

Ultraviolet B-Induced DNA Damage in Human Epidermis Is Modified by the Antioxidants Ascorbic Acid and D- α -Tocopherol

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DNA damage caused by ultraviolet (UV) irradiation is considered the main etiologic factor contributing to the development of skin cancer. Systemic or topical application of antioxidants has been suggested as a protective measure against UV-induced skin damage. We investigated the effect of long-term oral administration of a combination of the antioxidants ascorbic acid (vitamin C) and D- α -tocopherol (vitamin E) in human volunteers on UVB-induced epidermal damage. The intake of vitamins C and E for a period of 3 mo significantly reduced the sunburn reaction to UVB irradiation. Detection of thymine dimers in the skin using a specific antibody revealed a significant increase of this type of DNA damage following UVB exposure. After 3 mo of antioxidant administration, significantly less thymine dimers were induced by the UVB challenge, suggesting that antioxidant treatment protected against DNA damage.

Key words: human skin/ultraviolet radiation/antioxidants/DNA damage/thymine dimers
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Skin cancer is very frequent in Caucasians, and its incidence is increasing steadily. This development is caused inter alia by demographic changes (increased life expectancy) and by the intake of photosensitizing drugs (Placzek *et al*, 1999). These two effects together with an increased ultraviolet (UV) exposure by changes in the recreational behavior are a major cause of skin cancer. The exposure to UV radiation promotes the development of squamous cell carcinoma (SCC) and its precursor lesions, actinic keratoses. Epidemiologic data also imply UV as a major factor in the etiology of melanoma and basal cell carcinoma (BCC), although the role of the cumulative UV dose is less clear for these cutaneous malignancies than for SCC.

Both DNA damage caused by direct absorbance of UV radiation and indirect DNA damage contributed by reactive oxygen species (ROS) may lead to mutations, which can result in UV-induced skin cancer. The most prominent direct DNA damages are the cyclobutane pyrimidine dimers, i.e. thymine dimers, and the (6-4) photoproducts. Indirect DNA damage is inferred by ROS such as singlet oxygen ($^1\text{O}_2$) and free radicals such as the superoxide anion radical ($^{\bullet}\text{O}_2^-$), the perhydroxyl radical (HO_2^{\bullet}), or the hydroxyl radical ($^{\bullet}\text{OH}$).

Administration of antioxidants like ascorbic acid and D- α -tocopherol that scavenge ROS has been promoted as a strategy to decrease UV-induced skin damage and ultimately to also prevent skin cancer. In this study, we investigate the protective effect of long-term oral administration of antioxidant vitamins against UV-induced epidermal damage in human volunteers. We find that a combination

of vitamins C and E decreased the sunburn reaction and protected epidermal cells against the induction of thymine dimers.

Results

Subjects took 1 g of ascorbic acid and 500 IU of D- α -tocopherol twice daily over a period of 3 mo. None of the volunteers complained of any adverse events. The mean vitamin C serum level at the beginning was 11.6 ± 4.8 mg per liter (normal range: 4–20 mg per liter). After 1 mo of treatment vitamin C blood concentration was increased to 19.3 ± 7.5 mg per liter ($p < 0.001$). This increase remained stable, levels being 19.0 ± 6.8 mg per liter after 2 mo and 18.2 ± 6.8 mg per liter after 3 mo. Vitamin E serum levels were 21.0 ± 12.2 mg per liter at baseline (normal range: 5–20 mg per liter) and increased to 36.0 ± 14.4 mg per liter after 1 mo ($p < 0.001$). After 2 or 3 mo, they were 39.6 ± 17.9 or 38.8 ± 17.8 mg per liter, respectively. These data demonstrate that serum levels of vitamins E and C can be increased significantly by exogenous oral intake.

To assess the potential protective effects of antioxidant treatment against UVB-induced sunburn, we determined the minimal dose of UVB required to elicit a clearly demarkable erythema (reddening) of the skin on a previously unexposed body site (minimal erythema dose (MED)) before and after the 3 mo course of vitamins C plus E. After 90 d of antioxidant administration, the median MED rose from 80 to 113 mJ per cm^2 ($p = 0.002$, Wilcoxon's test; Fig 1) demonstrating that oral vitamins E plus C decreases the skin's susceptibility to sunburn.

Abbreviation: UV, ultraviolet

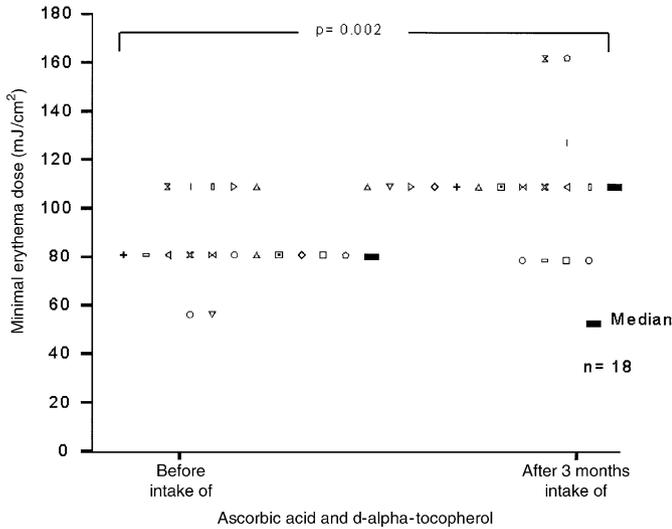


Figure 1
Minimal erythema dose before and after antioxidant treatment.

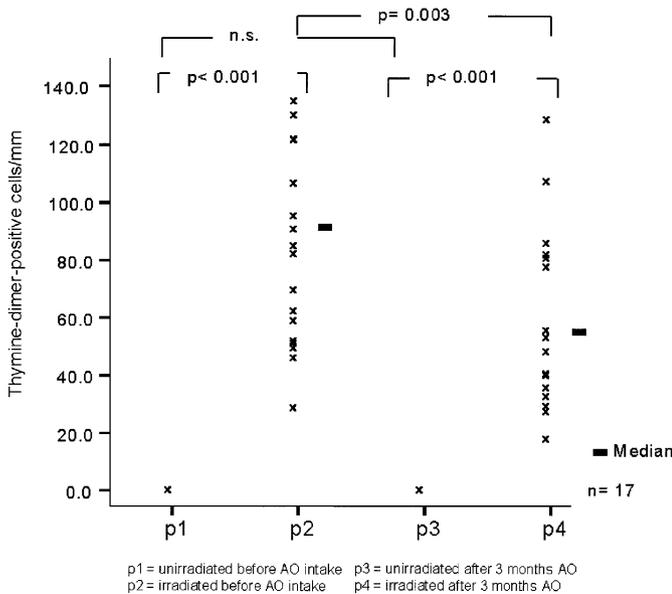


Figure 2
Thymine-dimer-positive cells per mm epidermis before or after 3 mo antioxidant (AO) administration (NS, not significant).

Biopsy specimens were taken from uninvolved skin prior to irradiation and from a sunburned (2-fold MED) test field 24 h after UVB exposure. Thymine dimers were visualized by immunohistochemistry using a monoclonal antibody. Prior to UVB irradiation, no thymine-dimer-positive cells could be detected in the epidermis. Twenty-four hours after irradiation the median number of thymine-dimer-positive cells per mm epidermis was 82.0, demonstrating the induction of DNA damage by a 2-fold MED of UVB. Following 3 mo of antioxidant treatment, the number of thymine-dimer-positive cells after 2 MED UVB reduced to 48.2 per mm ($p = 0.003$, Wilcoxon's test), showing that the protection provided by antioxidants against clinical sunburn was reflected on the molecular level by a protective effect against UV-inflicted DNA damage (Fig 2).

Discussion

This study shows that antioxidants modify skin reactivity to UVB *in vivo*. The long-term combined oral intake of ascorbic acid and D- α -tocopherol may lead to a significant physiologic increase of antioxidant capacity of human skin. This was demonstrated by a reduction of sunburn reaction and thymine dimers in human skin.

We used a combination of ascorbic acid and D- α -tocopherol, because there is evidence for a synergistic interaction of vitamins C and E in antioxidant defense: UV-activated molecules oxidize cellular components; particularly, they induce a chain reaction of lipid peroxidation in membranes rich in polyunsaturated fatty acids. D- α -tocopherol, by acting as an antioxidant, is oxidized to the tocopheroxyl radical in this process (Chow, 1991; Fryer, 1993), and it is regenerated by ascorbic acid to D- α -tocopherol (Packer *et al*, 1979). This synergism has been shown with unsaturated lipids in micelles (Barclay *et al*, 1983) or in phosphatidylcholine liposomes (Niki *et al*, 1985). Furthermore, when compared with other vitamin E compounds, α -tocopherol has the best bioavailability (Burton and Traber, 1990) and reaches the highest concentration in epidermis (Fuchs *et al*, 2003).

A protective effect of topical or systemic antioxidants against UV-induced skin damage has been demonstrated in several experimental systems (reviewed in Pinnell, 2003). Several studies have documented photoprotective effects when vitamin E was applied to animal skin. Topical α -tocopherol protected rabbit or mouse skin against UV-induced erythema (Roshchupkin *et al*, 1979; Record *et al*, 1991; Trevithick *et al*, 1992). In mice, topical vitamin E decreased UV-induced effects such as lipid peroxidation (Lopez-Torres *et al*, 1998), cutaneous photoaging (Bissett *et al*, 1990; Jurkiewicz *et al*, 1995), immunosuppression (Gensler and Magdaleno, 1991; Yuen and Halliday, 1997; Steenvoorden and Beijersbergen v Henegouven, 1999), and photocarcinogenesis (Bissett *et al*, 1990; Gensler and Magdaleno, 1991; Yuen and Halliday, 1997; Burke *et al*, 2000). Topical application of vitamin E to human skin has also been found to reduce UVB- and PUVA-induced skin erythema (Potapenko *et al*, 1980, 1984).

Topical L-ascorbic acid protected porcine skin from UVB- and UVA-phototoxic injury as measured by erythema and sunburn cell formation (Darr *et al*, 1992), and also protected against UV-induced immunosuppression and systemic tolerance to contact allergies in mice (Nakamura *et al*, 1997).

Topical application of combined vitamins C and E on porcine skin provided significant protection against erythema, sunburn cell formation, and thymine-dimer formation. Ascorbic acid or D- α -tocopherol alone were also protective against erythema and sunburn cell formation but the combination was superior. Only the combination of vitamins C and E reduced thymine dimers (Lin *et al*, 2003).

In mice, systemic vitamin E reduced MED, lipid oxidation, and incorporation of thymine dimers in DNA (Record *et al*, 1991). Systemic application of vitamin C decreased the incidence and delayed the onset of the malignant skin lesions (Dunham *et al*, 1982).

Few studies have assessed the photoprotective potential of systemical antioxidant treatment in humans. Ingestion of

400 IU D- α -tocopherol acetate per d or of a selenium-copper-vitamin complex (containing 40 mg vitamin E per d) did not significantly influence the MED (la Ruche and Césarini, 1991; Werninghaus *et al*, 1994). But we demonstrated in a previous study that the combined oral administration of vitamins C and E provided protection against UV-induced erythema after only 1 wk (Eberlein-König *et al*, 1998). This finding was confirmed by Fuchs (1998) and Fuchs and Kern (1998) who further showed that either vitamin alone was ineffective.

In this study, we show that by extending the administration of ascorbic acid and D- α -tocopherol to a period of 3 mo the protective effect against UVB-induced erythema could be increased even further: whereas after 1 wk of oral vitamins C and E, the median MED was increased by 21%, the increase was 41% after 3 mo. The decreased sensitivity to sunburn was paralleled by an increase of blood levels of vitamins C and E. A significant rise of vitamin serum concentration was found after only 1 mo of administration; beyond this point no further increase of levels was found. Serum levels achieved by oral intake for 1 mo were on average higher than levels after only 1 wk in our previous study. This suggests that saturation levels of vitamins E and C in serum are not yet reached after 1 wk, and that the increased protective effect after long-term administration of vitamins E and C is caused by higher serum (and, possibly, tissue) concentrations.

Proceeding from the protective effect of antioxidants on the sunburn response to UVB, we assumed that administration of vitamins C and E might also protect against UV-induced DNA damage, as has been shown previously with topical antioxidant treatment in pig skin (Lin *et al*, 2003). In accordance with this, we found a reduction of thymine dimers also in UVB-irradiated human skin after 3 mo of systemic administration of antioxidants.

In summary, our findings suggest that twice daily oral administration of ascorbic acid plus D- α -tocopherol represents a convenient and well-tolerated prophylactic treatment against the hazardous effects of solar UV irradiation. Further studies are required to confirm whether the photoprotective effects of this treatment result indeed in protection against the development of skin cancer.

Patients and Methods

Study design Twelve males and six females (21–77 y old) were included in the study, according to the Declaration of Helsinki principles after informed written consent had been obtained. Experiments were approved by the Ethikkommission des Fachbereichs Medizin of the Ludwig-Maximilians-Universität München. Sixteen participants had skin type II, and two had skin type III (according to Fitzpatrick). Fourteen patients were recruited during follow-up examinations at the outpatient department of oncology. The patients had a history of melanoma, BCC, or SSC. Four patients had no UV-induced skin cancer. All participants agreed to refrain from sun exposure and from vitamin supplements for 2 wk before and during the study. UV sensitivity by determining the MED was assessed at the start of the trial, and again after 90 d of oral intake of ascorbic acid 2 g per d and D- α -tocopherol 1000 IU per d (both Kloesterl Apotheke, Munich, Germany). Blood vitamin concentrations were measured at the beginning and every 30 d during the trial.

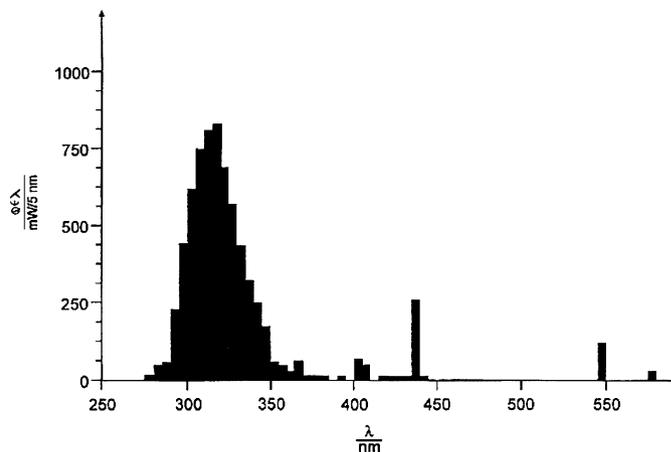


Figure 3
Wavelength spectrum of TL 20 W/12 fluorescent tubes.

UV irradiation was performed using fluorescent tubes with a main emission between 275 and 365 nm and a maximum at about 315 nm (TL 20 W/12, Philips, Hamburg, Germany); the spectral distribution is shown in Fig 3. Irradiance was 1.0 mW per cm² for UVB and 0.4 mW per cm² for UVA at a distance of 40 cm as measured by an integrating instrument (UV-Meter, Waldmann, Villingen-Schwenningen, Germany). On the lower back, 10 circular areas each measuring 1.8 cm² were exposed to increasing UVB doses (20, 28, 40, 57, 80, 113, 124, 160, 175, and 200 mJ per cm²). The MED was the lowest dose causing a sharply defined, homogeneous erythema 24 ± 2 h after exposure. The second test after 90 d of administration of antioxidants was carried out on the other side of the lower back.

Biopsies were taken from unirradiated skin and the day after from the UVB-irradiated site that had received a 2-fold MED before and after 3 mo intake of antioxidants. Only 17 patients agreed to the biopsies.

Evaluation of ascorbic acid and α -tocopherol levels Evaluation of ascorbic acid and α -tocopherol levels in the serum/plasma was carried out by high-pressure liquid chromatography. For vitamins C and E analysis, we used commercial kits (ascorbic acid: Immundiagnostik, Bensheim, Germany; α -tocopherol: Chromsystems, Munich, Germany).

Immunohistochemistry For detection of thymine dimers, we used the labeled streptavidin-biotin method (LSAB 2 System, Alkaline Phosphatase; Dako, Carpinteria, California) applying a monoclonal mouse anti-thymine-dimer antibody (Kamiya Biomedical Company, Seattle, Washington). Paraffin-embedded sections were deparaffinized and then incubated in a moist chamber with 0.1% trypsin (Sigma, Steinheim, Germany) for 30 min at room temperature. After washing with distilled water, unspecific binding was blocked by incubation at room temperature for 20 min with 20% fetal calf serum (PAA, Linz, Austria) in Tris-buffered saline (TBS). Then sections were covered with anti-thymine-dimer antibody (1:80) at room temperature for 90 min. Sections were rinsed with TBS three times and incubated with biotinylated secondary antibody (ready-to-use, enclosed in the LSAB 2 System, Alkaline Phosphatase; Dako) in PBS buffer for 7 min and, after washing, with streptavidin-conjugated alkaline phosphatase (ready-to-use, enclosed in the LSAB 2 System, Alkaline Phosphatase; Dako) in PBS buffer for further 7 min. Sections were rinsed again and staining was achieved by incubation for 10 min with Fuchsin chromogen. Counterstaining was performed with hematoxylin blue, and samples were embedded in Kaiser's glycerol gelatine.

Statistical evaluation Statistical analysis was performed with the Student's *t* test (blood vitamin levels) and Wilcoxon's test for paired comparisons (MED, thymine dimers) with the assistance of the

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