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Original Contribution

MODULATION OF UV-LIGHT-INDUCED SKIN INFLAMMATION BY D-ALPHA-TOCOPHEROL AND L-ASCORBIC ACID: A CLINICAL STUDY USING SOLAR SIMULATED RADIATION

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Abstract—*Objective:* In this clinical trial we studied whether oral supplementation with D-alpha-tocopherol (α-Toc), L-ascorbic acid (Asc), or α-Toc combined with Asc influenced the solar simulated radiation (SSR) induced skin inflammation in healthy volunteers. *Methods:* We investigated the following groups in a prospective, randomized and placebo controlled study: Group (1) α-Toc 2 g / day, group (2) Asc 3 g / day, group (3) α-Toc 2 g / day combined with Asc 3 g / day, and group (4) placebo. Before and 50 days after supplementation we analyzed α-Toc and Asc concentrations in keratinocytes. The dose response curve of UV erythema was determined by reflectance spectrophotometry and the minimal erythema dose (MED) by visual grading before and after supplementation. *Results:* 50 days after supplementation α-Toc keratinocyte levels were increased in groups (1) and (3), Asc concentrations were elevated in groups (2) and (3), and the a/γ-Toc ratio increased in groups (1) and (3). The dose response curve of UVR induced erythema showed a significant flattening and the MED increased from 103 $\pm$ 29 mJ/cm^2 (before supplementation) to 183 $\pm$ 35 mJ/cm^2 (after supplementation) in group (3), while there were no significant changes in groups (1) and (2) after vitamin supplementation. *Conclusion:* α-Toc and Asc act synergistically in suppression of the sunburn reaction. © 1998 Elsevier Science Inc.

Keywords—Photoprotection, Antioxidants, Tocopherol, Ascorbate, Free radicals, Reactive oxygen species, Skin, Inflammation

INTRODUCTION

The skin is continuously exposed to environmental insults, one if not the most important stress factor is solar radiation. Solar radiation causes a variety of biological effects on the skin, including inflammation, pigmentation, immunomodulation, photoaging and cancer [1]. The sunburn reaction is the most studied effect and has been well documented clinically and histologically. The mediators which induce this clinical response are only partially defined. ROS generated by endogenous photosensitizers [2,3], or released from inflammatory cells [4], prostaglandine endoperoxides [5], nitric oxide and peroxynitrite [6,7], as well as prooxidant cytokines such as TNF-α, IL-1 and IL-6 have been identified as mediators of the UVR induced inflammatory response [8–11]. Consequently, administration of antioxidants may be a promising strategy to counteract solar light induced skin inflammation. α-Toc and Asc are physiological antioxidants and potential photoprotective agents [12–22]. The American Academy of Dermatology has developed a guideline of care for photoaging/photodamage and recommended topical antioxidants as a medical treatment of photodamage [23]. In cutaneous photoprotection a safe and effective systemic antioxidant supplement (nutrient) is desirable, because it could provide convenient and prophylactic use at population levels [24]. The purpose of this study was to evaluate the effects of dietary α-Toc and Asc mono- and combination therapy on the sunburn reaction and to measure keratinocyte concentration of the vitamins before and after supplementation.

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MATERIALS AND METHODS

Study subjects

Forty healthy volunteers (20–47 years old) with skin types II Fitzpatrick were selected for this study [25]. Exclusion criteria were smoking, heavy alcohol intake,

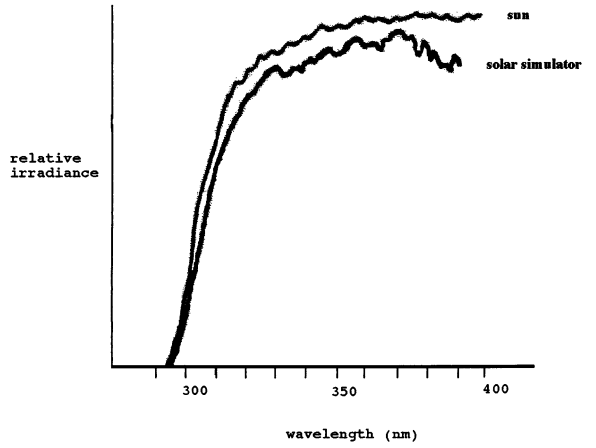

Fig. 1. Spectral irradiance of the solar simulator and of UV reaching the earth's surface. The output of the solar simulator was generated between 300 and 400 nm using a calibrated Optronic 742 spectro radiometer. Solar spectrum related to a cloudless sky, July 5th, southern Germany.

sun bathing, exposure to artificial UVR, and intake of minerals, vitamins or other antioxidant supplements. The human subjects were randomly divided into four groups. Group (1): 10 subjects receiving orally α-Toc 2 g / day over 50 days, group (2): 10 subjects taking Asc 3 g / day over 50 days, group (3): 10 subjects taking α-Toc 2 g / day combined with Asc 3 g / day over 50 days, and group (4): 10 subjects taking placebo. Optovit®, Cetebe® and placebo were given in equal doses in the morning and evening with meals. The subjects in group 4 could not identify that they were taking placebo, but the subjects in groups 1-3 could ascertain that they were taking Optovit®, Cetebe® or both together. Written consent to participate in the study was provided by each individual.

UV irradiation

An oriel xenon arc solar simulator (Oriel Corp., Stratford, CT, U.S.A.) with a spectral output in the UV region (Schott UV filter WG 320, 1.0 mm) very similar to natural sunlight was used as the source of UVR (Fig. 1). The lamp irradiance was monitored 30 cm from the source with a hand held wide band radiometer (7183 Oriel photodetector module equipped with a 7070 Oriel photodiode readout, Oriel Corp.) calibrated against an Optronic 742 spectroradiometer giving a dose rate of 3.5 mW cm^{-2} in the UVB (310 nm). Irradiation times were calculated accordingly. Ten small areas (2 cm^2) of the untanned buttock skin were exposed to increasing doses (25, 50, 75, 100, 125, 150, 200, 250, 300, 400 $mJcm^{-2}$) of SSR for determination of the dose response curve. The dose response curve of SSR induced erythema was determined before and after antioxidant supplementation. The erythema was measured by reflectance spectrophotometry using a Dermatospectrometer (Cortex Technologya, Hadsund, Denmark) 24 h after UV exposure. The degree of erythema is quantitated as an erythem index [26]. The subjects were lying prone on a couch for measurements of UV erythema by spectrophotometry, and the measuring head was placed very softly and perpendicularly to the skin. The minimal erythema dose (MED) was determined visually and defined as the lowest dose causing a sharply circumscribed homogenous erythema 24 h after SSR exposure.

Chemicals and vitamins

BHT, SDS, n-hexane, Asc and pronase from Streptomuces griseus were purchased from Sigma Chemical Company, Munich, Germany. α-Toc and γ-Toc were provided from Hoffmann-La Roche, Nutley, NJ. The vitamins supplemented were Optovit fortissimum^R (1 capsule contains 500 mg D-alpha-tocopherol; Hermes, München, Germany), Cetebe® (1 capsule contains 500 mg L-ascorbic acid; Fink, Bühl, Germany), and placebo (lactose capsules).

Preparation of buccal mucosal keratinocytes

Buccal mucosal keratinocytes were collected before supplementation and 50 days after antioxidant supplementation by using an established non-invasive method [27]. Keratinocyte harvest was performed 12 h after the last vitamin intake. Briefly, the subjects were asked to rinse their mouth with drinking water, then brush the inside of their cheeks with a toothbrush, 20 times on each side. Subsequently the cheeks were rinsed with 20 ml of physiological saline solution twice and the toothbrush was washed with 10 ml of saline solution. The rinsing and washing fluids were collected and centrifuged in an Hettrich EBA8 centrifuge at maximum speed for 5 min to obtain a pellet. The pellet was transferred into a 1 ml Eppendorf cup, made up to volume and stored on liquid nitrogen. By this method large amounts of keratinocytes are readily prepared (on average 250 mg wet weight cells per subject). Histologic examination of the pellet showed presence of keratinocytes derived from the stratum spinosum and granulosum. Protein was determined with the Folin reagent [28].

Antioxidant analysis

Frozen buccal mucosal keratinocytes were thawed at room temperature, the pellet was suspended in 500 μl phosphate buffered (10 mM, pH 7.0) physiological saline and sonified in eppendorf cups on ice with a

Table 1. Keratinocyte Tocopherol and Ascorbate Concentration (Mean ± SD), Concentration Range (min–max) and MED (Mean ± SD) Before and After Vitamin Supplementation

Patients	α-Toc before ng/mg protein	MED before mJcm-2	α-Toc after ng/mg protein	MED before mJcm-2	γ-Toc before ng/mg protein	γ-Toc after ng/mg protein	α/γ-Toc before ng/mg protein	α/γ-Toc after ng/mg protein
Group 1 (TOC)	54.27 ± 27.66 (12.4 – 110.5)	108 ± 31	117.50 ± 52.07* (37.3 – 180.1)	133 ± 45	10.57 ± 5.49 (2.2 – 18.9)	7.73 ± 3.85 (1.6 – 12.9)	6.04 ± 3.49	15.15 ± 9.39*
Group 3 (TOC + ASC)	48.43 ± 31.38 (15.4 – 120.7)	103 ± 29	100.08 ± 54.11* (27.4 – 189.9)	183 ± 35#	9.04 ± 4.88 (3.4 – 25.8)	6.91 ± 4.76 (1.9 – 12.19)	5.18 ± 3.15	17.41 ± 11.19**
Group 4 (control)	52.66 ± 37.39 (12.0 – 137.9)	100 ± 33	59.86 ± 29.02 (15.3 – 113.1)	113 ± 31	10.15 ± 6.36 (3.8 – 24.8)	9.86 ± 4.25 (5.5 – 19.7)	6.32 ± 4.69	6.61 ± 3.45

	Asc before nmol/mg protein		Asc after nmol/mg protein	
Group 2 (ASC)	5.74 ± 2.83 (1.60 – 10.40)	95 ± 26	11.28 ± 6.13** (2.6 – 18.8)	120 ± 31
Group 3 (TOC + ASC)	6.06 ± 3.71 (2.7 – 12.4)		13.20 ± 6.85*** (1.9 – 21.7)	
Group 4 (control)	5.19 ± 2.45 (2.3 – 9.4)		5.66 ± 3.00 (2.74 – 11.3)	

*$p < .01$; **$p < .02$; ***$p < .04$; #$p < .004$.

Branson sonifier under nitrogen gas to avoid antioxidant decomposition. After centrifugation in an Eppendorf centrifuge 5415 at maximum speed for 2 min the pellet was used as the membrane fraction for simultaneous determination of α-Toc and γ-Toc. The supernatant was used as the cytosol fraction for analysis of Asc by HPLC using electrochemical detection [29]. For tocopherol analysis the buccal mucosal keratinocyte membranes were processed as decribed [30]. In brief, the pellet was combined with 1 mg BHT and 200 μl of 1% protease solution (pronase from Streptomuces griseus) and incubated at 37°C for 30 min. After the incubation, 400 μl of 1% SDS in ethanol containing 0.1% BHT was added and the samples were vortex mixed for 60 s. Then, 500 μl of n-hexane containing 0.1% BHT was added, vortex mixed for 60 s, and centrifuged in an Eppendorf centrifuge for 2 min. The upper hexane layer was removed and the extraction was repeated once. The two hexane layers were combined and dried under nitrogen gas. α-Toc and γ-Toc were analyzed by HPLC using electrochemical detection [31].

Statistics

The experimental data points showed a non-Gaussian distribution, therefore non-parametric tests were selected for statistical analysis. The unpaired Kruskal-Wallis test was used for comparison of multiple independent data points, and the Wilcoxon-Mann-Whitney test was applied for comparison of two independent data points. A $p < 0.05$ was considered significant.

RESULTS

In group (1) 9 of 10 subjects, in group (2) 10 of 10 subjects, in group (3) 9 of 10 volunteers, and in group (4) 9 of 10 volunteers completed the study protocol and could be evaluated. There were no reports of any systemic or cutaneous side effects.

In groups (1) and (3) α-Toc concentrations increased significantly in buccal mucosal keratinocytes after vitamin supplementation, and the γ-Toc concentration decreased (statistically not significant), (Table 1). The ratio of α/γ-Toc increased significantly in groups (1) and (3) and Asc levels increased significantly in groups (2) and (3) after supplementation. The antioxidant status of group (4) did not change during the study (Table 1).

In group (4) a mean MED was produced by 100 ± 33 $mJcm^{-2}$ UVB, which did not change significantly after 50 days of placebo administration (113 ± 31 $mJcm^{-2}$ UVB), and was not significantly different from the MED's of group (1) (108 ± 31 $mJcm^{-2}$ UVB), group (2) (95 ± 26 $mJcm^{-2}$ UVB) and group (3) (103 ± 29 $mJcm^{-2}$ UVB) before supplementation (Table 1). After 50 days of daily administration of α-Toc combined with Asc, the MED increased significantly in this group from 103 ± 29 (before supplementation) to 183 ± 35 $mJcm^{-2}$ UVB. The MED's in group (1) and group (2) were slightly increased after supplementation with either α-Toc (133 ± 45 $mJcm^{-2}$ UVB), or Asc (120 ± 31 $mJcm^{-2}$ UVB) (Table 1), however the differences were statistically not significantly different from the MED's before supplementation.

Before supplementation the dose response curves of the SSR induced erythema were not different in groups

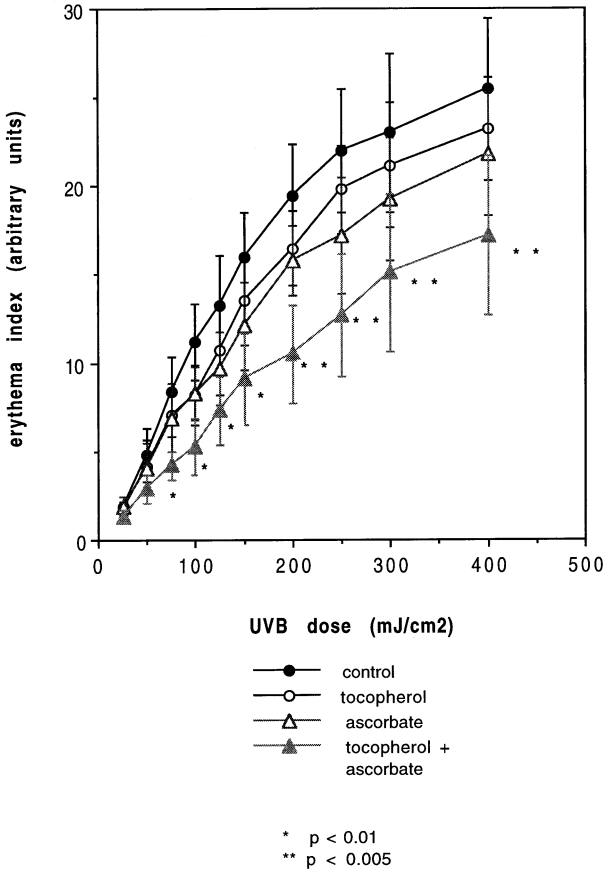

Fig. 2. Dose response curves obtained 24 h after irradiation of individuals with a solar simulator, showing the increase in erythema index. Points are means ± SD.

(1-4), and they did not change during the study in group (4) (data not shown). After supplementation there were no significant differences in the dose response curves between groups (1), (2) and (4), (Fig. 2). However, in (group 3) the dose response curve showed a significant flattening after vitamin supplementation, which was most evident at higher UV doses (Fig. 2).

DISCUSSION

α-Toc and Asc bioavailability in keratinocytes

In this clinical trial it was not feasible to analyze freshly collected human specimens and to obtain skin samples from every patient, which involves an invasive technique. We therefore analyzed α-Toc and Asc in buccal mucosal keratinocytes, which can be collected non-invasively [27]. Their antioxidant level is a good indicator of the skin keratinocyte antioxidant content [32].

The results of this clinical trial show that systemic supplementation with megadoses of α-Toc, or α-Toc combined with Asc increased vitamin E levels in keratinocytes significantly (Table 1). Supplementation with Asc, or Asc combined with α-Toc also increased the keratinocyte concentration of vitamin C significantly (Table 1). These findings are in good agreement with other clinical studies. A significant increase in Asc concentration after vitamin C supplementation (1 g/day over 4 wk) was observed in human gingiva [33]. In adipocytes of patients supplemented with 800 mg DL-alpha-tocopherol per day Handelman et al. observed an increase in α-Toc, a decrease in γ-Toc and an increase in α/γ-Toc tissue levels [34]. The interindividual differences were quite large in our subjects, but intraindividual differences in vitamin concentrations were rather small (mean ± 10%, data not shown). Similar high interindividual variations in tocopherol concentrations were observed in adipocytes [34], and buccal mucosal keratinocytes in humans after vitamin supplementation [30,32]. We observed a decrease in γ-Toc (statistically not significant) and a significant increase in the a/γ-Toc levels in keratinocytes of α-Toc supplemented individuals (Table 1). The decrease in tissue γ-Toc during supplementation with α-Toc may be caused by competitive intestinal uptake and/or plasma transport and opposing tissue delivery mechanisms.

Relatively little is known about α-Toc and Asc biokinetics in human tissues (particularly in skin) following a single or repetitive oral applications [35,36]. Animal studies indicate that it may take several weeks to reach a new steady state level of α-Toc in the skin in response to changes in dietary intake. The skin half live time (50% turn over) of α-Toc was determined in rats supplemented with dietary trideuterated D-alpha-tocopherol acetate (36 mg/kg diet) and found to be about 23 days [37]. It was suggested that this value might underestimate the actual time for a 50% turnover of α-Toc, because the animals underwent significant growth during the experiment [37]. In mice supplemented with a megadose of α-Toc (10.000 IU/kg diet) skin tocopherol levels slowly increased during supplementation, reaching a plateau after 4 wk [38]. A study investigating the distribution of tritium-labeled α-Toc following a single intravenous injection of chylomicra bound α-Toc implicated, that there is rapid distribution to the skin (within 1 h) and significant accumulation (over 20 days). Significant amounts of α-Toc were excreted through the skin (presumably) through sebaceous glands) on the skin surface [39]. It may be extrapolated from these results that in human skin the concentration of α-Toc increases only slowly during supplementation therapy.

A clinical study in children showed that following a single oral dose of 600 mg D-α-Toc, both plasma and erythrocyte α-Toc levels reached a maximum within

24 h, while buccal mucosal keratinocytes achieved maximum concentration at 4-6 days [40]. Human buccal mucosal keratinocytes reached maximum concentration of α-Toc by day 7, following oral administration of α-Toc (800 IU first 4 days, 400 IU thereafter) [30]. We are not aware of any published data on α-Toc biokinetics in human dermis. It was estimated that about 2 years are required for α-Toc to reach a new steady state level in human adipose tissue in response to changes in oral intake [34].

Protection from the sunburn reaction

Our results showing no statistically significant protection from SSR induced skin erythema by megadose supplementation with α-Toc or Asc monotherapy (Table 1, Fig. 2) is in good agreement with other clinical studies. In a double blind and placebo controlled study, no clinical or histological difference in the acute UVB response to a threefold MED could be detected in human volunteers supplemented with daily α-Toc (400 IU / day, over 6 months) [41]. Although a long supplementation time was used in this study, no significant increase of α-Toc in the skin was found. However, combined daily administration of 2 g Asc and 1g α-Toc over 8 days provided small but significant protection from UVB/UVA radiation induced erythema in human subjects [42]. Systemic Asc has been regarded as having poor and questionable effectiveness in preventing the sunburn reaction [43,44]. In EPP patients (photosensitivity disease) oral administration of 1g Asc daily for 4 wk resulted in a tendency of better sun-tolerance, however the photoprotective effect did not reach statistical significance [45].

As already demonstrated by Eberlein-König et al. [42], the supplementation of α-Toc combined with Asc suppressed the SSR induced skin erythema significantly, particularly at high UV doses. α-Toc is a powerful inhibitor of lipid peroxidation and is oxidized to the tocopheroxyl radical and other products during this process. Asc regenerates α-Toc from the tocopheroxyl radical [46–48], and this recycling action presumably contributes to the synergistic effect of combined Asc and α-Toc supplementation observed in our study.

The main differences between between our trial and the study of Eberlein-König et al. were that our subjects (1) received a much higher α-Toc dose (3g/day vs. 1000 IU/day), (2) the supplementation period was much longer (50 vs. 8 days), (3) we also studied mono-supplementation with α-Toc and Asc, and determined keratinocyte vitamin concentrations. In the study of Eberlein-König et al. an increase in UVR sensitivity in the placebo group was observed (80 $mJcm^{-2}$ vs. 68.5 80 $mJcm^{-2}$), the cause for this is unknown [42]. Increased UVR sensitivity in placebo subjects was not observed in our study.

Higher dose of systemic α-Toc, longer supplementation time and constant UV sensitivity of the study subjects during the study period may partially explain the greater degree of photoprotection observed in our study (MED increase 78%), when compared with the results of Eberlein-König et al. (MED increase 21%).

Caveats

General objectives of photoprotection are prevention and inhibition of acute and chronic sequelae of solar radiation. The most studied effect is inhibition of sunburn and the efficacy of sun screens is usually evaluated by the degree of inhibition of UVB and/or UVA erythema and/or pigment response. The ultimate objective in photoprotection is prevention from immunosuppression, aging and cancer. Measures to protect against sunburn were recommended to prevent the occurrence of UVR induced skin cancer [1,49]. However, the molecular pathways leading to UVR induced inflammation are presumably different from the biochemical cascade causing immunosuppression, aging and photocarcinogenesis [50]. The relationship between MED, sunburn and photocarcinogenesis is not well characterized and UVR induced erythema may be an inappropriate biological endpoint and invalid indicator for assessing the protective efficacy of antioxidants from photoimmunosuppression [51], or photocarcinogenesis [52]. A clinical study is currently in progress analyzing the potential of combined α-Toc and Asc in protection from photoimmunosuppression.

CONCLUSIONS

Our study clearly shows that dietary supplementation with megadoses of α-Toc combined with Asc protects from the sunburn reaction. However, the feasibility of a long term megadose supplementation with these vitamins is questionable. Oral supplementation of α-Toc and Asc in megadoses (2 g α-Toc, 3 g Asc) is not restricted by serious side effects [53,54], but the sun protection factor (SPF) achieved was only about 2. Topical sunscreens can provide a much higher degree of photoprotection. However, systemic photoprotection is convenient and overcomes some of the problems associated with the topical use of sunscreens. Dietary modification resulting in only a moderate protection factor could have a significant protective effect over a lifetime [1]. Presently studies are in progress analyzing the photoprotective effects of low-dose supplemenation with α-Toc combined with Asc.

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ABBREVIATIONS

Asc—L ascorbic acid, ascorbate
BHT—2.6-Di-tert.butyl-p-cresol
EPP—erythropoetic protoporphyria
HPLC—high performance liquid chromatography
IL-2—interleukin-2
IL-6—interleukin-6
MED—minimal erythema dose
ROS—reactive oxygen species
SDS—sodium dodecylsulfate
SPF—sun protection factor
SSR—solar simulated radiation
TNF-α—tumor necrosis factor-alpha
α-TOC—D-alpha-tocopherol
γ-Toc—gamma-tocopherol
UVR—ultraviolet radiation
UVA—ultraviolet A (320–400 nm)
UVB—ultraviolet B (280–320 nm)