Acta Derm Venereol (Stockh) 1996; 76: 264–268

Effectiveness of Antioxidants (Vitamin C and E) With and Without Sunscreens as Topical Photoprotectants

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Considerable interest has been recently generated concerning the use of natural compounds, anti-oxidants in particular, in photoprotection. Two of the best known anti-oxidants are vitamins C and E, both of which have been shown to be somewhat effective in different models of photodamage. Very little has been reported, however, on the effectiveness of a combination of the two (known to be biologically the more relevant situation); nor have there been detailed studies on the ability of these anti-oxidants to augment commercial sunscreen protection against UV damage. We report that (in swine skin) vitamin C is capable of additive protection against acute UVB damage (sunburn cell formation) when combined with a UVB sunscreen. A combination of both vitamins E and C provided very good protection from a UVB insult, the bulk of the protection attributable to vitamin E. However, vitamin C is significantly better than vitamin E at protecting against a UVA-mediated phototoxic insult in this animal model, while the combination is only slightly more effective than vitamin C alone. When vitamin C or a combination of vitamin C and E is formulated with a commercial UVA sunscreen (oxybenzone), an apparently greater than additive protection is noted against the phototoxic damage. These results confirm the utility of anti-oxidants as photoprotectants but suggest the importance of combining the compounds with known sunscreens to maximize photoprotection.

(Accepted December 12, 1995.)

Acta Derm Venereol (Stockh) 1996; 76: 264–268.

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The skin is subject to constant exposure to oxidative stress. This can be the result of drugs, environmental pollutants or radiation, to name a few. Ultraviolet radiation (UV) is a major contributor to adverse reactions in the skin; many of these reactions are due to the UV-induced generation of reactive oxygen species. The consuming public is becoming increasingly aware of the potential dangers inherent in exposure to the various wavelengths of UV radiation. If it proves correct that there is a growing depletion of atmospheric ozone, considerably higher doses of short wavelength UVB would be predicted to be absorbed by the skin. As a by-product of attempts to protect against this, increased exposure to longer wavelength UVA and visible radiation is occurring as high SPF UVB sunscreens are allowing longer periods of sun exposure, recreational or otherwise. Additionally, although no firm data is available, legitimate concerns are being raised over whether long-term use of chemical sunscreens is ultimately going to prove detrimental to the user (1).

In the past several years, attention has turned to the potential of using topically applied antioxidants as photoprotectants, alone or as adjuncts to sunscreens. We, and others, have reported that vitamin C (L-ascorbic acid), applied topically, moderates some UVB-induced damage to skin (erythema, sunburn cell formation, tumor incidence) (2,3). We also showed that vitamin C was quite effective at inhibiting a psoralen-UVA (PUVA)-mediated phototoxic reaction to swine skin (2).

Vitamin E is another pre-eminent nutritional anti-oxidant. Several groups have investigated the utility of this anti-oxidant as a topical photoprotectant with positive results (3–5), though a recent study using oral vitamin E was inconclusive (6). Interestingly, although intuitively a natural experiment to run, the effect of topical application of a combination of vitamins C and E on photodamage has not to our knowledge been reported. Additionally, although suggestive evidence is prevalent (7), very little data has been forthcoming documenting beneficial effects of antioxidants, when combined with sunscreens, in inhibiting cutaneous photodamage. In this report, we present results which indicate that vitamin C can augment the protection of a sunscreen para-aminobenzoic acid (PABA) against UVB damage, in an apparently additive fashion. The combination of vitamin C with vitamin E is shown to also be effective at UVB protection. Finally, while vitamin E, at the concentration used, showed little or no effect against PUVA damage, it marginally increased the protection seen with topical vitamin C. In conjunction with a UVA/B sunscreen (oxybenzone), significantly improved PUVA protection was noted for vitamin C or a combination of vitamins C and E, appearing to be more than additive.

MATERIALS AND METHODS

L-ascorbic acid, α-tocopherol, hydroxypropyl cellulose (average MW 300,000) and 1,2, propanediol (propylene glycol) were purchased from Aldrich. PABA and 2-hydroxy-4-methoxybenzophenone (oxybenzone) were purchased from Sigma. Lotion formulations (UVB experiments) contained ascorbic acid in an aqueous solution containing 20% propylene glycol (v/v) and 1% hydroxypropylcellulose (w/v). In model UVB sunscreen experiments, PABA was dissolved with the vitamin C in the aqueous phase to 0.25% (w/v). The vitamin C/oxybenzone lotion (and control vitamin C lotion for those experiments) were made in an ethanol/propylene glycol/water (45:35:25) formulation. Oxybenzone was chosen because, while not absorbing at longer UVA wavelengths, it effectively filters wavelengths in the PUVA action spectrum as well as being a commercially utilized sunscreen. Cream formulations were supplied by Union Carbide Corp., or were made by blending desired ingredients into the commercial moisturizing cream, Theraplex®. Both gave products of similar characteristics. Vitamin E or oxybenzone was dissolved in absolute ethanol before blending into the cream base. Concentration of anti-oxidants and/or sunscreens used were chosen after initial UVB/PUVA dose-response studies. In all animals tested, there was absolutely no evidence of irritation due to the anti-oxidant per se.

Animals

Due to the similarity of their skin to humans, domestic Yorkshire swine (males weighing 30–45 kg) were used in this study. They were

 ISSN 0001-5555

housed in barns at the North Carolina State Veterinary School and fed a standard diet. Prior to experiments, animals were anaesthetized with ketamine/xylazine (2 mg/kg, IM) and their backs clipped with animal shears. Experimental sites (10 cm^2) were delineated with a felt-tipped marker. Animals were typically pretreated for 3 days with $\approx$0.1 ml/site. Details are described in Figs. 1–4. During irradiation and prior to biopsying, all animals were placed under halothane anaesthesia. Typically, four animals were used per experiment.

UV source

For UVB studies, unfiltered Westinghouse FS-40 fluorescent bulbs (4 housed in a planar array) were placed above a restrained animal at a distance of approximately ten inches. While not ideal in terms of matching solar spectrum, this source allowed for uniform irradiation of the rather large surface area on the animals. The UV intensity at the experimental sites was measured to be $\approx$1.5 mW/cm^2 using a National Biological Corporation Model UVB LMH06C photodetector. In PUVA experiments, a similar set-up was used, but with GE F40BL fluorescent bulbs. The measured intensity for the UVA was $\approx$2 mW/cm^2 using an International Light IL440 detector. In these experiments, 8-methoxypsoralen was formulated at a 0.1% concentration (w/v) in 95% ethanol. This solution was applied to the sites at 10 $\mu g/cm^2$, 1 h prior to exposure and 30 min prior to the final treatment with antioxidants/sunscreens.

Histology

In UVB studies, experimental sites were punch-biopsied (4 mm) in triplicate, 24 h post-exposure. The biopsies were fixed in formalin and processed for routine histology. For analysis, duplicate hematoxylin and eosin stained sections were cut from the middle of each biopsy specimen taken from experimental sites and analyzed in a blinded fashion for "sunburn cells" (basal keratinocytes having pyknotic nuclei as well as eosinophilic cytoplasm). These counts were normalized to the 4-mm punch diameter. The average number of sunburn cells per given condition was calculated in this way from four animals per experiment.

In PUVA studies, sites were treated similarly and biopsies taken as above (at 48 h post-irradiation, however). Because analysis of sunburn cells was difficult from untreated sections in many instances due to focal epidermal necrosis and lymphocytic infiltrates, a semi-quantitative scoring scale was devised (Table I). The doses used in PUVA experiments were determined from initial dose-response experiments and chosen to allow assessment of "more than additive" protection in combination sunscreen and anti-oxidant-treated sites. All sections were scored in a blinded fashion and scores validated by a second independent analyst.

Spectrophotometry

Ultraviolet absorbance spectra were generated using a dual beam Shimadzu Model UV 260 scanning spectrophotometer. Over the wavelength range scanned, the vehicle formulations had no UV absorbance, nor did they alter those of the added anti-oxidants.

RESULTS

Augmentation of UVB sunscreen-mediated skin protection by vitamin C

The ability of antioxidants to safely augment the sunburn protection by sunscreens was investigated. As shown in Fig. 1, vitamin C, while not significantly protective on its own in these experiments, was able to significantly increase a UVB sunscreen's protective ability against the UVB-induced formation of sunburn cells. Experiments using oxybenzone gave similar results. A combination of vitamin C and E also significantly protected the pig skin from UVB damage; most of the protection attributed to vitamin E (Fig. 2). Our previous works with experimental fluorescent UVB bulbs showed that the protection afforded by topical vitamin C was not due to its ability to absorb the damaging wavelengths (2). Some of this latter vitamin's protective abilities, however, no doubt stem from its ability to absorb wavelengths in the UVB region emitted from the fluorescent bulbs (generating the tocopherol radical (8)). Interestingly, in several preliminary experiments, vitamin E's ability to inhibit SBC formation appeared to peak at $\approx$1% concentration (w/v), while inhibition of erythema was dose-dependent (up to 3%), suggesting some dissociation

Table I. *Histopathological scale for PUVA-mediated phototoxicity*

In cases where 1–4 sunburn cells were noted, a value of 0.5 was assigned. In other cases, the half unit allowed for more precise quantification and distinction of a more intense reaction (but not enough to be diagnostic for the next grade). Non-treated, non-exposed sites failed to reveal any alterations and thus served as controls. Qualitatively, a histopathological score of 1 was equal to the minimal phototoxic dose (MPD).

Histopathological score	Histological characteristics
0	No histopathological change
1	Several keratinocytes (more than 4) with brightly eosinophilic cytoplasm and dyskeratotic nuclei (sunburn cells)
2	"Sunburn cells" plus vacuolated keratinocytes at the dermal-epidermal junction (D/E)
3	Same as 2 plus separation at the D/E junction as a blister with $<1/3$ of specimen width involved
4	Same as 3 plus $>1/3$ of specimen width involved
5	Epidermal necrosis and neutrophilic leukocyte infiltrate

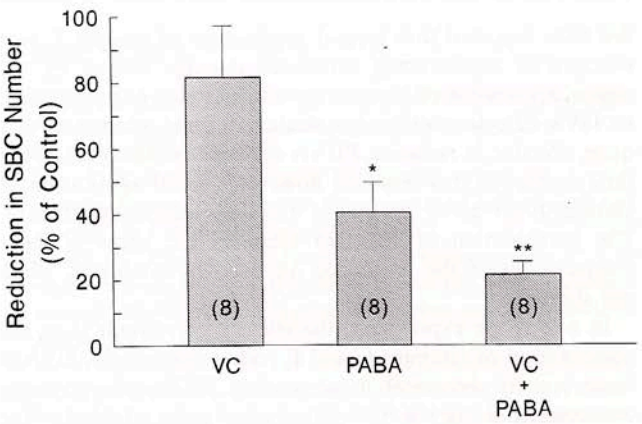

Fig. 1. Additive protection by vitamin C against UVB-induced sunburn cell formation when combined with a UVB sunscreen (PABA). Experimental sites (10 cm^2) were treated with the indicated preparations 2$\times$/day for 3 days and 30 min prior to irradiation with $\approx$3–4 MED of UVB radiation ($\approx$300 mJ/cm^2). Biopsies were taken at 24 h and sunburn cells enumerated as described in Methods. SBC values from treated sites were compared to paired vehicle-treated sites (treated as 100%) to calculate reduction in SBC numbers. At doses used, vehicle treated sites averaged 55.0 SBC's/4 mm biopsy. *: $P<0.01$ vs vehicle-treated sites; **:$p<0.05$ vs sunscreen alone. Concentrations used (w/v): Vitamin C: 10%; PABA: 0.1%.

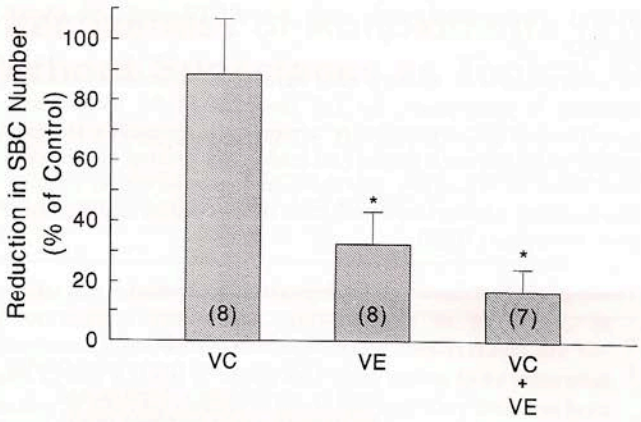

Fig. 2. Effect of a combination of vitamin C and E on the prevention of UVB-induced sunburn cell formation in swine skin. Treatments and calculations are as in Fig. 1. At doses used, vehicle-treated sites averaged 28.3 SBC/4 mm biopsy. *: $p < 0.05$ vs vehicle-treated sites. Concentrations used (w/v): Vitamin C: 10%; Vitamin E: 3%.

between the anti-oxidant property of the vitamin and its "sunscreen" potential (data not shown).

Augmentation of UVA/B sunscreen-mediated skin protection by vitamin C with and without vitamin E

We have reported that topical application of vitamin C was effective at ameliorating cutaneous damage caused by the topical application of 8-methoxypsoralen followed by exposure to UVA (2). In preliminary studies, topical vitamin C was quite effective in reducing PUVA damage to skin over a 96-h time course. In this instance, however, topical application of vitamin E (at up to 2% (w/v)) was only minimally effective. The combination of the two vitamins did show a slight enhancement of the protection afforded by vitamin C (data not shown).

In a separate experiment, the efficacy of vitamin C or the combination of vitamins C and E with a commercial UVA/B sunscreen (oxybenzone) in preventing PUVA phototoxicity was assessed using the histopathological index (Table I). The results are illustrated in Fig. 3. Vitamin C, again, was effective itself in lessening the phototoxic damage. The chosen level of oxybenzone was also quite efficacious under these experimental conditions. When combined, the inhibition of skin damage was even greater than seen with either compound individually (Panel A). Panel B in Fig. 3 illustrates that a combination vitamin C/vitamin E formulation was, as noted before, slightly more protective against PUVA damage than vitamin C alone (see panel A). In combination with oxybenzone, however, an apparently greater than additive effect is seen in the protection. In support of this, enumeration of "sunburn cells" (impossible from unprotected sites) shows comparable values for oxybenzone and the vitamin C/vitamin E-treated sites (see Table II). The combination of the antioxidants with the sunscreen gave virtually complete protection, again suggesting a more than additive effect.

This protection does not appear to be due to any increased UVA absorbency of the combination product. Thus, from Fig. 4 it can be appreciated that there is absolutely no increased UV absorbance of the vitamin C/E plus oxybenzone formulation compared to oxybenzone alone at any of the wavelengths

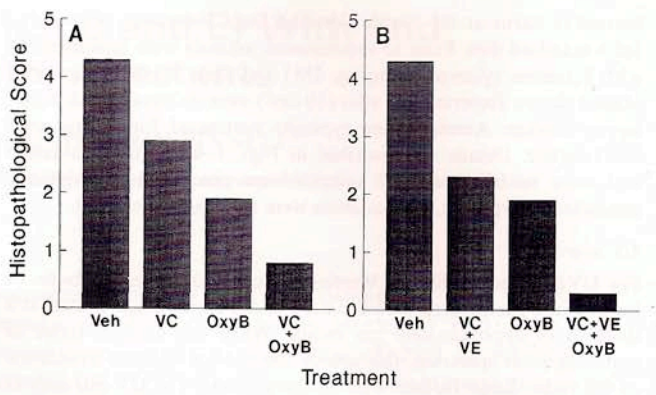

Fig. 3. Combined effect of vitamin C (Panel A), or of vitamin C plus E creams (Panel B) with the UVA sunscreen (oxybenzone) in inhibiting PUVA damage to swine skin. Experimental sites were treated once a day for 3 days and 30 min prior to a 6-min (≈ 720 mJ/cm^2) UVA exposure (which was 60 min after psoralen application). Sites were also post-treated once a day. Biopsies were taken 48 h post-exposure, processed for histology and graded according to the criteria in Table I. Values are the mean of the average scores from the four animals. Concentrations used (w/v): VC:10%, VE:2%, oxybenzone:0.25%.

Table II. *Increased protection from PUVA-induced phototoxicity by a combination of antioxidant vitamins and sunscreen*

Sunburn cells were enumerated as described in Materials and Methods. Vitamin C concentration: 10% (w/v); Vitamin E: 2% (w/v); Oxybenzone: 0.25% (w/v). Treatment regimen was as in Fig. 3.

Experimental condition	Number of 'Sunburn cells'/ 4 mm biopsy
Vehicle-treated (4)	TMTC
Vitamin C/vitamin E-treated (4)	59.5 ± 16.7
Oxybenzone-treated (4)	$31.2 \pm 11.9^{++}$
VC/VE + oxybenzone-treated (4)	$4.3 \pm 1.1^{*,+}$

TMTC: Too many to count (>100 SBC's, epidermal necrosis); $^{++}$: not significantly different from VC/VE-treated ($p > 0.1$); *: $p < 0.05$ compared to oxybenzone-treated; +: $p < 0.01$ compared to VC/VE-treated. Numbers in parenthesis indicate animal number. Values given are the mean ± standard error of averaged values for the four animals.

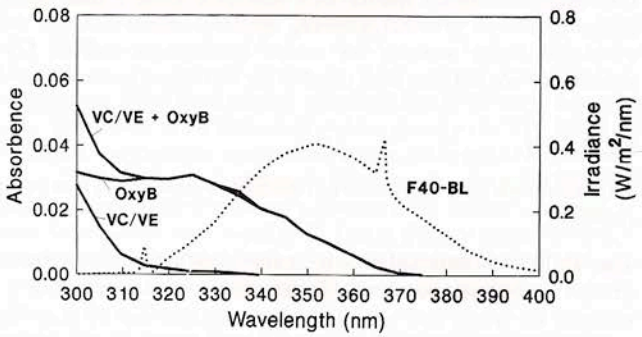

Fig. 4. Absorbance spectrum of anti-oxidant cream formulation ± oxybenzone versus emission spectrum from UVA fluorescent bulbs (GE F40 BL). Absorbance spectra were measured in three ethanolic solutions using a Shimadzu model 260 spectrophotometer. Emission spectra were taken from the manufacturer's specifications. Formulations used in Fig. 3 were diluted 4×10^3 times into absolute ethanol.

emitted from the UVA fluorescent bulbs. Thus, it can be concluded that a biological property of the anti-oxidants must be responsible for the increased protection against the phototoxic insult.

DISCUSSION

Results from this study indicate that "natural" anti-oxidants can be useful by themselves or as additions to sunscreen preparations. Suggestions to this effect have appeared in the literature (7), but more studies are needed to critically evaluate the mechanisms of protection in the combination products.

These experiments were run at fixed, moderately high, UV doses ($\approx$3–4 MED and MPD for these animals) and obviously only vitamins C and E were evaluated. Lower, suberythemogenic doses of UVB have been shown to be cumulatively damaging and in such a model, antioxidants alone might show even greater protection than reported here (9,10). Additionally, combinations of other antioxidants could be included, which theoretically would broaden the protective net. However, it must be noted that under any circumstances, one cannot predict that antioxidants should be totally protective, particularly against shorter wavelength UVB-mediated damage. Too much of the energy from these wavelengths is directly absorbed by biomolecular targets, DNA being the most important, without the need for reactive oxygen intermediacy. At some point, even with complete protection against the "free radical" components of UVB toxicity, sufficient cellular damage will occur, leading to compromised function and ultimately cell death. Thus, UVB sunscreens will no doubt remain an essential component of photoprotection.

Of considerable interest is the protection noted against PUVA-mediated skin damage by the combination of the anti-oxidants with the UVA absorbing sunscreen. We have used a PUVA model to assess UVA photoprotection, a model which is often used in photoprotection studies (11–13), though not without some drawbacks, e.g. UVA protection factors, based on MPD determination is true and applicable only for the prevention of 8-MOP phototoxic reactions (action spectrum equals 320 nm–360 nm) (14). Certainly, both UVA and PUVA share an oxygen dependence for some of their biological effects (15–17) and seem particularly relevant for studying antioxidants in this regard. While not as energetic or acutely damaging as UVB, the longer wavelength UVA is no longer thought to be innocuous. It penetrates much deeper into skin, where it can interact with virtually all skin components notably eliciting phototoxic reactions (18), and with chronic exposure, altering connective tissue morphology and chemistry (19,20). Recently, it was reported that the causative wavelengths responsible for the induction of malignant melanoma may well lie in the UVA spectrum (21), giving rise to more than cosmetic concerns about the potential dangers in exposure to these longer wavelength UV rays. Recent work also hints that sunscreens alone may not be sufficient to inhibit melanoma growth (22).

With increasing public use of potent UVB sunscreens of SPF 15 or greater, large numbers of people are exposing themselves to unnaturally high levels of UVA because of their ability to remain in the sun considerably longer before sunburning. It is, therefore, important to find safe methods of protecting against these wavelengths. As with the UVB sunscreens, nothing is known about potential health risks associated with a life-time of use of chemically synthesized absorbers, so using naturally occurring antioxidants, certainly at least as adjuncts to commercial sunscreens, would appear to be a worthwhile endeavor.

In conclusion, this study provides evidence that antioxidants (particularly vitamin E) are effective in inhibiting UVB damage to porcine skin. Vitamin C can add to that protection and that provided by a UVB sunscreen. In a PUVA model of UVA-mediated skin damage, vitamin C was the more effective of the two anti-oxidants at photoprotection. Extrapolating, when using only the antioxidant vitamins in a formulation, broad spectrum protection may be achieved due to the differential protection noted above. However, when combined with a commercial UVA/B sunscreen, the antioxidants (vitamin C alone or the combination of C and E) provided excellent skin protection. Tests with other models of UVA damage are necessary, however, to substantiate this. With a rising concern over potential problems associated with UVA exposure, these combinations may be beneficial in preventing pathologies ranging from photosensitivity reactions to cancer to photoaging of the skin.

ACKNOWLEDGEMENT

We thank Dr. Nathan Adams and Dr. George Brode of Union Carbide Corporation for providing formulations used in one of the experiments and Drs. Diane Breviere, Tom Manning and Hideko Kamino for support during the studies.

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