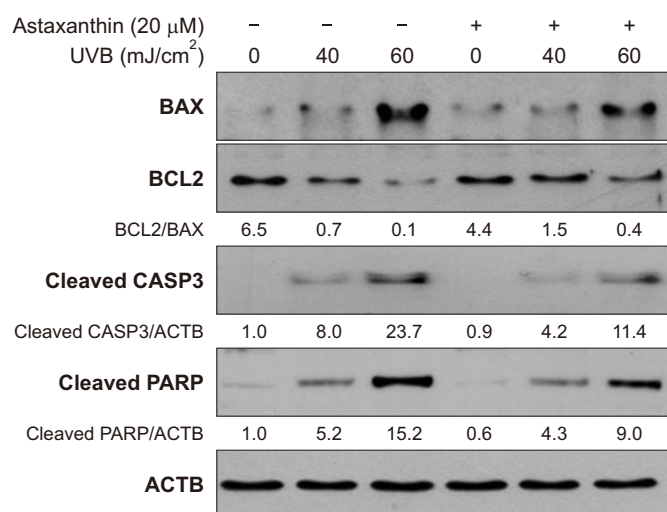


Fig. 4. The effects of astaxanthin on apoptosis after ultraviolet B (UVB) radiation. Cells were treated with or without astaxanthin (20 μ M) for 24 hours and then irradiated with UVB (0, 40, and 60 mJ/cm^2). Cell lysates were prepared, and protein levels were analyzed by western blotting analysis using *BAX*, *BCL2*, cleaved *CASP3*, cleaved *PARP*, and *ACTB* antibodies. Densitometry data standardized to *ACTB* are presented below the band. The expression of *BAX* and *BCL2* was consistent and are expressed as a ratio. The data shown are representative of three independent experiments.

present in crustaceans through *in vitro* experiments used NHEKs. In most papers, the effectiveness of astaxanthin was demonstrated using HaCaT cell. But NHEKs is more similar to human skin than HaCaT cells, because they are derived from normal epithelial tissue of human. Astaxanthin was not cytotoxic to NHEKs even at a high concentration of 30 μ M, and the antioxidant effect of removal of intracellular ROS by UVB was assessed. With UVB 60 mJ/cm^2 , there was no increase in ROS and so no effect of astaxanthin, but this was because the ROS did not increase due to cell death from the high UVB index. Also, flow cytometry analysis was conducted to confirm the apoptosis caused by UVB, and the expression of *CASP3*, *PARP*, *BAX*, and *BCL2* was measured through western blot assays to confirm expression of apoptosis-related genes. Astaxanthin effectively reduced the apoptosis caused by UVB.

The UV-induced apoptosis pathway was activated in response to cellular stress such as ROS and DNA damage. ROS can activate p53 and/or c-Jun N-terminal kinase (JNK), which activate pro-apoptotic effectors *BAX*, *BAK*, and *BID* that can inhibit the functions of anti-apoptotic proteins (Bcl-2)^{14,15}. ROS

cause oxidation of cardiolipin, which relinquishes cytochrome c, resulting in membrane permeabilization and release of cytochrome c¹⁶. Cytochrome c and the *APAF1* together form the apoptosome, a protein complex that recruits and activates *CASP9*¹⁷. This *CASP9* is cleaved and activates other effector caspases such as *CASP3*¹⁸. This results in *CASP3*-mediated cleavage of the DNA repair-associated enzyme *PARP*. Cleavage of *PARP* might be involved at least indirectly in triggering apoptosis¹⁹.

The results of this study support the possible antioxidant effects of astaxanthin on the skin through local application or systemic absorption. And the protective effect of astaxanthin is caused by inhibiting apoptosis through an intrinsic apoptotic pathway. Astaxanthin reduces ROS generation and apoptosis caused by UV and so can have beneficial effects on the skin. Several studies have reported that the biological activities of astaxanthin such as antioxidant, anti-inflammation, anti-lipid peroxidation, anti-diabetic, wound healing, and immune-enhancing effects have a positive influence on skin damage due to DNA repair and anti-cancer effects^{7,20-22}. Therefore, astaxanthin can be useful for maintaining skin health and protecting UVB-induced skin damage. However, there is a lack of research on absorption methods or effective dosage of astaxanthin in cutaneous physiology. Further research is required to investigate the metabolic pathways of *in vitro* and *in vivo* models to confirm the positive effects on skin health. The absence of comparison with other efficacy-proven antioxidants, such as epigallocatechin gallate or resveratrol²³⁻²⁵, was another limitation of this study. Further studies are needed to clarify the antioxidant efficacy of astaxanthin using various investigative methods with positive controls.

In conclusion, astaxanthin is of high value as a mitigating agent for cell aging due to UV exposure and has possibility as an ROS scavenger.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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