See related Commentary on page viii

# Dietary Lutein/Zeaxanthin Decreases Ultraviolet B-Induced Epidermal Hyperproliferation and Acute Inflammation in Hairless Mice

Salvador González, Susi Astner, Wu An, David Goukassian,* and Madhu A. Pathak

Wellman Laboratories of Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; *Department of Dermatology, Boston University Medical School, Boston, Massachusetts, USA

**Lutein and zeaxanthin are carotenoids found in green leafy vegetables with interesting antioxidant properties. They are present in high concentrations in the fovea centralis of the human retina and their role in the prevention of age-related macula degeneration has been reported. We have investigated the effect of orally administered lutein and zeaxanthin in the cutaneous response to ultraviolet B irradiation. Female hairless SKh-1 mice receiving 0.4% and 0.04% lutein plus zeaxanthin-enriched diet for 2 wk were exposed to single doses of ultraviolet B radiation. Skin biopsies were taken at 24 and 48 h after irradiation and analyzed for the presence of apoptotic cells, proliferating cells, and expression of proliferating cell nuclear antigen. Our results show a clear ultraviolet-induced dose-dependent inflammatory response. Orally administered 0.4% lutein and zeaxanthin decreased significantly the edematous cutaneous response ($p < 0.01$) as determined by the reduction of the UVB-induced increase of ear bifold thickening. Additionally, dietary carotenoids were efficient in reducing the ultraviolet B-induced increases in the percentage of proliferating cell nuclear antigen ($p < 0.05$), bromodeoxyuridine ($p < 0.05$), and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling-positive cells ($p < 0.01$). These data demonstrate that oral supplementation of lutein and zeaxanthin diminishes the effects of ultraviolet B irradiation by reducing acute inflammatory responses and ultraviolet-induced hyperproliferative rebound.** ***Key words: antioxidants/carotenoids/lutein and zeaxanthin/oral photoprotection. J Invest Dermatol 121:399–405, 2003***

The acute responses of human skin to solar ultraviolet (UV) radiation (λ, 290–400 nm) are recognized as a form of inflammatory reaction that are mediated by several possible mechanisms, including: (1) a direct action of absorbed photons on DNA of viable nuclei of skin cells (Cadet and Vigny, 1990; Cadet *et al*, 1992; Tedesco *et al*, 1997); (2) the generation of reactive oxygen species (ROS) and free radicals (e.g., superoxide anion ($O_2^-$), singlet oxygen ($^1O_2$), hydroxy (OH), or peroxy radicals (OOH)) (Pathak and Stratton, 1968; Athar *et al*, 1992; Dalle Carbonare and Pathak, 1992); and (3) the generation of prostaglandins ($PGD_2$, $PGE_2$), histamines, leukotrienes, and other cytokines (Black *et al*, 1980; Hawk *et al*, 1983; Pentland and Needleman, 1986; Kupper, 1990; Soter, 1990; Norris *et al*, 1993; Tedesco *et al*, 1997). It is conceivable that these UV-induced reactions represent oxidative stress reactions of DNA bases, lipids, and proteins mediated by the formation of free radicals and ROS, liberation of membrane phospholipids, membrane lipid peroxidation, and subsequent formation of prostaglandins as well as other cytokines by cyclooxygenase- and lipoxygenase-mediated pathways (Black *et al*, 1980; Hawk *et al*, 1983; Pentland and Needleman, 1986; Pentland and Jacobs, 1991).

Several studies have been reported to indicate that a number of UV-mediated cutaneous inflammatory reactions (e.g., sunburn, skin phototoxic reaction involving drug-induced photosensitization, epidermal edema, and vesicle formation) can be partially inhibited or minimized by systemic administration of antioxidants, such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), β-carotene, polyphenolic antioxidants and isoflavones, and other compounds (Mathews-Roth *et al*, 1970, 1972; Greenberg *et al*, 1990; Gerrish and Gensler, 1993; Wang *et al*, 1994; Darr and Pinnell, 1997). Although this search has recently widened to include cosmetic benefits, nutraceutical and therapeutic interventions (e.g., in skin photoaging, skin carcinogenesis), only a few compounds of antioxidant nature (e.g., polyphenolic antioxidants silymarin from milk thistle, epigallocatechin-3-gallate from green tea, and isoflavone) appear to be promising in the chemoprevention of UV-induced skin damage (Wang *et al*, 1994, 1998; Wei, 1998; Lahiri-Chatterjee *et al*, 1999).

In recent years, our interest has broadened for minimizing or preventing the acute and chronic reactions of skin to UV radiation using hitherto poorly recognized antioxidants of plant origin such as lutein and zeaxanthin to prevent or minimize the harmful effects of UV radiation involving ROS. Although β-carotene has been used in the past against sunburn reaction and porphyrin-induced cutaneous photosensitization reaction in

Manuscript received September 23, 2002; revised December 5, 2002; accepted for publication March 12, 2003

Address correspondence and reprint requests to: Dr Salvador González, Wellman Laboratories of Photomedicine, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, 40 Blossom St, Bartlett Hall 814, Boston, Massachusetts 02114, USA. Email: sgonzalez3@partners.org

Abbreviations: BrdU, bromodeoxyuridine; LI, labeling index; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; .TUNEL, terminal deoxynucleotidyl transferase nick end labeling.

0022-202X/03/$15.00 · 

patients with erythropoietic protoporphyria and healthy individuals (Mathews-Roth *et al*, 1970, 1972), its use has been shown to be of limited success in UV-induced inflammation (Darr and Pinnell, 1997). Lutein, a major xanthophyll carotenoid, is structurally related to β-carotene and appears to have superior antioxidant properties compared with β-carotene (O'Connor and O'Brien, 1998). Lutein and zeaxanthin are found in high concentrations in several fruits and green-leafed vegetables, such as broccoli, spinach, kale, cabbage, and green mustard leaves, etc. Additionally, these xanthophyll carotenoids have been identified to accumulate in human skin (Wingerath *et al*, 1998).

In this communication, we report a potentially beneficial *in-vivo* property of this naturally occurring antioxidant agent in photoprotection of murine skin against harmful effects of UVB radiation. These photoprotective effects include: inhibition of UVB-induced acute inflammatory responses, decreased number of UVB-induced apoptotic sunburn cells, reduction in UV-induced hyperproliferative response as shown by decreased number of proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU)-positive cells in the epidermis of lutein and zeaxanthin fed mice. Because UV-induced inflammation and hyperproliferation (Black *et al*, 1980; Hawk *et al*, 1983; Pentland and Needleman, 1986; Pentland and Jacobs, 1991) have been strongly involved in UVB carcinogenesis, inhibitory effects of dietary lutein and zeaxanthin on acute inflammation and epidermal hyperproliferation may have a potential implication for the prevention of carcinogenesis in chronically irradiated skin.

## MATERIALS AND METHODS

**Mice and diets** Twenty-five female SKH-1 mice, 6 to 8 wk of age, were purchased from Charles River Laboratories (Wilmington, Massachusetts). Mice were housed in our animal housing facility under controlled temperature and humidity, and alternating 12 h light and dark cycles for a period of 2 wk before use. Mice received drinking water *ad libitum*. They were housed into groups of four animals per cage and put into three different groups according to the diet administered. Mice of group I ($n = 9$) were fed with the standard diet of Purina Rodent Chow 5001; mice of group II ($n = 8$) were fed with Purina Rodent Chow 5001 enriched with 0.04% lutein and zeaxanthin and mice of group III ($n = 8$) received Purina Rodent Chow 5001 enriched with 0.4% lutein and zeaxanthin. Crystalline FloraGLO brand lutein was provided in a stable form by Kemin Foods, L.C. (DesMoines, Iowa). This material contains lutein and zeaxanthin in a ratio of approximately 20:1. FloraGLO brand lutein is a purified source of lutein and zeaxanthin, which is manufactured and sold commercially according to a patented process. (US patent no. 5382 714) and is the only pure product available for oral supplementation, and was used in our studies without further separation. The diets were prepared by Research Diets Inc. (New Brunswick, New Jersey) and packed under vacuum in evacuated and sealed plastic bags, each containing 100 g of diet in pellet form. The dietary bags enriched with 0.4% and 0.04% lutein and zeaxanthin were refrigerated at 4°C to preserve the carotenoids. These concentrations were recommended by nutritional experts (Professor Curran-Celentano, personal communication). Mice were fed on alternate days for a total of 14 d prior to UVB exposure. The study aimed to evaluate the photo protective effects of dietary lutein supplementation was approved by our Institutional Animal Care and Ethic Committee and carried out in observance of the NIH Guidelines for the care and use of laboratory Animals.

**UVB exposure** Mice were irradiated using a Spot Light Source Radiation (Model L2859-07, Hamamatsu Photonics K. K., Japan), equipped with a cut-off filter at 295 nm. UV radiation was delivered through a bundle of quartz optical fibers with a spot size of 1 cm in diameter. The emitted UVB radiation was measured with a spectroradiometer (model 742, Optronic Laboratories Inc., Orlando, Florida). The spectral output of this lamp was maximal at 310 nm with the majority of the UVB energy emitted below 320 nm. The filtered UV radiation was calibrated and measured with the aid of an IL1700 research radiometer equipped with an SED 240 UVB detector probe (International Light, Newburyport, Massachusetts).

Two weeks before UVB exposure, all mice were tattooed on lateral skin of the dorsum surface for carefully locating the irradiation exposure sites and for obtaining skin biopsies of the exposed skin sites. The mice were then exposed at duplicate sites to single doses of 40, 80, and 160 mJ per $cm^2$ of UVB radiation at the previously marked skin areas of the dorsal surface. Of note, 1 minimum erythema dose for skin phototype III is about 30 mJ per $cm^2$.

**Skin edema** To assess the effect of p.o. administered lutein and zeaxanthin on UVB-induced skin inflammation and edema, ears of mice were exposed to 40 and 160 mJ per $cm^2$ of UVB radiation and the increases in skin-bifold thickness of the irradiated ears were measured at 24 h in a blinded manner. In doing this, the ear skin double fold thickness was determined using a Vernier Caliper micrometer (Scienceware, Switzerland). The increase in ear-bifold thickness of ears was calculated by subtracting the baseline values obtained prior to UV exposure.

**Isolation of skin samples** From each experimental group, half of the experimental animals receiving UVB radiation were killed at 24 h and the remaining were killed at 48 h after UVB irradiation using IACUC (Institutional Animal Care and Use Committee) approved techniques of euthanasia. In each animal, 6 mm skin biopsies were obtained from the UVB-exposed skin sites and from nonirradiated skin. One skin specimen obtained from each exposure dose was immediately fixed in 10% buffered formaldehyde for paraffin-embedded sections, and the other skin specimen was immediately stored at −80°C for western blot studies.

**Terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay** UVB-irradiated skin specimens were examined for the presence of apoptotic cells by the TUNEL assay. This assay identifies apoptotic cells and allows to quantify reliably the number of TUNEL-positive cells. We used ApopTag Fluorescein In Situ Apoptosis detection kit (S7110, Intergenco, Inc., Purchase, New York; www.intergenco.com). This kit labels digoxigenin-labeled 3′-OH ends of the genomic DNA. Briefly, 5 μm sections were deparaffinized with xylene, treated with series of graded alcohols, washed once in phosphate-buffered saline and subsequently treated with proteinase K (Sigma, St Louis, Missouri; www.sigma-aldrich.com) (20 μg per mL) for 15 min at 37°C. After proteinase digestion, sections were incubated with 75 μL equilibration buffer for 10 s. Sections were then incubated in a humidified chamber with terminal deoxynucleotidyl transferase enzyme for 1 h at 37°C. Incubation of one section with phosphate-buffered saline was used as a negative control. The sections were then soaked in working strength stop/wash buffer, agitated for 15 s and rinsed in three changes with phosphate-buffered saline. After rinsing, sections were incubated with anti-digoxigenin fluorescein for 30 min at room temperature. Finally, sections were rinsed with three washes of phosphate-buffered saline and counterstained with propidium iodide/anti-fade. The slides were viewed under a fluorescence microscope equipped with a $\lambda = 380$–420 nm emission filter (Axiophot, Göttingen, Germany). TUNEL-positive cells were counted using a 40 × objective lens on five randomly selected fields per section. A total of three sections per sample were analyzed.

**Immunohistochemistry staining for PCNA** Immunohistochemical assays were performed using mouse anti-human PCNA antibody clone PC10 (cat. no. M 0879, DAKO, Glostrup, Denmark) and a M.O.M. kit staining procedure (Vector Laboratories, Burlingame, California; www.vectorlabs.com). Briefly, 5 μm thick sections were deparaffinized and hydrated slowly with xylene and graded alcohol series and subsequently rinsed for 5 min in water. After appropriate antigen unmasking by using standard techniques, the endogenous peroxidase activity was blocked by incubating the skin sections with 3% hydrogen peroxide in water for 5 min. Then avidin/biotin blocking was applied and sections were incubated for 1 h in working solution of M.O.M. mouse immunoglobulin blocking reagent. Next, sections were incubated with mouse anti-human PCNA monoclonal antibody diluted 1:1000 in M.O.M. diluent for 4 h at room temperature. Slides were rinsed with phosphate-buffered saline, and subsequently incubated with working solution of M.O.M. biotinylated anti-mouse IgG reagent for 10 min at room temperature. ABC reagent (Vectastain, Vector Laboratories) was added to sections for 5 min 3,3′-diaminobenzidine (Sigma) peroxidase substrate solution was used as a chromogen followed by a light counterstaining with Mayer's hematoxylin (Vector Laboratories) for 1 min, cleared with xylene, mounted with a coverslip and examined under a light microscope. Cells showing a brown nuclear reaction indicated proliferating cells and were considered PCNA positive. The slides were examined and scored under a light microscope. PCNA-positive and PCNA-negative cells in every randomly selected field were counted using a 20 × objective lens. Labeling index was calculated as the percentage of

PCNA-positive keratinocytes specifically in the basal cell layer. A total of five fields per slide and three slides per sample were evaluated.

**BrdU incorporation into DNA** BrdU, a thymidine analog that is incorporated into proliferating cells mainly during the S-phase of DNA synthesis, was detected by a biotinylated mouse monoclonal anti-BrdU antibody (Olsen and Kirkhus, 1989) (Clone BU 33, product no. B2531, Sigma) and a M.O.M. kit staining procedure (Vector Laboratories). Briefly, all animals were intraperitoneally injected with BrdU (Sigma) in normal saline (50 mg per kg) and killed 1 h after injection. Sections from all skin samples were prepared as described above for PCNA immunohistochemistry. Endogenous peroxidase was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. DNA denaturation was achieved by incubating sections in 2 M HCl for 30 min at 37°C. Slides were rinsed thoroughly in phosphate-buffered saline and incubated in a moist chamber with 0.1% trypsin for 10 min at 37°C; they were then incubated with mouse monoclonal anti-BrdU antibody. Subsequent steps were performed similar to PCNA staining described above. The slides were examined and scored under a light microscope using a 20 × objective lens. The BrdU labeling index (LI) was calculated from the number of stained BrdU-positive cells per 100 basal cells counted in each field. A total of five fields per section, and three sections per sample were analyzed.

**Western blot analysis** Epidermal sheets were separated by incubating skin biopsies in 6 M NaBr solution for 1 to 2 h. Only separated epidermis was processed for homogenization and subsequent western blot analysis. Next, proteins were extracted with a lysis buffer consisting of 0.25 M Tris–HCl (pH 7.5), 0.375 M NaCl, 2.5% sodium deoxycholate, 1% Triton X-100, 25 mM $MgCl_2$, 1 mM phenylmethyl sulfonyl fluoride, and 0.1 mg aprotinin per mL as described by Yaar *et al* (1991). Equal amounts of protein extract (50 μg), quantified by Bradford method were processed for western blot analysis (Goukassian *et al* 2000). Antibody reactions were performed with 1:200 dilutions of affinity-purified mouse monoclonal and rabbit polyclonal antibodies. Anti-PCNA (Ab-2) (Oncogene Science Inc., Cambridge, Massachusetts) was used as primary antibody. The secondary rabbit and donkey anti-mouse antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, California) were used at 1:2000 dilution. Equal protein loading in each lane was confirmed by hybridization with a 1:2000 dilution of b-actin antibody (Santa Cruz Biotechnology, Inc.). Antibody binding was detected by ECL detection kit (Amersham, Piscataway, New Jersey), followed by autoradiography (Kodak X-Omatic AR, Rochester, New Jersey). Autoradiographs of western blots were scanned (Microtek Scan Maker II, Taiwan) and band intensity was quantified after background subtraction by using densitometric program "Sigma gel" (Jandel Scientific, Corte Madera, California). Normalization for loading of the lanes was determined by Coomassie blue staining using multiple lanes.

**Statistical analyses** Results are expressed as the mean ± SD. Data were evaluated by two-tailed unpaired Student's t test and analysis of variance using the StarView statistical program. The level of statistical significance was taken as $p < 0.05$.

## RESULTS

**Oral administration of lutein and zeaxanthin suppresses UVB-induced skin inflammation** To evaluate the protective effect of oral administration of lutein and zeaxanthin against UVB-induced inflammation we measured ear skin bifold-thickness. Animals fed with diet enriched with lutein and zeaxanthin at 0.4% (striped bars) were found to have a significantly suppressed edematous response induced by UVB radiation ($p < 0.001$). Values of ear thickness increase in millimeters after UVB exposure of untreated and lutein and zeaxanthin-treated mice are shown in **Fig 1**.

**Oral administration of lutein and zeaxanthin decreases the number of UVB-induced apoptotic keratinocytes** The presence of apoptotic keratinocytes was assessed in skin biopsies obtained at 24 and 48 h after UVB irradiation by using the TUNEL assay. Immunofluorescence microphotographs of representative control irradiated skin (**Fig 2*a1***) and irradiated skin from mice fed with 0.04% (**Fig 2*a2***) and 0.4% (**Fig 2*a3***) lutein and zeaxanthin supplemented diets are shown. When a dose of either 80 or 160 mJ per $cm^2$ of UVB irradiation was

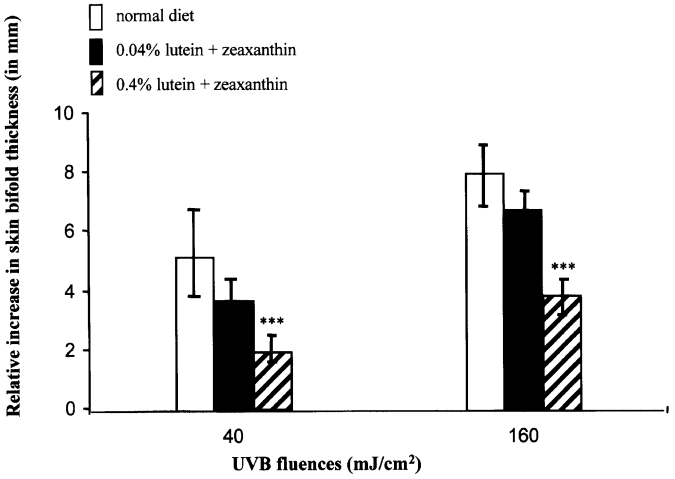


**Figure 1. Effects of dietary 0.4% and 0.04% lutein and zeaxanthin supplementation against UVB-induced ear swelling as outlined in *Materials and Methods*.** The clear bar corresponds to control animals of group I, fed with normal diet, the black bar corresponds to animals of group II receiving dietary lutein and zeaxanthin in a concentration of 0.04% and the striped bar correlates to group III receiving a dietary supplementation of 0.4% lutein and zeaxanthin. X-axis shows UVB fluences in mJ per $cm^2$, Y-axis indicates the increase in ear bifold thickness in mm (***$p < 0.001$ *vs* control, irradiated mice of group I, fed with normal diet).

used, almost all the cells in epidermis became TUNEL positive (data not shown) and the number of apoptotic cells per field in the epidermis of control (clear bars, **Fig 2*b***) irradiated animals increased in a dose-dependent manner. In mice fed with lutein and zeaxanthin at 0.04% (black bars, **Fig 2*b***) for 2 wk before UVB irradiation, the number of TUNEL-positive cells induced by 40 mJ per $cm^2$ of UVB radiation was significantly reduced ($p < 0.01$). This reduction was even greater (by 210%, $p < 0.001$) when mice were fed with lutein and zeaxanthin at 0.4% (striped bars **Fig 2*b***). This reduction in the number of apoptotic cells was similar in specimens obtained at 24 and 48 h (**Fig 2*b***).

**Oral administration of lutein and zeaxanthin decreases the number of UVB-induced PCNA-positive cells in murine skin** To determine whether dietary administration of lutein and zeaxanthin affects epidermal cell proliferation after UVB irradiation, skin biopsies from irradiated and nonirradiated skin of mice were examined for PCNA expression by immunohistochemical and western blot analyses. In nonirradiated skin of mice receiving a normal diet, the PCNA immunoreactivity was limited to the basal layer (**Fig 3*a1***). Strong nuclear immunostaining was detected throughout the entire epidermis of UV irradiated control (**Fig 3*a2***) and mice fed with 0.04% (**Fig 3*a3***) and 0.4% (**Fig 3*a4***) lutein and zeaxanthin. Significantly higher numbers of PCNA-positive cells, however, were observed in control *versus* lutein and zeaxanthin fed animals (**Fig 3*a2*,*a3*,*a4***) consistent with UV-induced hyperproliferative response. To estimate the PCNA labeling index in our studies we counted only the basal PCNA-positive keratinocytes. At 24 and 48 h after exposure to UVB radiation, there was a UVB dose-dependent increase in PCNA labeling index (**Fig 3*b***). Oral administration of diets enriched with lutein and zeaxanthin at 0.04% (black bars) or 0.4% (striped bars) for 2 wk before irradiation with 40 mJ per $cm^2$ reduced the percentage of basal PCNA-positive cells by 35% and 14%, respectively. Compared with control fed animals (clear bars), PCNA labeling index was significantly reduced for all UVB doses at both time points ($p < 0.05$) (**Fig 3*b***). PCNA protein expression was also examined by western blot analysis. (**Fig 4*b***) There was a statistically significant decrease compared with control in PCNA protein levels in 0.4% (striped bars) lutein and zeaxanthin fed

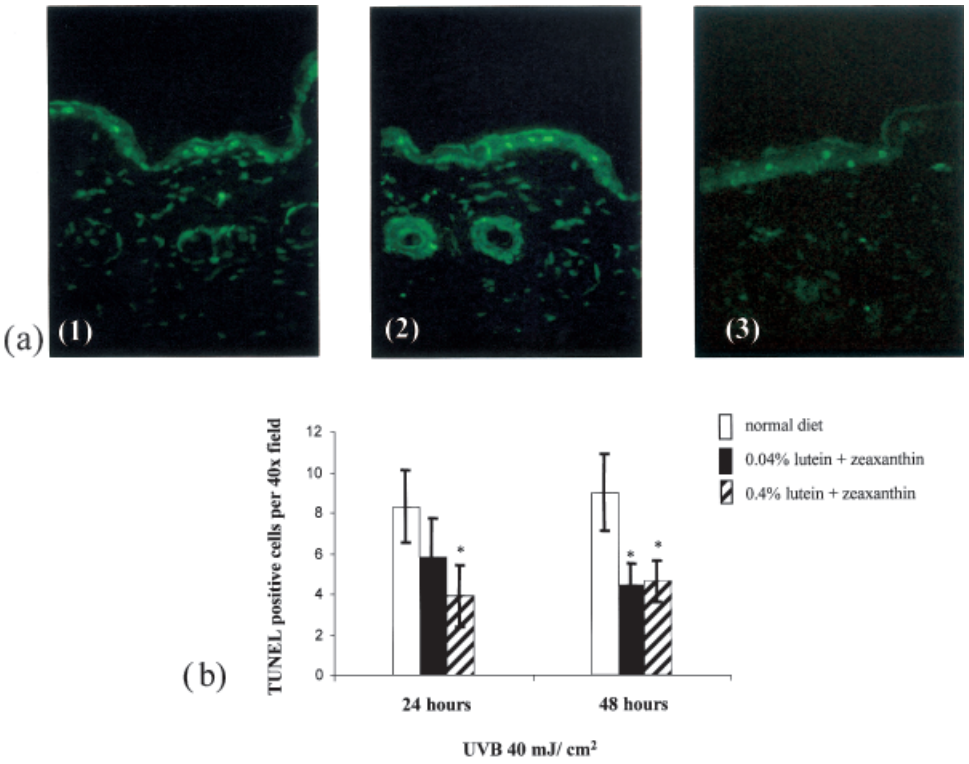


**Figure 2. Effects of oral administration of 0.4% and 0.04% lutein and zeaxanthin on UVB-induced increase in TUNEL-positive cells.** Female SKh-1 mice were fed with either normal diet or diet supplemented with lutein and zeaxanthin and killed at 24 h or 48 h after UVB irradiation. (*a*) TUNEL-stained sections from skin sites of unirradiated (*a1*) and irradiated control (*a2*) animals, and animals treated with 0.04% (*a3*) and 0.4% (*a4*) lutein and zeaxanthin after exposure to 40 mJ per $cm^2$ of UVB radiation. Original magnification × 200. (*b*) Graphic representation of the percentage of TUNEL-positive cells observed at 24 h (*left panel*) and 48 h (*right panel*) after exposing skin sites to 40 mJ per $cm^2$. Clear bar represents data of group I, who received no supplementation with lutein and zeaxanthin, the black bar corresponds to group II receiving dietary lutein and zeaxanthin in a concentration of 0.04% and the striped bar corresponds to group III with a dietary supplementation of 0.4% lutein and zeaxanthin. X-axis represents UVB doses at both 24 and 48 h time points after the UVB exposure and Y-axis represents the number of TUNEL-positive cells per 40X field of view. Each bar represents the mean value of six mice ± SD (*$p < 0.05$)

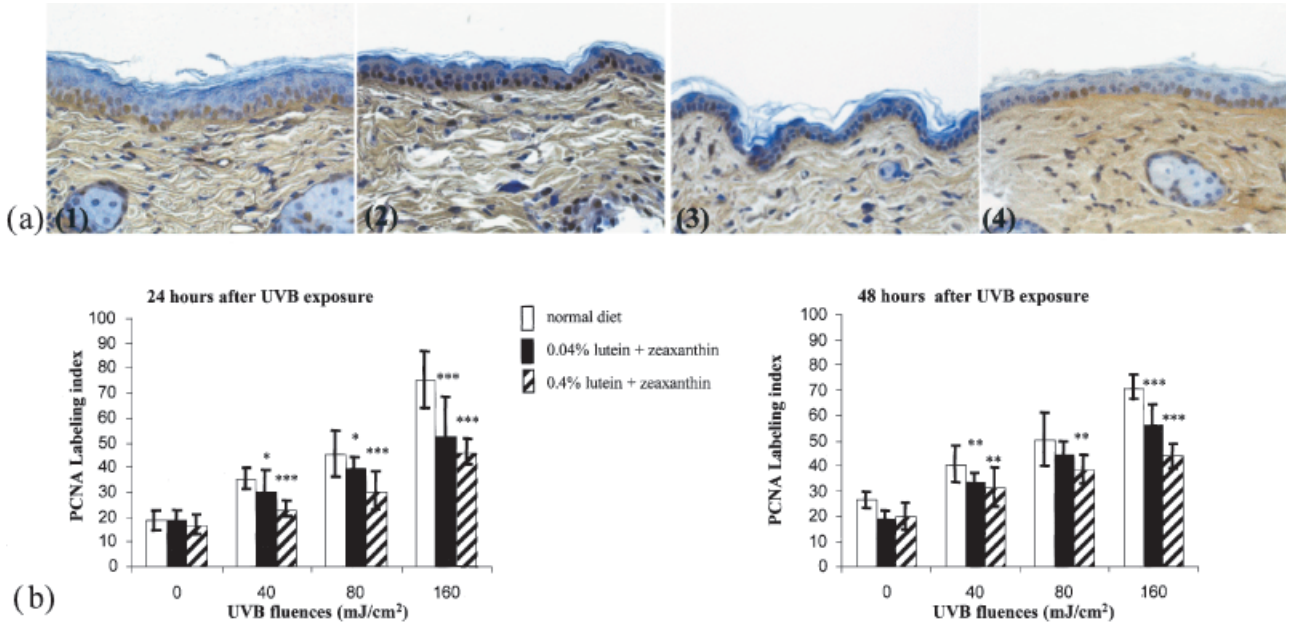


**Figure 3. Effects of oral administration of lutein and zeaxanthin on UVB-induced increase in PCNA-positive cells.** (*a*) Mice were killed at 24 h or 48 h after irradiation. Skin biopsies were taken immediately after exposure for PCNA immunohistochemical staining. PCNA-stained sections from unirradiated (*a1*) and irradiated (*a2*) control animals and from irradiated animals treated with 0.04% (*a3*) and 0.4% (*a4*) lutein and zeaxanthin (24 h after 40 mJ per $cm^2$ of UVB radiation). Original magnification × 200. (*b*) Graphical representation of the immunohistochemical analysis of PCNA protein expression in UVB-irradiated mouse skin fed with either normal diet or 0.4% and 0.04% lutein and zexanthin enriched diet. Clear bar represents data of group I, who received no supplementation of diet with lutein and zeaxanthin, the black bar corresponds to group II receiving dietary lutein and zeaxanthin in a concentration of 0.04% and the striped bar correlates to group III with a dietary supplementation of 0.4% lutein and zeaxanthin. X-axis represents different UVB fluences at both 24 h (*left panel*) and 48 h (*right panel*) after the UVB exposure and Y-axis represents PCNA labeling indices. Each bar represents the mean of six mice, *bars*, SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

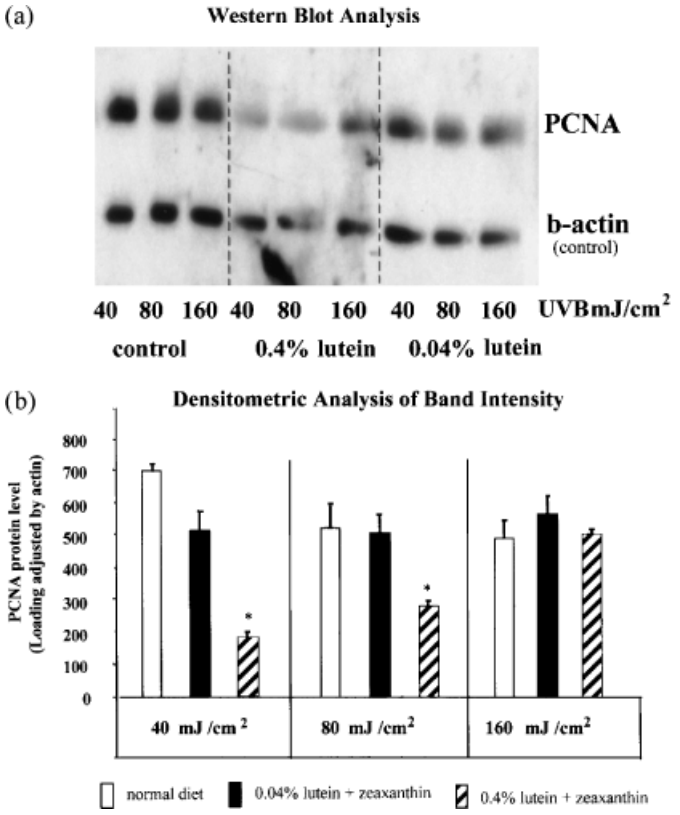


**Figure 4. (*a*) Western blot analysis of PCNA and b-actin protein expression in unirradiated and UVB irradiated skin of mice fed with either normal diet or lutein and zeaxanthin enriched diet at various irradiation doses and at different time points.** (*b*) Western blot analysis of autoradiographs from each of the three experiments were subjected to densitometric analysis of band intensity. X-axis represents the different UVB fluences, Y-axis indicates the PCNA protein level. Loading was adjusted for b-actin protein expression. Clear bars represent mice of group I without dietary supplementation, black bars show the results of group II with 0.04% lutein and zeaxanthin and the striped bars represents animals of group III with 0.4% dietary lutein and zeaxanthin. Each bar represents the mean of three animals, *bars*, SD (*$p < 0.05$).

animals irradiated with 40 and 80 mJ per $cm^2$ UVB. At lower (40 mJ per $cm^2$) UVB dose mice fed with 0.04% (black bars) lutein and zeaxanthin also showed an approximately 29% decrease in PCNA protein levels, which was not statistically significant (**Fig 4*b***, left panel). Lutein and zeaxanthin supplementation did not have any effect on PCNA protein levels after the highest (160 mJ per $cm^2$) UVB dose (**Fig 4*b***, right panel). Of note, slightly different results of PCNA expression in immunohistochemical and western blot analysis, are most likely due to our methodologic approach of counting PCNA-positive cells only in the basal layer (immunochemistry), whereas the entire epidermal sheets were processed for western blot analysis.

**Oral administration of lutein and zeaxanthin reduces UVB-induced increase in BrdU incorporation into basal epidermal keratinocytes** To evaluate the number of proliferating basal keratinocytes that are predominantly in the S-phase of cell cycle we measured the BrdU incorporation into the DNA of basal keratinocytes.

In unirradiated mice skin, only a fraction of basal keratinocytes was found in the S-phase, as shown by BrdU incorporation (**Fig 5*a1***). UVB exposure alone increased BrdU incorporation into epidermal DNA in a dose-dependent manner and these increases were 2- to 4-fold the baseline values (**Fig 5*a2***). Significantly lower numbers of BrdU-positive cells were observed in animals fed with 0.04% (**Fig 5*a3***) and 0.4% (**Fig 5*a4***) lutein and zeaxanthin at all given UVB doses. To calculate the BrdU incorporation index only the fraction of basal BrdU-positive cells were counted. The percentage of BrdU-positive basal cells was reduced in samples obtained from lutein and zeaxanthin fed mice 24 and 48 h after UVB irradiation (**Fig 5*b***). This decrease in the percentage of BrdU-positive basal keratinocytes was lutein and zeaxanthin concentration dependent. At 24 h (left panel) and 48 h (right panel) after UVB exposure, the increase in the percentage of BrdU-positive cells was significantly reduced for all UVB doses tested in mice receiving 0.04% (black bars) and 0.4% (striped bars) lutein and zeaxanthin ($p < 0.05$).

## DISCUSSION

To our knowledge, this is one of the first *in vivo* studies evaluating at cutaneous cellular level the protective effects of oral intake of lutein and zeaxanthin against epidermal hyperproliferation and inflammation induced by single exposure doses of UVB radiation. An acute exposure of human or murine skin to UVB radiation from natural sunlight or artificial light sources results in various biologic responses, through the direct formation of cyclobutane pyrimidine dimers and (6–4)pyrimidine pyrimidone dimers in DNA (Cadet and Vigny, 1990; Cadet *et al*, 1992). UVB exposure also results in the formation of free radicals and ROS that damage DNA and non-DNA cellular targets. Among the latter, lipid-rich cellular membranes are particularly vulnerable to the deleterious effects of ROS. These biologic changes include cell cycle arrest, apoptosis, cell proliferation, and reparative hyperplasia during the early adaptive response and are considered to be involved in the pathophysiology of the UV-induced erythema, premature skin aging, and skin cancer (Norris *et al*, 1993; Young, 1993). In recent years, our research interest has been focusing on minimizing the adverse acute and chronic UV responses using natural antioxidants known to act as radical scavengers and radical quenchers *in vivo* (González and Pathak, 1996; González *et al*, 1997). In this study, we showed that supplementation of diets with 0.4% and 0.04% lutein and zeaxanthin for a period of 2 wk reduced UV-induced increases in the number of apoptotic cells and proliferating cells in the epidermis of the hairless albino (SKh-1) mouse model when exposed to graded doses of UVB radiation.

Only few studies in the past several years have reported the beneficial antioxidant properties of certain carotenoids in photoprotection of skin against UV radiation (Mathews-Roth *et al*, 1970, 1972; Stahl *et al*, 2000). Recently, Granstein and his coworkers[1] reported an anti-inflammatory effect of lutein in the skin of mice exposed to UV radiation. Mice were fed diets containing 0.04% and 0.4% lutein for 2 wk; ears were then exposed to UVB radiation to induce inflammation. The thickness of ear skin of mice before and 24 h after UV radiation was evaluated. The ear swelling in response to UV radiation was significantly inhibited in the mice fed lutein diet relative to the controls receiving no dietary supplementation of lutein and zeaxanthin. In our studies reported here we have confirmed these observations. Stahl *et al* (2000) examined the effect of carotenoids (mainly β-carotene) and carotenoids plus vitamin E on the skin of human volunteers on the development of erythema after exposure to UV radiation. The erythematous response was significantly reduced after 8 wk of supplementation with a carotenoid mixture that included β-carotene and a small fraction of lutein. The ability of β-carotene, lutein, and astaxanthin to protect against UVA-induced oxidative stress in cultured rat kidney fibroblasts was also recently reported by O'Connor and O'Brien (1998). Activities of antioxidant enzymes, such as catalase and superoxide dismutase, as well

[1]Granstein RD, Faulhaber D, Ding W: Lutein inhibits UVB radiation-induced tissue swelling and suppression of the induction of contact hypersensitivity (CHS) in the mouse. *J Invest Dermatol* 117(2):497, 2001 (Abstr.)

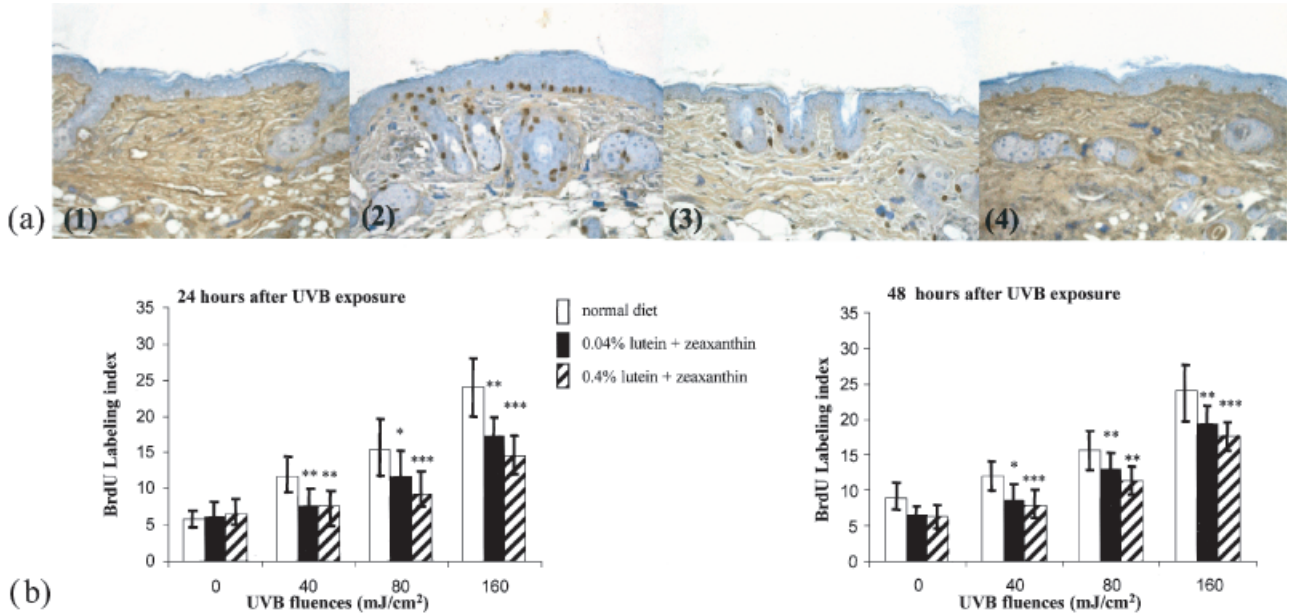


**Figure 5. Effects of oral administration of lutein and zeaxanthin on UVB-induced increase in BrdU-positive cells.** Female SKh-1 mice were fed with either normal diet or diet supplemented with 0.4% and 0.04% lutein and zeaxanthin. (*a*) BrdU IP injection was performed 1 h before killing the animals. Mice were killed at 24 h or 48 h after irradiation and skin biopsies were obtained for immunohistochemical staining as described in *Materials and Methods*. BrdU-stained sections from unirradiated (*a1*) and irradiated (*a2*) control animals and from irradiated animals treated with 0.04% (*a3*) and 0.4% (*a4*) lutein and zeaxanthin (24 h after 40 mJ per $cm^2$ of UVB radiation). Original magnification × 200. (*b*) Graphical representation of the percentage of BrdU-positive basal keratinocytes of mice fed with lutein and zeaxanthin. The percentage of BrdU-positive cells was significantly decreased in animals receiving dietary lutein and zeaxanthin. X-axis represents different UVB fluences at both 24 h (*left panel*) and 48 h (*right panel*) after the UVB exposure and Y-axis represents BrdU labeling indices. Clear bar represents data of group I, who received no supplementation with lutein and zeaxanthin, the black bar represents data of group II receiving dietary lutein and zeaxanthin in a concentration of 0.04% and the striped bar corresponds to group III with a dietary supplementation of 0.4% lutein and zeaxanthin. Each bar represents the mean value of six mice. *bars*, SD (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

as thiobarbituric reactive substance were evaluated to ascertain enzymatic changes resulting from oxidative stress induced by UVA radiation. β-carotene, lutein, and astaxanthin were found to protect against enzymatic changes with astaxanthin exhibiting superior photoprotective properties (O'Connor and O'Brien, 1998).

Lutein and zeaxanthin, nonprovitamin A carotenoids, are the predominant carotenoids found in human retina and their concentration is greatest in the fovea centralis of the macula lutea of human retina where they constitute the macular pigment and serve to decrease the risk of age-related macular degeneration (Beatty *et al*, 1999; Pratt, 1999) and to protect the retinal pigment epithelium against a photooxidative damage initiated in part by light absorption (Snodderly, 1995; Broekmans *et al*, 2002). There is good evidence that this protection is due mainly to their antioxidant properties (Handleman and Dratz, 1986; Zhang *et al*, 1991; Taylor *et al*, 1993; Sujak *et al*, 1999). *In vitro* lutein and zeaxanthin has been shown to be more effective than β-carotene in inhibiting auto-oxidation of cellular lipids (Zhang *et al*, 1991) and in further protecting against oxidant-induced cell damage (Martin *et al*, 1996).

It is becoming more apparent that many of the maladies that affect human skin are the result of direct and indirect environmental interaction invariably caused by UV radiation. Excessive and often uncontrolled exposure to terrestrial solar radiation is harmful to human skin and causes acute sunburn, damage to epidermal and dermal cells, induction of cell death, immune suppression, and eventually skin cancer (Young, 1993). Currently the most widely accepted method of photoprotection against UV radiation is the use of two or more topical sunscreens containing UVB and UVA absorbing chemicals that are nonphotolabile (e.g., octyl methoxy cinnamate, octyl dimethyl amino benzoate, benzophenones, or avobenzone (parsol 1789), etc.) with or without admixture of UV scattering and reflecting chemicals (e.g., ZnO, $TiO_2$ in micronized form) incorporated in a water-resistant base. Other approaches, including chemicals with antioxidant properties such as vitamin C, vitamin E, β-carotene, etc., are also advocated (Thompson *et al*, 1993; Naylor *et al*, 1995; Green *et al*, 1999; Pathak *et al*, 1999; Stahl *et al*, 2000). The *in vivo* presence of effective antioxidants such as lutein and zeaxanthin in the skin prior to UVB irradiation may reduce the deleterious effect of irradiation, most likely by decreasing the generation of UV-induced ROS (Athar *et al*, 1992; Dalle Carbonare and Pathak, 1992). Although β-carotene has been examined in our laboratories in the past (Mathews-Roth *et al*, 1970; 1972; Greenberg *et al*, 1990), we feel that the antioxidant lutein is less labile and thus better than β-carotene in minimizing the photooxidative changes in the skin. Comparative studies using lutein and β-carotene, however, have to be done to verify the better photoprotective properties of lutein and zeaxanthin. Because UV-induced inflammation and hyperproliferation (Pentland *et al*, 1999; An *et al*, 2002) has been strongly involved in UVB carcinogenesis, inhibitory effects of dietary lutein and zeaxanthin on acute inflammation, and epidermal hyperproliferation may have a potential implication for the prevention of carcinogenesis in chronically UV-exposed skin.

*The authors want to thank Martine Sifakis and William Farinelli for excellent technical assistance and Kemin Foods L.C. (DesMoines, IA) for funding this study through a research grant from The Skin Cancer Foundation, New York.*

## REFERENCES

An KP, Athar M, Tang X, *et al*: Cyclooxygenase-2 expression in murine and human non-melanoma skin cancers: Implications for therapeutic approaches. *Photochem Photobiol* 76:73–80, 2002

Athar M, Agarwal R, Bickers DR, Mukhtar H: Role of reactive oxygen species. In: Mukhtar H (ed.). *Pharmacology of the Skin.* Boca Raton, FL: CRC Press Inc., 1992; p 269–279

Beatty S, Boulton M, Henson D, Koh HH, Murray IJ: Macular pigment and age related macular degeneration. *Br J Ophthalmol* 83:867–877, 1999

Black AK, Fircham N, Greaves MW, Hensby CN: Time course changes in levels of arachidonic acid and prostaglandins D2, E2, and F2 alpha in human skin following ultraviolet B irradiation. *Br J Clin Pharmacol* 10:453–457, 1980

Broekmans WMR, Berendschot TTJM, Klöpping-Ketelaars IAA, *et al*: Macular pigment density in relation to serum and adipose tissue concentrations of lutein and serum concentrations of zeaxanthin. *Am J Clin Nutr* 76:595–603, 2002

Cadet J, Vigny P: The photochemistry of nucleic acids. In: Morrison H (ed.). *Bioorganic Photochemistry.* New York: John Wiley & Sons, 1990; p 3–272

Cadet J, Anselmino C, Douki T, Voituriez L: Photochemistry of nucleic acids in cells. *J Photochem Photobiol B Biol* 15:277–298, 1992

Dalle Carbonare M, Pathak MA: Skin photosensitizing agents and the role of reactive oxygen species in photoaging. *J Photochem Photobiol B Biol* 14:105–124, 1992

Darr D, Pinnell SR: Reactive oxygen species and antioxidants protection in photodermatology. In: Lowe NJ, Shaat NA, Pathak MA (eds). *Sunscreens.* New York: Marcel Dekker, Inc., 1997; p 155–173

Gerrish KE, Gensler HL: Prevention of photocarcinogenesis by dietary vitamin E. *Nutr Cancer* 19:125–133, 1993

González S, Pathak MA: Inhibition of ultraviolet-induced formation of reactive oxygen species, lipid peroxidation, erythema, and skin photosensitization by *Polypodium leucotomos*. *Photodermatol Photoimmunol Photomed* 12 (2):45–56, 1996

González S, Pathak MA, Cuevas J, Villarrubia VG, Fitzpatrick TB: Topical or oral treatment of an extract of *Polypodium leucotomos* prevents acute sunburn and psoralen-induced phototoxic reactions as well as depletion of Langerhans cells in human skin. *Photodermatol Photoimmunol Photomed* 13:50–60, 1997

Goukassian DA, Gad F, Yaar M, Eller MA, Nehal US, Gilchrest BA: Mechanisms and implications of the age-associated decrease in DNA repair capacity. *FASEB J* 14:1325–1334, 2000

Green A, Williams G, Neale R, *et al*: Daily sunscreen application and betacarotene supplementation in prevention of basal-cell and squamous-cell carcinomas of the skin: A randomised controlled trial. *Lancet* 354:723–729, 1999

Greenberg ER, Baron JA, Stukel TA, *et al*: The Skin Cancer Prevention Study Group. A clinical trial of beta-carotene to prevent basal cell and squamous-cell cancers of the skin. *N Engl J Med* 323:789–795, 1990

Handleman GJ, Dratz EA: The role of antioxidants in the retina and retinal pigment epithelium and the nature of prooxidant-induced damage. *Adv Free Radic Biol Med* 2:1–89, 1986

Hawk JLM, Black AK, Jaenicke KF, *et al*: Increased concentration of arachidonic acid, prostaglandins E2, D2, and 6-oxo-F1alpha, and histamine in skin following UVA irradiation. *J Invest Dermatol* 80:496–499, 1983

Kupper TS: Role of epidermal cytokines. In: Oppenheim JJ, Shervach GM (eds). *Immunophysiology.* New York: Oxford University Press, 1990; p 285–305

Lahiri-Chatterjee M, Katiyar ST, Mohan RR, Agarwal R: A flavonoid antioxidant, silymarin affords exceptionally high protection against tumor promotion in the *sencar* mouse skin tumorigenesis model. *Cancer Res* 59:622–632, 1999

Martin KR, Failla ML, Smith JC Jr: Beta-carotene and lutein protect HepG2 human liver cells against oxidant-induced damage. *J Nutr* 126:2098–2106, 1996

Mathews-Roth MM, Pathak MA, Fitzpatrick TB, Harber LC, Kass EH: Beta-carotene as a photoprotective agent in erythropoietic protoporphyria. *N Engl J Med* 282:1231–1234, 1970

Mathews-Roth MM, Pathak MA, Parrish JA, Fitzpatrick TB, Kass EH, Toda K, Clemens WA: A clinical trial of the effects of oral beta-carotene on the responses of human skin to solar radiation. *J Invest Dermatol* 59:349–353, 1972

Naylor MF, Boyd A, Smith DW, Cameron GS, Hubbard D, Neldner KH: High sun protection factor sunscreens in the suppression of actinic neoplasia. *Arch Dermatol* 131:170–175, 1995

Norris PG, Gange RW, Hawk JLM: Acute effects of ultraviolet radiation on the skin. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds). *Dermatology in General Medicine.* New York: McGraw-Hill, Inc., 1993; p 1651–1658

O'Connor I, O'Brien N: Modulation of UVA light-induced oxidative stress by β-carotene, lutein and astaxanthin in cultured fibroblasts. *J Dermatol Sci* 16:226–230, 1998

Olsen WM, Kirkhus B: The epidermal cell kinetic response to ultraviolet B irradiation combines regenerative proliferation and carcinogen associated cell cycle delay. *Photochem Photobiol* 50 (3):391–397, 1989

Pathak MA, Fitzpatrick TB, Nghiem P, Aghassi DS: Sun-protective agents. formulations, effects, and side effects. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds). *Dermatology in General Medicine.* New York: McGraw-Hill, Inc., 1999; p 2742–2763

Pathak MA, Stratton K: Free radicals in human skin before and after exposure to light. *Arch Biochem Biophys* 39:445–448, 1968

Pentland AP, Jacobs SC: Bradykinin-induced prostaglandin synthesis is enhanced in keratinocytes and fibroblasts by UV injury. *Am J Physiol* 261:R543–R547, 1991

Pentland AP, Needleman P: Modulation of keratinocyte proliferation in vitro by endogenous prostaglandin synthesis. *J Clin Invest* 77:246–251, 1986

Pentland AP, Schoggins JW, Scott GA, Khan KN, Han R: Reduction of UV-induced skin tumors in hairless mice by selective COX-2 inhibition. *Carcinogenesis* 20:1939–1944, 1999

Pratt S: Dietary prevention of age-related macular degeneration. *J Am Optom Assoc* 70:39–47, 1999

Snodderly DM: Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *Am J Clin Nutr* 62 (6 (Suppl.):1448S–1461S, 1995

Soter NA: Acute effects of ultraviolet radiation on the skin. *Semin Dermatol* 9:11–15, 1990

Stahl W, Heinrich U, Jungmann H, Sies H, Tronnier H: Carotenoids and carotenoids plus vitamin E protect against ultraviolet light-induced erythema in human. *Am J Clin Nutr* 71:795–798, 2000

Sujak A, Gabrielska J, Grudzinski W, Borc R, Mazurek P, Gruszecki WI: Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: The structural aspects. *Arch Biochem Biophys* 371:301–307, 1999

Taylor A, Jacques PF, Dorey CK: Oxidation and aging: Impact on vision. *J Toxicol Ind Health* 9:349–371, 1993

Tedesco AC, Martínez L, González S: Photochemistry and photobiology of actinic erythema. Defensive and reparative cutaneous mechanisms. *Braz J Med Biol Res* 30:561–575, 1997

Thompson SC, Jolley D, Marks R: Reduction of solar keratoses by regular sunscreen use. *N Engl J Med* 329:1147–1151, 1993

Wang ZY, Huang MT, Lou YR, *et al*: Inhibitory effects of black tea, green tea, decaffeinated black tea, and decaffeinated green tea on ultraviolet B light-induced skin carcinogenesis in 7,12-dimethylbenz[a]anthracene-initiated SKH-1 mice. *Cancer Res* 54:3428–3435, 1994

Wang Y, Zhang X, Lebwohl M, DeLeo V, Wei H: Inhibition of ultraviolet B (UVB)-induced c-fos and c-jun expression in vivo by a tyrosine kinase inhibitor genistein. *Carcinogenesis* 19:649–654, 1998

Wei H: Photoprotective action of isoflavone genistein: models, mechanisms, and relevance to clinical dermatology. *J Am Acad Dermatol* 39 (2 Part 1):271–272, 1998

Wingerath T, Sies H, Stahl W: Xanthophyll esters in human skin. *Arch Biochem Biophys* 355:271–274, 1998

Yaar M, Grossman K, Eller MS, Gilchrest BA: Evidence for nerve growth factor mediated paracrine effects in human epidermis. *J Cell Biol* 115:821–828, 1991

Young AR: Chronic effects of ultraviolet radiation on the skin. Experimental aspects. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF (eds). *Dermatology in General Medicine.* New York: McGraw-Hill, Inc., 1993; p 1658–1660

Zhang L-X, Cooney RV, Bertram JS: Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: Relationship to their cancer chemopreventive action. *Carcinogenesis* 12:2109–2114, 1991