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UV photoprotection by combination topical antioxidants vitamin C and vitamin E

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Background: Virtually all plants and animals protect themselves from the sun using vitamins C and E.

Objective: The purpose of this study was to see if a combination of topical vitamins C and E is better for UV protection to skin than an equivalent concentration of topical vitamin C or E alone.

Methods: We developed a stable aqueous solution of 15% L-ascorbic acid (vitamin C) and 1% α-tocopherol (vitamin E). We applied antioxidant or vehicle solutions to pig skin daily for 4 days. We irradiated (1-5× minimal erythema dose) control- and antioxidant-treated skin using a solar simulator with a 295-nm band-pass filter. On day 5, we measured antioxidant protection factor, erythema, sunburn cells, and thymine dimers.

Results: The combination of 15% L-ascorbic acid and 1% α-tocopherol provided significant protection against erythema and sunburn cell formation; either L-ascorbic acid or 1% α-tocopherol alone also was protective but the combination was superior. Application during 4 days provided progressive protection that yielded an antioxidant protection factor of 4-fold. In addition, the combination of vitamins C and E provided protection against thymine dimer formation.

Conclusion: Appreciable photoprotection can be obtained from the combination of topical vitamins C and E. We suggest that these natural products may protect against skin cancer and photoaging. (J Am Acad Dermatol 2003;●:000-000.)

The skin naturally uses antioxidants to protect itself from the damaging effects of sunlight.[1] Skin predominantly uses L-ascorbic acid (vitamin C) to protect the aqueous environment and α-tocopherol (vitamin E) to protect lipid structures including membranes.[2] In many biologic systems, vitamins C and E work synergistically; when vitamin E becomes oxidized by free radicals, it is regenerated in the membrane by vitamin C.[3-5]

Previously, we have reported that L-ascorbic acid applied topically to the skin reduces the photoinjury produced by both UVB and UVA irradiation.[6] The critical role of α-tocopherol for the antioxidant protection of the stratum corneum lipid bilayer and protection against stratum corneum protein oxidation has been recently reviewed.[7] In this study, we report on a combination of antioxidants, L-ascorbic acid and α-tocopherol, optimized with regard to concentration and pH for maximum photoprotective effects. We demonstrate that this combination of vitamins C and E is superior to either antioxidant alone. The combination provides for a 4-fold protection when compared with vehicle against erythema produced by solar-simulated irradiation and inhibits the generation or accumulation of thymine dimers in skin.

MATERIALS AND METHODS
Chemicals

An aqueous vehicle of 15% L-ascorbic acid (Merck, Darmstadt, Germany) and/or 1% dl-α-tocopherol (Roche, Nutley, NJ) was formulated con-

taining 5% Brij 30 (Uniqema, New Castle, Del) and 15% ethanol. All solutions were adjusted to pH 3.2. For the purposes of these experiments, concentrations of ingredients and pH of the solution chosen for testing was formulated to provide maximum percutaneous absorption of L-ascorbic acid and α-tocopherol.

Experimental design

Experiments were performed in weanling white Yorkshire pigs. In conducting research using animals, the investigators adhered to the guide for the care and use of laboratory animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (National Institutes of Health, publication No. 86-23, revised 1996).

UV irradiation

A 1000-W UV radiation (UVR) source (Lightning Cure 200, Hamamatsu, Japan) was used for delivering solar-simulated radiation to pig skin. The lamp was combined with a dichroic mirror assembly reflecting most of the visible and infrared emission, to reduce the heat load on the skin, and with a 1-mm WG295 Schott selective UVB band-pass filter (295 nm) to eliminate wavelengths less than 295 nm. A 1-cm diameter liquid light guide was connected to the exit port of the lamp housing to deliver energy to the surface of the skin. The light guide was positioned just above the surface of the skin. The intensity used in the experiment was 5 mW/cm^2 of UVB as measured by a research radiometer (IL1700, International Light, Newburyport, Mass). At this irradiance, there was about 40 mW/cm^2 of UVA; because of the much greater erythemal effectiveness of UVB, the latter is expected to be the dominant wave band in the observed biologic effects.

Treatment and irradiation procedure

Yorkshire pigs were clipped 24 hours before exposure. The antioxidant or vehicle formulations (500 mL) were applied to each patch of back skin (7.5 × 10 cm) daily for 4 days. To determine the minimal erythema dose (MED), on day 3 we gave 30 to 100 mJ/cm^2 at 10-mJ/cm^2 intervals of solar-simulated UVR to untreated skin. On day 4, MED was determined as the lowest dose that induced perceptible erythema with distinct borders (ordinarily 40-60 mJ/cm^2). From 1 to 5× MED at 1×-MED intervals of solar-simulated UVR was given in triplicate to each 7.5- × 10-cm area of back skin. On day 5, the antioxidant protection factor was calculated for each formulation as the ratio of the MED in antioxidant-treated skin in comparison with untreated skin. Each treatment area was photographed using polarizing filters to minimize surface reflection. Each irradiated spot was biopsied with an 8-mm skin punch.

Measurement of erythema

By using 8- × 12-inch color photographic enlargements, erythema was measured with a chromameter (ColorMouse Too, Color Savvy Systems Ltd, Springboro, Ohio). Previously, we found that skin erythema varied appreciably depending on blood flow to the area. By photographing the area, the depth of erythema was documented at a moment in time and could be reliably measured in high-quality photographic enlargements. Three separate sites from each irradiated spot on photographs were chosen to measure the average erythemal response. Nonirradiated adjacent skin was measured for comparison. Erythema was measured in the "a*" mode as instructed by the supplier. The difference of the a* value between irradiated skin and nonirradiated skin determined the erythema.

Measurement of sunburn cells

Skin biopsy specimens were fixed in 10% neutral-buffered formalin and processed for routine histology. Hematoxylin-eosin–stained center-cut sections of each biopsy specimen were analyzed for sunburn cells (keratinocytes with pyknotic nuclei having an eosinophilic cytoplasm). The entire 8-mm center section of the histologic ribbon was analyzed and the results expressed as sunburn cells/mm. When photodamage is extensive, it may be difficult to precisely define a sunburn cell in the presence of epidermal necrosis. For analysis, whenever sunburn cells could not be accurately identified, an upper limit of 35 sunburn cells/mm was used.

Thymine dimer immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through a graded alcohol-water series. After equilibrium in phosphate-buffered saline, slides were incubated in 1.5% horse serum to block nonspecific binding. Antithymidine dimer antibody, Clone KTM-53 (Kamiya Biomedical, Seattle, Wash) was diluted 1/50 in 15% horse serum and applied for 30 minutes at 37°C. Antibody binding was detected using a kit (avidin-biotin ABC-elite kit, Vector, Burlingame, Calif) as instructed by the vendor. Bound peroxidase was also visualized using a kit (AEC substrate kit, Vector). Slides were then counterstained with hematoxylin and mounted using aqueous mounting medium (Biomedia, Foster City, Calif).

Statistics

Results are expressed as mean ± SD (n = 3). The P values were calculated by Student t test.
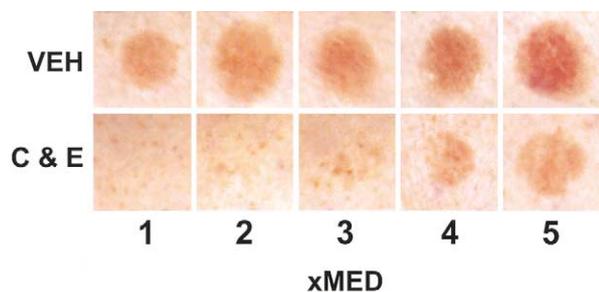
Fig 1. Vehicle (*VEH*) and combination vitamins C and E (*C & E*) were applied to pig skin daily for 4 days. Skin was irradiated with solar-simulated UV irradiation, 1 to 5 minimal erythema dose (*MED*) at 1-MED intervals. Erythema was determined 1 day later.

RESULTS

In these experiments, concentrations of ingredients and pH of the solution chosen for testing was formulated to provide maximum percutaneous absorption of L-ascorbic acid and α-tocopherol. We have previously reported that skin levels of L-ascorbic acid were maximal after 3 days of application of 15% L-ascorbic acid in aqueous solution at pH 3.2.[8] Under these conditions, L-ascorbic levels increased in tape-stripped skin from 56 to 1145 pmol/mg skin. Levels of α-tocopherol in tape-stripped skin after topical application of 1% solution for 24 hours increased from 14 to 31 pmol/mg skin as determined by high-pressure liquid chromatography. Higher levels of α-tocopherol up to 5% were no more effective at delivering α-tocopherol into skin.

Antioxidant protection factor

Four days of combination 15% L-ascorbic acid and 1% α-tocopherol provided 4-fold protection against erythema (Fig 1), whereas 15% L-ascorbic acid or 1% α-tocopherol alone protected 2-fold when compared with vehicle-treated skin.
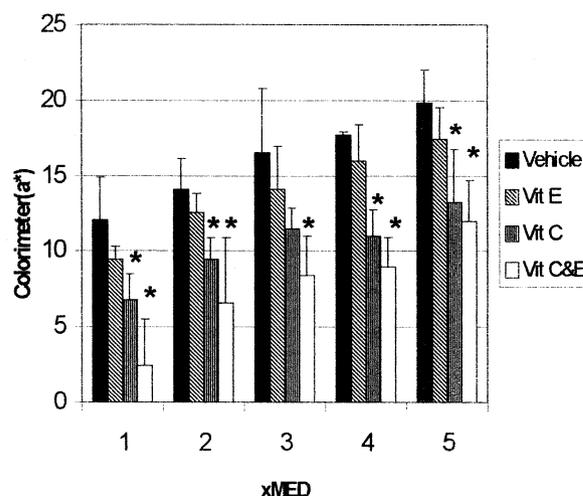
Erythema

Chromameter readings confirmed the visual erythema evaluations (Fig 2). The combination of 15% L-ascorbic acid and 1% α-tocopherol significantly reduced erythema at 1, 2, 3, 4, and 5 MEDs. L-ascorbic acid alone was significantly effective at 1, 2, 4, and 5 MEDs. α-Tocopherol alone lessened erythema but this effect was not statistically significant.

Sunburn cells

Enumeration of sunburn cells revealed statistically significant reductions at 1 and 2 MEDs by combination 15% L-ascorbic acid and 1% α-tocopherol, and L-ascorbic acid and α-tocopherol alone (Fig 3). At 3 and 4 MEDs, only the combination of L-ascorbic acid and α-tocopherol was significantly protective.
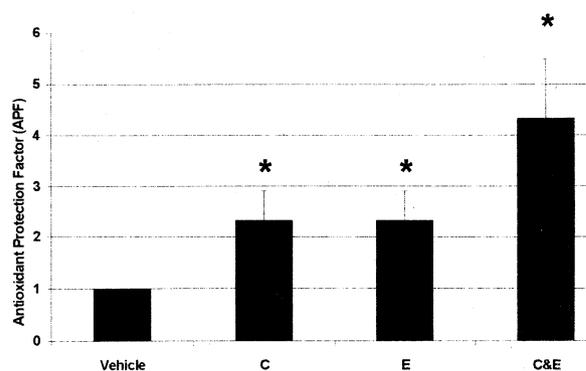
Fig 2. Vehicle, vitamin C (*C*), vitamin E (*E*), and combination (*C&E*) were applied to pig skin daily for 4 days. Skin was irradiated with solar-simulated UV irradiation, 1 to 5 minimal erythema dose (MED) at 1-MED intervals. Clearly delineated erythema was determined 1 day later. Antioxidant protection factor was determined as ratio of MED in antioxidant-treated skin in comparison with control skin. Mean ± SD (n = 3). *$P < .05$.

Fig 3. Vehicle, vitamin (*Vit*) C, Vit E, and combination (*Vit C&E*) were applied to pig skin daily for 4 days. Skin was irradiated with solar-simulated UV irradiation, 1 to 5 minimal erythema dose (MED) at 1-MED intervals. Erythema was measured by colorimeter. Mean ± SD (n = 3). *$P < .05$.

Thymine dimers

Solar-simulated irradiation generated numerous thymine dimers as determined by immunohistochemistry. Combination 15% L-ascorbic acid and 1% α-tocopherol almost completely protected against this DNA alteration (Fig 4).

Kinetics

To determine the kinetics of photoprotection of combination 15% L-ascorbic acid and 1% α-tocoph-

Fig 4. Vehicle and combination vitamins C and E (*C & E*) were applied to pig skin daily for 4 days. Skin was irradiated with 1 minimal erythema dose of solar-simulated UV irradiation. One day later skin was biopsied and sunburn cells were delineated in tissue sections stained with hematoxylin-eosin.
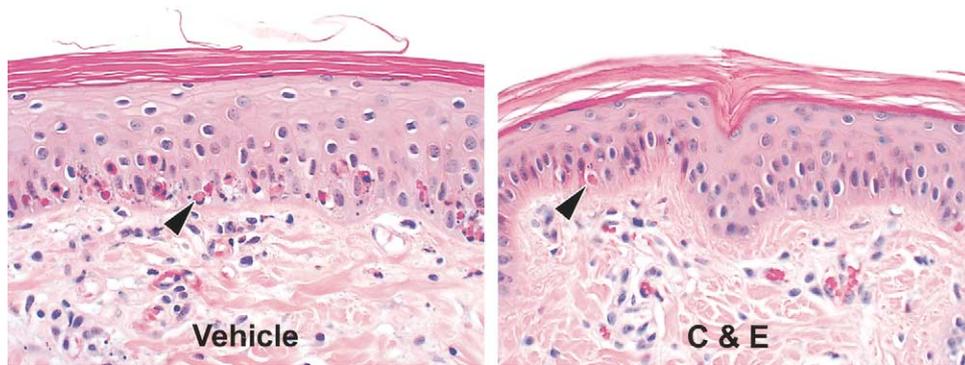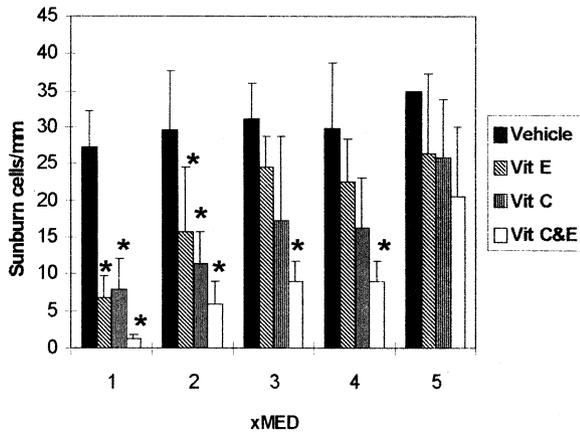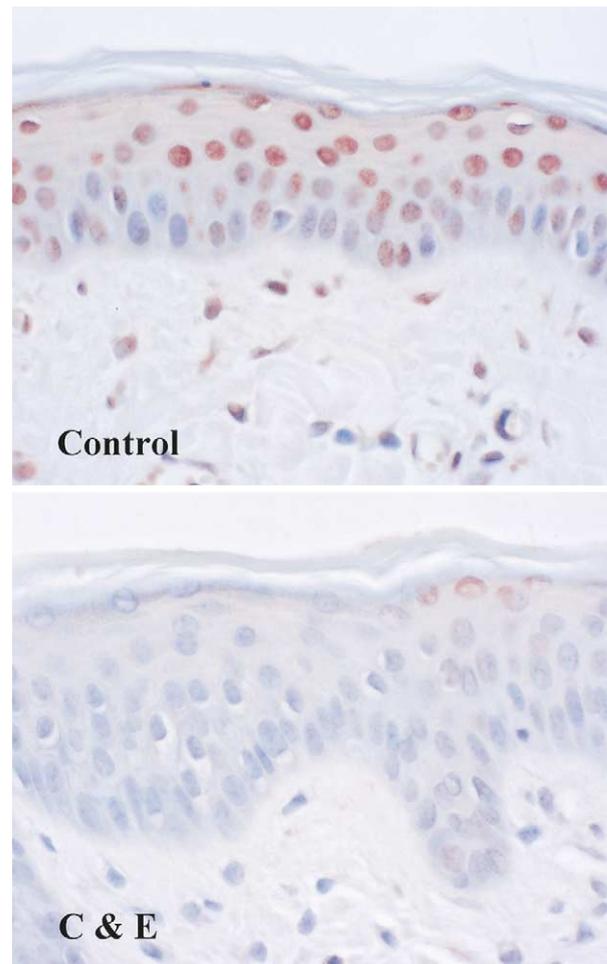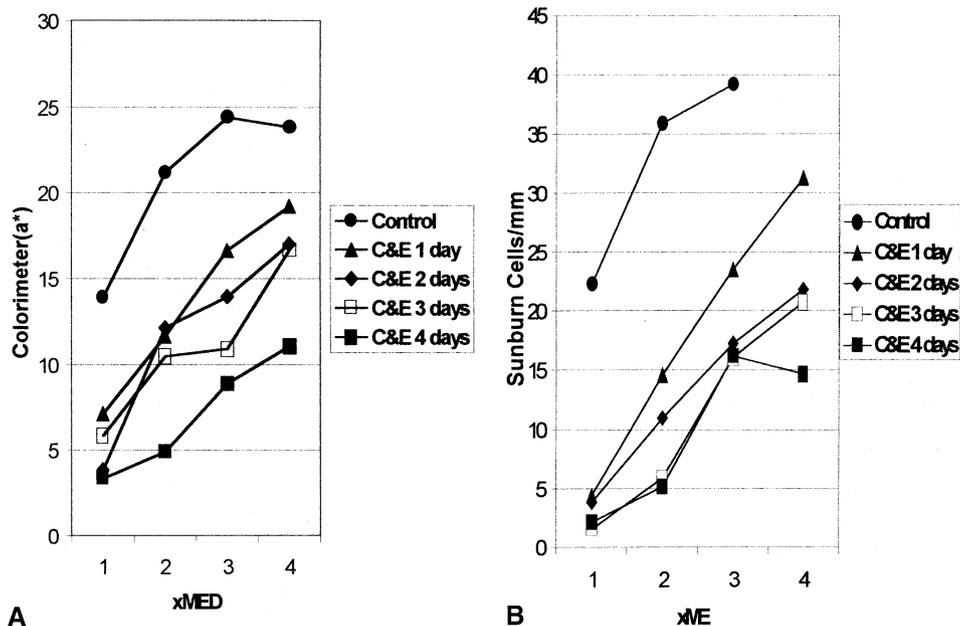

Fig 5. Vehicle, vitamin (*Vit*) C, Vit E, and combination (*Vit C&E*) were applied to pig skin daily for 4 days. Skin was irradiated with solar-simulated UV irradiation, 1 to 5 minimal erythema dose (MED) at 1-MED intervals. Sunburn cells were counted and are expressed as cells per millimeter of epidermis. Mean ± SD (n = 3). *$P <$ .05.

erol, applications were made at 1, 2, 3, and 4 days. Progressive protection as measured by erythema and sunburn cells was observed (Fig 5). To determine if the dosage was critical, the amount applied during 4 days (2 mL) was applied 30 minutes before irradiation (Fig 6). Single application of the entire amount (2 mL) produced appreciable but incomplete protection when compared with application in split doses for 4 consecutive days. Additional protection may have required time for percutaneous absorption. Single application of 0.5 mL and 2 mL resulted in appreciable but approximately the same protection (Figs 5 and 6).

Fig 6. Combination vitamins C and E (*C & E*) was applied to pig skin daily for 4 days. Treated and control skin was irradiated with 4 minimal erythema doses of solar-simulated UV irradiation. One day later thymine dimers were determined by immunohistochemistry.

Fig 7. Combination vitamins C and E (*C&E*) were applied to pig skin daily for 1 to 4 days. Skin was irradiated with solar-simulated UV irradiation 1 to 4 minimal erythema dose (*MED*) at 1-MED intervals. Results of colorimeter readings of erythema (**A**) and sunburn cells (**B**) 1 day after irradiation. Error bars have been eliminated for clarity. Mean ± SD (n = 3). In comparison with control *P* <.05 for all observations.
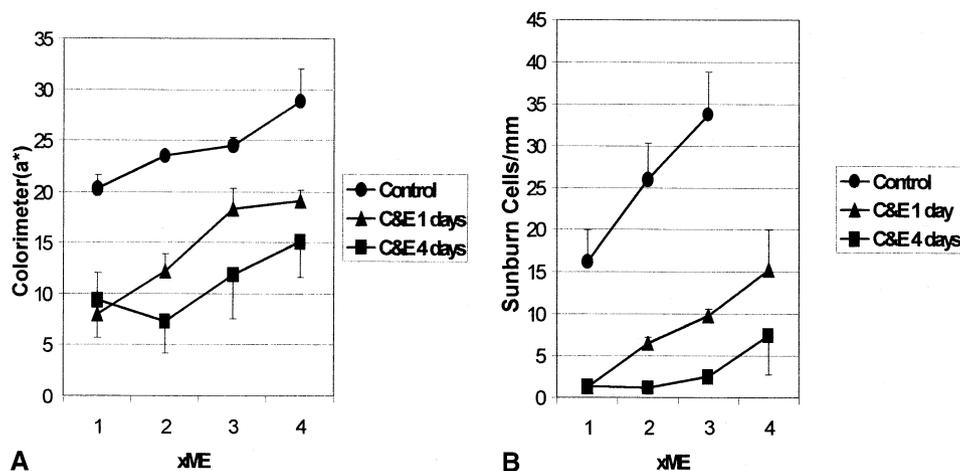
Fig 8. Combination vitamins C and E (*C&E*) were applied to pig skin (500 mL) daily for 4 days or entire amount (2000 mL) as single application. Skin was irradiated with solar-simulated UV irradiation, 1 to 4 minimal erythema dose (*MED*) at 1-MED intervals. Results of colorimeter readings of erythema (**A**) and sunburn cells (**B**) 1 day after irradiation. Mean ± SD (n = 3). In comparison with control, *P* < .05 for all observations.

DISCUSSION

Antioxidants protect the skin against damage produced by UV irradiation.[1,9] L-ascorbic acid is the major fluid-phase antioxidant, glutathione protects the intracellular compartment, and vitamin E and ubiquinol protect membranes. On a molar basis, L-ascorbic acid is the predominant antioxidant in skin; its concentration is 15-fold greater that glutathione, 200-fold greater than vitamin E, and 1000-fold greater than ubiquinol/ubiquinone.[2] Concentrations of antioxidants are higher in epidermis than dermis: 6-fold for L-ascorbic acid and glutathione, and 2-fold for vitamin E and ubiquinol/ubiquinone. In aged and photoaged skin, levels of α-tocopherol

and L-ascorbic acid were reduced significantly, by as much as 60% to 70%.[10] UV irradiation depleted antioxidants; ubiquinol and vitamin E were the most photosensitive, whereas L-ascorbic acid was relatively resistant.[11] Antioxidants work in concert in the skin[9,12]; after oxidation, the lipophilic antioxidants ubiquinol and vitamin E are regenerated by L-ascorbic acid, which in turn is regenerated by glutathione.[9,13,14]

Virtually all plants and animals synthesize L-ascorbic acid. Human beings have lost this capacity as a result of a mutation in L-gulono-χ-lactone oxidase that renders the gene nonfunctional.[15] Instead, human beings must consume L-ascorbic acid in their diet.[16] Although many persons consume large oral supplements of this vitamin, biologic control mechanisms limit the amount that can be absorbed and subsequently transported to skin. Therefore, the only way to get large concentrations into skin is to apply L-ascorbic acid topically, in this way targeting the area to be protected. To achieve percutaneous delivery, the pH of the formulation must be below 3.5 to accomplish protonation of the ascorbic acid. Previously we have determined that maximal concentrations into skin can be achieved with daily applications of 15% L-ascorbic acid for 3 days.[8] We have previously reported that topical L-ascorbic acid is capable of protecting skin against erythema produced by UVB and UVA irradiation by a mechanism unrelated to absorption of the UVR.[6] Moreover, we have demonstrated that topical L-ascorbic acid can provide protection against UVB-induced immunosuppression and systemic tolerance to contact allergens.[17]

In biologic systems, L-ascorbic acid is an extremely effective antioxidant capable of neutralizing superoxide anion,[18] hydroxyl radical,[19] singlet oxygen,[20] and peroxynitrite.[21] In addition to its antioxidant properties, L-ascorbic acid is necessary for collagen synthesis. It is an essential cofactor for prolyl hydroxylase and lysyl hydroxylase, enzymes critical for collagen structure and cross-linking.[22] L-ascorbic acid induces collagen gene transcription[23] and is essential for proper wound healing.[24,25] L-ascorbic acid may inhibit elastin synthesis[26] and, therefore, could be useful for photoaged skin where elastin synthesis is increased.[24,25,27] L-ascorbic acid is a useful skin lightener; it inhibits tyrosinase.[28] L-ascorbic acid is important for epidermal barrier function;[29,30] it stimulates sphingolipid production.[31]

Vitamin E is the body's major lipid soluble antioxidant and functions to protect membranes from free radical attack. It is our major peroxyl radical scavenger and ends the chain reaction damage caused when free radicals attack membranes. Vitamin E is particularly abundant in the stratum corneum of skin, and is delivered there in sebum.[7] It protects the outer layers of skin against pollutants and UV light; UV light causes depletion.[32] The lipophilic nature of vitamin E makes it attractive for application to and percutaneous absorption into skin.[33] Topical vitamin E applied to skin protected against UV-induced erythema,[34] lipid peroxidation,[35] and photoaging changes[36-38] in mice. Topical application protected against UV-immunosuppression.[38-41] Topical vitamin E prevented photocarcinogenesis in mice[40,42] and UV-induced thymine dimer formation.[43] In addition to its photoprotective effects, vitamin E inhibits melanogenesis; its major effect may be through inhibition of tyrosine hydroxylase.[44]

This study demonstrates that synergistic photoprotection can be achieved when vitamin C is combined with vitamin E. Although both L-ascorbic acid and α-tocopherol are prone to oxidize in solution, combining the hydrophilic and lipophilic antioxidants together helps to stabilize the formulation. It is not entirely clear why an antioxidant formulation would have a major effect on thymine dimer formation. Thymine dimer formation results from direct absorption of UVB by DNA.[1,45] DNA may also be the chromophore for erythema.[46] Protection by combination L-ascorbic acid and α-tocopherol apparently is not a sunscreen effect.[47] Although α-tocopherol has modest UVB absorption ([molar extinction of 3500 {mol/l}$^{-1}$/cm] and lmax at 290 nm),[48] we were unable to detect absorption above 295 nm in our solution. L-ascorbic acid does not absorb UV light above 295 nm.[6] Moreover, in our study, application of a 4-fold amount of vitamins C and E before irradiation was no more protective than a single application (Figs 5 and 6); if the protective effect were a sunscreen absorption phenomenon, one would expect more protection from more product. Topical α-tocopherol has been demonstrated to prevent photocarcinogenesis.[40,42] McVean and Liebler [43,49] studied the photocarcinogenic effect of α-tocopherol and demonstrated that it protected DNA photodamage by preventing thymine dimers. They reported better protection with α-tocopherol than derivatives of α-tocopherol with similar UVR absorption and better protection than several sunscreen chemicals.[49] Their studies suggested that cellular uptake was necessary for the protective effect. UVR generates reactive oxygen species in skin (UVA > UVB), resulting in oxidation of proteins.[50] DNA repair enzymes appear to be especially prone to UVA damage,[51] which may lead to inefficient excision of pyrimidine dimers. Topical antioxidants may help to prevent this damage. Chen et al[52] dem-

onstrated, in UV-irradiated mice, a 55% reduction of pyrimidine dimer formation in epidermal p53 by topical α-tocopherol. However, they saw little change in effect from 1 to 10 hours after irradiation and reasoned that it was unlikely that the effect related to repair. Although we cannot completely eliminate a sunscreen effect to explain our data with vitamins C and E, we believe that the effect is minimal. In support, protection against photocarcinogenesis and pyrimidine dimer formation has been reported using other antioxidants including green tea polyphenols[53,54] and silymarin[55] in the apparent absence of UVR absorption.

Photochemistry requires absorption of photons by a chromophore. Transurocanic acid is a major chromophore for generation of singlet oxygen in skin.[56,57] Its maximum concentration is in the upper epidermis and stratum corneum. The peak UV spectrum for singlet oxygen generation from urocanic acid is about 345 nm. The stratum corneum antioxidant system is strategically placed to deal with this insult to the skin barrier.

Photoprotection against erythema has been previously reported by combination vitamins C and E administered systemically[58,59] or applied topically to skin.[47,60] This combination was particularly effective at preventing the tanning response and immunosuppression of contact hypersensitivity.[61] Because α-tocopherol is the major antioxidant of the outer layers of skin, its lipophilic nature lends itself to topical application; absorption occurs in minutes.[39] Simultaneous application of L-ascorbic acid may be an advantage because it adds a coantioxidant to the outer layers of skin. It may then be available to regenerate α-tocopherol whenever it is oxidized, regenerating it for further protection.

Because L-ascorbic acid and α-tocopherol are relatively unstable in cosmetic formulations, these compounds have been esterified to improve stability. These esters are readily converted when taken orally, but not necessarily changed to active compounds when topically applied to skin. Indeed, neither magnesium ascorbyl phosphate nor ascorbyl-6-phosphate topically applied appreciably changed skin levels of L-ascorbic acid.[8] Likewise, esters of α-tocopherol including acetate, and succinate are only poorly converted and are functionally ineffective when used topically for skin.[62,63]

CONCLUSION

We demonstrate that protection against UV irradiation can be achieved with a solution containing a combination of vitamins C and E. Combination antioxidants may be particularly efficacious in that they may eliminate toxic free radicals by transfer of single hydrogen atoms rather than electrons.[14] In this way, they may reduce damaging free radicals while minimizing the reduction of molecular oxygen to superoxide and adding additional free radical stress. Antioxidants do not work individually in the skin but work synergistically in an integrated and regulated way to protect against oxidative stress.[9,12] Combining these 2 antioxidants, vitamins C and E, can provide useful supplementation to sunscreen protection against photocarcinogenesis and photoaging damage produced by the sun.

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